



University
of Glasgow

Irvine, David Arthur (2013) *Defining novel therapeutic targets in chronic myeloid leukaemia stem cells: targeting self-renewal through hedgehog pathway inhibition*. PhD thesis.

<http://theses.gla.ac.uk/4584/>

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

**Defining Novel Therapeutic Targets in Chronic Myeloid
Leukaemia Stem Cells; Targeting Self-Renewal
Through Hedgehog Pathway Inhibition**

David Arthur Irvine

A thesis submitted for the degree of Doctor of Philosophy to the
University of Glasgow March 2013

College of Medical, Veterinary and Life Sciences
Institute of Cancer Sciences

Dedication

This work is dedicated to the memory of my younger brother, Michael Irvine - alumnus of this university, life-long companion, confidant, raconteur and fellow adventurer; I wish there had been more time.

Michael Irvine BSc (Hons) MBChB MRCS MRCGP
29th April 1977 - 10th July 2011

Summary

Chronic myeloid leukaemia (CML) is a clonal disorder that arises following a reciprocal genetic translocation event between chromosomes 9 and 22 $t(9;22)(q34;q11)$ in a haemopoietic stem cell. This produces a constitutively active, oncogenic tyrosine kinase, BCR-ABL which leads to deregulated mitotic signalling, inhibition of apoptosis and altered normal cellular adhesion and stromal interaction. As a result there is expansion of leukaemic stem cell numbers and accumulation of leukaemic myeloid progenitors. Untreated there will be acquisition of further genetic abnormalities leading to disease progression, blast crisis and death.

The development of tyrosine kinase inhibitors (TKI) (e.g. imatinib, dasatinib, nilotinib, bosutinib and ponatinib) that block BCR-ABL kinase activity has radically improved the management of CML leading to unprecedented levels of cytogenetic and molecular responses in chronic phase (CP) patients. However a substantial minority of patients fail to reach optimal treatment targets, TKI resistance can develop over time and even optimally responding patients after prolonged treatment with TKI continue to harbour primitive leukaemic cells that have the capacity to cause disease relapse or progression.

One reason for disease persistence is that primitive CML cells are resistant to pharmacologically achievable concentrations of TKI and are not actually dependent on BCR-ABL kinase signaling for survival. Therefore eradication of the CML clone will require alternative therapeutic strategies involving novel pharmacological targets against these primitive CML cells. In this work, two complementary approaches to define novel survival mechanisms and characterise new potential therapeutic targets in CML are described.

Gene expression microarray technology was used to measure gene expression differences between the most highly purified and rigorously defined stem and progenitor populations from chronic and advanced phase CML patients at diagnosis compared with normal volunteers. Flow activated cell sorting (FACS) was used to purify haemopoietic stem cells [HSC] $CD34^+38^-Lin^-CD45RA^-CD90^+$ and progenitor populations (multipotent progenitors [MPP] $CD34^+38^-Lin^-CD45RA^-CD90^-$; common myeloid progenitors [CMP] $CD34^+38^+Lin^-CD123^+CD45RA^-$; granulocytic/monocytic progenitors [GMP] $CD34^+38^+Lin^-CD123^+CD45RA^+$ and

megakaryocytic/erythroid progenitors [MEP] CD34⁺38⁺Lin⁻ CD123⁻ CD45RA⁻) from primary CML samples and samples derived from normal volunteers. FISH analysis confirmed that >90% cells from all sub populations harboured the *BCR-ABL* transgene and that this was most highly expressed in the HSC population. Global analysis of the microarray data indicated that the normal gene expression profile was deregulated in CML. While mature progenitor populations (GMP and MEP) from CML or normal haemopoiesis were very similar at a transcriptional level there were significant differences in expression between normal HSC and CML HSC. These differences include broad up regulation of many aspects of cellular machinery required for proliferation, DNA replication and repair and segregation and mitosis. There was also down regulation of various cell adhesion molecules. Strikingly, there was comparative down regulation of many genes associated with stem cell function, self-renewal and maintenance of the HSC population in CML HSC compared to normal HSC. Thus CML HSCs appear to have a more mature transcriptional phenotype, with similarities to the normal MPP and CMP populations. In addition to this, CML stem and progenitor cells were noted to have reduced expression of HLA molecules and other antigen presentation mediators. Thus from these studies, CML HSC appear to be mature, readily able to enter cell cycle but deficient in expression of various mediators of self-renewal and antigen presentation.

Targeting the mechanisms that regulate self-renewal might offer a mechanism of targeting CML HSC. One pathway that has an important role in influencing stem cell self-renewal is the Hedgehog (Hh) signalling pathway. In embryogenesis this pathway is critically involved in patterning but remains active in adult tissue contributing to tissue homeostasis, regeneration and healing. In adults it is known to contribute to oncogenesis and is critical for the propagation of CML in murine models but appears to be dispensable in normal haemopoiesis. If self-renewal could be inhibited in CML, it follows that there would be reduced primitive CML HSC available for disease propagation. Therefore parallel studies were performed to investigate whether Hh signalling was active in CP CML HSC and progenitor cells and whether inhibition of Hh signalling alone or in combination with conventional TKI treatment might eradicate CML HSC.

Targeted expression analysis confirmed that critical mediators and targets of Hh signalling were enriched in both normal and CML primitive haemopoietic populations. There was differential expression of several Hh signalling mediators

and targets in primary CML populations. Most notably there was reduced expression of the key negative regulators of Hh signalling (*GLI3* and *SUFU*) in CP and advanced phase CML. There was also up regulation of several targets of Hh signalling (*GLI1*, *PTCH1*, *CCNB1/2*, *FOXM1* and *STIL*) in CML compared with normal populations.

Inhibition of Hh signalling with the clinical grade smoothed (SMO) inhibitor, LDE225 led to modest and variable reduction in expression of the Hh target *GLI1* in CD34⁺ CML HSC. Short term *in vitro* culture of CP CML CD34⁺ cells with LDE225 ± nilotinib did not affect viable cell counts, exert an additive anti-proliferative effect, inhibit cell cycle progression nor augment TKI mediated apoptosis. Additionally CFSE cell tracking experiments up to 12 days confirmed the lack of effect of LDE225 on cell division kinetics and maturity as measured by surface expression of CD34. However if SMO inhibition predominantly exerted influence through modulation of self-renewal activity in CP CML HSC then targeted inhibition of SMO would not alter measures of apoptosis, proliferation or cell cycle progression in these short term *in vitro* experiments and assays designed to interrogate the number and function of self-renewing progenitors would be required to discern the effect of LDE225 on CML HSC function.

Pre-treatment with LDE225 did not affect primary colony formation in colony forming cell (CFC) assays reflecting the fact that primary colony formation is largely driven by committed progenitor proliferation and not HSC self-renewal. However, serial replating assays demonstrated that LDE225 reduced secondary and tertiary colony formation (an *in vitro* surrogate of self renewal activity). Furthermore, while pre-treatment with nilotinib increased apparent re-plating capacity of CD34⁺ CP CML cells, combination treatment with LDE225 and nilotinib led to the greatest reduction in CD34⁺ CP CML cells with replating capacity. Similarly, pre-treatment of CD34⁺ CP CML cells with LDE225 alone or in combination with nilotinib led to reduction of residual primitive haemopoietic cells as measured by long term colony initiating cell (LTC-IC) assay, while pre-treatment normal CD34⁺ cells with LDE225 alone or in combination with nilotinib did not affect LTC-IC read-out.

It is well recognised that stem cell fate decisions are formulated in the context of complex microenvironmental signalling. To assess the effect of long term exposure to LDE225 ± nilotinib on CP CML CD34⁺ cells in the presence of

microenvironmental factors, CD34⁺ CP CML cells were directly inoculated into pre-prepared mixed M210B4 and SL/SL stromal co-cultures and cultured in the presence of LDE225 alone or in combination with nilotinib over 5 weeks prior to assessment of residual colony forming cells. Prolonged exposure to LDE225 resulted in a significant reduction in residual colony forming cells.

In conclusion these studies indicate that Hh signalling is active in CP CML HSC and that the abrogation of Hh signalling through SMO inhibition alone or in combination with conventional TKI treatment is a potentially useful therapeutic strategy to target resistant CML HSC. These findings provided the impetus for collaborative murine modelling studies and together these have provided the preclinical background for clinical trials of Hh signalling in combination with TKI in CML.

Acknowledgements

In the preparation of this thesis I have had the opportunity to reflect on my experiences over the past few years; it has been a long but enjoyable and ultimately rewarding journey due to the influence of my friends and colleagues, past and present, at the Paul O' Gorman Leukaemia Research Centre and I am truly appreciative of their camaraderie and support.

I am deeply indebted to by supervisors Dr Mhairi Copland and Professor Tessa Holyoake for their guidance and unflagging support over the years, for the opportunity to participate in the exciting and inspirational work of the Paul O' Gorman Leukaemia Research Centre and for the excellent academic training that I have been fortunate to receive during the completion of this work. I was privileged to have Dr Copland as my primary supervisor and would particularly like to offer her my sincerest thanks for the opportunity to work with her on this project and for her patient supervision throughout. I would also like to thank Dr David Vetrie, my advisor and Professor Ravi Bhatia in the City of Hope, California for their collaboration, invaluable suggestions, advice and encouragement.

I would like to gratefully acknowledge contributions from other members of the laboratory particularly Helen Wheadon, Heather Jorgensen, Alan Hair, Alison Michie, Vignir Helgason, Francesca Pellicano, Ashley Hamilton, Ross Kinstrie and Sheela Abraham; and thank them for their assistance, innumerable helpful discussions and sharing of technical expertise. Additionally I would like to thank Elaine Allan and Heather Morrison for their assistance with BCR-ABL FISH. Profound thanks also to Jennifer Richmond, friend and former colleague, for her help with many aspects of this work and her support throughout.

I would also like to express my gratitude to my clinical colleagues in Glasgow for their encouragement, to the patients who contributed their samples for research and to the Chief Scientists Office in Scotland who funded my clinical academic training fellowship, affording me the opportunity to complete this work.

Finally, it goes without saying that this endeavour would not have been completed without the constant support of my "long suffering" fiancée Frances, my parents - to whom I owe everything - and my friends.

Related Publications

Chronic myeloid leukaemia stem cells display alterations in expression of genes involved in oxidative phosphorylation. Flis K, [Irvine D](#), Copland M, Bhatia R, Skorski T. *Leukaemia & Lymphoma*. 2012 Dec;53(12):2474-8

Targeting hedgehog in haematological malignancy. [Irvine DA](#), Copland M. *Blood*. 2012 Mar 8;119(10):2196-204.

Restricted access to second generation tyrosine kinase inhibitors in the UK could result in suboptimal treatment for almost half of chronic myeloid leukaemia patients: results from a West of Scotland and Lothian population study. Gallipoli P, Shepherd P, [Irvine D](#), Drummond M, Holyoake T. *British Journal of Haematology* 2011 Oct;155(1):128-30

Optimising chronic myeloid leukaemia therapy in the face of resistance to tyrosine kinase inhibitors - a synthesis of clinical and laboratory data. [Irvine DA](#), Heaney NB, Holyoake TL. *Blood Rev*. 2010 Jan;24(1):1-9

Recurrence of lymphoid blast crisis over 20 years after successful sibling allo-SCT for CML: short lived complete cytogenetic response to imatinib. [Irvine DA](#), Shepherd JD. *Bone Marrow Transplant*. 2009 Aug;44(4):267-8

In press / preparation

Personalized synthetic lethality induced by targeting RAD52 in leukemias identified by gene mutation and expression profile. Kimberly Cramer-Morales, Margaret Nieborowska-Skorska, Kara Scheibner, Michelle Padgett, [David A Irvine](#), Tomasz Sliwinski, Kimberly Haas, Jaewoong Lee, Huimin Geng, Darshan Roy, Artur Slupianek, Feyruz V Rassool, Mariusz A Wasik, Wayne Childers, Mhairi Copland, Markus Muschen, Curt I Civin, Tomasz Skorski - in press, BLOOD/2013/501072

Inhibition of Chronic Phase Chronic Myeloid Leukemia Stem Cells by the combination of the Hedgehog Pathway Inhibitor LDE225 and Nilotinib. [David A. Irvine](#), Bin Zhang, Heather Morrison, Jennifer Richmond, Yinwei Ho, Paul Manley, Tessa L. Holyoake, Ravi Bhatia, Mhairi Copland - in preparation

Declaration

This work represents original work carried out by the author unless otherwise stated and has not been submitted in any form to any other University.

David Irvine

March 2013

Table of Contents

Dedication.....	ii
Summary.....	iii
Acknowledgments.....	vii
Related Publications	viii
Declaration	ix
Table of Contents.....	x
List of Figures.....	xv
List of Tables	xviii
List of Abbreviations	xix
1 Introduction	20
1.1 Overview of Haemopoiesis	21
1.1.1 Hierarchical haemopoiesis and the concept of stem cells	21
1.1.2 Defining the haemopoietic hierarchy	22
1.1.3 Progenitor differentiation in mouse and man	28
1.2 Stem Cell Fate.....	30
1.2.1 Haemopoietic stem cell kinetics.....	31
1.2.2 Haemopoietic stem cell survival	33
1.2.3 Self-renewal	34
1.2.4 Control of stem cell fate.....	37
1.2.5 External regulation of stem cell fate.....	41
1.2.6 External signalling through developmental pathways	45
1.2.7 Self-renewal and malignancy	58
1.2.8 Developmental signalling in malignancy.....	60
1.2.9 The concept of cancer stem cells	63
1.3 Chronic Myeloid Leukaemia	68
1.3.1 Chronic myeloid leukaemia cell of origin	69
1.3.2 Molecular biology of BCR-ABL	70
1.3.3 Treatment of CML.....	72
1.3.4 The CML stem cell.....	81

1.4	Microarray Analysis in Haematology.....	87
1.4.1	Gene expression analysis	87
1.4.2	Anatomy and function of a gene expression microarray	88
1.4.3	Data handing - the bioinformatics pipeline	90
1.4.4	Limitations and validation of microarray analysis.....	92
1.4.5	Microarrays in normal and malignant haemopoiesis.....	93
1.5	Aims.....	99
1.5.1	Specific objectives	100
2	Materials and Methods	101
2.1	Materials	102
2.1.1	Tissue culture	102
2.1.2	Flow Cytometry.....	107
2.1.3	Molecular Biology	108
2.1.4	Immunofluorescence	110
2.1.5	Microarray	111
2.2	Methods.....	112
2.2.1	Drugs and reagents	112
2.2.2	Standard cell culture conditions.....	112
2.2.3	Patient samples	112
2.2.4	Cryopreservation of cells	113
2.2.5	Cell recovery post cryopreservation	113
2.2.6	Cell counting and viability assessment	114
2.2.7	Primary CD34 ⁺ cell culture experiments	114
2.2.8	Cell line culture	114
2.2.9	Flow Cytometry.....	115
2.2.10	Short term cellular techniques	124
2.2.11	Functional Assessment of Haemopoietic Progenitors	126
2.2.12	Molecular Biology Techniques.....	133
3	Gene Expression Analysis of CML Stem and Progenitor Cells.....	141
3.1	Introduction.....	142
3.2	Cell Preparation, FACS and Preparation of RNA.....	144
3.3	FISH Analysis of Sorted Populations	146
3.4	Affymetrix Gene Expression Microarray	147
3.5	Validation of Microarray Findings using qRT-PCR and the Fluidigm Platform.....	148

3.6	Results.....	149
3.6.1	Global gene expression analysis.....	149
3.6.2	Contrasts and comparative gene expression analysis.....	152
3.6.3	Comparison between normal and leukaemic HSC.....	160
3.7	Summary and Conclusions.....	181
4	Expression of Components and Targets of the Hh Signalling Pathway in CML.....	187
4.1	Introduction.....	188
4.2	Results.....	191
4.2.1	Expression of Hh constituents and targets in primary CP CML CD34 ⁺ cells in comparison to normal CD34 ⁺ cells.....	191
4.2.2	Gene expression analysis of Hh signalling molecules in CML compared with normal stem and progenitor populations.....	193
4.2.3	Analysis of key Hh signalling mediators and targets.....	193
4.2.4	Analysis of hedgehog ligand expression.....	197
4.2.5	<i>BMP4</i> expression.....	198
4.2.6	Gene expression analysis of Hh signalling molecules in CML compared with normal haemopoiesis from gene expression microarray.....	199
4.2.7	<i>BCR-ABL</i> expression in CML LSC and progenitor sub-populations.....	201
4.3	Discussion.....	202
5	Effect of Specific Inhibitors of Hh Signalling on CP CML in Short Term <i>in vitro</i> Culture.....	207
5.1	Introduction.....	208
5.1.1	Measuring the activity of Hh signalling in cells.....	209
5.1.2	Hh modulation of cell cycle and proliferation.....	209
5.1.3	Hh as a survival signal.....	210
5.1.4	Measuring Hh activity and its effect in CML haemopoiesis.....	210
5.2	Results.....	212
5.2.1	LDE225 and LEQ506 cause dose dependent reduction in luminescence in a GLI responsive luciferase reporter assay.....	212
5.2.2	SMO inhibition with LDE225 reduces expression of downstream Hh target genes in K562 cells.....	213
5.2.3	SMO inhibition with LDE225 reduces expression of GLI1 in CD34 ⁺ CP CML cells.....	214

5.2.4	SMO inhibition with LDE225 alone and in combination with nilotinib does not influence viability in CP CML CD34 ⁺ cells in short term <i>in vitro</i> culture	217
5.2.5	SMO inhibition with LDE225, alone and in combination with nilotinib, does not influence proliferation of CP CML CD34 ⁺ cells in short term <i>in vitro</i> culture	218
5.2.6	SMO inhibition with LDE225, alone and in combination with nilotinib, does not cause apoptosis in CP CML CD34 ⁺ cells in short term <i>in vitro</i> culture	219
5.2.7	SMO inhibition with LDE225, alone and in combination with nilotinib, does not influence cycle distribution of CP CML CD34 ⁺ cells in short term <i>in vitro</i> culture	220
5.2.8	SMO inhibition with LDE225, alone and in combination with nilotinib does not influence cell division kinetics or retention of primitive cell surface markers on CP CML CD34 ⁺ cells	221
5.3	Discussion	229
6	Effect of LDE225, a Specific Inhibitor of Hh Signalling, on Stem Cell Function of CP CML HSC	233
6.1	Introduction	234
6.2	Assessment of Stem Cell Numbers and Self-Renewal	234
6.3	Results	236
6.3.1	Inhibition of Hh signalling with LDE225 does not affect primary CFC formation but significantly reduces secondary re-plating efficiency	236
6.3.2	LDE225 in combination with nilotinib significantly reduces LTC-IC frequency in CP CML	238
6.3.3	LDE225 in combination with nilotinib significantly reduces LTC-IC frequency following prolonged treatment in stromal co-culture	240
6.4	Discussion	243
6.5	Conclusion	246
7	General Conclusion	247
7.1	Summary of Results	248
7.2	A Combinatorial Approach to Therapy	248
7.3	Current and Future Directions in CML	250
7.4	The Potential Role of Hh Inhibitors in the Treatment of CML	252
7.5	Concluding Remarks	253

8	Appendices	255
8.1	Appendix 1: Samples, Cell Number and Total RNA for Microarray and Validation Experiments.....	256
8.2	Appendix 2: Collaborative <i>in vivo</i> Murine Modelling Studies.....	257
8.2.1	Introduction.....	257
8.2.2	Inhibition of Hh signalling in primary CML samples and murine models of CML.....	258
8.3	Collaborative Results.....	259
9	Bibliography	266

List of Figures

Figure 1-1: Simplified model of human haemopoiesis.....	30
Figure 1-2: Possible stem cell fates.	31
Figure 1-3: The potential fates of a dividing stem cell.	37
Figure 1-4: The mechanism of Hh signalling.....	49
Figure 1-5: Interactions between selected cell fate determinants.....	57
Figure 1-6: Possible mechanisms of Hh signalling in malignancy.....	62
Figure 1-7: Diagram indicating possible mechanisms for targeting the CML stem cell by influencing HSC behaviour.	82
Figure 1-8: Anatomy and function of an Affymetrix Genechip	89
Figure 1-9: The bioinformatics pipeline indicating the process from samples hybridisation, through statistical analysis and ultimate biological interpretation. ...	92
Figure 2-1: Annexin V / Viabrobe apoptosis assay.....	118
Figure 2-2: High resolution cell cycle analysis.	119
Figure 2-3: Cell division tracking by dye dilution.	122
Figure 2-4: Experimental schema for the LTC-IC assay.....	130
Figure 2-5: Schematic for CFC re-plating assay.	131
Figure 2-6: Schematic diagram of Taqman [®] chemistry	135
Figure 2-7: Schematic and representative example of Taqman [®] qRTPCR	137
Figure 3-1: Haemopoietic hierarchy and cell sorting strategy.	146
Figure 3-2: PCA of non CML and CP CML populations.	150
Figure 3-3: PCA of non CML and CP CML HSC/LSC and MPP populations.	151
Figure 3-4: Principal components analysis of AP CML populations.	152
Figure 3-5: Diagram showing potential microarray contrasts.	153
Figure 3-6: Comparison of gene expression along each haemopoietic hierarchy and between each corresponding sub-population isolated from normal and CP CML patients.....	154
Figure 3-7: Relative expression of the primary cell surface antigens used to sort the HSC populations.	155
Figure 3-8: Heat map demonstrating expression of haemopoiesis related genes across HSC and progenitor populations from normal and CP CML samples.	156
Figure 3-9: Heat map representing the expression level of various genes associated with antigen presentation in normal haemopoiesis and in CP CML. .	159

Figure 3-10: Ingenuity® pathway analysis comparing normal HSC and CP CML LSC.....	162
Figure 3-11: Ingenuity® pathway analysis comparing normal HSC and CP CML LSC showing the most significantly differentially expressed canonical pathways.	163
Figure 3-12: Selected cell cycle active genes that were differently expressed between normal and CP CML LSC.	167
Figure 3-13: Validation of microarray expression levels of selected cell cycle active genes by qRTPCR.....	168
Figure 3-14: Selected genes with a role in DNA recombination and repair.	170
Figure 3-15: Selected genes with roles in stem cell fate, differentiation and self-renewal that were differentially regulated in the microarray comparison.....	176
Figure 3-16: Validation of microarray expression levels of selected stem cell fate, differentiation and self-renewal genes by qRTPCR.	177
Figure 3-17: Relative similarity of CP CML LSC and normal HSC and progenitors.	180
Figure 4-1: Key components of Hh signalling.....	189
Figure 4-2: Comparison of Hh pathway related gene expression in PB CD34 ⁺ cells in CP CML versus normal haemopoiesis.	192
Figure 4-3: Relative expression of key targets and modulators of Hh signalling in sorted sub-populations (HSC, CMP, GMP and MEP) from PB CD34 ⁺ cells derived from patients with CP, AP or BC CML at diagnosis compared with normal sub-populations.	195
Figure 4-4: Relative expression of additional targets of Hh signalling across sub-populations in normal, CP, AP and BC CML haemopoiesis.	196
Figure 4-5: Relative expression of <i>SHH</i> ligand across sub-populations in normal, CP, AP and BC CML haemopoiesis.....	197
Figure 4-6: Relative expression of <i>BMP4</i> across sub-populations in normal, CP, AP and BC CML haemopoiesis.....	198
Figure 4-7: Microarray comparison of selected Hh related genes.	200
Figure 4-8: <i>BCR-ABL</i> expression in CP and advanced CML stem and progenitor cells.....	201
Figure 5-1: SMO activation and inhibition in Hh responsive TM3 reporter cell line.	213

Figure 5-2: Gene expression analysis of the BC CML cell line K562 following exposure to LDE225.	214
Figure 5-3: Quantitative RTPCR measuring expression of several constituents and targets of Hh signalling in CD34 ⁺ CP CML cells after (A) 24h and (B) 72h culture in SFM in the presence of LDE225.	215
Figure 5-4: Quantitative RTPCR measuring expression of <i>GLI1</i> in CD34 ⁺ CP CML cells after 72h culture in SFM in the presence of LDE225.....	216
Figure 5-5: Quantitative RTPCR measuring expression of <i>GLI1</i> and other downstream targets of Hh signalling in CD34 ⁺ CP CML cells after 24h culture with rSHH.	217
Figure 5-6: Viable CD34 ⁺ CP CML cells following exposure to LDE225 +/- nilotinib.....	218
Figure 5-7: Chronic phase CML CD34 ⁺ cell proliferation in presence of LDE225 at 72h.....	219
Figure 5-8: Apoptosis assays in CP CML CD34 ⁺ cells exposed to LDE225 and/or nilotinib.....	220
Figure 5-9: High resolution cell cycle analysis in CP CML CD34 ⁺ cells exposed to LDE225 and/or nilotinib.....	221
Figure 5-10: Representative CFSE flow cytometry plot indicating the effect of LDE225 and nilotinib on CD34 ⁺ CML CP cells.....	223
Figure 5-11: CFSE stained viable cells following culture for 4, 8 and 12d in SFM+HGF, untreated or with nilotinib and LDE225 alone and in combination....	224
Figure 5-12: Percentage recovery of input cells following culture for 4, 8 and 12d in SFM+HGF untreated or with nilotinib and LDE225 alone and in combination.	226
Figure 5-13: CFSE stained cells following culture for 4d in SFM+LGF untreated, with nilotinib, LDE225 or rSHH alone and in combination.	228
Figure 6-1: Relative frequency of total CFC in clonogenic colony assays.....	236
Figure 6-2: Secondary and tertiary colony formation following re-plating of primary colonies.....	237
Figure 6-3: Secondary colony formation following re-plating of primary colonies derived from CP CML CD34 ⁺ cells exposed to LDE225 ± nilotinib over 72h.	238
Figure 6-4: LTC-IC recovery from CP CML CD34 ⁺ cells exposed to LDE225 ± nilotinib over 72h.....	239
Figure 6-5: LTC-IC recovery from normal CD34 ⁺ cells exposed to LDE225 ± nilotinib over 72h.....	240

Figure 6-6: CFC recovery from CD34 ⁺ CP CML cells following prolonged stromal co-culture in the presence of LDE225 ± nilotinib.....	242
Figure 8-1: NSG murine model engraftment with normal and CP CML CD34 ⁺ cells after pre treatment with LDE225 +/- nilotinib.....	260
Figure 8-2: Expression of Hh mediators and targets in the <i>Scf-tTa-BCR-ABL-GFP</i> ⁺ inducible transgenic mouse model of CML at baseline and following treatment.	262
Figure 8-3: Studies in the <i>Scf-tTa-BCR-ABL-GFP</i> ⁺ inducible transgenic mouse model of CML.....	264

List of Tables

Table 1-1: Murine HSC phenotypes	24
Table 1-2: Human HSC phenotypes	27
Table 1-3: List of selected niche factors and their influence on stem cell fate.	43
Table 1-4: Selected additional targets of Hh signalling and GLI activation from disparate gene expression studies.....	50
Table 1-5: Accepted levels of haematologic, cytogenetic and molecular response to treatment in CML.....	74
Table 1-6: ELN criteria detailing minimum required response to TKI therapy according to time from initiation.	74
Table 3-1: Immunophenotype and number of samples the progenitor populations analysed.	145
Table 3-2: FISH for BCR-ABL translocation in samples utilised for gene expression studies.	147
Table 3-3: Differentially expressed genes between normal HSC and MPP.....	157
Table 3-4: Expression of selected antigen presentation genes in normal versus CP CML CMP.	159
Table 3-5: Differential expression of selected cell cycle / proliferation genes in CP CML LSC compared to normal HSC.	166
Table 3-6: Differential expression of selected DNA replication, recombination and repair genes (CP CML LSC compared with normal HSC).....	171
Table 3-7: Differential expression of selected antigen presentation genes (CP CML LSC compared with normal HSC).....	172
Table 3-8: Differential expression of selected genes with roles in stem cell fate, differentiation and self-renewal (CP CML HSC compared with normal HSC).	178
Table 3-9: Differential expression of selected genes in CP CML LSC compared with normal MPP.....	181
Table 7-1: Clinical trials in CML utilising various small molecule SMO inhibitors in combination with conventional TKI therapy.....	250

1 Introduction

1.1 Overview of Haemopoiesis

The haemopoietic system is incredibly dynamic; its central feature is constant, massive cell turnover. In the average adult human it is estimated that around one million blood cells are produced and destroyed every second simply to maintain homeostasis (Ogawa, 1993). The mechanisms that serve to fulfil and sustain this enormous requirement throughout the life of an organism, whilst avoiding catastrophic error, have been the object of intense investigation and debate over the past 50 years. These endeavours have yielded important insights, not only into the process of haemopoiesis itself, but also into the development of other tissues, into aspects of regeneration and healing and lastly into the pathological processes leading to aberrant haemopoiesis and oncogenesis.

1.1.1 Hierarchical haemopoiesis and the concept of stem cells

It has been apparent since the middle of the last century that reconstitution of an irrevocably damaged haemopoietic system in mice or other mammals, including humans, was possible through the transplantation of bone marrow (BM) cells (Lorenz et al., 1951; Thomas and Epstein, 1965; Thomas et al., 1957). However, whether the required cells descended from a single parental haemopoietic cell or whether there were different parental cells for each lineage was not initially clear. Series of elegant experiments taking full advantage of advances in scientific understanding and technology in many fields have painstakingly dissected the haemopoietic system of mouse and man and have culminated in our current hierarchical model of haemopoiesis (Bhatia et al., 1997; Dick et al., 1997; Hogan et al., 2002; Kiel et al., 2005; Majeti et al., 2007; Morrison and Weissman, 1994; Notta et al., 2011; Spangrude et al., 1988).

It is generally accepted that a small population of haemopoietic stem cells (HSC) gives rise to a committed proliferative progenitor cell population and ultimately, through successive cell divisions, to progenitors with increasingly restricted potential leading to production of the terminally differentiated effector cells that comprise the blood system. This model owes its core premise to the work of Aleksandr Maximow who postulated the existence of a common precursor haemopoietic cell in 1909, but definitive experimental verification and subsequent elaboration is attributed to Till, McCulloch and many others over the last 50 years (Maximow, 1909; Till and McCulloch, 1961).

In order for this model to explain how the haemopoietic system is sustained throughout life, HSCs must possess two key properties; firstly, the capacity to give rise to all the constituents of the haemopoietic system (multipotency); and secondly, the capacity to re-duplicate without differentiation, thereby maintaining the stem cell pool (self-renewal). Experimental confirmation of the existence of cells possessing these unique characteristics was first provided by Till and McCulloch who observed colonies of haemopoietic cells in the spleens of lethally irradiated mice following transplantation. These colonies (colony forming unit-spleen, [CFU-S]) contained multipotent cells, some with the capacity to produce further colonies in the spleens of secondary transplant recipients (Becker et al., 1963; Till and McCulloch, 1961; Wu et al., 1967).

Since the identification of these cells, there have been extensive efforts to develop assays to investigate the behaviour, proliferative capacity, differentiation potential and self-renewal properties of HSCs and their progeny. Technical advances, primarily in monoclonal antibody production, fluorescence activated cell sorting (FACS) and refinements in the manufacture and purification of essential haemopoietic growth factors (GFs), not to mention the development of progressively more robust *in vitro* and *in vivo* stem and progenitor cell assays have led to a rapid increase in our understanding of the behaviour of HSC and their progeny.

1.1.2 Defining the haemopoietic hierarchy

The cell population defined by Till and McCulloch was quickly realised to be a heterogeneous population including HSC, but largely comprised of more mature, committed haemopoietic cells that lacked multipotency and self-renewal capacity. In order to better understand stem cell behaviour and unravel the mechanisms controlling stem cell behaviour it is critical to begin with a pure stem cell population. The challenge since then has been to attempt the purification of increasingly functionally homogeneous populations of HSC and progenitor cells from haemopoietic tissue through the parallel application of cell sorting strategies and functional assays.

1.1.2.1 Defining the haemopoietic hierarchy in the mouse

Despite the obvious differences between mice and humans in terms of size, life span and proliferative requirement in the haemopoietic system, our

progressively refined model of murine haemopoiesis has greatly informed our understanding of human haemopoiesis. Prospective selection for populations enriched for stem cell activity can be performed using various strategies including fluorescent dye exclusion due to higher expression of ATP binding cassette (ABC) multidrug transporters (Zhou et al., 2001), by specific enzymatic activity e.g. aldehyde dehydrogenase (ALDH) (Storms et al., 1999), or since HSC are largely not cycling, through their relative quiescence through retention of DNA, mitochondrial or protein binding dyes (Goodell et al., 1996). Following the advent of reliable flow cytometry and sorting procedures coupled with the availability of high quality monoclonal antibodies it has become common to use FACS to sort populations based on surface phenotype and then to characterise their stem cell activity in a functional assay e.g. their capacity to cause long term complete haemopoietic reconstitution in a lethally irradiated mouse (Bhatia et al., 1997; Cashman et al., 1997; Dick et al., 1997; Hogan et al., 2002; Kiel et al., 2005; Majeti et al., 2007; Morrison et al., 1997a; Morrison and Weissman, 1994; Murray et al., 1995; Notta et al., 2011; Spangrude et al., 1988). Using these technologies and various surface markers, tiny cell populations with different purities and repopulation characteristics have been identified in murine BM. Spangrude and colleagues were the first to demonstrate that they could isolate a population containing all the cells with the capacity for long term engraftment - long term HSC (LT-HSC) - in the murine haemopoietic system by isolating cells positive for stem cell antigen-1 (Sca-1) but negative for mature lineage markers and with low expression of thymocyte antigen-1 (Thy-1). This population was again heterogeneous with these cells making up only a tiny minority of the whole (Spangrude et al., 1988). Subsequent refinement, adding various additional markers, e.g. the expression of the mast/stem cell GF receptor (c-Kit/CD117) or lack of expression of the markers of commitment (e.g. FMS-like kinase-2 [Flk-2/CD135]) and the lack of surface protein CD34 yielded purer LT-HSC enrichment. These techniques also demonstrated the presence of multipotent populations with more limited repopulation capacity: - short term HSC (ST-HSC) with only transient repopulation capacity (< 16 weeks) and a multipotent progenitor population (MPP) (Morrison et al., 1997a; Morrison and Weissman, 1994). Recently use of further surface phenotypic markers, in particular recognition that the signalling lymphocyte activation molecules (SLAM) antigens are differentially expressed in primitive murine haemopoietic cells has allowed further enrichment (Kiel et al., 2005; Yilmaz

et al., 2006). Table 1-1 below summarises the surface phenotype of various HSC populations.

Phenotype	Approx. ratio of cells with LT-HSC activity	Reference
Lin ⁻ , Sca-1 ⁺ , Thy-1 ^{lo}	1 in 10	(Spangrude et al., 1988)
Lin ⁻ , Sca-1 ⁺ , c-Kit ⁺ (LSK)	<1 in 10	(Okada et al., 1992)
Lin ⁻ , Sca-1 ⁺ , Thy-1 ^{lo} c-Kit ⁺ (KTSL)	1 in 5	(Wagers et al., 2002)
CD34 ⁻ , Sca-1 ⁺ , Lin ⁻ , c-Kit ⁺ (+ hoescht 33342 staining)	1 in 5 (1 in 1.1)	(Osawa et al., 1996b) (Matsuzaki et al., 2004)
CD150 ⁺ , CD48 ⁻ , Sca-1 ⁺ , Lin ⁻ , c-Kit ⁺	1 in 2	(Kiel et al., 2005)

Table 1-1: Murine HSC phenotypes

Table indicating predominant phenotypic populations of murine HSC studied and the approximate enrichment of cells with long term engraftment capacity in each phenotype.

It is a testament to the power of these techniques, as well as further validation of the hierarchical nature of haemopoiesis, that murine HSC populations can now be prospectively enriched to near purity (Benveniste et al., 2003; Kiel et al., 2005; Matsuzaki et al., 2004; Osawa et al., 1996a).

1.1.2.2 Defining human HSCs and their progeny

Successful therapeutic human BM transplantation has been performed since 1958; however the identities of the cell or cells responsible for durable engraftment was not initially clear (Thomas et al., 1957). Attempts to isolate human HSCs have progressed utilising broadly similar strategies to those detailed above; that is by prospective phenotypic isolation of populations enriched for HSC activity followed by their functional characterisation in *in vitro* or *in vivo* quantitative assays. It would be fair to say, however, that our understanding of early human haemopoiesis remains significantly behind that of the murine counterpart. One of the reasons for this relates to the complexities of developing quantitative assays of human stem cell activity. This requires either stromal supported, prolonged *in vitro* assays or xenotransplantation repopulation assays using immuno-compromised mice.

1.1.2.3 Human HSC assays

The most rigorous *in vitro* surrogate assay for stem cell activity is the long term culture-initiating cell (LTC-IC) assay and this identifies and quantifies a population of cells that harbour a variable capacity to initiate and sustain haemopoiesis with BM or stromal support for prolonged periods (60 days or

longer) (Eaves et al., 1991; Hao et al., 1996; Sutherland et al., 1994; Sutherland et al., 1991; Sutherland et al., 1993a; Sutherland et al., 1993b; Sutherland et al., 1995; Sutherland et al., 1990). While these assays are widely used and are useful *in vitro* assays, they are limited by lack of capacity to demonstrate multipotency (as the culture conditions will not support lymphoid development). They are also unable to demonstrate reconstitution of the full haemopoietic system or directly measure self-renewal activity. In order to truly demonstrate HSC activity, it is necessary to demonstrate full reconstitution of the haemopoietic system *in vivo*. While clinical stem cell transplantation demonstrates the capacity for long term engraftment in humans using BM and selected peripheral blood (PB) sub-populations, quantitative assays require surrogate transplant models (Weissman, 2000).

Several groups have utilised various immunocompromised murine models (severe combined immune deficiency [SCID] mice, non-obese diabetic [NOD] - SCID mice and more recently NOD-SCID gamma chain deletion [NSG]) mice as xenotransplantation recipients to assay haemopoietic reconstitution activity *in vivo* (Bhatia et al., 1997; Cashman et al., 1997; Dick et al., 1997; Hogan et al., 2002; Lapidot et al., 1992; Shultz et al., 2005). These studies functionally identified a candidate HSC population known as the SCID repopulating cell (SRC) in human BM, mobilised PB and cord blood (CB) that could be measured in limiting dilution, demonstrated reconstitution of all features of the haemopoietic system and was potentially transplantable into secondary and tertiary recipients indicating the capacity for self-renewal. A limitation of these xenotransplantation assays is that the read-out depends on recipient factors which are unrelated to the characteristics of the cells under study, including method of administration and the degree of immunocompetency in the recipient mice. This is clearly illustrated by the improved efficiency of repopulation witnessed in the more profoundly immunocompromised NSG mice compared to other models (Shultz et al., 2005).

1.1.2.4 Phenotypic characterisation of human HSC

Phenotypic characterisation of putative HSC populations in human tissue began with attempts to generate antibodies allowing the identification of early haemopoietic cells and led to the discovery of CD34, a surface glycoprotein highly expressed in primitive haemopoietic tissue but absent in mature differentiated cells. Its function has not been established although it is thought to be involved in

adhesion and possibly signalling. Its main utility is as a surrogate marker for primitive haemopoietic cells. Approximately 1-4% of BM haemopoietic cells express CD34⁺ and this population is multipotent, highly enriched for colony forming cells in *in vitro* assays in addition to harbouring virtually all the cells with the capacity to effect long term haemopoietic reconstitution in murine xenograft models, primates and humans (Krause et al., 1996; Lu et al., 1993b; Sutherland et al., 1989; Sutherland et al., 1990; Udomsakdi et al., 1992c). Recognition of CD34 as a single phenotypic marker that allowed identification and enrichment of this HSC-containing population was a crucial event in human HSC research and has been of immense value both in a research and a clinical context. Despite its clinical utility it has been clear for many years from functional studies that this population is heterogeneous, containing both multipotent and lineage restricted cells and cells with long term and short term repopulating capacity in xenotransplant recipients (Hogan et al., 2002; Sutherland et al., 1989). Further characterisation of this population has been performed using other surface markers notably CD38 and CD90. Between 90 and 99% of CD34⁺ haemopoietic cells also express CD38 (cyclic ADP ribose hydrolase - a glycoprotein with roles in cell adhesion, signal transduction and regulation of intracellular calcium), however, CD34⁺CD38⁺ cells do not form the more primitive fraction and it is the CD34⁺CD38⁻ fraction which is enriched for LTC-IC and particularly SRC activity (Bhatia et al., 1997; Hao et al., 1996; Huang and Terstappen, 1994). Additional studies demonstrated that while CD34⁺38⁺ cells produced rapid transient engraftment that was not transplantable into secondary hosts, the CD34⁺38⁻ fraction produced stable long term engraftment that was more comprehensive (including lymphoid lineage cells, specifically T cells) and could be transplanted into secondary recipients (Bhatia et al., 1997; Conneally et al., 1997). Expression of CD90 (Thy1) has been used by various groups to identify a primitive haemopoietic population (Baum et al., 1992; Craig et al., 1993; McCune et al., 1988; Murray et al., 1995). It is a glycosylphosphatidylinositol (GPI)-linked cell surface protein with a single variable immunoglobulin domain. In humans it is expressed on stem cells from various organ systems including the liver, neurological system and BM (Craig et al., 1993). Its expression correlates with an immature phenotype with CD90⁺ cells representing a fraction of the CD34⁺38⁻ population (Craig et al., 1993; Majeti et al., 2007). Its expression declines in the presence of markers of maturity and lineage commitment. Populations exhibiting CD90 in addition to CD34 are highly enriched

for LTC-IC and SRC and have demonstrated greater efficiency in repopulating not only murine haemopoiesis but human haemopoiesis as well (Bhatia et al., 1997; Craig et al., 1993; Majeti et al., 2007; Michallet et al., 2000; Murray et al., 1995).

It is generally held that LTC-IC represent a more mature functional population than the SRC. Likewise while CD34⁺ cells are well characterised they are a heterogeneous population comprising cells with and without stem cell activity. The CD34⁺38⁻ population is more primitive but still heterogeneous with approximately 1 in 650 CD34⁺38⁻ cells exhibiting SRC activity (Bhatia et al., 1997). Majeti and colleagues further sub-divided this population using expression of CD90 and CD45RA (Majeti et al., 2007). They defined a population of candidate HSC (CD34⁺38⁻90⁺lin⁻45RA⁻) that exhibited multipotency and long term repopulation capacity in about 1 in 10 cells but also contained virtually all the cells with capacity to engraft secondary recipients and a second population (CD34⁺38⁻90⁻lin⁻45RA⁻) - akin to the MPP population in mice. While exhibiting multipotency and long term repopulation capacity, these cells were less able to engraft secondary recipients (Majeti et al., 2007). The HSC population was shown to give rise to the putative MPP population demonstrating it to be more primitive than the latter (Majeti et al., 2007). More recently, in an attempt to further refine this population of HSC isolated, Notta *et al*/found that expression of the cell adhesion and signalling molecule CD49f was associated with enriched long term HSC activity and when combined with rhodamine exclusion in addition to the phenotype above, could refine the HSC population isolated to about 1 in 5, although this was variable between samples (Notta et al., 2011). Table 1-2 summarises these populations, their frequency and gives an indication of their approximate HSC enrichment.

Phenotype	Proportion of BM HSC	Approx. ratio of cells with LT-HSC activity	Reference
Whole BM	100%	1 in 1x10 ⁶	(Wang et al., 1997)
CD34 ⁺	1-4%	1 in 9.3x10 ⁵	(Bhatia et al., 1997)
CD34 ⁺ , CD38 ⁻	0.01-0.4%	1 in 650	(Bhatia et al., 1997)
CD34 ⁺ , CD38 ⁻ , CD90 ⁺ , CD45RA ⁻	0.003-0.013%	1 in 10	(Majeti et al., 2007)
CD34 ⁺ , CD38 ⁻ , CD90 ⁺ , CD45RA ⁻ , CD49f ⁺ , rhodamine exclusion	N/A	1 in 5	(Notta et al., 2011)

Table 1-2: Human HSC phenotypes

Table indicating phenotypic populations of human HSC studied and the approximate enrichment of cells with long term engraftment capacity in each phenotype.

Further refinements of this model of haemopoiesis are available from meticulous clonal tracking of HSC in transplantation experiments where different clones were noted to have differing contributions to re-establishing haemopoiesis. While some were dominant at early stages, others, while present, remained dormant or had a substantially delayed contribution. Thus phenotypically similar HSC have different self-renewal behaviours in transplantation (Dick et al., 2001; Mazurier et al., 2004; McKenzie et al., 2006).

1.1.3 Progenitor differentiation in mouse and man

The massive amplification required to deliver a sufficient number of haemopoietic cells to maintain homeostasis occurs through a series of highly proliferative but increasingly lineage restricted progenitor intermediates. Notwithstanding differences in cell phenotype, similar hierarchies of progenitor cells have been described in humans and in mice. It is now generally accepted that MPP give rise to the oligo-potent progenitors the common lymphocyte progenitor (CLP) and the common myeloid progenitor (CMP), each with the limited capacity to form committed daughter lymphoid or myeloid progenitors respectively (Akashi et al., 2000; Galy et al., 1995; Kondo et al., 1997; Manz et al., 2002). Following differentiation into CMP, either megakaryocyte/erythroid progenitors (MEP) or granulocytic/monocytic progenitors (GMP) are formed, each with the capacity to give rise to erythrocytes or megakaryocytes and granulocytes or monocytes respectively (Manz et al., 2002; Pronk et al., 2007). Maturation, survival and proliferation are supported by combinations of exogenous GFs and regulated by expression of specific transcriptional programmes at critical junctures during development. This strict differentiation pathway has been challenged in recent years by mounting evidence that, as HSC mature, they may develop a lymphoid or myeloid bias earlier than had previously thought (Challen et al., 2010) and that early myeloid and lymphoid development may not be completely disparate. For example, in murine systems the existence of multipotent lymphoid-primed MPP (LMPP; LSKCD34⁺FLT3⁺) population with lymphoid and GMP potential but reduced capacity to give rise to MEP have been described. Recent evidence suggests that a similar population may exist in humans (Adolfsson et al., 2005; Kawamoto et al., 2010; Goardon et al., 2011). In other studies myeloid cells were derived from lymphoid specified cells and vice versa (Doulatov et al., 2010; Dykstra et al., 2007).

The advent of *in vitro* clonogenic assays and purified haemopoietic GFs allowed characterisation of the broad stages between the primitive multipotent populations and the mature effector cells that constitute the murine blood system (Ogawa, 1993). These assays demonstrate the ultimate developmental fate of a progenitor cell held in single cell suspension in semi-solid medium under the influence of selected cytokine GFs. In the context of myeloid differentiation, the colonies formed are comprised of a mixture of cell types dependent on the developmental potential of the originator cell and are either mixed (granulocytic, erythroid, megakaryocyte, monocytic; [CFU-GEMM], granulocytic, monocytic; [CFU-GM]) or single cell type colonies (granulocytic [CFU-G], monocytic [CFU-M] or erythroid burst [BFU-E] or erythroid [CFU-E]) which broadly reflect the stages of myeloid differentiation described above.

Thus, haemopoiesis can be regarded as a highly hierarchical process where multipotency and self-renewal are the properties of a small number of tightly regulated cells that gives rise to increasingly committed progenitors. The enormous cell turnover is serviced by the massive amplification that occurs through this system. The fate of the cells close to the top of the hierarchy is critical as even small imbalances would be expected to reverberate through the entire hierarchy (Figure 1-1).

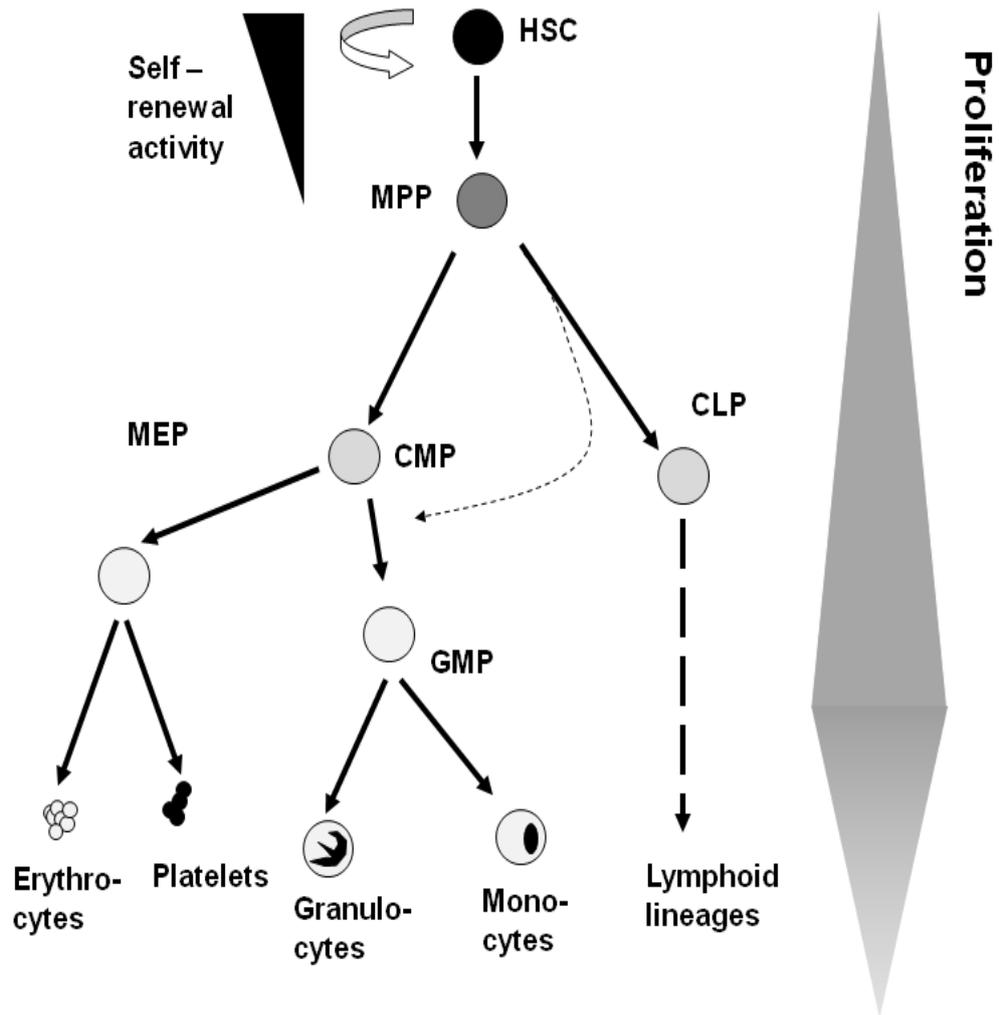


Figure 1-1: Simplified model of human haemopoiesis

Simplified model of human haemopoiesis modified from Doulatov *et al* and Majeti *et al*. (Doulatov *et al.*, 2012; Majeti *et al.*, 2007)

1.2 Stem Cell Fate

Potential fates of an HSC at any given moment are maintenance of quiescence, entering cell cycle, apoptosis or senescence (see Figure 1-2). When an HSC divides, the outcome can either be commitment to differentiation or self-renewal. As discussed earlier in this chapter, self-renewal is a fundamental property of HSC. In order to sustain a steady supply of haemopoietic cells throughout the life of an organism, the HSC pool must be maintained and in order to achieve this, HSC loss through apoptosis, senescence or commitment to differentiation must be balanced by replenishment of the HSC population. This is achieved through self-renewal.

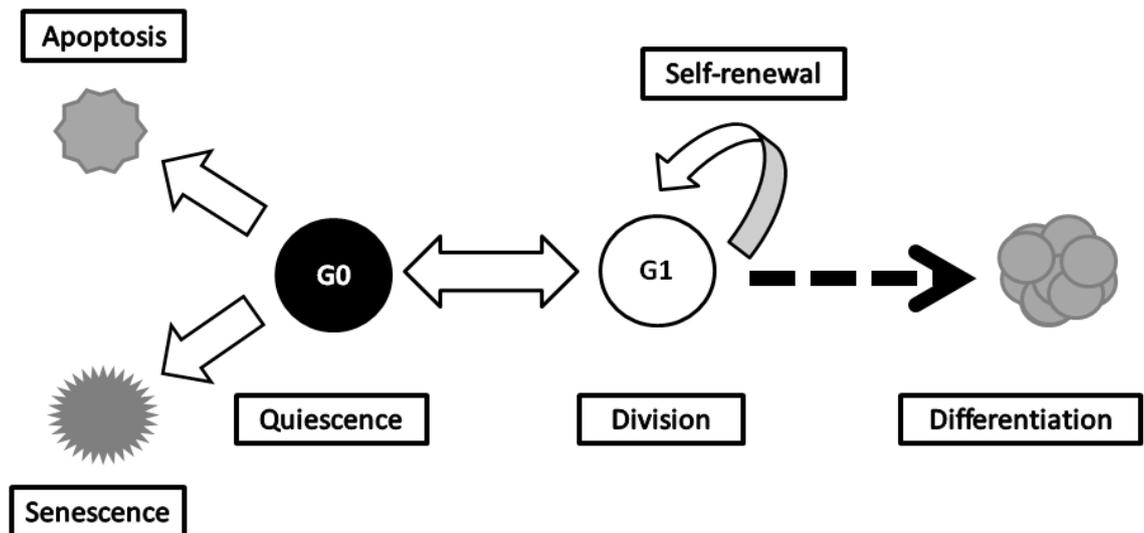


Figure 1-2: Possible stem cell fates.

HSCs are largely quiescent; entry into cell cycle requires a choice between self-renewal and differentiation. Alternative fates include senescence or apoptosis.

1.2.1 Haemopoietic stem cell kinetics

It is clear that HSCs comprise a rare population in contrast to the massive turnover required to maintain haemopoiesis. Curiously, despite the massive variation in size and longevity, evidence suggests that total HSC numbers are relatively conserved between mammals (Abkowitz et al., 2002). In humans, it is estimated that there are between 11,200 and 125,000 HSC (Gordon et al., 2002). While in early developmental haemopoiesis, HSC are thought to be predominantly in active cell cycle, in steady state adult haemopoiesis the majority of HSC settle into a state of quiescence; that is in reversible G_0 cell cycle arrest and enter cell cycle comparatively infrequently (Bowie et al., 2006; Cheshier et al., 1999). Furthermore, different HSC may have distinct cycling profiles. It has been suggested that at least two differing populations exist with a rare particularly dormant population of HSC cycling very infrequently compared to a more active fraction (Cheshier et al., 1999). One study demonstrated that 90% of murine Lin^- , $Sca-1^+$, $Thy-1^{lo}$, $c-Kit^+$ HSC had incorporated BrDU within 30 days (d) of exposure and 99% by 6 months, indicating that virtually all HSC had divided at least once in this time frame with about 25% in cycle at any given time (Cheshier et al., 1999). Another study suggested an average division rate of 1:2.5 weeks (Abkowitz et al., 2000). Wilson *et al*/found evidence of dual populations of HSC (LSK SLAM CD34⁺) with a dormant population dividing on average every 145d and a more active population dividing every 38d (Wilson et al., 2008). Studies in humans indicate

HSC division occurs much less frequently - approximately once every 40 weeks (Catlin et al., 2011; Shepherd et al., 2004).

Preservation of the quiescent state and the maintenance of HSC activity are intrinsically linked; robust repopulation and self-renewal activity segregates to the quiescent fraction (Cheshier et al., 1999; Passegue et al., 2005). Under normal conditions quiescence is required to maintain a pool of viable stem cells throughout the life of an organism. When this is disrupted through manipulation of internal or external signalling, the HSC population can be exhausted (Cheng et al., 2000b). Thus HSC quiescence and proliferation are carefully controlled through a myriad of cell intrinsic and extrinsic (microenvironmental) mechanisms which feed into the cell cycle machinery.

Key control of the cell cycle resides with cyclin proteins active at specific phases of the cycle and the opposing effects of cyclin dependent kinases (CDK) and cyclin dependent kinase inhibitors (CDKI). The maintenance of HSC in G_0 is through high levels of hypophosphorylated retinoblastoma (Rb) transcriptional repressor which prevents expression of E2F transcription factors and inhibits progression out of G_0 (Wikenheiser-Brokamp, 2006). Triple deletion of Rb, p130 and p107 causes HSC expansion, loss of function and exhaustion (Viatour et al., 2008). Emergence from G_0 to G_1 relies predominantly on the D cyclins (CCND) and CDK4/6 interaction and this is positively regulated by the v-akt murine thymoma viral oncogene homolog 1 (AKT)/phosphatidylinositide 3-kinase (PI3K) pathway. Therefore PI3K/AKT signalling must be suppressed in HSC to maintain quiescence (Warr et al., 2011). This occurs through phosphatase and tensin homolog (PTEN) signalling which restrains PI3K activity and allows forkhead box O (FOXO) family transcription factors to transit to the nucleus where they have a predominantly cell cycle inhibitory function through activating the expression of CDKI p21^{cip} and p27^{kip} and reducing intracellular reactive oxygen species (ROS) levels (Massague, 2004; Miyamoto et al., 2007; Tothova et al., 2007). The main families of CDKI are cip/kip and Ink4. Elements of both families are expressed in HSC; CDKI p21^{cip}, p27^{kip} and p57^{kip2} inhibit G_1 and S phase entry. Deletion of p21^{cip1} and p57^{kip2} in HSCs causes HSC expansion but loss of long term repopulation potency (Cheng et al., 2000a; Matsumoto et al., 2011). Lastly of the Ink4 family of CDKI proteins which repress G_0 - G_1 transition, p18^{ink4c} is strongly expressed in HSC and its abrogation appears to increase stem cell numbers and activity suggesting that it has a critical role in repressing HSC division (Bowie et

al., 2007; Passegue et al., 2005). These key intracellular mechanisms are not isolated but respond to extracellular signalling and microenvironmental conditions allowing the activity of HSC to adjust to prevailing external conditions.

There are various advantages to this organisational structure. Relative quiescence preserves the HSC pool over time. It also protects the HSCs from damage through genotoxic insults e.g. through exposure to radiation or chemicals and minimises the accumulation of mutations during DNA replication in the self-renewing population. While largely quiescent, the HSC pool could potentially be recruited rapidly if required for regeneration of the blood system under stress e.g. following chemotherapy or blood loss (Cheshier et al., 2007; Morrison et al., 1997b). The committed progeny are then swept into differentiation programmes becoming proliferating progenitors with massive amplification potential and ultimately giving rise to the terminal effector cells.

1.2.2 Haemopoietic stem cell survival

Clearly, in order to participate in haemopoiesis an HSC must survive. Apoptosis is critically important in embryogenesis and initial body patterning and in adult HSC it offers one possible mechanism to delete aged, abnormal or damaged clones prior to significant contribution to the haemopoietic cell pool. The DNA damage repair apparatus plays an important role in HSC survival signalling. HSC have robust DNA damage and cell cycle checkpoint controls and genomic damage has been shown to limit stem cell activity and induce activation of apoptosis and senescence pathways (Alenzi et al., 2009; Oguro and Iwama, 2007). Likewise, intracellular ROS levels influence HSC and high levels of ROS are associated with increased likelihood of oxidative damage to the cell and its genome. High levels of ROS activate DNA damage pathways, e.g. through p53 (Vazquez et al., 2008). High levels of ROS e.g. in ataxia telangiectasia mutated knock-out ($ATM^{-/-}$) mice have been associated with reduced stem cell activity and the activation of apoptosis and senescence pathways (Ito et al., 2004; Kazuhito et al., 2008; Oguro and Iwama, 2007).

Cell intrinsic anti-apoptotic factors also have a role in HSC as evidenced by the effect of over-expression of B-cell CLL/lymphoma 2 (BCL2) in murine HSC which led to protection from apoptotic stimuli, expanded the HSC population and increased its self-renewal potential (Domen et al., 2000). Likewise, expression of the anti-apoptotic protein myeloid cell leukemia sequence 1, BCL2-related (MCL1)

is a critical requirement for human HSC survival and signalling through fms-related tyrosine kinase 3 (FLT3) is an important survival signal in primitive human HSC as it causes up regulation of MCL1 expression (Kikushige et al., 2008; Opferman et al., 2005).

HSC require other survival signals from the environment to avert apoptotic cell death. They require a minimal cytokine exposure to survive. In one study, over-expression of BCL2 alone was not capable of preventing apoptosis in murine HSC populations in serum free culture; however the addition of KIT ligand prevented apoptosis but initiated differentiation (Domen and Weissman, 2000). This is the case for many “classical” cytokines e.g. thrombopoietin (TPO), interleukin (IL) 3, IL6 or granulocyte/monocyte colony stimulating factor (GMCSF), underlining the complexities in understanding and replicating the milieu that supports stem cell maintenance *in vivo* (Domen and Weissman, 2000; Oguro and Iwama, 2007).

An alternative HSC fate is the occurrence of senescence. In contrast to the reversible quiescent state, senescence represents an irreversible state of cell cycle arrest in metabolically active cells (Collado et al., 2007). HSC can divide a finite number of times as evidenced by their limited re-transplantation capacity (5 in murine models) and replicative senescence may occur due to telomere attrition as a consequence of successive divisions and age-related chromatin alterations (Harrison and Astle, 1982 ; Zimmermann and Martens, 2008). Senescence also occurs as a consequence of unrectified DNA damage. Senescence is mediated through pRB, p53, p14^{ARF} and p16^{INK4a} and is negatively regulated by the action of polycomb protein complexes (Hidalgo et al., 2012; Sauvageau and Sauvageau, 2010). Recent evidence suggests that cellular response to ROS, which increase with HSC age is another important mediator of senescence in HSC (Brown et al., 2013; Ito et al., 2006).

1.2.3 Self-renewal

Self-renewal is a fundamental property of HSC. Self-renewal, very simply, is the ability of a multipotent stem cell to undergo division such that the progeny include at least one cell with a similar level of multipotency to the original stem cell. This can be accomplished by symmetrical or asymmetrical division. Symmetric division results in two identical daughter cells either committed to differentiation (not self-renewal) or each retaining the stem cell properties of the mother cell; in

either case the total number of stem cells in the population is increased or decreased accordingly. Asymmetrical division results in the production of one daughter stem cell and one cell committed to differentiation thus asymmetric division has no net effect on the size of the stem cell pool.

The possible mechanisms behind asymmetric division are interesting. Asymmetric stem cell division has been most extensively studied in stem cell populations in lower organisms e.g. *Drosophila* (Knoblich, 2008; Lin and Schagat, 1997). These studies have provided the basic model through which asymmetric division is thought to occur. In *Drosophila* larvae, the neuroblast cells divide symmetrically along the epithelial surfaces that they are proximate to, and in these divisions the mitotic spindle is oriented along the apical / basal axis (Knoblich, 2008). When the mitotic axis rotates 90° and mitosis occurs perpendicular to the original plane, asymmetrical division occurs producing a differentiating neuronal progenitor cell (ganglion mother cell) and a copy of the original neuroblast (Knoblich, 2008; Lin and Schagat, 1997). This asymmetry is caused by cell polarity proteins which accumulate on the side destined to become the remaining neuroblast and this causes accumulation of cell fate determinants (e.g. numb) at the side destined to differentiate (Knoblich, 2008). These are examples of cell intrinsic autonomous asymmetric division. An alternate model is suggested by the maturation of *Drosophila* germ cells. Here germ-line stem cells exist in close proximity to supportive hub cells. When the germ-line stem cell divides, it does so perpendicular to the face that is in contact with the hub cells. The outermost daughter cell differentiates whereas the innermost cell still in contact with hub cell signalling retains self-renewal properties (Fuller and Spradling, 2007). Thus asymmetric division occurs through the influence of external microenvironmental signalling. Interestingly, disruption of the apparatus that regulates these processes leads to aberrant unrestrained self-renewal and accumulation of stem cells in a process with similarities to carcinogenesis (Knoblich, 2008).

While evidence of stem cell expansion (hence symmetric division) can be obtained from examining the development of the early haemopoietic system and inferred through the capacity of small numbers of transplanted cells to reconstitute the haemopoietic system in the transplant recipient, specific evidence that asymmetric division occurs in HSC has been difficult to garner (Iscoe and Nawa, 1997; Osawa et al., 1996b). Direct demonstration of asymmetric division has been difficult to achieve and whether asymmetric division occurs at all in HSC has been

a subject of debate. Given that the haemopoietic system must both maintain homeostasis and respond to haemopoietic stress, it is attractive to speculate that stem cell expansion through symmetric division primarily has a role in the initial expansion of HSC in the development of the haemopoietic system and in response to haemopoietic stress e.g. post transplantation, whereas asymmetric division is primarily involved in homeostasis of the HSC pool under steady state conditions. However this need not necessarily be the case, homeostasis could also be achieved by balanced symmetric divisions or indeed through initial symmetric divisions with the progeny subsequently following different fates as a consequence of differing external factors.

Several *in vitro* studies of human and murine HSC division at the single cell level have demonstrated that the resultant progeny can have different developmental fates, providing indirect evidence of asymmetric division (Brummendorf et al., 1998; Ema et al., 2000; Giebel et al., 2006; Leary et al., 1985; Punzel et al., 2002; Suda et al., 1984; Takano et al., 2004). Direct evidence of this phenomenon was provided by recent work by Wu *et al*/who reported the division behaviour of murine primitive haemopoietic cells in real time using a Notch driven GFP reporter system (Wu et al., 2007). They found evidence of both symmetric and asymmetric division in these cells, demonstrated that asymmetric division corresponded with segregation of the cell determinant Numb and the balance of self-renewal or asymmetric divisions compared with differentiation divisions could be influenced by external signalling depending on the type of supportive stroma used (Wu et al., 2007). Likewise Beckman *et al*/demonstrated that several other proteins e.g. CD53, CD63 and CD71 segregated in a subgroup (20%) of dividing human HSC (Beckmann et al., 2007) suggesting a potential role for these molecules in asymmetrical division. Additionally Ting *et al*/recently demonstrated that expression of the cell polarity gene, adaptor-related protein complex 2, alpha subunit 2 (AP2A2), in murine HSC enhances stem cell function without markedly affecting the size of the HSC population (in contrast to several other candidates that enhanced self-renewal and caused expansion of the HSC pool) and therefore postulated that it is an effector of asymmetric self-renewal in HSC (Ting et al., 2012). Furthermore they demonstrated its asymmetric localisation in HSC during division suggesting a possible mechanistic role in this type of self-renewal (Ting et al., 2012).

Thus, there is evidence that asymmetric division can occur in mammalian HSC and that the underlying mechanisms may be similar to those described in *Drosophila*, i.e. influenced by cell fate determinants distributing unequally within an HSC prior to division. Whether this in turn is driven by external factors e.g. stromal adhesion, is an interesting but as yet incompletely addressed question in HSC. Figure 1-3 summarises the mechanics of stem cell self renewal division.

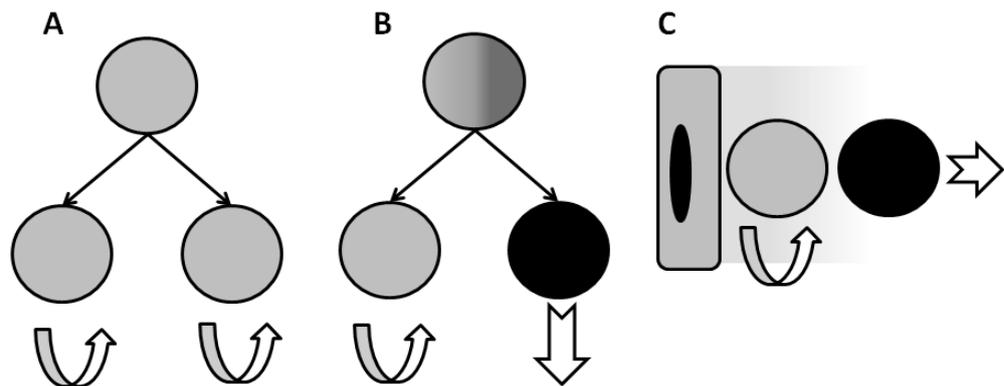


Figure 1-3: The potential fates of a dividing stem cell.

(A) represents symmetric division, where the dividing HSC (grey) gives rise to 2 daughter cells with the same level of multipotency and capacity to self-renew, resulting in expansion of the HSC pool. (B) and (C) represent asymmetric divisions, where the dividing HSC gives rise to 1 daughter cell with the same level of multipotency and capacity to self-renew and one daughter cell committed to differentiation (black). In (B) asymmetric division has arisen due to asymmetric localisation of cell determinants within the parent HSC and in (C) asymmetric division has arisen as a consequence of proximity and interaction with a third “niche” cell.

1.2.4 Control of stem cell fate

The balance between self-renewal and differentiation must be highly regulated to maintain a stable HSC pool and allow expansion for regenerative purposes. This is achieved by a combination of external signals originating from the microenvironmental supporting cells and stroma e.g. direct cell-cell contact and developmental signalling pathways such as wingless (Wnt), Notch and Hedgehog (Hh), prevailing chemical conditions such as oxygen tension, in addition to internal cues related to epigenetic modifications and chromatin priming.

1.2.4.1 Cell intrinsic control of self-renewal

Accumulating information regarding the processes contributing to self-renewal within stem cells in general and HSC in particular has led to improvements

in our understanding of these mechanisms. As would be expected, many are critical in embryonic development of haemopoiesis and have been highlighted in genetic manipulation studies or in studies designed to analyse the effect of leukaemic translocations. While not comprehensive, the next section presents a few important examples.

1.2.4.1.1 HOX genes

The family of homeobox (HOX) genes are a highly conserved and closely related set of genes encoding transcription factors with a fundamental role in embryonic development and patterning (Spitz and Duboule, 2008). They exist in four clusters (A,B, C, and D), each comprising several different individual members with additional members dispersed throughout the genome and have diverse, overlapping roles in the development and maintenance of homeostasis in different tissues including haemopoietic tissue (Abramovich and Humphries, 2005). Several members of this family are highly enriched in primitive HSC and are down regulated with maturity (Pineault et al., 2002; Sauvageau et al., 1994). Functions have been defined in various murine and human knockout (KO) and over-expression models (Argiropoulos and Humphries, 2007). While several HOX members appear to have a role in haemopoietic development and differentiation, others have a role in the maintenance of HSC (Argiropoulos and Humphries, 2007). HOXB4 has been shown to be critical in the maintenance and expansion of HSC (Antonchuk et al., 2001; Antonchuk et al., 2002; Sauvageau et al., 2004; Sauvageau et al., 1994). Over expression of HOXB4 results in significant expansion of functional murine and human HSC without HSC exhaustion (Antonchuk et al., 2001; Antonchuk et al., 2002; Sauvageau et al., 1995). It has been proposed that HOXB4 drives HSC towards symmetrical self-renewal division. Deletion of HOXB4 does not have as definitive a phenotype. Development of the haemopoietic system occurs in HOXB4 KO mice and the resultant HSC exhibit only mild impairment of function (Bijl et al., 2006; Brun et al., 2004). Similarly, HOXA9 is also highly expressed at the HSC level (Sauvageau et al., 1994). Deletion of HOXA9 leads to a significant reduction in HSC function while over-expression enhances function but additionally leads to leukaemogenesis (Bijl et al., 2006; Lawrence et al., 2005; Thorsteinsdottir et al., 2002).

Expression of HOX genes is regulated by the trithorax group of proteins including mixed-lineage leukaemia (MLL) and the caudal type homeobox (CDX)

homeobox genes (Lengerke et al., 2007; Schuettengruber et al., 2007). Expression of MLL is critical for ordered haemopoiesis and deficiency leads to profound haemopoietic developmental abnormalities during embryogenesis and an inability to maintain the HSC population in adults (Ernst et al., 2004; Jude et al., 2007; McMahon et al., 2007). In contrast, while the CDX system is critical in development of the haemopoietic system and CDX4 is expressed preferentially in primitive haemopoietic precursors, conditional deletion of CDX4 in adult haemopoiesis does not lead to marked haemopoietic deficiency, suggesting either a lesser role or greater degree of redundancy in CDX function in adult haemopoiesis (Bansal et al., 2006; Koo et al., 2010).

Downstream co-factors of HOX proteins include Meis homeobox 1 (MEIS1) and pre-B-cell leukaemia homeobox 1 (PBX1) which interact directly with the HOX proteins to modify their activity (Abramovich and Humphries, 2005; Shanmugam et al., 1999). Expression of these entities maintains or increases self-renewal in murine haemopoietic cells (Ficara et al., 2008; Unnisa et al., 2012; Wang et al., 2006). Furthermore, deletion studies of these co-factors resulted in profound abnormalities in the developing haemopoietic system and the self-renewal capacity of early primitive haemopoietic cells (Abramovich and Humphries, 2005), suggesting a non-redundant role in modulating the output of the complex HOX transcriptional system.

1.2.4.1.2 Polycomb genes and epigenetic regulation

Epigenetic control of gene transcription, i.e. the modification of the tertiary structure of DNA through biochemical modification of the chromatin structure, e.g. acetylation, ubiquitination and methylation of histone proteins, allows silencing or activation of transcription of large numbers of genes simultaneously (Cedar and Bergman, 2011). This epigenetic control has been recognised to be critically important in developmental processes and in the control of stem cell fate (Cedar and Bergman, 2011). Polycomb protein complexes are important epigenetic regulators and are large multi-protein complexes that function as transcriptional repressors and serve to establish patterns of gene repression (Denell and Frederick, 1983). In *Drosophila*, one of their roles is regulating embryonic patterning through activation and repression of *hox* transcription factors along the length of the embryo, thus specifying body shape (Lewis, 1978). In mammals there are two main polycomb complexes, PRC1 and PRC2 each with differing

functions (Lund and van Lohuizen, 2004; Majewski et al., 2010; Sauvageau and Sauvageau, 2010). The PRC1 complex is heterogeneous and comprises combinations including the following - chromobox homolog proteins (CBX) 2/4/8, BMI1 polycomb ring finger oncogene (BMI1), polycomb group ring finger 2 (PGRF2/MEL18), polyhomeotic homolog (PHC) 1/2/3 and ring finger proteins (RING) 1A/1B; depending on the cellular context (Morey and Helin, 2010). The PRC2 complex comprises enhancer of zeste homolog (EZH) 1/2, embryonic ectoderm development protein (EED), suppressor of zeste 12 homolog (SUZ12) and phd finger protein (PHF) 1 (Morey and Helin, 2010). The silencing effect of the PRC2 complex is due to EZH2 mediated tri-methylation of histones (H3K27) (Morey and Helin, 2010). This also allows recruitment of PRC1 which can have additional transcriptional repression activities. PRC complexes are known to play an important role in the maintenance of embryonic stem (ES) cells (Lee et al., 2006), but evidence is also accumulating for an important role in the maintenance of self-renewal in various somatic stem cells, including HSC, where they are largely involved in suppression of genetic programmes that would result in proliferation, apoptosis or senescence and differentiation. (Iwama et al., 2004; Lessard and Sauvageau, 2003; Morey and Helin, 2010; Park et al., 2003; Radulovic et al., 2013; Rizo et al., 2009). In the context of HSC, PRC1 components have a repressive effect on the expression of the *Ink4a* locus, mitigating expression of the CDKIs $p16^{\text{Ink4a}}$ and $p19^{\text{Arf}}$ (Park et al., 2003). It has been postulated that this activity allows HSC to enter cell cycle when required, as unrestrained expression of these CDKI resulted in cell cycle arrest, senescence or apoptosis (Park et al., 2003). BMI1 is enriched in HSC compared with progenitor cells (Lessard et al., 1998). It is clear that expression of the PRC1 component BMI1 is required for maintenance of HSC function (Park et al., 2003). HSC from BMI1 deficient mice have severely impaired self-renewal activity, as do human CD34^+ cells following repression of BMI1 (Rizo et al., 2009). Additionally, over expression of BMI1 is associated with increased symmetrical division and self-renewal potential (Iwama et al., 2004; Lessard and Sauvageau, 2003; Rizo et al., 2009). Another component of PRC1, PGRF2, appears to be expressed at higher levels in progenitors and is associated with differentiation in this context (Kajiume et al., 2004). Additionally PGRF2 deletion has been shown to accentuate self-renewal capacity in HSC through promotion of HOXB4 expression (Kajiume et al., 2004). Components of the PRC2 complex also have demonstrable roles in

haemopoiesis. Over-expression of the PRC2 component, EZH2 confers enhanced long term repopulating capacity in HSC, preventing their exhaustion in serial transplantation (Kamminga et al., 2006). On the other hand, deletion studies did not confirm a non-redundant role in this process as EZH2^{-/-} mice have normal HSC activity (O'Carroll et al., 2001). It has recently been proposed that EZH1 may compensate for EZH2 loss in EZH2 deficient adult HSC (Mochizuki-Kashio et al., 2011). A model for polycomb function in HSC has thus been proposed suggesting that PRC1 and PRC2 may have differing and opposing roles in haemopoiesis and activate discrete transcriptional programmes (Majewski et al., 2010).

1.2.5 External regulation of stem cell fate

In the preceding section we discussed selected cell intrinsic processes that serve to control self-renewal activity. However if HSC self-renewal is responsive to haemopoietic stress then these processes must be influenced by external factors. It has long been clear that this is the case. Self-renewal is intrinsically linked to the environment and to extracellular signalling, particularly to signalling along embryonic developmental pathways. In this section I provide an overview of key elements of these processes and the environment in which they occur.

1.2.5.1 The stem cell niche

The BM is a specialised environment with a profound influence on haemopoietic development. It is a protected, profoundly vascular and intensely cellular space. Cortical bone forms the hard mechanical shell of the BM, the interior comprises a meshwork of bony trabeculae, cellular spaces, fat and stromal tissue. The inner surfaces of the bone cavity and the bony trabeculae within are lined with endosteal cells and tissue including osteoblasts. The substance of the BM is highly vascular, being penetrated by a web of blood vessels and vascular sinusoids providing nutrients, draining venous blood and allowing passage of myelinated and unmyelinated nerves.

It has been clear for many years that the BM structure is integral to the haemopoietic process. The environment is not homogenous, and the distribution of the structures within the BM create specialised microenvironments, where there is local enrichment for specific cell types that have critical roles in the support of haemopoiesis (Calvi et al., 2003; Kiel et al., 2007; Zhang et al., 2003).

Trentin and colleagues first demonstrated that the differentiation fate of haemopoietic progenitors could be influenced by different tissue environments (Trentin, 1971). The concept of specialised areas within the BM in which HSC reside was first formalised by Schofield in 1978 as a potential explanation for the variability found in the CFU-S population (Schofield, 1978). He proposed that CFU-S cells only exhibited HSC behaviour when resident in a stem cell niche; a specific microenvironment that provided the necessary interactions that would prevent the HSC from differentiating but allow division when dislodged from this environment. The concept of a stem cell niche was subsequently supported by investigations in other model systems, e.g. the *Drosophila* ovary and testes where specialised local environments or cell interactions were required to allow stem cell maintenance (Xie and Spradling, 2000). The modern interpretation of a stem cell niche is of a geographical and functional nexus that integrates signalling that influences HSC behaviour. Some of the most important factors are shown in Table 1-3.

Entity	Effect on HSC population	References
Oxygen tension	Hypoxia maintains HSC quiescence	(Jing et al., 2012) (Suda et al., 2011)
Ca ²⁺	Loss of Ca ²⁺ receptor reduces BM HSC content	(Adams et al., 2006)
N-cadherin	Expressed on both HSCs and osteoblasts, postulated to be required for direct adhesion and signalling	(Zhang et al., 2003) (Calvi et al., 2003)
PTH	Endocrine source, increases notch signalling through jagged, expands HSC population	(Calvi et al., 2003)
BMP / BMPR1A	KD of <i>BMPR1A</i> in osteoblasts led to increased numbers of osteoblasts and HSC, BMP ligands have dose-dependent effects on HSC.	(Zhang et al., 2003) (Bhatia et al., 1999)
TGFβ	Maintains HSC quiescence, produced by BM Schwann cells	(Yamazaki et al., 2011)
Ang1 / Tie2	Maintains quiescence, upregulates N-cadherin on HSC	(Arai et al., 2004)
CXCR4 / CXCL12	Critical in adhesion / migration, produced by variety of BM cells e.g. osteoblasts, CXCR12 abundant reticular (CAR) cells, Nestin ⁺ mesenchymal stem cells (MSC). Abrogation leads to disordered migration and reduced HSC numbers	(Sugiyama et al., 2006) (Semerad et al., 2005)
TPO	Paracrine and endocrine delivery, deficiency leads to contraction of HSC population	(Yoshihara et al., 2007) (Qian et al., 2007)
Osteopontin	Produced by osteoblasts and HSC, maintains HSC quiescence	(Nilsson et al., 2005)
Hh	variable effects	(Bhardwaj et al., 2001) (Trowbridge et al., 2006) (Gao et al., 2009) (Hofmann et al., 2009)
Jagged 1 (JAG1) / Notch	variable effects	(Calvi et al., 2003) (Duncan et al., 2005) (Mancini et al., 2005) (Stier et al., 2002)
Wnt	variable effects	(Nemeth and Bodine, 2007) (Oh, 2010)

Table 1-3: List of selected niche factors and their influence on stem cell fate.

The nature of the stem cell niche and its constituent parts has been subject to intense investigation over the past 30 years. Broadly speaking, two anatomical sites have been proposed; the endosteal area in close proximity to osteoblasts - termed the 'osteoblastic niche' and the perivascular areas - termed the 'perivascular niche' (Calvi et al., 2003; Kiel et al., 2007; Zhang et al., 2003). The endosteal areas are found along the surface of trabecular and cancellous bone and are rich in osteoblasts and osteoclasts, Ca²⁺ and may be relatively hypoxic. The perivascular areas are found surrounding the vasculature and sinusoids that penetrate the BM and are in close proximity to vascular endothelial cells, MSC and perivascular cells.

Evidence for an endosteal niche extends from studies demonstrating enrichment of primitive quiescent haemopoietic cells in endosteal areas (Nilsson et al., 2001). Calvi *et al.* and Zhang *et al.* both proposed the osteoblasts as the key cell supporting haemopoiesis (Calvi et al., 2003; Zhang et al., 2003). Both groups induced excess osteoblast production in murine models and were able to demonstrate that this excess was accompanied by an excess of functional HSC in the BM through direct interaction with N-cadherin (Zhang et al., 2003) and indirect interaction through JAG1 (Calvi et al., 2003), a Notch specific ligand. This led them to postulate an endosteal niche where HSC were maintained in a state of relative quiescence, in physical contact with osteoblasts via N-cadherin expression (Zhang et al., 2003). Certainly the osteoblast has several features that make it an attractive candidate niche cell. It has been demonstrated that HSC localise to the endosteal areas in close proximity with osteoblasts. Osteoblasts express many of the signalling molecules that have subsequently been determined to be important in influencing HSC fate, e.g. JAG1 (Calvi et al., 2003), N-cadherin; (Zhang et al., 2003), ANG1 (Arai et al., 2004), TPO (Yoshihara et al., 2007) and CXCL12 (Semerad et al., 2005) and are capable of supporting haemopoiesis in *in vitro* culture (Taichman and Emerson, 1994).

On the other hand, the studies suggesting endosteal localisation have been criticised as 5'-bromo-2-deoxyuridine (BrDU) or carboxyfluorescein succinimidyl ester (CFSE) labelling (used to identify quiescent cells) may be insufficiently sensitive or specific (Kiel et al., 2007). Although osteoblasts produce factors that are critical in defining stem cell fate, they are not the exclusive source of these agents and the role of close adhesion between HSC and osteoblasts has recently been questioned (Kiel and Morrison, 2008). Furthermore, reduction in osteoblast number or function has not necessarily resulted in parallel reduction in HSC quantity (Visnjic et al., 2004; Wilson and Trumpp, 2006). However, osteoblasts themselves are part of a hierarchically organised structure of maturing cells involved in bone metabolism with osteoblastic progenitors giving rise to osteoblasts and ultimately osteocytes embedded within trabecular bone. The capacity to support haemopoiesis may be limited to specific subgroups within this lineage (Raaijmakers et al., 2010). This concept is supported by recent work by Raaijmakers *et al.* who demonstrated that deletion of *dicer-1*, a critical component of miRNA and RNA processing, in osteoblastic precursors but not mature osteoblasts, resulted in disruption of normal haemopoiesis that was associated

with the microenvironment and not the haemopoietic cell lineages (Raaijmakers et al., 2010).

Evidence for a perivascular niche extends from studies demonstrating that the majority of the most primitive murine HSC (SLAM) are to be located surrounding the vascular sinusoids of the BM (Kiel et al., 2007). These perivascular areas contain multiple different cells that contribute to HSC fate. The proposal of a perivascular niche for HSC is perhaps not surprising as endothelial cells and the haemopoietic system are closely related and develop in parallel in embryogenesis (Cumano and Godin, 2007; Huber et al., 2004; Medvinsky and Dzierzak, 1996). One question that has yet to be resolved, however, is whether the seeming enrichment of primitive HSC in the perivascular areas is due to stem cell trafficking through the vasculature rather than representing a stable population (Kiel et al., 2005; Wright et al., 2001).

Recent evidence has suggested that the majority of HSC are closely associated with mesenchymal cells such as Nestin⁺ MSC or CXCR12 abundant reticular (CAR) cells (Mendez-Ferrer et al., 2010; Omatsu et al., 2010; Sugiyama et al., 2006). Both cell types contribute to the osteoprogenitor lineage and express HSC maintenance-related molecules. These cells are found in both perivascular and endosteal regions and represent a potential rationalisation of the above distinct niche model. Furthermore autonomic nerve, particularly sympathetic nerve, signalling input has a role in HSC maintenance (Katayama et al., 2006). Nestin⁺ MSCs interact with sympathetic nerves and translate signals to HSC allowing HSC egress and trafficking (Mendez-Ferrer et al., 2008; Mendez-Ferrer et al., 2010).

1.2.6 External signalling through developmental pathways

Another level of external regulation that has drawn increasing interest are the embryonic regulators of stem cell fate that are critical to the developing embryo, through establishment of initial patterning and organogenesis. These ligand dependent signalling pathways e.g. Notch, Wnt and Hh are fundamentally required for normal embryogenesis and are critical to the developing haemopoietic system. Evidence has accumulated since their discovery that they may also play a significant role in the adult organism, particularly during stress or regeneration, as signalling through these pathways can stimulate expansion of the stem cell population. It is also increasingly appreciated that they have a role in

oncogenesis. Here the Hh signalling pathway and its role in the haemopoietic system is reviewed in detail followed by a brief discussion of Wnt and Notch signalling.

1.2.6.1 The Hh pathway and normal haemopoiesis - an example of stem cell fate regulation through developmental signalling

The Hh signalling pathway was initially discovered by Nusslein-Wolhard and Weischaus in 1980 through a genetic screen for factors influencing *Drosophila* embryonic patterning (Nusslein-Volhard and Wieschaus, 1980). Characterisation of the *hh* gene revealed that it encodes a secreted protein expressed in a repeated pattern across the *Drosophila* embryo. Absence of this hh protein prevented normal segmentation and gave the *Drosophila* embryo a characteristic “prickly” appearance reminiscent of a curled hedgehog. As studies progressed, it became clear that Hh signalling is a conserved process between species and is critically important in vertebrate embryogenesis where it is required for development of internal organ, midline and neurological structures, limb patterning and development of the haemopoietic system. It has been well established that absence of functional Hh signalling causes dysmorphisms such as holoprosencephaly (cyclopia), limb abnormalities and improper biological system and organ development (Chiang et al., 1996; Roessler et al., 1996).

Hedgehog signalling exerts its biological effect through controlling the behaviour of stem cells within the target tissue. Hedgehog concentration gradients are generated and, in concert with or in opposition to other similar signalling pathways, exert a topological and time-dependent influence on the apoptosis, proliferation and differentiation programmes of local stem and progenitor cells. Thus populations of tissue-specific stem and progenitor cells can be driven to expand or contract and be guided towards a particular specialised function (Blank et al., 2008; Cridland et al., 2009; Dyer et al., 2001; Roessler et al., 1996).

Following its critical role in embryogenesis, Hh signalling remains active in a more limited extent throughout the life of the organism, where it plays a key role in the maintenance and expansion of somatic stem cell populations through modulation of self-renewal activity. It is important in response to injury, tissue stress, healing and regeneration (Beachy et al., 2004; Ingham and Placzek, 2006; Ruiz i Altaba et al., 2007; Trowbridge et al., 2006).

1.2.6.2 The mechanism of Hh signalling

Hedgehog is a ligand-dependent signalling pathway. Secretory cells produce three possible isoforms of the Hh ligand; Sonic (SHH), Desert (DHH) or Indian (IHH). These are initially 45kDa precursor proteins which are cleaved and subjected to cholesterol and palmitoyl modification to produce an active N-terminal fragment of 19kDa (Beachy et al., 1997; Lee et al., 1994). These proteins are similar in structure and may be partially redundant in function; however, they also have tissue-specific roles in the developing organism and in the adult stem cell microenvironment (Pathi et al., 2001). Sonic HH influences development of many tissues in the embryo, IHH is produced in haemopoietic tissue and bone and has a role in early haematological development and DHH, is expressed in the testes and nervous system where deficiency leads to male sterility and peripheral nerve defects (Bitgood et al., 1996; Chiang et al., 1996; Dyer et al., 2001).

Hedgehog signalling requires strict spatial limitation and tight modulation. Numerous proteins are involved in receiving and transmitting the Hh signal to the nucleus; positive feedback loops accentuate and maintain the signal and negative feedback loops switch off the pathway following signal activation. The central components of the pathway comprise a pair of 12-span transmembrane receptor proteins - patched (PTCH) 1 and 2; a 7-span transmembrane protein - smoothed (SMO) and the family of glioma (GLI) zinc finger transcription factors; GLI1, GLI2, and GLI3 (see Figure 1-4). Unusually, the Hh receptor PTCH is a negative regulator of pathway activity whereas the immediately downstream element SMO is the major positive regulator. In the resting state, PTCH represses SMO activity, but following Hh binding this repression is removed (Robbins and Hebrok, 2007). The mechanism of signal transduction between PTCH and SMO in vertebrates is not known but the two receptors do not appear to interact directly. Patched proteins bear a striking homology to a trans-membrane lipid transporter protein (Neimann Pick C1 protein [NPC1]) and SMO appears to belong to the G-protein coupled receptor family (Ayers and Therond, 2010; Ioannou, 2000). Current evidence suggests that PTCH influences SMO activity through oxysterol trafficking. Oxysterols activate Hh signalling through SMO, whereas secretion of pro-vitamin D3 is inhibitory (Bijlsma et al., 2006; Corcoran and Scott, 2006). Following receptor ligand interaction, PTCH is internalised and SMO accumulates on the plasma membrane and interacts with the GLI family of transcription factors

(Deneff et al., 2000). While GLI3 is predominantly a transcriptional repressor, GLI2 exists in both a full length active form and a truncated repressor form (Hui and Angers, 2011; Sasaki et al., 1999). Activated SMO alters the balance between these forms (Hui and Angers, 2011; Ruiz i Altaba et al., 2007). In many vertebrate cell types this signalling occurs within the primary cilium (Corbit, 2005; Rohatgi et al., 2007). SMO and GLI proteins accumulate there through interaction with the intra-flagellar transport (IFT) proteins facilitating activation and onward transduction of the Hh signal (Corbit, 2005; Hui and Angers, 2011; Kim et al., 2009). However this is dispensable in haemopoietic cells as they appear not to possess primary cilia although, interestingly, they do express IFT proteins (Finetti et al., 2009).

When Hh signalling is “off” GLI2 and GLI3 are retained in the cytoplasm by a protein complex including the inhibitory molecule suppressor of fused (SUFU) and are phosphorylated by non-specific kinase activity mediated by glycogen synthase kinase 3 β (GSK3 β), casein kinase (CKI) and protein kinase A (PKA) (Humke et al., 2010; Kise et al., 2009; Pan et al., 2009; Tempe et al., 2006). Following phosphorylation, GLI2 and GLI3 undergo C-terminal ubiquitination and partial proteasome-mediated proteolysis to the truncated inhibitory form prior to nuclear translocation, resulting in repression of the pathway (Tempe et al., 2006). In the presence of Hh ligand, GLI2 and GLI3 phosphorylation is prevented and full length active forms of the transcription factors translocate to the nucleus (Pan et al., 2009; Sasaki et al., 1999). This activates the transcription of downstream targets that include both positive (*GLI1*) and negative (*PTCH1/2*) regulatory elements which drive the Hh transcription programme and down regulate ongoing signalling, respectively (Hui and Angers, 2011). Figure 1-4 summarises the mechanism of Hh signalling.

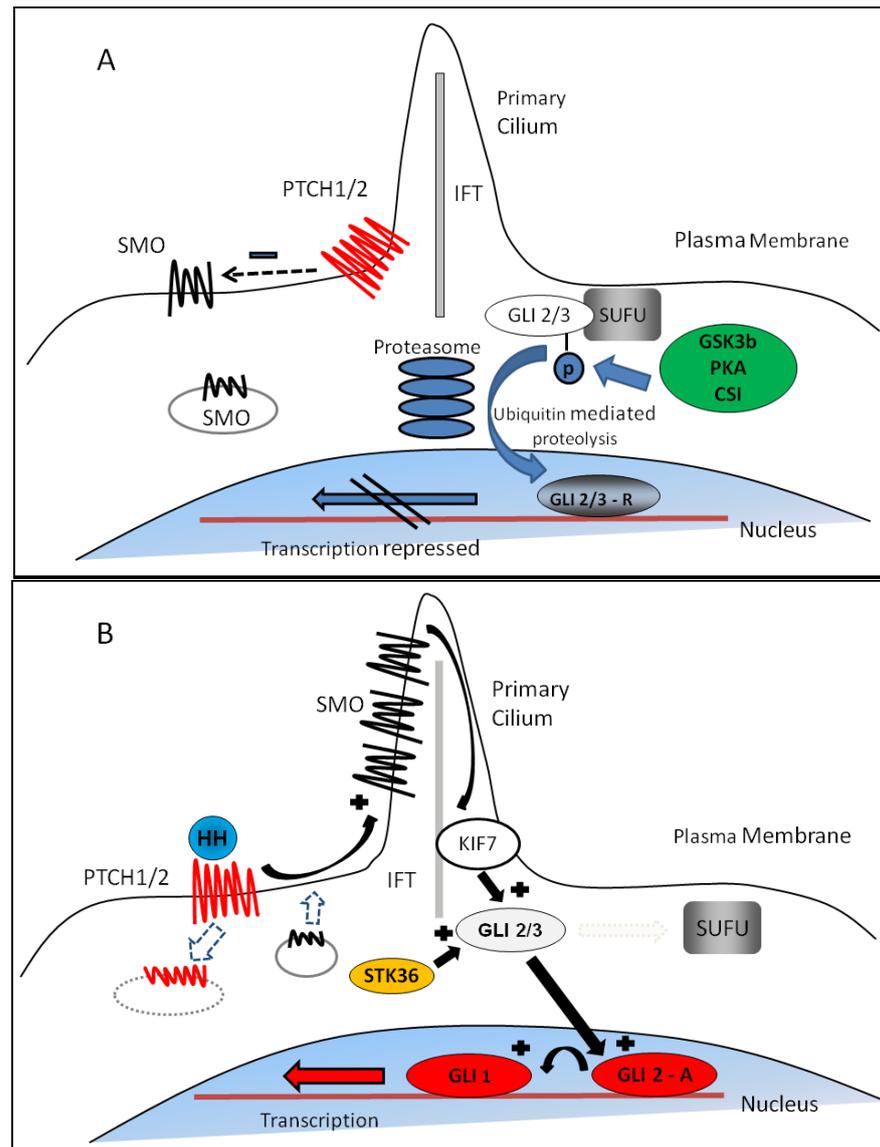


Figure 1-4: The mechanism of Hh signalling

In the resting state (a), PTCH 1/2 is expressed on the plasma membrane and acts to repress SMO activity by preventing its expression and localisation to the primary cilium. GLI2/3 transcription factors are within a complex including SUFU - an inhibitor of Hh signalling. This conformation promotes nonspecific phosphorylation of the C terminus by GSK3 β , CSI and PKA resulting in E3 ubiquitin ligase activity and subsequent partial proteasomal proteolysis to the C terminal truncated repressor form. Following translocation to the nucleus the repressive forms of GLI2 (GLI2-R) and particularly GLI3 (GLI-3R) potently inhibit the Hh transcriptional programme. Interaction of Hh ligand with PTCH promotes PTCH internalisation and degradation, and releases the repression of SMO causing its accumulation within the primary cilium. Active SMO in the primary cilium stabilises the full length forms of GLI2 (GLI2-A) and GLI3 (GLI3-A) and accentuates the effect of other positive regulators of Hh signalling, including serine threonine kinase 36 (STK36) and kinesin family member 7 (KIF7) which may be involved in translocation of GLI into the primary cilium. Following translocation to the nucleus GLI2-A potently activates transcription of downstream Hh targets including *GLI1* and *PTCH1*, and influences chromatin conformation, apoptosis, cell cycle activity and differentiation. Figure reproduced from Irvine and Copland (Irvine and Copland, 2012).

More broadly, Hh signalling manifests change through regulation of target gene expression. Several targets of Hh signalling have been identified in various tissues and can be broadly categorised into those regulating cell cycle and proliferation, survival, self-renewal and cell fate determination, adhesion and epithelial-to-mesenchymal transition (EMT) - see Table 1-4.

Functional role	Gene	Reference
Proliferation	<i>N-MYC</i>	(Kenney et al., 2003)
	<i>CCND1 & 2</i>	(Yoon et al., 2002), (Trowbridge et al., 2006)
	<i>CCNB1</i>	(Barnes et al., 2001)
	<i>FOXM1</i>	(Teh et al., 2002)
	<i>p53</i>	(Yoon et al., 2009)
	<i>IGFBP6</i>	(Yoon et al., 2002)
	<i>CDC25B</i>	(Katoh and Katoh, 2009)
Stem cell associated	<i>BMI1</i>	(Liu et al., 2006)
	<i>JAG2</i>	(Katoh and Katoh, 2009)
	<i>BMP4</i>	(Bhardwaj et al., 2001)
	<i>SOX2</i>	(Takanaga et al., 2009)
	<i>NANOG</i>	(Po et al., 2010)
	<i>Osteopontin</i>	(Yoon et al., 2002)
	<i>Nkx2.2</i>	(Vokes et al., 2007)
Adhesion	<i>CXCR4</i>	(Yoon et al., 2009)
Survival	<i>BCL2</i>	(Regl et al., 2004)
EMT	<i>FOXC2</i>	(Katoh and Katoh, 2009)
	<i>TWIST2</i>	(Isohata et al., 2009)
	<i>SIP1</i>	(Isohata et al., 2009)
	<i>ZEB1/2</i>	(Katoh and Katoh, 2009)

Table 1-4: Selected additional targets of Hh signalling and GLI activation from disparate gene expression studies

It should be noted that the majority of these studies were performed in neurological tissue and response to Hh signalling is likely to vary in a tissue dependent manner. However these data do provide insight into the potential mechanisms through which Hh signalling might regulate stem cell fate in haemopoietic tissue.

Lastly, there is recent provocative work that identifies mechanisms of non-canonical signalling in Hh-responsive cells. PTCH may interact directly with cell proliferation and apoptosis machinery independently of SMO and, in the absence of Hh signalling, can act to restrain proliferation and promote apoptosis through CCNB1 and caspase activity, respectively (Robbins et al., 2012). In a second model of non-canonical Hh signalling, SMO may act via Rho GTPases, independently of GLI transcription factors, to regulate the cytoskeleton and intracellular Ca²⁺ concentration in some tissues (Robbins et al., 2012).

1.2.6.3 *Hedgehog signalling and haemopoiesis*

Hedgehog signalling has multiple complex roles in both adult and embryonic haemopoiesis depending on the stage of development and whether the haemopoietic system is under regenerative pressure. Indian HH expressed by endodermal cells specifies haemopoietic differentiation at the earliest stages of primitive haemopoiesis and has roles in haemopoietic and vascular development (Cridland et al., 2009; Dyer et al., 2001). However 50% of *lhh* null mice survive, suggesting that *lhh* functions are partially redundant (St-Jacques et al., 1999). Definitive haemopoiesis is the second wave of haemopoietic activity, characterised by the development of HSC. This process is initiated in the aorta-gonad-mesonephros region but relocates to the foetal liver and subsequently the BM (Cumano and Godin, 2007). Studies in zebrafish and mice indicate that Hh signalling has a role in establishing definitive haemopoiesis (Cridland et al., 2009; Gering and Patient, 2005; Peeters et al., 2009). Furthermore, Hh ligand has been shown to expand definitive haemopoietic progenitors in mice and humans (Bhardwaj et al., 2001; Cridland et al., 2009; Kobune, 2004).

Early studies of adult haemopoiesis suggested that Hh signalling caused expansion and increased functional activity of the HSC compartment (Bhardwaj et al., 2001; Trowbridge et al., 2006). More recently, there has been controversy over the role, if any, that Hh signalling plays in adult haemopoiesis due to the differing results of murine studies dissecting the role of Hh mediators in adult haemopoiesis.

Dierks *et al* and Trowbridge *et al* both demonstrated that *Ptch1* heterozygosity (hence Hh activation) was associated with increased stem cell number, faster haematologic recovery post challenge with 5-fluorouracil and increased transplantability in murine models (Dierks et al., 2008; Trowbridge et al., 2006). Additionally, Trowbridge and co-workers found that LT-HSCs were ultimately exhausted through constant Hh activation (Trowbridge et al., 2006). In contrast to this; Dierks *et al* found no diminution of LT-HSC function (Dierks et al., 2008). Siggins *et al* examined adult mice subject to conditional deletion of *Ptch1* in various tissues including primitive haemopoietic cells, demonstrating that conditional deletion of *Ptch1* increased circulating primitive haemopoietic cells but did not result in enhanced engraftment in transplantation experiments or exhaustion of long term re-populating capacity (Siggins et al., 2009). Furthermore, their experiments suggested that *Ptch1* deletion only altered proliferation and

mobilisation of HSC when occurring within the supporting BM stroma as, when specific deletion limited to HSC was performed, these differences disappeared. This suggests that the effects are through signalling extrinsic to the HSC and not a direct affect of Hh on the HSC themselves (Siggins et al., 2009).

These discrepancies may be partly explained by the alternative experimental approaches employed. Dierks *et al*/used foetal liver HSC from *Ptch1*^{+/-} embryos, whereas Trowbridge *et al*/studied adult *Ptch1*^{+/-} HSC (Dierks et al., 2008; Trowbridge et al., 2006). In these studies, *Ptch1* deletion was present during embryogenesis. In the germ-line mutant, *Ptch1* heterozygosity would be a feature of all cells and tissues rather than being confined to the haemopoietic system. In contrast, Siggins *et al*/employed conditional and targeted approaches utilising the *Mx-Cre* system. They induced deletion of *Ptch1* in haemopoietic tissue of mature mice and also used the stem cell leukaemia (SCL) enhancer to target *Ptch1* deletion to adult murine HSC (Siggins et al., 2009).

Because absence of *Smo* is lethal, analysis of *Smo* null mutants is not possible. Four groups have investigated the effect of *Smo* deletion, either through recovery of *Smo* null HSC from foetal liver or through two different conditional deletion methodologies (Dierks et al., 2008; Gao et al., 2009; Hofmann et al., 2009; Zhao et al., 2009). Dierks *et al*/found no significant difference in the behaviour of foetal HSC recovered from *Smo* null mice compared to normal mice (Dierks et al., 2008). Gao *et al*/and Hoffman *et al*/both used the *Mx1-Cre* system to create a murine model where *Smo* deletion could be induced. Neither found any significant difference in the short or long term measures of haemopoiesis following deletion of *Smo* and therefore abrogation of Hh signalling (Gao et al., 2009; Hofmann et al., 2009). In contrast, Zhao *et al*/demonstrated profound reduction in stem cell activity as indicated by secondary transplant capacity following *Vav*-driven *Cre-Lox* mediated conditional *Smo* deletion (Zhao et al., 2009).

While on the surface these conflicts appear irreconcilable, the alternative experimental strategies go some way to explaining these differences. The *Mx1-Cre* system allows deletion in adulthood and predominantly affects haemopoietic and liver cells whereas the *Vav-Cre-Lox* system is active throughout embryogenesis and is less selective, affecting both haemopoietic and endothelial tissue, raising the possibility that Hh signalling was disrupted in a wider range of tissues and at

an earlier time point (Dierks et al., 2008; Gao et al., 2009; Hofmann et al., 2009; Zhao et al., 2009).

Genetic studies have also been performed on the main downstream mediators of Hh signalling. Merchant *et al.* characterised the effect of *Gli1* deletion on haemopoiesis in a *Gli1* null murine model (Merchant et al., 2010). No abnormalities in gross haemopoiesis were found, however there was a reduction in proliferation within the stem and myeloid progenitor compartments as well as reduction in myeloid differentiation and delayed recovery following 5-fluorouracil exposure.

Hedgehog acts in concert with other morphogenic signals to influence development of T and B lymphocytes. Thymic stroma and T cells both express Hh pathway mediators (Andaloussi et al., 2006; Outram et al., 2009). Early studies suggested that Hh signalling negatively regulated T cell development (Outram et al., 2000), however subsequent work indicates that Hh signalling has a positive regulatory role in early T cell development (Andaloussi et al., 2006; Outram et al., 2009; Shah et al., 2004; Siggins et al., 2009; Uhmman et al., 2007; Uhmman et al., 2011). Both IHH and SHH affect T cell proliferation and maturation (Outram et al., 2009; Shah et al., 2004). Conditional deletion of *Smo* at different levels of T cell development, performed by Andaloussi *et al.*, suggested that abrogation of *Smo* expression mainly affects primitive thymocytes, leading to thymic atrophy and profound defects in early T cell survival, proliferation and maturation (El Andaloussi et al., 2006). Uhmman *et al.* studied a *Ptch1* conditional deletion murine model and demonstrated that KO of *Ptch1* led to profound loss of mature T and B cells (Uhmman et al., 2007; Uhmman et al., 2011). Siggins *et al.* demonstrated that *Ptch1* deletion led to significant apoptosis in pre-B cells, blocked T cell specification in BM T cells and caused apoptosis of CD4⁺CD8⁺ T cells due to deletion of *Ptch1* in the supporting microenvironment (Siggins et al., 2009; Uhmman et al., 2011).

Overall it appears that Hh signalling has effects on the haemopoietic system dependent on developmental stage and cell lineage. These effects are due to a complex interplay between the cells and their microenvironment at certain developmental stages rather than being purely haemopoietic cell intrinsic mechanisms. Thus, while Hh signalling may still have a role in normal adult haemopoiesis, from the data above, it is likely to be dispensable, at least in steady state conditions.

1.2.6.4 Other key developmental pathways

Other key ligand dependent embryonic development pathways with ongoing roles in adult haemopoiesis include Notch and Wnt signalling. Like Hh signalling their existence and developmental importance were also established in lower species particularly *Drosophila* (Mohr, 1919; Rijsewijk et al., 1987).

1.2.6.4.1 Notch signalling

The importance of this pathway was established in *Drosophila*. Notch signalling has been shown to act through lateral inhibition. Notch signalling in one cell influences the fate of an adjacent similar cell e.g. maintaining stem cell-like activity in primitive progenitor cells or, as we have already seen, through asymmetrical localisation of cell fate determinants prior to mitosis (Bray, 2006). Notch proteins are highly conserved cell surface receptors. In humans there are 4 Notch receptors (NOTCH 1-4) and 5 ligands Delta-like (DLL1,3,4) and Jagged (JAG1,2) which interact with varying specificity (Pajcini et al., 2011). Ligand binding to the extracellular portion of the receptor leads to cleavage of the intracellular portion of the transmembrane receptor through the action of ADAM10 and γ -secretase enzymes (Bray, 2006). The intracellular portion of the Notch receptor (NICD) can then transit to the nucleus where it forms an activation complex with core binding factor 1 (CBF1) which complexes with various other factors, including mastermind-like (MAML), stimulating transcription of its downstream targets (Baron, 2003). Notch signalling is critical in embryonic haemopoiesis. Deletion of the gene for the ligand *Jag1* and receptor *Notch1* indicate that these components of the Notch pathway are crucial, whereas others, e.g. *Jag2* and *Notch 2-4*, appear not to be imperative in this context (Kumano et al., 2003; Pajcini et al., 2011; Robert-Moreno et al., 2008). In adult haemopoiesis the role of Notch signalling remains controversial. Notch1 is expressed in normal HSC but also in mature progenitor populations and in mature effector cells of lymphoid and myeloid lineages. There is a requirement for Notch signalling in maturing T cells, but Notch may also influence B cell development (Duncan et al., 2005; Milner and Bigas, 1999). Some studies have indicated that Notch signalling can expand human and murine HSC populations; however others have challenged this notion. Soluble JAG1 appeared to expand CD34⁺38⁻ cells and immobilised DLL1 ligand (in combination with traditional supportive cytokines) caused significant expansion of transplantable HSC from human cord blood in *in*

in vitro culture systems (Delaney et al., 2010; Karanu et al., 2000). In contrast, murine models with conditional deletions of various components of Notch signalling e.g. Notch1 and Jag1 failed to demonstrate significant effects on the HSC population (Mancini et al., 2005; Radtke et al., 1999).

1.2.6.4.2 Wnt Signalling

WNTs are secreted glycoproteins that initiate signalling through their receptors, a family of 7-span transmembrane proteins termed frizzled (FZD) that exhibit some homology to the Hh membrane activator SMO (Malhotra and Kincade, 2009). Wnt signalling was first defined in *Drosophila* where it serves as a segment polarity gene required for correct embryonic patterning (Malhotra and Kincade, 2009; Rijsewijk et al., 1987). In mammals, Wnt signalling is complex, comprising multiple ligands, receptors and accessory molecules acting along several different and potentially opposing intracellular signalling cascades (Macdonald et al., 2007; Staal and Luis, 2010). Furthermore, Wnt signalling is dose, time and cell context specific rendering the study of Wnt signalling in haemopoiesis particularly challenging (Luis et al., 2012). Nevertheless considerable effort has been applied to try to unravel its effect in the development and maintenance of the haemopoietic system (Luis et al., 2012).

Canonical Wnt signals are transduced when a WNT molecule interacts with an appropriate FZD receptor (Malhotra and Kincade, 2009). This interaction prevents degradation of cytoplasmic β -catenin and allows its transit to the nucleus where it interacts with members of the TCF/LEF transcription factor family (Malhotra and Kincade, 2009). In combination with other nuclear accessory factors, this allows the transcription of target genes. When Wnt signalling is not present cytoplasmic β -catenin interacts with a negative regulatory complex comprised of GSK3 β , AXIN and adenomatous polyposis coli (APC) (Malhotra and Kincade, 2009). This complex targets cytoplasmic β -catenin for rapid destruction preventing nuclear translocation (Luis et al., 2012). Components of Wnt signalling are expressed in primitive and various lineages of differentiated haemopoietic tissue, suggesting a role in haemopoietic development (Malhotra and Kincade, 2009; Reya and Clevers, 2005; Staal et al., 2008). Indeed Wnt signalling is essential for T cell development and proliferation in the thymus (Malhotra and Kincade, 2009; Staal et al., 2008). The effect of Wnt signalling in HSC remains controversial as *in vitro* culture experiments and gain- or loss-of-function studies of

key elements of the canonical Wnt pathway have yielded differing effects on HSC number and function. Willert *et al* and Reya *et al* indicated that murine normal or *Bcl2* transgenic HSC populations either exposed *in vitro* to purified WNT3A and SCF, or transfected with a constitutively active form of β -catenin underwent significant expansion while retaining self-renewal capacity (Reya et al., 2003; Willert et al., 2003). Likewise, HSC from *Wnt3a*^{-/-} murine foetal liver reconstituted the haemopoietic system in primary transplant but had severely impaired secondary transplant capacity, suggesting impaired self-renewal potential (Luis et al., 2009). In contrast, other murine models expressing high levels of β -catenin ultimately resulted in HSC exhaustion and block of erythroid differentiation through reduction in expression of *HoxB4*, *Cdkn1a*, *Bmi1* and *PU.1* mediated Gata1 down regulation (Kirstetter et al., 2006). Additionally, conditional deletion of both β -catenin and its close homologue γ -catenin in adult HSC using the *Mx-Cre* system had no apparent effect on stem cell number or function in one study but another using the *Vav-Cre* conditional deletion system to delete β -catenin in embryonic haemopoiesis found significant impairment of self-renewal in HSC (Zhao et al., 2007). In some respects these results mirror the inconsistencies found in the similar experiments interrogating Hh signalling (Gao et al., 2009; Hofmann et al., 2009; Merchant et al., 2010).

1.2.6.5 Integration of self-renewal signalling

As the above discussion illustrates, internal and external mechanisms that serve to control HSC function are complex and comprise a plethora of inputs that act in a stage, cell and environment specific manner. Although discussed individually, *in vivo* such control is mediated by a complex network of complementary signalling and cross talk across various pathways that are integrated at the HSC level resulting in cell fate outcome. There is evidence that these pathways interact at multiple levels influencing the level of signalling from these and other pathways culminating in a finely modulated activation/repression of broad downstream transcriptional programmes influencing HSC fate. For example, GSK3 β activity influences Notch, Wnt and Hh signalling (Espinosa et al., 2003; Kise et al., 2009; Staal et al., 2008; Tempe et al., 2006). Wnt activity influences Hh activity and vice versa (Mullor et al., 2001). Wnt signalling can influence the stability of *GLI1* mRNA allowing accumulation of GLI1 whereas downstream targets of Hh include Wnt ligands (Maeda et al., 2006). Downstream

targets of Notch include components of the Hh and Wnt pathways (Li et al., 2012b). Hedgehog signalling can influence BMP signalling and both Wnt and BMP act cooperatively to affect CDX/HOX activity in embryonic haemopoiesis (Bhardwaj et al., 2001; Lengerke et al., 2008). Figure 1-5 presents a map based on selected key regulatory molecules in these pathways and their potential interactions.

Additionally, the level of signalling from each pathway plays an important role, indeed it has been proposed that HSC response to these ligands may vary depending on the concentration to which they are exposed in a manner analogous to the effect of gradient of diffusible morphogens in the developing embryo (Luis et al., 2012; Ruiz i Altaba et al., 2007). Thus factors influencing level of ligand secretion and distance from the secreting cell are likely to be important. The picture that emerges is one of converging signals with differing effects dependent on the composition and strength of each input and this reinforces the importance of the specific cellular relationships within the niche.

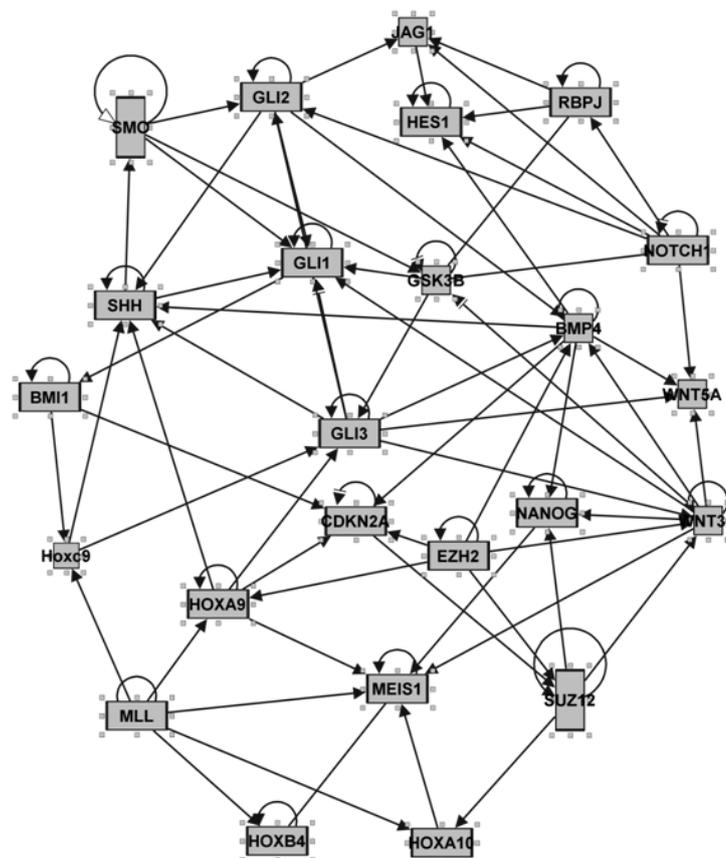


Figure 1-5: Interactions between selected cell fate determinants

Interaction map generated from Ingenuity Pathway Analysis® highlighting the complex network of interactions that exist between selected stem cell fate determinants.

1.2.7 Self-renewal and malignancy

Given the central role of self-renewal control in maintenance of haemopoiesis it is not surprising that aberrant expression of components of these pathways is associated with haematological malignancy.

1.2.7.1 Aberrancy in the intracellular control of self-renewal

Aberrant expression of the *HOX* gene family and associated factors has long been associated with leukaemogenesis (Abramovich et al., 2005; Argiropoulos and Humphries, 2007; Imamura et al., 2002). These genes are frequently involved in leukaemic translocations seen in patients with myeloid and lymphoid leukaemias and are frequently associated with a poor prognosis (Abramovich et al., 2005; Argiropoulos and Humphries, 2007; Drabkin et al., 2002; Golub et al., 1999; Imamura et al., 2002). Furthermore a causative role has been suggested by murine over-expression models indicating that excess expression of various several HOX genes (e.g. *HoxA9*, *HoxA10* or *HoxB3*) results in a leukaemic phenotype (Argiropoulos and Humphries, 2007; Thorsteinsdottir et al., 2002). Perhaps the classical example of a leukaemogenic HOX translocation is the translocation of the N-terminal portion of nucleoporin 98kDa (NUP98) to the C-terminus of several HOX partners; the resultant fusion protein confers excess HOX-related gene expression and activity with resultant leukaemic transformation (Gough et al., 2011). Expression of *Nup98-HoxA9* or *HoxD13* is sufficient to cause leukaemia in murine models and is capable of conferring extensive self-renewal capacity to myeloid progenitors (Gough et al., 2011; Pineault et al., 2005).

Aberrancy in factors controlling or influencing HOX gene expression is also involved in leukaemia. The CDX family of transcription factors influence HOX gene expression in normal haemopoiesis, *CDX4* is preferentially expressed in primitive HSC compared with more mature progenitors (Bansal et al., 2006). Additionally, *CDX4* is expressed in almost 25% of human acute myeloid leukaemia (AML) and *CDX2*, while not normally expressed in haemopoiesis, was aberrantly expressed in 90% of human AML samples in one study (Scholl et al., 2007). In murine models, transfection of *Cdx2* or *Cdx4* resulted in deregulated expression of various HOX family members, increased serial replating capacity *in vitro* and induced leukaemia in murine models (Bansal et al., 2006; Scholl et al., 2007).

Deregulated MLL expression is a common feature of both AML and acute lymphoid leukaemia (ALL) (Bernt and Armstrong, 2011). It is a promiscuous

partner in leukaemogenesis. Typically the N-terminal region of MLL associates with the C-terminal region of one of over 70 different translocation partners, forming a novel oncogenic transgene which affects the transcription of a wide array of downstream gene targets (Marschalek, 2011). Unsurprisingly, given the role of MLL in HOX regulation, deregulation of MLL expression often results in deregulation of HOX gene (e.g. HOXA9) and co-factor (e.g. MEIS1) expression (Fine et al., 2004). Whether HOX expression is necessary in MLL driven leukaemia may depend on the specific translocation partner. In one murine study investigating the role of *MLL-ENL*; *HoxA9* was required, and indeed forced expression of *HoxA9* and *Meis1* could substitute for the presence of *MLL-ENL* (Zeisig et al., 2004). However in another model investigating the role of another translocation, *MLL-AF9*, *HoxA9* expression was not required (Kumar et al., 2004). Furthermore analysis of MLL leukaemias indicate that while some MLL translocations drive HOX expression and confer enhanced self-renewal, others appear not to affect, at least *in vitro*, measures of self-renewal (Marschalek, 2011). It has been observed in murine and human studies that leukaemias induced by MLL translocations have a longer latency in mice and that these translocations may be present for a long time prior to manifestation of leukaemia in humans therefore suggesting that cooperating leukaemic mutations are required (Bernt and Armstrong, 2011).

Similarly polycomb proteins have a significant role in malignancy. Aberrant expression is seen in a variety of contexts including haemopoietic neoplasia (Martin-Perez et al., 2010; Radulovic et al., 2013; Sauvageau and Sauvageau, 2010). Over-expression of BMI1 is seen in myeloid disease (e.g. myelodysplastic syndrome [MDS], AML and chronic myeloid leukaemia [CML]), and in CML correlates with disease progression (Mohty et al., 2007; Radulovic et al., 2013). Additionally, other members of the PRC1 e.g. RING1A/1B are deregulated in MDS/AML (Radulovic et al., 2013). The core PRC2 complex members are also aberrantly expressed in several haematological malignancies. For example, *EZH2*, *SUZ12* and *EED* deletion are found in various haemopoietic neoplasms and *EZH2* over-expression is seen in both Hodgkin (HL) and non-Hodgkin lymphoma (NHL) (Martin-Perez et al., 2010; Raaphorst et al., 2000; van Kemenade et al., 2001). Thus aberrant activities of both PRC1 and PRC2 components have potential roles in malignancy (Rajasekhar and Begemann, 2007; Sparmann and van Lohuizen, 2006). While the aberrant expression of polycomb genes is not thought to be

sufficient to cause malignancy as a sole agent, they are seen as critical co-factors in transformation, repressing expression of key genetic programmes that facilitate tumourigenesis and a common polycomb repressive signature has been proposed where aberrant polycomb activity prevents the activation of the normal differentiation pathways or confers/augments stem cell properties (Martin-Perez et al., 2010). It has also been proposed that a critical level of polycomb activity is required in HSC, with too little leading to a tendency to differentiate or senesce and too much leading to unrestrained expansion or the conferral of stem cell-like properties and the development of malignancy (Sauvageau and Sauvageau, 2010).

1.2.8 Developmental signalling in malignancy

Just as there are emerging roles for the key developmental signalling pathways Notch, Wnt and Hh in self-renewal, it is becoming increasingly clear that signalling through these pathways is intrinsically involved in the pathogenesis of multiple human cancers including various haemopoietic malignancies.

1.2.8.1 *Hedgehog signalling -a paradigm for developmental signalling in malignancy*

Abnormal Hh signalling is associated with diverse human malignancies (Teglund and Toftgard, 2010). Its oncogenic properties were first identified through the realisation that the genetic abnormality causing Gorlin's syndrome (associated with an excess risk of rhabdomyosarcoma, medulloblastoma and basal cell carcinoma [BCC]) is an inherited inactivating *PTCH* mutation (Gorlin, 2004). Sporadic cases of the same malignancies are frequently associated with either inactivating mutations of *PTCH* or activating mutations of *SMO*, indicating that *PTCH* and *SMO* act as tumour suppressor and oncogene, respectively (Teglund and Toftgard, 2010). Further work confirmed that GLI transcription factors were responsible for driving tumour formation (Grachtchouk et al., 2000).

Recent evidence suggests that Hh signalling may have distinct mechanisms of action in different tumour environments (Ruiz i Altaba, 2008). Indeed, Hh related mechanisms of oncogenesis can be divided into those that are due to constitutive activation of the pathway and those that retain a requirement for ligand dependent signalling. For example, in human sporadic BCC and medulloblastoma, the most common lesions in Hh signalling are gain-of-function *SMO* mutations or

loss-of-function *PTCH* mutations (Reifenberger et al., 2005; Wolter et al., 1997). Other mutations have been recognised, for example inactivating mutations in the negative regulator *SUFU* in medulloblastoma (Taylor et al., 2002).

Mutations of Hh signalling components are comparatively rare in other malignancies, however there is evidence that Hh signalling remains important as tumour cells respond to Hh ligand secreted from the surrounding microenvironment. This mechanism has been demonstrated in various tumour types e.g. lymphoma, glioma, small cell lung cancer and colon cancer (Dierks et al., 2007; Ehtesham et al., 2007; Varnat et al., 2009; Watkins et al., 2003). Furthermore, while Hh signalling is important in pancreatic cancer tumourigenesis, this is not through direct signalling to tumour cells but rather, at least in part, due to Hh ligand production by tumour cells interacting with the local stroma leading to a more permissive microenvironment (Tian et al., 2009; Yauch et al., 2008).

Thus Hh signalling appears to be associated with development and maintenance of malignancy through (i) ligand independent signalling due to cell intrinsic mutations or (ii) autocrine / paracrine signalling between tumour cells and stroma or vice versa (Ruiz i Altaba, 2008). Figure 1-6 illustrates these possibilities.

Following activation, either by acquisition of an enhancing mutation or through ligand binding, there is induction of expression of downstream mediators in the receiving cell. Hedgehog, as we have discussed, influences a wide range of downstream transcriptional targets with multiple potentially oncogenic roles e.g. cell cycle, proliferation, apoptosis, transformation and self-renewal (Kato and Kato, 2009). Dierks *et al.* found that Hh signalling from supportive stroma in the E μ -Myc model of lymphoma acted as a survival signal through Bcl2 (Dierks et al., 2007). Additionally, direct evidence from glioblastoma and breast cancer suggests that functional BMI1 is required for Hh-mediated oncogenesis, suggesting that its role, and therefore the role of polycomb repression is important in this context (Liu et al., 2006; Michael et al., 2008). The effect that Hh signalling has on microenvironmental stromal cells is not known at present; however various soluble factors including WNTs, TGF β and insulin-like GFs are produced in response to Hh signalling. Therefore, it seems possible that such signalling would induce production of these factors in the receiving stromal cells (Harris et al., 2011).

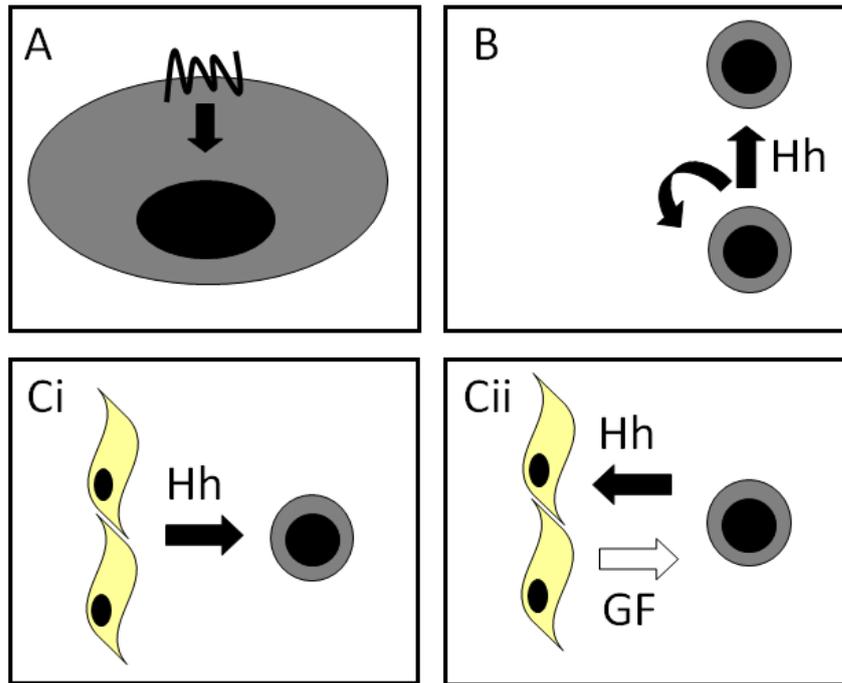


Figure 1-6: Possible mechanisms of Hh signalling in malignancy.

A: Activating mutations of positive regulators such as *SMO* or inactivating mutations of pathway inhibitors such as *PTCH*, *HHIP* or *SUFU* cause constitutive Hh signalling in the affected cell as seen in medulloblastoma or BCC (Teglund and Toftgard, 2010). B: Autocrine/paracrine signalling between cancer cells. C: Interaction with the microenvironment; in Ci malignant cells are supported by Hh ligands produced by the stromal microenvironment e.g. lymphoma and myeloma as reported by Dierks *et al* (Dierks *et al.*, 2007), in Cii the signalling is reversed; malignant cells secrete Hh ligands causing supporting cells to provide a more permissive environment through provision of additional growth or survival signals as has been described in pancreatic cancer (Tian *et al.*, 2009; Yauch *et al.*, 2008).

1.2.8.2 Notch and Wnt signalling in oncogenesis

Notch signalling has been associated with diverse solid malignancies and plays a key role in the pathogenesis of haematologic malignancies such as T-ALL, chronic lymphocytic leukaemia and various lymphomas. The relevance of Notch signalling in the pathogenesis of T-ALL is particularly well characterised and is due to mutation or chromosomal translocation resulting in constitutive activation of NOTCH1 (South *et al.*, 2012). In other contexts Notch has been suggested to have tumour suppressor capacity, e.g. in the skin where Notch appears to retard Hh and Wnt signalling, Notch deficient mice have increased Gli2 expression and a tendency to develop BCC (Shao *et al.*, 2012), underlining the complexity and tissue-specific nature of developmental pathway signalling.

Similarly, Wnt signalling is up regulated in various malignancies and in some instances appears to be necessary for propagation of the tumour (Clevers, 2006; Lustig and Behrens, 2003; Polakis, 2012). Wnt pathway mutations are frequently seen in human malignancy. The most obvious example of this is familial adenomatous polyposis coli, an inherited condition leading to the generation of multiple intestinal polyps and ultimately colon cancer which occurs due to mutation of the key Wnt regulator APC (Morin et al., 1997). APC is also mutated in sporadic colorectal cancer. Sporadic mutations of AXIN, another key inhibitor of Wnt signalling, are seen in sporadic hepatocellular cancer and colorectal cancer (Clevers, 2006). Non mutation-related up regulation of Wnt signalling is also frequently found in cancer. This frequently occurs as a consequence of epigenetic silencing of Wnt inhibitory regulators and can be mediated by the action of PRC2 polycomb complexes (Polakis, 2012). Recent evidence also suggests a role in several haemopoietic malignancies including AML and CML (Eaves and Humphries, 2010; Luis et al., 2012; Wang et al., 2010; Zhao et al., 2007). The role of Wnt in CML will be discussed later. Wnt signalling is often active in murine models of AML and primary AML samples. In murine models, deletion of β -catenin significantly reduced development and transplantation of AML driven by *MLL-AF9* or *HoxA9* (Eaves and Humphries, 2010; Luis et al., 2012; Wang et al., 2010).

1.2.9 The concept of cancer stem cells

Malignant cells within a tumour are heterogeneous as a result of following pre-existing differentiation programmes, genetic differences that arise as a result of clonal evolution and as a result of environmental influence (Anderson et al., 2011; Ding et al., 2012; Greaves and Maley, 2012). However, a concept that has gained traction over recent years is that malignancies, like the normal tissues from which they arise, may have a hierarchical structure comprised of comparatively differentiated cells, transit amplifying cells or progenitors and more primitive cells with stem cell properties. In this model, the capacity for tumour initiation and propagation is limited to the small number of very primitive cells within the larger tumour bulk. These cancer stem cells (CSCs) exhibit similar characteristics to normal stem cells including the ability to undergo symmetric and asymmetric self-renewal divisions, thereby maintaining or expanding their number in addition to giving rise to more differentiated progeny (Clarke et al., 2006; Jordan et al., 2006; Reya et al., 2001). If malignancies are organised in this manner, then the propagation of the tumour relies on a comparatively small population of cells with

specific and critical oncogenic activity within the tumour bulk. Therefore, traditional measures of response which are largely aimed at measuring residual tumour mass following treatment will not accurately define the effect of a treatment on the malignant stem cell population and will therefore not necessarily reflect likelihood of progression or future relapse, two key clinical measures of treatment efficacy. This notion was supported by early work observing the behaviour of teratomas (Pierce et al., 1959). However, the advent of cell sorting techniques, monoclonal antibodies and stem cell assaying techniques *in vitro* and *in vivo*, parallel to a desire to better understand the molecular and cellular basis for the limitations of treatment in many malignancies has allowed the phenotypic and functional characterisation of these populations and led to a resurgence of interest in this area.

1.2.9.1 Identification of CSC populations

The existence of a CSC population in AML was first described by Bonnet and Dick in 1997. It was demonstrated that the capacity to propagate the disease resided in the most primitive CD34⁺CD38⁻ cell populations in the AML samples that were analysed. Since then, similar approaches have been used to isolate putative CSC populations from other haematological malignancies e.g. CML, myeloma, lymphoma and solid tumours such glioblastoma, breast and prostate cancer (Bonnet and Dick, 1997; Brennan et al., 2010; Holyoake et al., 1999; Jones et al., 2009; O'Brien et al., 2010; Peacock et al., 2007). Evidence that these populations were rare extended from the number of cells required to recapitulate the disease in secondary transplant xenograft models. Our understanding of the biology of CSC activity has expanded over recent years and with it the CSC model has become increasingly complex. Additionally, the model has been questioned in terms of its broad applicability - is it a general model of oncogenesis or does it apply only to a few specific examples? (Magee et al., 2012). Furthermore the rarity of CSCs in some malignancies has been questioned following the implementation of more immunocompromised xenograft models. In one study, melanoma CSC were found to be far less rare than previously thought when the immunological barriers to transplantation were reduced by the utilisation of the profoundly immunocompromised murine NSG model as a recipient (Quintana et al., 2008). Likewise leukaemic CSCs (LSC) appear more frequent in syngeneic models, although the frequencies reported are variable (Kelly et al., 2007; Somerville and Cleary, 2006).

1.2.9.2 *Origin of CSCs*

While some cancers can arise from the normal stem cell compartment, through acquisition of mutations or deregulation of the self-renewal machinery, others appear to result from the reacquisition of stem cell characteristics in progenitor cells. Using AML as a model for discussion, in the original demonstration of LSCs in AML, the LSC activity was primarily found in the primitive CD34⁺38⁻ compartments, suggesting an HSC origin (Bonnet and Dick, 1997; Lapidot et al., 1994; Ishikawa et al., 2007). These findings were supported by evidence that leukaemic translocations were found in both lymphoid and myeloid lineages. However it has subsequently been demonstrated that although LSC activity is often enriched in the CD34⁺38⁻ cell population. In some AMLs there is significant LSC activity in more mature progenitor populations e.g. CD34⁺38⁺ (Eppert et al., 2011; Taussig et al., 2008). Goardon *et al.* recently demonstrated the presence of distinct subsets of AML cells with LSC activity in the CD38⁻ and CD38⁺ haemopoietic progenitor fractions corresponding to lymphoid primed multipotent progenitors (LMPP) and GMP, respectively, with the latter derived from the former (Goardon et al., 2011). These findings suggest that the reacquisition of stem cell properties in these cells may be required for AML leukaemogenesis. Forced reacquisition of stem cell characteristics has been demonstrated experimentally by the generation of LSCs from committed murine progenitors following insertion of a leukaemogenic translocation (*AML-ETO*, *MLL-AF9*, *MLL-ENL* or *MOZ-TIF2*) (Huntly et al., 2004; Krivtsov et al., 2006; Somerville and Cleary, 2006; Steffen et al., 2011; Cozzio et al., 2003). A contrasting model is found in chronic phase (CP) CML (discussed in detail in subsequent sections) where CSCs reside within the HSC population (Holyoake et al., 1999). However in myeloid blast crisis (mBC) CML, the prevalent LSC population is derived from the progenitor GMP population, suggesting that one of the drivers for progression is the reacquisition of HSC characteristics by leukaemic progenitors (Jamieson et al., 2004).

It is possible that both mechanisms could exist in parallel, with an initial LSC population within the stem cell compartment giving rise to malignant progenitor progeny of which some ultimately develop CSC characteristics. This might explain why some malignancies with long latencies e.g. CML, progress to advanced disease with a more aggressive phenotype.

1.2.9.3 Mechanisms of resistance to therapy

Stem cells have several characteristics that provide comparative protection to therapeutic intervention. As discussed in normal HSC, quiescence protects from DNA damage sustained through mitosis and will protect against standard chemotherapeutic approaches. Evidence has generally pointed to the fact that LSCs exist in a state of relative quiescence compared to their progeny (Alison et al., 2012; Guan et al., 2003; Guzman et al., 2001; Holyoake et al., 1999; Terpstra et al., 1996). More recently, this has been challenged by Somerville *et al*/who found a cycling CSC population in AML (Somerville et al., 2009). Additionally, HSC/LSC express high levels of ABC multi-drug transporters, preventing accumulation of intracellular toxins (Alison et al., 2012; Dean et al., 2005; Gottesman et al., 2002; Scharenberg et al., 2002). Furthermore, LSCs are relatively resistant to anti-apoptotic stimulæ, exhibit other cell survival behaviours such as autophagy and may be subject to genomic instability and subsequent mutation, particularly under selection pressure (Helgason et al., 2011; Sallmyr et al., 2008). Failure to effectively target these cells results in a residual LSC population and the likelihood of future relapse.

1.2.9.4 Control and function of CSCs

Factors with a role in the generation and maintenance of CSC include the developmental signalling pathways Wnt, Notch and Hh. Evidence is emerging that their oncogenic activity may be due to their effect on CSC populations. Wnt signalling is critical for LSC activity in AML and CML (Jamieson et al., 2004; Wang et al., 2010). As has been discussed, signalling along these pathways impacts on the activity of key intracellular regulators of stem cell fate. Hh signalling in CSC is an area of rapidly expanding interest. Hedgehog signalling has a demonstrable role in the maintenance of CSC characteristics in several malignancies e.g. glioma, medulloblastoma, gastrointestinal cancer and breast cancer, in addition to several haemopoietic malignancies including myeloma and CML which is discussed in detail later (Clement et al., 2007; Dierks et al., 2008; Liu et al., 2006; Peacock et al., 2007; Varnat et al., 2009; Zhao et al., 2009). In accordance with the suggestion that Hh signalling influences CSC through key downstream mediators of cell fate, the effect of Hh signalling in breast cancer is dependent on the action of BMI1 (Liu et al., 2006).

The obvious clinical extension of this theory is that, for the majority of these CSC driven cancers, cure will likely require the eradication of the CSC population. One approach is through targeting these developmental pathways to abrogate their effects on survival, proliferation and influence on self-renewal in CSCs. Targeting self-renewal is a particularly attractive notion as, if CSC self-renewal behaviour could be specifically reduced or completely abrogated, in the absence of significant effect on the un-diseased HSC population, the CSC clone would be gradually extinguished. The demonstration that CSCs are dependent on developmental signalling for acquiring / maintaining CSC behaviour suggests that inhibitors of these pathways may be excellent CSC targeting therapies.

1.2.9.5 Targeting the Hh pathway - a paradigm for therapeutic self-renewal inhibition

While the Hh signalling pathway is only one of several different pathways with overlapping roles in the complex processes that govern self-renewal behaviour, it is the lead pharmaceutical target. This is due to the fact that SMO - the main positive regulator of Hh signalling - is readily targetable, in contrast to Notch or Wnt, similar pathways lacking an identifiable and directly targetable positive regulator. Additionally the identification of the naturally occurring SMO inhibitor, cyclopamine, has provided a molecular “prototype” on which the structure of synthetic inhibitors could be based (Chen et al., 2002; Incardona et al., 1998).

Cyclopamine was identified some 50 years ago following investigation into a spate of birth defects, including cyclopia, in lambs born in sheep farms across Idaho. It transpired that pregnant ewes were grazing on the corn lily “*Veratrum Californicum*” which is a rich source of steroidal alkaloids that include cyclopamine. Cyclopamine was subsequently found to be a potent inhibitor of SMO with anti-tumour activity in *in vitro* and *in vivo* systems. (Chen et al., 2002) It exerts its effect through directly binding to the hepta-helical bundle of SMO, preventing the conformational change that would normally occur when PTCH repression was released (following Hh ligand binding) and causing the redistribution of SMO away from the plasma membrane (Incardona et al., 2002). Although cyclopamine is an effective inhibitor of Hh signalling, it is unstable, relatively non-specific and has significant toxicities and therefore has not been effectively employed clinically (Heretsch et al., 2010). However, these results provided impetus to synthesise and test derivatives of cyclopamine and screen large scale synthetic molecule libraries

for agents with anti-SMO activity. Several years on, there are now a number of clinical grade SMO inhibitors undergoing pre-clinical evaluation or early stage clinical trials in various malignancies.

An orally active agent, GDC-0449, showed significant (>50% response) clinical efficacy against Hh-driven malignancies such as advanced or metastatic BCC in a phase 1 clinical trial (Von Hoff et al., 2009). A subsequent study reported that other solid malignancies did not respond, although the participants had advanced, multiply-treated malignancies with relatively short follow-up limiting the likelihood of a positive result (LoRusso et al., 2011). In both cases, SMO inhibition was well tolerated with little toxicity (predominantly low grade fatigue, hyponatremia and dysgeusia); no maximum tolerated dose was reached. Notably, there was a lack of haematological toxicity in treated patients confirming the observations that Hh signalling is not required for normal adult haemopoiesis (LoRusso et al., 2011; Von Hoff et al., 2009).

1.2.9.6 Targeting Hh in haematological malignancy

There is great interest in utilising anti-Hh therapies in haematological malignancies. This extends from accumulating evidence suggesting that Hh signalling may be critical in the genesis, maintenance, progression and relapse of various haematological conditions through both direct and indirect mechanisms as described above. Attention has particularly focused on CML where Hh signalling has been implicated in CSC persistence despite conventional therapy.

1.3 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia is a clonal myeloproliferative disorder characterised by massive myeloid expansion, accumulation of differentiating granulocytic precursors and terminally differentiated effector cells leading to the key clinical features at presentation of marked PB granulocytosis, basophilia, splenomegaly and often thrombocytosis and anaemia (Lichtman et al., 2006).

Chronic myeloid leukaemia is a relatively rare disease, accounting for about 15% of all leukaemias. It has a world-wide incidence of about 1-2 per 100,000/year (Lichtman et al., 2006). While CML does occur in children and young adults, its incidence increases with age, peaking in the seventh decade (Lichtman et al., 2006). There is no concordance between twins or significant evidence of a familial susceptibility (Lichtman et al., 2006). The only

environmental aetiological agent that has been recognised is exposure to ionising radiation. This relates to the increased incidence of CML observed in survivors of the atomic bomb explosions that terminated World War 2 or the medicinal use of spinal radiation in sufferers of ankylosing spondylitis (Lichtman et al., 2006).

Untreated, the clinical course of CML is one of inevitable progression from a stable CP lasting about 5 years from diagnosis, where there is gradual accumulation of leukaemic myeloid progenitors, to accelerated phase (AP), characterised by accumulation and clonal evolution of increasingly primitive myeloid precursors in the blood or BM before terminating in BC with rapid accumulation of immature myeloid or lymphoid precursors resembling acute leukaemia (Lichtman et al., 2006). Transformation to BC directly from CP is also recognised.

Our current understanding of the pathogenesis of CML is informed by the important contributions of various investigators. Nowell and Hungerford first recognised the presence of the pathognomonic cytogenetic alteration, a shortened chromosome 22 - termed the Philadelphia chromosome (Ph) in CML cells (Nowell and Hungerford, 1960). Rowley demonstrated that this cytogenetic abnormality was due to a balanced translocation between chromosomes 9 and 22. De Klein *et al*, Bartram *et al* and Groeffen *et al* demonstrated that this translocation caused the juxtaposition of the Abelson (c-ABL) tyrosine kinase from chromosome 9q with the break point cluster region (BCR) on chromosome 22q (Bartram et al., 1983; de Klein et al., 1982; Groeffen et al., 1984a; Groeffen et al., 1984b). Lugo *et al* demonstrated that this event caused the expression of a constitutively active oncogenic tyrosine kinase BCR-ABL, while McLaughlin *et al* and Daley *et al* first demonstrated that expression of BCR-ABL was sufficient for leukaemic transformation *in vitro* and *in vivo* (Daley and Baltimore, 1988; Daley et al., 1990a; Lugo et al., 1990; McLaughlin et al., 1987).

1.3.1 Chronic myeloid leukaemia cell of origin

Chronic myeloid leukaemia is thought to arise following the malignant transformation of a single HSC. Several lines of evidence lead to the conclusion that CML is a clonal stem cell disease. Firstly, the presence of the Ph chromosome in multiple haemopoietic lineages suggests that it must be present in a sufficiently primitive cell to give rise to all myeloid lineages in addition to some B cells. Furthermore, molecular analysis of the precise translocation points in

different cell types from the same patient demonstrated that they were identical and consequently likely to be clonally derived from a primitive multipotent originator cell (Groffen et al., 1984b; Martin et al., 1980). Secondly, the presence of a single isoform of glucose-6-phosphate dehydrogenase (G6PD) in leukaemic cells, but not other non haemopoietic cells derived from women carrying both isoforms of G6PD (Fialkow et al., 1967; Fialkow et al., 1977). Thirdly, study of X-linked chromosomal inactivation patterns of hypoxanthine phosphoribosyltransferase (HPRT) in the leukaemic cells of heterozygous females demonstrated clonality (Yoffe et al., 1987). Whether the cell of origin may be more primitive still is an extremely interesting question. While it is clear that BCR-ABL driven haemopoiesis contributes to myelopoiesis, cells of the lymphoid lineage have also been demonstrated to descend from the LSC as patients may have a mix of BCR-ABL⁺ and BCR-ABL⁻ B cells (Nitta et al., 1985; Takahashi et al., 1998). Furthermore, while most mature T cells and NK cells are BCR-ABL⁻, BCR-ABL expressing T cell progenitors have been described suggesting that the leukaemic clone also contributes to B and T cell lymphoid haemopoiesis, but that the progeny may not expand or may undergo apoptosis (Haferlach et al., 1997; Jonas et al., 1992; Takahashi et al., 1998). Lastly, haemangioblast-like BCR-ABL⁺ cells have been reported in some studies suggesting the possibility of an even more primitive cell of origin (Fang et al., 2005; Gunsilius et al., 2000; Wu et al., 2009).

1.3.2 Molecular biology of BCR-ABL

The initiating translocation event in CML causes the juxtaposition of a variable length of the 5' end of the BCR region with exon a2 of the c-ABL gene. Most commonly, the mRNA produced from transcription of this area results in the production of a 210kDa chimeric protein, p210^{BCR-ABL} (Melo, 1996). Why this results in oncogenic transformation can be related to the normal functions of the individual components and the molecular changes that occur as a consequence of the translocation. The c-ABL gene is conserved between humans and lower species indicating its pivotal biological role (Van Etten, 1999). The c-ABL gene encodes a 140kDa protein. The N-terminal portion has homology with SRC family proteins containing 3 SRC homology (SH) domains and a myristoylated residue at the N-terminus (Van Etten, 1999). The first SH domain harbours tyrosine kinase activity (Van Etten, 1999). Other domains include those that allow direct interaction with other proteins; regions that undergo autophosphorylation and phosphorylation by other protein kinases, nuclear localisation and DNA and actin

binding (Van Etten, 1999). Under normal conditions, c-ABL is present in the cytoplasm, where it is associated with the cytoskeleton and is a substrate for various cytoplasmic kinases, and the nucleus where it has the capacity to interact with nuclear proteins, e.g. RAD51, and directly bind DNA in response to tightly regulated intracellular signalling processes (Van Etten, 1999). Under physiological conditions the tyrosine kinase function of c-ABL undergoes auto-inhibition by the myristoylated N-terminal region and conformational changes induced by interaction via SH2 and SH3 domains (Hantschel et al., 2003; Maru, 2012; Nagar et al., 2003). Overall its key roles appear to be integration of cell cycle, apoptosis and environmental signalling (Van Etten, 1999).

The BCR region encodes a ubiquitously expressed cytoplasmic 160kDa protein which acts as a serine threonine kinase via a domain at the N-terminus (Maru and Witte, 1991). Other domains include a dimerisation domain, central and C-terminal domains that allow interaction with RAS, RHO and p21^{RAC} signalling factors (Deininger et al., 2000). Additionally, phosphorylation at Y177 allows activation of the RAS signalling pathway via GRB2 (Deininger et al., 2000).

The translocation event results in constitutive production of the chimeric protein BCR-ABL. Fusion of BCR prevents normal auto-inhibition of c-ABL activity and furthermore potentiates c-ABL tyrosine kinase activity including auto-phosphorylation through oligomerisation (Maru, 2012; Quintas-Cardama and Cortes, 2009). Additionally, the cellular localisation of BCR-ABL is abnormal; BCR-ABL is retained in the cytoplasm allowing exclusive interaction with the cytoskeleton, cytoplasmic signalling pathways and preventing its interaction with nuclear proteins and DNA (Wetzler et al., 1993).

Cytoplasmic BCR-ABL deregulates a variety of critical cellular processes through interaction with cytoplasmic proteins. While it is beyond the scope of this work to discuss in detail the multiplicity of BCR-ABL interactions, its net effect is to confer a survival and proliferation advantage on cells through deregulated mitotic signalling and GF independence (e.g. via activation of RAS/RAF/MAPK, PI3K/AKT or JAK/STAT signalling), inhibition of apoptosis machinery (via BCL2, BCLXL and BAD expression) and altered cellular adhesion or stromal interaction (through reduced CXCR4 expression and cytoskeletal interactions via F-actin, focal adhesion molecules such as paxillin and β 1-integrin), (Deininger et al., 2000; Li et al., 2007b; Maru, 2012; Quintas-Cardama and Cortes, 2009). As a result there is expansion of leukaemic stem and progenitor cells.

1.3.3 Treatment of CML

The major treatment goal in CML is to reduce the leukaemic burden, normalise haemopoiesis and avert progression to advanced phase disease. Prior to the advent of specific tyrosine kinase inhibitors (TKIs) for BCR-ABL, treatment of CML relied on cytoreduction with conventional chemotherapeutics (e.g. busulphan, hydroxycarbamide and cytarabine), interferon based regimens and allogeneic transplant (Lichtman et al., 2006). Allogeneic transplantation is an effective and potentially curative therapy due to the exquisite sensitivity of CML cells to graft versus leukaemia interactions, however lack of donor availability and high levels of associated morbidity and mortality (particularly in older patients) limited its use (Gratwohl et al., 2006). For those unable to undergo transplantation, interferon-containing regimens appeared to target CML cells, induced a complete cytogenetic response (CCyR - absence of Ph⁺ cells in a minimum of 20 metaphases) in a minority of patients (13%) and had a survival advantage compared to purely cytoreductive regimens (around 70% 10 year survival in optimally responding patients) (Bonifazi et al., 2001).

The development of TKI, such as imatinib, dasatinib, and nilotinib that block BCR-ABL kinase activity has radically improved the management of CML, leading to unprecedented levels of cytogenetic and molecular responses in CP patients (Druker et al., 2006; Kantarjian et al., 2011; Kantarjian et al., 2012).

1.3.3.1 Defining response to treatment

Several studies have suggested that patient outcome in CML correlates with speed and depth of response to treatment (Druker et al., 2006; Hanfstein et al., 2012; Hochhaus et al., 2009; Hughes et al., 2010; Jabbour et al., 2011; Kantarjian et al., 2008; Quintas-Cardama et al., 2011). As treatments have become more effective, more stringent measures of leukaemic burden have been required and developed. Reaching a CCyR is clearly associated with improved prognosis in CML clinical trials (Druker et al., 2006; Hochhaus et al., 2009; Jabbour et al., 2011; Kantarjian et al., 2008; Quintas-Cardama et al., 2011). Degree of cytogenetic and molecular response is defined by the criteria set out in Table 1-5. Achieving a CCyR at 12 months is an important landmark as it correlates with progression free survival (PFS). In the IRIS trial, 97% of those with a CCyR at 12 months remained in CP compared to 81% of those with less than a partial cytogenetic response (PCyR) (Druker et al., 2006). Additionally, those that fail to achieve a CCyR at 1

year are subsequently less likely to do so (Iacobucci et al., 2006). Overall survival (OS) at 6 years correlated with the degree of cytogenetic response at 12 months: 92% for patients in CCyR, 89% for those in PCyR, and 63% for those failing to achieve at least a PCyR at 12 months (Hochhaus et al., 2009).

Likewise, early reduction in molecular evidence of disease as measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) quantification of residual BCR-ABL transcripts in PB is a favourable prognostic factor (Hanfstein et al., 2012; Hughes et al., 2010; Kantarjian et al., 2008; Marin et al., 2012; Palandri et al., 2009; Wang et al., 2003). Early reduction of BCR-ABL correlates with eventual achievement of a CCyR (Wang et al., 2003). Maintenance of a molecular response is associated with stability in cytogenetic response and a reduced risk of progression (Hughes et al., 2010; Hughes et al., 2003). Recently it has been suggested that long term CCyR, PFS and OS are predictable on the basis of initial response to imatinib with those not achieving a BCR-ABL level of less than approximately 10% within 3 months of commencing therapy less likely to achieve CCyR and having reduced PFS and OS (Marin et al., 2012). Thus modern indications of satisfactory response to therapy rely on cytogenetic and molecular assessment of disease status.

Response	Criteria	
complete haematologic response (CHR)	Normalisation of PB / resolution of splenomegaly	
minimal cytogenetic response (minimal CyR)	>65-95% Ph ⁺ in BM	
minor cytogenetic response (minor CyR)	>35-66% Ph ⁺ in BM	
partial cytogenetic response (PCyR)	>0-35% Ph ⁺ in BM	major cytogenetic response (MCyR)
complete cytogenetic response (CCyR)	0% Ph ⁺ in BM	
major molecular response (MMR ^{3log})	3 log reduction in <i>BCR-ABL</i> relative to control	
major molecular response (MMR ^{4.5log})	4.5 log reduction in <i>BCR-ABL</i> relative to control	
complete molecular response (CMR)	Undetectable <i>BCR-ABL</i> transcripts by qRT-PCR	

Table 1-5: Accepted levels of haematologic, cytogenetic and molecular response to treatment in CML.

The European Leukemia Network (ELN) recognised and formalised these factors into a set of treatment milestones which form the basis of modern CML monitoring and enable the objective assessment of response to therapy and recognition of patients who are responding sub-optimally or failing therapy (Table 1-6) (Baccarani et al., 2009).

Time from diagnosis	Response level	Response achieved
3 months	Optimal	CHR
	Sub-optimal	Less than CHR
	Failure	Stable disease or progression
6 months	Optimal	PCyR or better
	Sub-optimal	Less than PCyR
	Failure	No CHR / No CyR
12 months	Optimal	CCyR
	Sub-optimal	Less than CCyR
	Failure	Less than PCyR
18 months	Optimal	MMR
	Sub-optimal	Less than MMR
	Failure	Less than CCyR

Table 1-6: ELN criteria detailing minimum required response to TKI therapy according to time from initiation.

Loss of haematologic or cytogenetic response at any time is regarded as treatment failure as is the acquisition of a significant *BCR-ABL* kinase domain mutation. Loss of MMR and development of additional cytogenetic abnormalities in Ph⁺ cells is regarded as a suboptimal response (Baccarani et al., 2009). Patients are monitored every 3 months. In recent years with the advent of robust and standardised qRT-PCR, molecular monitoring is often substituted for repeated BM cytogenetics in responding / stable patients.

1.3.3.2 Imatinib

Imatinib was the first TKI used in the treatment of CML and changed the natural history of the disease. Its superiority was unequivocally demonstrated in a phase III trial of interferon and imatinib (IRIS; International Randomized study of Interferon and STI-571) in newly diagnosed patients with CML in CP. Ninety eight per cent of patients who received imatinib as an initial therapy achieved a CHR and 82% achieved a CCyR (Druker et al., 2006). At 60 months, estimated OS was 89% (Druker et al., 2006). Importantly, only an estimated 6% of all patients progressed to AP or BP (Druker et al., 2006).

This study has been criticised for censoring patients that discontinued imatinib (either for failing to respond adequately or due to toxicity) thereby creating a potential bias as these patients are likely to have had a poorer outcome. In another study, the probability of OS and likelihood of sustained CCyR while taking imatinib was 83% and 63%, respectively (de Lavallade et al., 2008). Other studies have indicated that the likelihood of sustaining a satisfactory response (according to ELN criteria above) to imatinib is closer to 50% in the general CML population (Lucas et al., 2008).

Thus a significant number of CP patients are not optimally benefiting from TKI therapy with imatinib, additionally imatinib responses in advanced phase disease are less impressive; a phase 2 study of imatinib demonstrated CCyR rates of 17% and 7% in AP and BC, respectively (Sawyers et al., 2002; Talpaz et al., 2002). Estimated 12-month PFS and OS rates for patients in AP were 59% and 74% (Talpaz et al., 2002). Patients in BC survived for a median of approximately 7 months (Sawyers et al., 2002).

1.3.3.3 Second generation TKIs

Potent second generation TKIs have since been developed and are in clinical use. Bosutinib, nilotinib and dasatinib have been studied in newly diagnosed patients and as second line agents following intolerance or treatment failure with imatinib.

Bosutinib is a dual SRC/ABL kinase inhibitor with increased potency against BCR-ABL and various SRC family kinases (e.g. gardiner -rasheed feline sarcoma viral oncogene homologue [Fgr], v-yes-1 Yamaguchi sarcoma viral related oncogene homolog [Lyn], sarcoma oncogene [Src] and spleen tyrosine kinase

[Syk]) (Puttini et al., 2006). Notably it has reduced inhibitory activity against c-Kit or PDGFR (Puttini et al., 2006). Bosutinib binds to the active and inactive conformations of BCR-ABL. The efficacy of bosutinib in imatinib resistant or intolerant patients has been assessed in phase 1/2 clinical trial (Cortes et al., 2011a). The majority of patients in this study did not have CHR at commencement and 78% achieved CHR with bosutinib. After a median 24 months follow up 52% had achieved a MCyR and 41% a CCyR. Of those that achieved a CCyR, 65% also achieved a MMR and 49% of imatinib resistant patients achieving MMR actually achieved a CMR (Cortes et al., 2011a). Thus those that did respond to bosutinib appeared to achieve deeper remissions.

Bosutinib has also been investigated in the primary treatment setting. The BELA trial compared bosutinib 500mg od with imatinib 400mg od. This trial demonstrated similar CCyR rates at 12 and 24 months post treatment (12 months: bosutinib 70% vs. imatinib 68%; 24 months: bosutinib 79% vs. imatinib 80%) but faster molecular responses and a higher cumulative rate of MMR (12 months: bosutinib 41% vs. imatinib 27%; 24 months: bosutinib 61% vs. imatinib 50%) (Cortes et al., 2012c; Cortes et al., 2011b).

Dasatinib is a dual SRC/ABL inhibitor with activity against BCR-ABL and a wide range of other intracellular kinases (Rix et al., 2007). Dasatinib binds to both the inactive and active states of ABL and is 325-fold more potent than imatinib *in vitro* (O'Hare et al., 2005). Efficacy of either 70mg twice daily (bd) or 100mg once daily (od) dasatinib in imatinib resistant and intolerant CP CML patients has been investigated in phase 2 clinical trials (Hochhaus et al., 2008a; Shah et al., 2008). The START C trial evaluated the efficacy of dasatinib in imatinib resistant or intolerant CP patients. MCyR was reached in 52% of the resistant cohort and 80% of the intolerant cohort. These responses were long lasting with over 97% of patients achieving MCyR maintaining this response at a median follow up of 15 months and with PFS and OS at 24 months of 80% and 94%, respectively (Hochhaus et al., 2008b). Treatment with 100mg od dasatinib appeared to be similarly effective in CP, but was less toxic (Shah et al., 2008). Equivalent trials have been performed in advanced phase CML indicating that dasatinib is effective in AP and BC CML (Kantarjian et al., 2009; Saglio et al., 2010), however, the recommended starting dose of dasatinib in advanced phase disease remains 70mg bd.

Given the clear therapeutic effect of dasatinib in the second line setting, phase 2 and subsequently phase 3 trials comparing dasatinib with imatinib in newly diagnosed CP CML patients were instigated. The phase 3 DASISION trial demonstrated that treatment with dasatinib 100mg od improved CCyR and MMR rates at 2 years compared to imatinib 400mg od (CCyR 86% vs. 82%; MMR 64% vs. 46% respectively) (Kantarjian et al., 2012). Additionally, more patients had deep molecular responses in the dasatinib arm (log 4.5 reductions in BCR-ABL transcripts 17% vs. 8%) (Kantarjian et al., 2012).

Nilotinib is a TKI with activity against BCR-ABL, KIT, PDGFR and ephrin receptor kinase (Manley et al., 2010). It is an analogue of imatinib and exerts its anti-leukaemic activity in a similar fashion, i.e. it binds to and stabilises the inactive conformation of ABL. However, because the topologic fit to ABL is improved, nilotinib has approximately 30-fold greater potency *in vitro* (Weisberg et al., 2005). A series of clinical studies investigating the clinical effectiveness of 400mg bd nilotinib in imatinib resistant or intolerant CML patients in CP, AP and BC have been performed (Giles et al., 2012; Kantarjian et al., 2007; le Coutre et al., 2008). A group of 321 CP patients intolerant or resistant to imatinib received nilotinib 200mg bd. Overall MCyR rates were 63% in the intolerant group and 56% in the resistant group. Again, these responses were durable with 84% remaining in MCyR for 18 months (Kantarjian et al., 2007). Estimated OS was reported as 91% at 18 months (Kantarjian et al., 2007). Equivalent trials have been performed in advanced phase CML indicating that nilotinib has effect in AP and BC (Giles et al., 2012; le Coutre et al., 2008).

Nilotinib has also been studied in newly diagnosed patients. The pivotal phase 3 clinical trial - ENESTnd compared two doses of nilotinib with imatinib for primary treatment of CP CML (Kantarjian et al., 2011). This trial indicated the superiority of either 300mg bd or 400mg bd nilotinib over imatinib in this setting with a significantly higher MMR rate at two years (nilotinib 300mg bd 71%, nilotinib 400mg bd 67% and imatinib 44%) (Kantarjian et al., 2011). Again, substantially more nilotinib treated patients were in deep molecular remissions (log 4.5 reduction in BCR-ABL transcripts nilotinib 300mg bd 26%, nilotinib 400mg bd 21% and imatinib 400mg od 10%) (Kantarjian et al., 2011).

Thus second generation TKI have demonstrated efficacy in both newly diagnosed and imatinib resistant or intolerant patients in all phases of CML. It is clear that these agents have the capacity to induce rapid cytogenetic and

molecular responses in newly diagnosed patients. This rapid reduction in leukaemic burden might be expected to reduce the likelihood of progression and indeed there is evidence for this in both frontline trials described above. Although the numbers of progressing patients was small, each trial reported a progression rate of approximately half that of the imatinib arm after 2 years follow up (BELA: 4 vs. 13 patients; DASISION: 6 vs. 13 patients and ENESTnd 5 vs. 12 patients) (Cortes et al., 2011b; Kantarjian et al., 2011). These agents were also able to rescue patients intolerant or poorly responsive to front line imatinib, therefore providing alternative therapeutic strategies in these patients. Bosutinib, nilotinib and particularly dasatinib are active in advanced phase CML however responses, like those seen with imatinib are less profound and more transient (Giles et al., 2012; Kantarjian et al., 2009; le Coutre et al., 2008; Saglio et al., 2010).

1.3.3.4 Resistance and persistence

Resistance to TKI and persistence of disease despite TKI therapy represent major clinical concerns. An estimated 16% of IRIS patients had primary (intrinsic) resistance to imatinib at 12 months (failure to achieve at least a PCyR) and 24% had primary resistance after 18 months (failure to achieve a CCyR) (O'Brien et al., 2003). Additionally, resistance can develop over time; in one study 16% of patients relapsed within years 1-3 because of secondary (acquired) resistance (Druker et al., 2006). The incidence of resistance and relapse is higher in patients with more advanced CML. While second generation TKIs can rescue a proportion of sub-optimally responding patients, review of the data above suggests that these responses are not universal and not necessarily robust (Giles et al., 2012; Hochhaus et al., 2008a; Kantarjian et al., 2009; Kantarjian et al., 2007; le Coutre et al., 2008; Saglio et al., 2010; Shah et al., 2008). Even in optimally responding patients treated with the most potent TKIs in clinical use about 29-36% of patients fail to achieve an MMR at 2 years (Kantarjian et al., 2011).

1.3.3.4.1 TKI resistance mechanisms

There have been strenuous efforts to elucidate the biological basis of TKI resistance. These mechanisms can be broadly divided into BCR-ABL-dependent mechanisms and BCR-ABL-independent mechanisms. While amplification of the BCR-ABL fusion gene has been associated with resistance to imatinib this is not common (Hochhaus et al., 2002). More clinically relevant are the presence of relatively TKI resistant clones. Imatinib binds to the ATP binding site of the

inactive conformation of BCR-ABL (Savage and Antman, 2002). Broadly speaking, point mutations afford resistance by blocking this interaction either directly through mutation of residues critical for TKI binding or (in the case of imatinib) indirectly by stabilising the protein in the active conformation (P-loop mutations) or preventing adoption of the inactive conformation (A-loop mutations) (Apperley, 2007). The BCR-ABL gene is affected by genomic instability resulting from its own kinase product, mutagenic ROS and by compromised DNA repair mechanisms leading to the development of point mutations that are then selected under the pressure of TKI therapy (Grant et al., 2010; Koptyra et al., 2006; Shah et al., 2002). A host of mutations are now described, with different *in vitro* sensitivities to TKI - ranging from mild insensitivity to virtually complete resistance in the case of the T315I mutation (Apperley, 2007; Shah et al., 2002). While some mutations may pre-exist treatment initiation and not all necessarily impact on prognosis, development of a clinically significant mutation will presage relapse in responding patients and will require alteration of therapy (Branford et al., 2003; Jabbour et al., 2006; Khorashad et al., 2008; Soverini et al., 2011). Fortunately the TKIs in current clinical use have differing potencies against the various BCR-ABL kinase domain mutations described thus expansion of a clone bearing a clinically significant mutation can often be managed by instigating treatment with an alternative TKI (Soverini et al., 2011). Until recently the major exception to this was development of the T315I mutation which is completely resistant to pharmacologically achievable concentrations of imatinib, dasatinib, nilotinib and bosutinib (Puttini et al., 2006; Soverini et al., 2011). However the recent development of the novel pan-BCR-ABL inhibitor, ponatinib, has provided the opportunity to target this mutation. Ponatinib has activity against all forms of BCR-ABL in addition to various other intracellular kinases including FLT3, SRC, LYN, c-KIT, FGFR and PDGFR (O'Hare et al., 2009). Furthermore it has demonstrated activity in CML, including patients harbouring a clone with the T315I mutation and was reasonably well tolerated in a phase 1 clinical trial (Cortes et al., 2012a). Its efficacy has been further investigated in the phase 2 PACE trial looking at heavily pre-treated patients and patients with T315I. In this study 50% of CP CML patients had achieved at least a MCyR at 9 months and this included 70% of those harbouring T315I (Cortes et al., 2012b).

Non BCR-ABL-related mechanisms of resistance include relative expression of plasma membrane transporter molecules and the influence of

parallel signalling pathways e.g. SRC kinase signalling, RAS signalling or JAK2 signalling. Initial studies suggested that imatinib is actively excreted from cells via the ABC family of transporters, ABCG2 and ABCB1 (MDR-1), therefore over-activity of these protein complexes might confer resistance through reducing intracellular imatinib concentrations. ABCB1 over-expression influenced imatinib concentration in K562 (Mahon et al., 2003). ABCG2 is over-expressed in CD34⁺ cells but does not affect intracellular imatinib levels (Jordanides et al., 2006). Second generation TKIs nilotinib and dasatinib are also substrates of ABCB1 and ABCG2 (Hegedus et al., 2009). However, thus far the clinical role for TKI efflux appears to be limited (Crossman et al., 2005; Mahon et al., 2003). Other *in vitro* and clinical studies have implicated imatinib import via the human organic cation transporter 1 (hOCT1) in resistance. Low expression of this complex is associated with reduced intracellular imatinib concentration and indeed has been found to have a negative prognostic influence in some studies (Thomas et al., 2004; White et al., 2007). Notably neither dasatinib nor nilotinib intracellular levels are affected by hOCT1 expression (Hiwase et al., 2008; White et al., 2006)

Cooperative signalling via other signalling pathways might cause TKI resistance. Indeed SRC family kinases have been implicated in resistance via direct activation of BCR-ABL. BCR-ABL⁺ CML cells cultured in the presence of imatinib or obtained from patients with imatinib-resistant CML have been shown to have increased expression and activity of the SRC kinases LYN and HCK (Dai et al., 2004; Donato et al., 2003; Mahon et al., 2008).

1.3.3.4.2 Disease persistence

It is recognised that even considering optimally responding patients, the majority continue to have evidence of persisting disease despite prolonged TKI therapy (Bhatia et al., 2003; Druker et al., 2006; O'Hare et al., 2007). Additionally, the majority of patients who achieve a CMR have persisting evidence of disease when more sensitive approaches are employed and suffer molecular relapse shortly after TKI withdrawal (Chomel and Turhan, 2011; Chu et al., 2011; Mahon et al., 2010; Ross et al., 2010; Sobrinho-Simoes et al., 2010). Thus it is clear that, in the majority of responding patients at least, there is a residual population of CML cells that are resistant to TKI therapy persist despite treatment and can cause disease relapse. This residual population is thought to include the CML LSC.

1.3.4 The CML stem cell

The organisation of CP CML is considered to be akin to that of normal haemopoiesis. The cell of origin as described above is an aberrant HSC with similar qualities of self-renewal and multipotency giving rise to leukaemic progenitors and differentiated cells. Surface phenotype has been considered to be indistinguishable from the normal HSCs with enrichment possible based on surface expression of CD34, CD38 and CD90 (Eaves et al., 1998a; Eaves et al., 1998b; Petzer et al., 1996). Recent work has suggested that there might be additional leukaemia-specific surface markers e.g. IL1-RAP which has been found to segregate with BCR-ABL expression (Jaras et al., 2010). Chronic myeloid leukaemia LSC have altered cell-cell adhesion, altered cell cycle kinetics and increased ROS levels as a consequence of expression of BCR-ABL (Deininger et al., 2000; Eaves et al., 1998b; Quintas-Cardama and Cortes, 2009). Both progenitor progeny and LSC are cycling faster than their normal counterparts (Eaves et al., 1998b). Analysis of PB and BM from patients in CP CML has suggested that there is significant progenitor expansion and mobilisation. However, hierarchically more primitive cells e.g. LTC-IC are present in equivalent quantities in the BM, but in excess in the peripheral circulation due to their altered adhesive qualities (Eaves et al., 1998b; Eaves et al., 1993). Chronic myeloid leukaemia cells also exhibit GF independence and do not undergo apoptosis in the absence of exogenous GF signalling due to autocrine secretion of IL3 (Holyoake et al., 2001).

Not all the LSCs are in cycle however - a quiescent (G_0) cell fraction can be identified in PB or BM specimens from patients with CML through CFSE or by Hoechst 33342 / Pyronin Y staining, even following culture in a proliferative cytokine cocktail (Holyoake et al., 1999). This population comprises approximately 0.5% of the total $CD34^+$ compartment and is competent to replicate the disease in xenograft transplantation studies (Holyoake et al., 1999). These LSCs have demonstrable resistance to TKI-mediated apoptosis *in vitro* and leads to an accumulation of quiescent leukaemic cells (Copland et al., 2006; Graham et al., 2002; Jorgensen et al., 2007). While various possible mechanisms have been suggested to explain lack of TKI-mediated apoptosis in LSC e.g. quiescence, low intracellular levels of TKI due to drug efflux, high levels of BCR-ABL expression or cooperative signalling via other related pathways (Jiang et al., 2007b), evidence is now emerging from *in vitro* and *in vivo* models suggesting that CML LSCs may not

actually be dependent on BCR-ABL kinase signalling for survival (Corbin et al., 2011; Hamilton et al., 2012). If this is the case then TKI inhibition alone will be unlikely to result in depletion of the leukaemic clone and cure of the disease and attention will require to be focused on alternative targets.

1.3.4.1 Targeting the CML stem cell

If, unlike their progeny, CML LSCs are not dependent on BCR-ABL signalling for survival, two questions arise: what are they dependent on and how might they be targeted? Intensive effort from various laboratories to better understand the biology of the CML LSC in CP and advanced phase disease has uncovered a multitude of important survival mechanisms. Investigation of these processes with a view to finding an Achilles heel in the CML LSC has been the challenge in recent years. One intriguing concept has been the direct targeting of mechanisms regulating self-renewal activity in CML LSC (Figure 1-7).

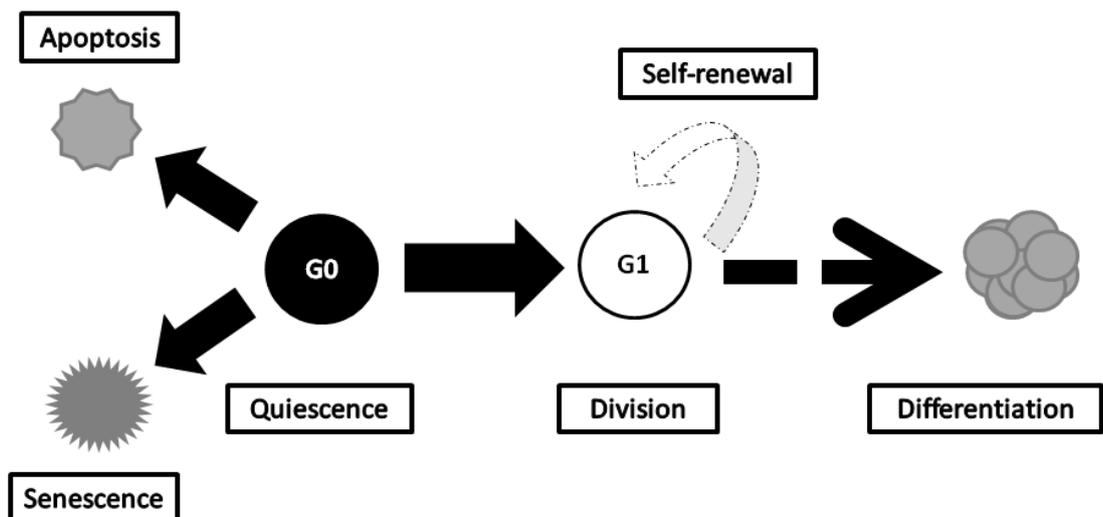


Figure 1-7: Diagram indicating possible mechanisms for targeting the CML stem cell by influencing HSC behaviour.

This diagram shows the potential fates of the quiescent LSC. Black block arrows indicate possible fates that could be accentuated, while faded arrows indicate fates that could be reduced or abrogated to deplete the LSC fraction. Thus, hypothetically, the quiescent CML HSC population could be reduced by accentuating apoptosis (either through inhibition of survival pathways or toxicity), causing senescence or driving quiescent cells into cell cycle but preventing or reducing self-renewal and/or accentuating differentiation.

1.3.4.2 Self-renewal in CML

Extending from the initial observation that expression of BCR-ABL was sufficient for oncogenesis was doubt as to whether BCR-ABL really was the sole

agent of malignant transformation (Daley et al., 1990b). If that was the case, BCR-ABL would have to confer extensive self-renewal activity. Therefore a key question is whether BCR-ABL, of itself, bestows self-renewal activity to cells lacking this quality or altered cell fate decisions at the Ph⁺ LSC level. There are several lines of evidence that strongly suggest that BCR-ABL does not confer self-renewal and that its net effect may be to reduce self-renewal activity. Firstly, the clonal nature of CP CML, the ordered haemopoietic hierarchy and the fact that BCR-ABL is expressed across most lineages argues against the possibility that self-renewal activity could be conferred by BCR-ABL alone. Secondly, while the CP CML LSC is thought to be phenotypically similar to normal HSC, it is not functionally equivalent. Chronic myeloid leukaemia LSCs can be assessed in the same *in vitro* and *in vivo* assays used to functionally define HSCs (Eaves et al., 1985b; Udomsakdi et al., 1992a). It has become apparent that CML LSCs have reduced *in vitro* measures of self-renewal and exhibit poorer engraftment and reduced transplantability *in vivo* compared to their normal counterparts or to other leukaemias (Jiang et al., 2002; Petzer et al., 1997; Schemionek et al., 2010; Udomsakdi et al., 1992b). Consistent with the previous point, conditional expression of BCR-ABL in murine models of CML resulted in reduced stem cell function in transplantation experiments (Schemionek et al., 2010). Thirdly, and most importantly, various viral transduction studies where BCR-ABL was introduced into haemopoietic tissue indicate that leukaemia occurs only in the presence of an HSC population. Thus transduction of whole BM or HSC populations may result in myeloid leukaemia but transduction of more stringently sorted populations of progenitors e.g. CMP or GMP uncontaminated with HSC will not. This is in direct contrast to other leukaemogenic translocations e.g. MOZ-TIF2 (Daley et al., 1990a; Elefanty et al., 1990; Huntly et al., 2004; Perez-Caro et al., 2009). These experimental findings are supported by the clinical observation that BCR-ABL transcripts are transiently detectable in normal adults who remain healthy (Biernaux et al., 1995; Bose et al., 1998). It can be inferred from these observations that the expression of BCR-ABL is only leukaemogenic in the correct cellular context; a cell with intrinsic or reacquired self-renewal capacity.

It has also been observed that NUP98-HOXA9 expression in primitive haemopoietic cells markedly alters the balance between symmetrical and asymmetrical divisions compared to uninfected normal controls, whereas BCR-ABL does not (Wu et al., 2007). This is significant as NUP98-HOXA9 is

associated with AML and BC CML and suggests that in NUP98-HOXA9 LSC, intrinsic self-renewal mechanisms are massively deregulated, whereas BCR-ABL⁺ LSC have a more normal phenotype keyed to maintenance rather than expansion of the stem cell compartment.

Thus the overall impression is that CP CML LSC can be considered to have intrinsically impaired self-renewal compared to normal HSC. It has been suggested that the BCR-ABL-mediated autocrine production of cytokines, with a predominant role in proliferation and differentiation and the observed increase in cycling LSC might be responsible for this phenotype (Sloma et al., 2010). Deregulation of other signalling pathways may also contribute, for instance PTEN is shown to be down regulated in CML LSC, since PTEN has a predominantly cell cycle suppressive role. This occurs through inactivation of PI3K/AKT signalling, therefore reduced expression of this molecule would be expected to result in increased proliferation (Peng et al., 2010). Likewise the FOXO family of transcription factors are inactivated by BCR-ABL signalling possibly via PI3K/AKT signalling (Naka et al., 2010a; Naka et al., 2010b). FOXO proteins are transcription factors that transit between the nucleus and the cytoplasm (Tothova et al., 2007). In the activated state they reside in the nucleus resulting in cell cycle inhibition and promotion of apoptosis signalling. Inactivation leads to their cytoplasmic relocation and degradation. FOXO3a expression has a potential role in the maintenance of the LSC phenotype, therefore down regulation of FOXO3a by BCR-ABL might represent another mechanism reducing the self-renewal potential of BCR-ABL bearing cells (Naka et al., 2010b). Many other pathways have also been implicated, for example hypoxia inducible factor 1a (HIF1a) is critical for CML LSC maintenance through its action on CDKI (e.g. p16^{ink4a}, p19^{arf} or p57) (Zhang et al., 2012b). Other pathways currently under scrutiny include TGFβ, ALOX5, BCL6 and JAK2 signalling (O'Hare et al., 2012).

Lastly, niche interactions are likely to play a significant role *in vivo*. BCR-ABL⁺ cells exhibit abnormal cell adhesion qualities and increased migration out of the BM. Expression of BCR-ABL causes reduced expression of CXCR4 and CML cells are therefore less able to respond to stromal derived factor 1 (SDF-1, CXCL12) and home to the niche where they would be exposed to this protective environment and the multitude of cell-to-cell interactions and GF/cytokine signalling that occurs therein (described in detail above) (Geay et al., 2005). This includes TGFβ, a mediator of HSC quiescence recently reported as having a

significant role in maintaining the CML LSC population via FOXO signalling (Naka et al., 2010b). It is noteworthy that TKI therapy reverses BCR-ABL effect on CXCR4 allowing redistribution to the protective niche (Jin et al., 2008).

The internal mechanisms conferring self-renewal in CP CML are likely to be those that are active in normal HSC and have been discussed in detail in previous sections. Certainly, expression of several HOX family members is evident in CML cells (Lawrence et al., 1995; Strathdee et al., 2007; Tedeschi et al., 2010; Wu and Minden, 1997). Hypermethylation of HOXA5 (associated with differentiation (Fuller et al., 1999)) was associated with advancing disease (Strathdee et al., 2007). Furthermore, Sloma *et al.* found that forced expression of a *NUP98-HOXA10* fusion gene led to increase in LSC, as measured by serial LTC-IC cultures or xenotransplantation into NSG mice, in both normal cord and CP CML cells (Sloma et al., 2013). This quality was largely restricted to the most primitive cell populations (CD34⁺38⁻ or CD34⁺/Rho⁻) (Sloma et al., 2013). Additionally BMI1, another key regulator of stem cell function is over-expressed in CML in comparison to non-malignant cells and co-expression with BCR-ABL in model systems is associated with expansion of the LSC population and enhanced self-renewal (Merkerova et al., 2007; Rizo et al., 2010; Sengupta et al., 2012a). Furthermore, BMI1 expression levels correlate with disease stage and prognosis (Bhattacharyya et al., 2009; Mohty et al., 2007). As discussed previously BMI1 is critical in normal HSC self-renewal where it represses key cell fate mediators e.g. INK4A locus CDKs, and itself is a downstream target of developmental signalling pathways such as Hh.

1.3.4.3 Embryonic developmental signalling in CML

One area of significant interest is the role of the ligand-dependent developmental pathways Wnt and Hh in the context of CML. The critical role of these pathways in directing and sustaining stem cell fate in HSC and CSC has been discussed in previous sections. While these factors have a critical role to play in the development of the haemopoietic system, their role is greatly diminished in fully developed adult haemopoiesis (Gao et al., 2009; Hofmann et al., 2009; Koch et al., 2008; Zhao et al., 2007). In contrast to normal haemopoiesis, recent evidence suggests that they continue to play an important role in CML LSC survival and propagation both in chronic and advanced phases of the disease.

This is illustrated by the work of Jamieson *et al*/who demonstrated that in BC CML, cells with LSC activity are found in the mature GMP progenitor population and that Wnt signalling through β -catenin was responsible for the reacquisition of self-renewal activity in these cells (Jamieson et al., 2004). Inhibition of β -catenin expression reduced BCR-ABL driven leukaemogenesis and transplantability in murine models underlining its potential importance in this context (Hu et al., 2008; Zhao et al., 2007). Furthermore, adhesion of CML cells to MSC in *in vitro* culture led to direct activation of β -catenin signalling via N-cadherin resulting in protection from TKI treatment (Zhang et al., 2013). As a consequence of these observations, pharmaceutical strategies aimed at inhibiting Wnt signalling are under evaluation as a potential therapeutic approach to CML (Heidel et al., 2012; Nagao et al., 2011).

Hedgehog signalling is increased in BCR-ABL⁺ stem and progenitor cells becoming more active with disease progression (Long et al., 2011; Radich et al., 2006). Two groups have explored the effect of *Smo* deletion in murine models of CML (Dierks et al., 2008; Zhao et al., 2009). Zhao *et al* used the *Vav-Cre-Lox* system to create *Smo* deficient mice from which to isolate *Smo*^{-/-} HSC whereas Dierks *et al* utilised foetal liver cells from *Smo* deficient mouse embryos. Both groups expressed BCR-ABL in these cells prior to transplant (Dierks et al., 2008; Zhao et al., 2009). Despite differing approaches, broadly similar results were obtained. *Smo* deletion reduced LSC numbers and reduced incidence of leukaemia with prolonged latency in primary transplantation and greatly reduced capacity to recrudescence disease in secondary hosts. Both groups performed complementary pharmacological inhibitor studies with cyclopamine demonstrating prolonged survival in diseased mice, reduced LSC population and lower functional activity *in vivo* and *in vitro* following cyclopamine treatment (Dierks et al., 2008; Zhao et al., 2009). Importantly, the combination of TKI therapy with cyclopamine resulted in the largest reduction in LSC *in vitro* and *in vivo* (Dierks et al., 2008). Furthermore, constitutive activation of *Smo* resulted in accentuation of leukaemogenesis (Zhao et al., 2009). Thus Hh signalling is active in CML (as opposed to normal homeostatic adult haemopoiesis) and has a clear role in the maintenance and expansion of the diseased LSC in these models of CML. Therefore Hh signalling presents a new potential therapeutic target in CML LSC. Early phase clinical evaluation of several targeted small molecule inhibitors of Hh signalling in CML is currently underway (see chapter 7, Table 7-1).

While the above work clearly demonstrates an important role for Wnt and Hh signalling in the maintenance of the LSC population in CML, it is curious that Wnt/Hh signalling is dispensable in normal HSC. It is tempting to speculate that CML LSC are more dependent on the cell fate signals delivered along these pathways by virtue of their intrinsically impaired self-renewal characteristics, perhaps mediated by BCR-ABL as discussed above. It is also striking that inhibition of either pathway independently has similar outcomes suggesting that, although there is significant similarity between both pathways, and both potentially interact at multiple levels, they are not redundant, and signals from both pathways are required to maintain the LSC phenotype (Dierks et al., 2008; Hu et al., 2008; Zhao et al., 2007; Zhao et al., 2009).

One criticism that might be levelled at these studies is that they principally define the role of Wnt and Hh signalling in the advanced phase of the disease as they largely used primary samples from BC patients and retroviral mediated BCR-ABL expression models of CML which have limitations in replicating CP disease (Koschmieder and Schemionek, 2011). As has been discussed above, there may be significant differences between BC and CP disease in terms of dominant LSC phenotype, self-renewal kinetics, requirement for Hh or Wnt signalling and response to their inhibition. Therefore, it is not clear that these results can be extrapolated to CP disease. This is significant because the majority of patients with CML are in CP and their numbers are increasing due to effective TKI therapy. Intervening with a directed stem cell therapy at an earlier stage might avert progression, limit chronic use of TKIs and through intervening at a point where disease burden is lower and accumulated additional mutations less prevalent might be expected to be more effective in eradicating the LSC population. This theme will be expanded on in subsequent chapters.

1.4 Microarray Analysis in Haematology

1.4.1 Gene expression analysis

Studying gene expression patterns involves measuring the quantity of different mRNA species in the cells or tissue of interest. Historically, this required Northern blotting / in situ hybridisation or targeted PCR (Raval, 1994). A precondition of these techniques was that the target sequence required being known and specific primers for that sequence developed and applied experimentally. A

corollary of this is that gene expression experiments were targeted at a small number of transcripts that were thought to be of interest, limiting the scope of these investigations. Few phenotypic changes result from changes in expression of a single or even a few genes. Most require sequential changes in large transcriptional networks, and to understand how complex networks of positive and negative interactions vary from cell to cell requires a more global approach. Conventional gene expression techniques are limited in this regard as they are labour intensive and therefore experiments designed to interrogate these complex interactions experiments can quickly grow beyond manageable proportions. Advances in manufacturing, chemical and biological sciences and computing and biostatistics have combined to enable the development of increasingly comprehensive and high throughput processes e.g. gene expression microarray analysis and next generation sequencing techniques (Mortazavi et al., 2008; Schena et al., 1995). Microarrays enable the simultaneous measurement of the abundance of tens to hundreds of thousands of transcripts simultaneously from a single sample and are therefore extremely powerful tools that have the ability to interrogate gene expression on a genome-wide scale. As the technology has matured, these methods have become more comprehensive, reproducible and affordable.

1.4.2 Anatomy and function of a gene expression microarray

Fundamentally a gene expression microarray provides a means of simultaneously analysing gene expression across a very large number of gene targets throughout the known genome. The basic principle that underlies this technology is hybridisation of a fluorescent or otherwise labelled target sample DNA or RNA fragment to unique and complementary probe sequence strands immobilised on a supporting structure.

The resultant signal (e.g. fluorescence output) can then be measured, related to the probe layout of the chip and the abundance of the target calculated. Microarray technology has moved forward at pace over the last 15 years and deserves some explanation (Auer et al., 2009; Bemmo et al., 2008; Dalma Weiszhausz et al., 2006; Forozaan et al., 1997; Ha et al., 2009; Lipshutz et al.; Robinson and Speed, 2007; Schena et al., 1995). The most common type of commercially available gene expression microarrays are oligonucleotide arrays such as the Affymetrix Genechip Gene ST 1.0 array. Here oligonucleotide probes

that have been designed to be complementary to a known or predicted open reading frame are synthesised directly onto a silicon chip by photolithography. Each probe sequence is similar in terms of optimal hybridisation temperature. A microarray chip is comprised of millions of “features”, themselves consisting of millions of identical unique 25mer oligomer probe sequences. Each target sequence is represented by several features scattered over the face of the chip. Additionally each chip has internal control probesets to assess sample labelling, hybridisation and background noise. The experimental samples are prepared; RNA is extracted, processed and labelled with a fluorescent probe prior to application to the array chip. Following incubation and washing, the fluorescent fragments of the original target are left hybridised to their specific probes. The chip can be visualised by laser scanning and the intensity of fluorescence at each feature can be calculated and is representative of the amount of target binding at each feature. The fluorescence at each identical feature can be summarised to provide a single overall value for fluorescence which, following normalisation and correction for background, is used to calculate the expression level of the target (Figure 1-8). As the technology has improved it has become possible to shrink down the size of the individual features increasing the density of genome coverage and thereby the sensitivity and reproducibility of the assay. Modern Affymetrix microarrays are designed to ensure that there are unique probe-set targets in every exon across the entire transcript, this reduces bias compared with older 3’ based arrays and ensures that alternate splicing variants are not missed.

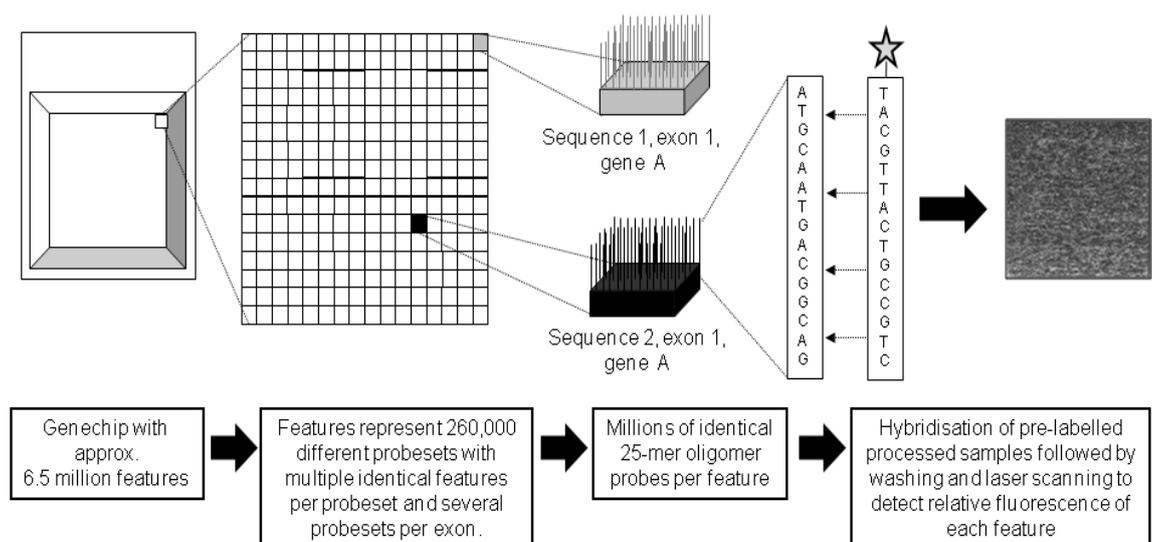


Figure 1-8: Anatomy and function of an Affymetrix Genechip

1.4.3 Data handing - the bioinformatics pipeline

High throughput, comprehensive experimental strategies like microarray analysis are extremely demanding in terms of data handling at all levels of analysis. A summary of the bioinformatics pipeline is provided in Figure 1-9. Following scanning, a raw image file is produced in which all the feature fluorescence intensities are digitally represented (DAT file). Initial quality control needs to be performed to ensure that sample preparation occurred correctly, that hybridisation occurred as expected and that the resulting images are complete with no physical flaws or obvious abnormalities. Each feature intensity value is related to its associated probe and all probe level intensities are combined (CEL file). This can be exported to a microarray analysis package for further analysis. Since comparison between different chips will be required in most experiments, the raw expression data in each chip needs to be normalised for non-specific background fluorescence and differing fluorescence intensities between chips. Additionally, the individual values for each probe set require to be summarised, providing a single value for each probe, exon target and finally each gene. There are a variety of different approaches to these requirements and biostatistics packages will perform these manipulations during import. Probably the most widely accepted method is to utilise robust multichip analysis (RMA) (Irizarry et al., 2003). This method is applied across the whole set of arrays in an experiment, correcting for background, normalising across arrays to cause the medians and distributions of each chip to be similar, performing log transformation of the probe intensity values followed by a probe-set summarisation process e.g. median polishing (Irizarry et al., 2003).

Following normalisation and summarisation, it is now possible to analyse the data for differences in gene expression. Proprietary software - for instance Partek Genomic Suite (Partek GS) have been developed to make this process less onerous. Broad representations of whole transcript differences between the different groups can be made using grouping strategies like principle components analysis (PCA) (Peterson, 2003). This enables the examination of the data as a whole between the groups of interest and allows broad conclusions to be drawn regarding the similarities or differences in the data sets. Specific comparisons can be made between different groups, (e.g. malignant / non-malignant or treated / untreated) to determine the differential expression level of any target and significance analysis performed using parametric (T-test or ANOVA) or non-

parametric (e.g. rank product analysis) tests. Given the comprehensive nature of array analyses, the treatment of multiple comparisons is particularly relevant and must be addressed in order to provide meaningful interpretation of the data. The problem briefly stated is that the more statistical comparisons that are made, the greater the likelihood that some will be found to be significant that are in fact due to random chance (false positive results). Some method of controlling for the number of comparisons is therefore required. One method is to employ Bonferonni's correction, which simply requires that to be considered significant, any member of a series of comparisons must have a p value of less than the accepted level of significance for the whole family divided by the number of members in the family (i.e. if $p < 0.05$ was considered significant for a family of 100 tests, each member would be significant with a p value of < 0.0005). This controls for the occurrence of false positives by preventing the sum of p values for the individual comparisons rising above the originally accepted significance level for the whole family. It is highly conservative and may lead to an unacceptably high false negative rate, particularly in the context of microarray and related analyses (Abdi, 2007). A different approach is to utilise a false discovery rate (FDR) correction. The FDR is the proportion of false positives that would be acceptable within the significant results returned (i.e. FDR 0.05 in 1000 significant tests implies that 50 are likely to be falsely positive). This is less conservative and in many circumstances is a more appropriate correction for hypothesis generating experiments like global gene expression analysis (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003).

By making contrasts between the experimental groups and controlling for the multiple comparisons undertaken, a list of differentially expressed genes can be produced. Such lists provide specific target expression information but can be difficult to synthesise. It may not be readily apparent how the differential expression of these genes is ultimately related to (a) each other or to (b) the phenotype of the cell. Biological understanding of the expression data requires it to be placed in a functional context (Thomas et al., 2007). Here gene annotation and ontological classification can be of assistance. Gene ontologies are consistent vocabularies that describe gene function in a cellular context across species. There are three broad classifications, cellular components, molecular function and biological process, and with each hierarchical step the classification becomes more specific. Thus gene expression data can be related to expected molecular,

biological or cellular function (www.geneontology.org). Finally the data can be analysed using known pathway interactions and relationships utilising pathway analysis tools e.g. Kyoto Encyclopedia of Genes and Genomes (KEGG) classification or Ingenuity Pathway Analysis (IPA), providing detailed information about relative gene expression of elements with consistent or antagonistic biological functions (www.genome.jp/kegg and www.ingenuity.com). In this way expression data can begin to provide a more comprehensive understanding of the transcriptional events that underpin the phenotype of the cell and a global insight into the interactions and networks that cooperate to influence these cells (see Figure 1-9 below).

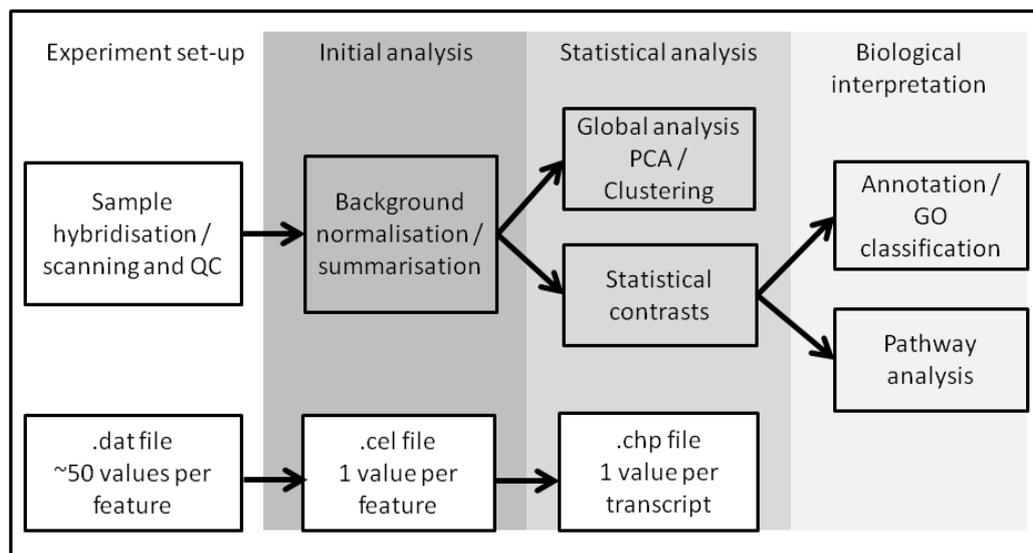


Figure 1-9: The bioinformatics pipeline indicating the process from samples hybridisation, through statistical analysis and ultimate biological interpretation.

1.4.4 Limitations and validation of microarray analysis

Microarray analysis is the first step in hypothesis generation. Gene expression activity can yield important insights into the biological activity of a cell population however no direct information regarding protein abundance or activity of any of the multitude of post transcriptional regulatory processes. Additionally the microarray platform utilised, how the data is treated and analysed statistically can have significant effects on the results obtained. This is highlighted by the lack of consistent data from similar studies in some instances (Fortunel et al., 2003). It is imperative that the findings are corroborated in some way. Corroboration might include demonstration of modulation of the relevant effector molecule e.g. at

protein level or the use of a secondary focused measure of gene expression e.g. qRT-PCR to ensure that similar results are obtained.

1.4.5 Microarrays in normal and malignant haemopoiesis

Microarray analysis of gene expression lends itself to the study normal and malignant HSC. The key advantages of this approach are that it can be employed on very limited quantities of starting material (a prerequisite in the study of very low abundance populations like relatively pure stem cell populations) and that a snapshot of the entire transcriptional activity of a cell population is provided allowing an appreciation of the prevailing biological processes that are active and the potential inter-relations and networks that are formed. Since their development, microarrays have been utilised extensively in the characterisation of normal and malignant haemopoiesis, classification of haemopoietic malignancies, analysis of factors that are associated with prognosis and progression and cellular responses to treatment (Forsberg et al., 2006; Golub et al., 1999; Nunoda et al., 2007; Rosenwald et al., 2002; Valk et al., 2004; Villuendas et al., 2006). Global microarray analysis has also been used to try and establish the central expression programmes that characterise stem cell behaviour in haemopoietic and other tissue stem cells (Fortunel et al., 2003; Ivanova et al., 2002; Ramalho-Santos et al., 2002). However, these studies did not provide concordant results (Fortunel et al., 2003). Subsequent analysis of HSC and their progeny have provided detailed information regarding the transcriptional activity associated with HSC populations and differentiation to progenitor populations (Chambers et al., 2007; Liu et al., 2007; Novershtern et al., 2011; Terskikh et al., 2003).

The apotheosis of this investigative approach is found in a recent study by Novershtern *et al.*/comparing gene expression across 211 human samples representing 38 prospectively isolated haemopoietic populations extending from HSC to mature differentiated lymphoid and myeloid cells (Novershtern et al., 2011). Transcriptional activity was divided into 80 groups or modules, many of which had lineage-specific function and proposed transcriptional networks operating to establish and sustain module expression in different cell populations (Novershtern et al., 2011). These results provide a comprehensive insight into the complex nature of transcriptional regulation occurring throughout haemopoiesis.

1.4.5.1 Gene expression studies in CML

Gene expression profiling by microarray analysis has been utilised extensively in the study of CML. The focus of these gene expression experiments has been to understand the differences between normal and CML haemopoiesis at the gene expression level, to investigate the effect of treatment and mechanisms of resistance to treatment, to understand the biology of disease progression and to try and produce clinically useful tools to predict response to treatment and prognosis. It is not necessarily easy to directly compare these studies, microarray technology advanced rapidly and therefore the platforms on which these experiments were performed was heterogeneous and the experimental design differed, likewise the cell populations used were disparate. Despite these problems there are certain broad themes that have repeatedly come to the fore. A brief discussion of the contribution of microarray study of primary CML cells and to cells derived from different stages of the disease is relevant.

1.4.5.2 Gene expression profiles associated with CML progression

An area of great interest relates to the transcriptional events that presage, accompany or drive disease progression in CML. The most comprehensive gene expression study in this area was performed by Radich *et al*, who looked at the expression profiles in CD34⁺ cells derived from 42 patients with CP CML, 17 patients with AP CML and 32 patients with BC CML (of whom 4 were in remission). Broadly speaking they found that - at a gene expression level - CML behaves like a 2 stage (chronic and advanced) rather than the accepted 3 stage model of progression (Radich et al., 2006). In comparison with CP CML, advanced CML exhibited profound up regulation in many gene groups involved in cellular metabolism, DNA replication, RNA transcription and processing, apoptosis, cell structure and adhesion and up regulation of several genes with potential oncogenic functions and down regulation of several tumour suppressors (Radich et al., 2006). They noted up regulation of preferentially expressed antigen in melanoma (*PRAME*; which has a known role in inhibition of myeloid differentiation), the oncogene Wilms tumor 1 (*WT1*), growth arrest-specific 2 (*GAS2*; involved in cell cycling and apoptosis), components of the IL3 receptor and the growth regulator suppressor of cytokine signalling 2 (*SOCS2*), whereas they noted down regulation of several heat shock proteins (*HSP*) (Radich et al., 2006). They also noted down regulation of various factors with potential roles in regulating

HSC fate in BC CML including FBJ murine osteosarcoma viral oncogene homolog (*FOS*), Cbp/p300-interacting transactivator 2 (*CITED2*) and the kruppel-like factor 4 (*KLF4*), the serine (or cysteine) proteinase inhibitors B8 and B10 and cell structure and adhesion molecules including cadherin (*CDH*) 1 and *CXCR4* (Du et al., 2012; Ishizawa et al., 2011; Kharas et al., 2007; Okada et al., 1999; Przygodzka et al., 2010; Radich et al., 2006; Sugiyama et al., 2006).

Critically, they also found that components of the developmental pathways were deregulated in advancing CML. Of particular interest *GLI2*, the Hh signalling mediator was the second most up regulated gene in their dataset and several members of the Wnt signalling pathway were differentially regulated lending credence to their putative role in advanced phase CML.

1.4.5.3 A CML gene expression signature

The earliest microarray studies in primary CML samples were in BM mononuclear cells (MNC). Nowicki *et al*/studied gene expression in 15 CML specimens (5 in CP, 5 in BC BM and 5 BC PB) compared to 3 normal BM and 4 PB specimens in a limited microarray (Nowicki et al., 2004). They found considerable heterogeneity between specimens and under half of the probesets in their array (5315/12530) gave useable signal, but nonetheless significant differences between normal and CML, according to disease stage and whether BM or PB derived (Nowicki et al., 2004). Many of the genes identified were known to be regulated by BCR-ABL. Broad categories included promotion of cell cycling, a mixed effect on cellular apoptosis apparatus, up regulation DNA repair apparatus, down regulation of adhesion mediators, up regulation of metabolic genes, differential regulation of mediators of chromosomal dynamics and reduced expression of mediators of the cellular response to pathogens (Nowicki et al., 2004).

Diaz-Blanco *et al*/and Kronenwatt *et al*/both employed CD34⁺ selection in BM samples to obtain a purer primitive cell population to investigate CP CML specific gene expression (Diaz-Blanco et al., 2007; Kronenwatt et al., 2005). Kronenwatt *et al*/compared 5 samples from patients with newly diagnosed CML with 10 normal volunteers using a limited array of 1185 probes; 158 genes were differentially regulated. Diaz *et al*/compared peripheral or BM CD34⁺ cells from 8 healthy donors with PB or BM from 9 patients with CP CML at diagnosis using a more comprehensive microarray of 8746 probes and found 1539 differentially

expressed genes (Diaz-Blanco et al., 2007). Again, in addition to up regulation of BCR-ABL signalling mediators and targets, both groups found significant up regulation of factors involved in driving the cell cycle and down regulation of several factors known to retard the cell cycle (Diaz-Blanco et al., 2007; Kronenwett et al., 2005). Both groups also demonstrated differential expression of cell adhesion molecules, reduction in expression of several factors involved in DNA damage response and detoxification, altered expression of various GFs and their receptors and differential expression of several key transcription factors (Diaz-Blanco et al., 2007; Kronenwett et al., 2005). Interestingly both groups also noted slight increases in the expression of key factors involved in the regulation of stem cell fate and self-renewal in CP CML CD34⁺ cells (Diaz-Blanco et al., 2007; Kronenwett et al., 2005). Kronenwett *et al.* also noted increased expression of neurologically related G protein signalling molecules such as neurotrophin 3 in addition to up regulation of the oncogenes *SKI* and *cMYC* but significant down regulation of *FOS*, V-jun avian sarcoma virus 17 oncogene homolog (*JUN*) and v-myb avian myeloblastosis viral oncogene homolog-like 2 (*MYBL2*) (Kronenwett et al., 2005). These authors also reported a marked disparity in the expression of key differentiation-associated genes between CP CML and normal CD34⁺ samples. This included marked up regulation of erythroid associated genes (e.g. haemoglobin, *GATA1*, kruppel-like factor 1 (*KLF1*) or the transferrin receptor), but significant down regulation of genes associated with a granulocyte/monocyte phenotype (e.g. myeloperoxidase [*MPO*], neutrophil elastase [*ELA2*] or CCAAT/enhancer binding protein δ [*C/EBP δ*] (Diaz-Blanco et al., 2007). They also noted increased expression of TGF β signalling related genes (Diaz-Blanco et al., 2007). Interestingly in both studies there was no significant difference between circulating or BM CD34⁺ CP CML cells at a transcriptional level (Diaz-Blanco et al., 2007; Kronenwett et al., 2005).

A major problem in these studies is the heterogeneity of the cell tissue under investigation. Gene expression data produced by microarray studies necessarily reflects the composition of the cell populations analysed and the expression data is essentially an average value from all cells within the population (Szaniszlo et al., 2004). While CD34⁺ selected populations were utilised in the two studies described, the CD34⁺ population is itself comprised of both stem and progenitor fractions and varied in composition between normal volunteers and CP CML patients. One would expect even greater diversity in the MNC populations

used by Nowicki *et al*, possibly reflected in the heterogeneity of their results (Nowicki et al., 2004). However the problem is illustrated particularly well by Diaz-Blanco *et al*/who found an excess of MEP and a significant reduction in HSC and GMP in CP CML samples compared to normal samples in their study. The gene expression profile thus obtained reflected these differences (Diaz-Blanco et al., 2007).

In order to further investigate the gene transcriptional profile in CP CML and avert the problems associated with sample heterogeneity, the same group performed an additional microarray experiment on stem and progenitor populations derived from the BM of 6 newly diagnosed patients with CP CML and 5 normal patients (Bruns et al., 2009). Using cell sorting, they collected HSC ($CD34^+/38^-$), CMP ($CD34^+/38^+/CD45RA^-/IL3R\alpha^lo$), GMP ($CD34^+/38^+/CD45RA^+/IL3R\alpha^lo$) and MEP ($CD34^+/38^+/CD45RA^-/IL3R\alpha^-$) populations from the BM MNC populations. They found, in terms of absolute numbers, CML and normal HSC were in approximate proportion, an excess of progenitors was seen in the CML populations with the most enriched populations being CMP and MEP. They then analysed the gene expression signature between sub-populations in normal and leukaemic samples. In this analysis the authors found many similarities with their previous study, for example, CML HSC had a more mature phenotype and was more akin to normal CMP progenitors, than normal HSC. The authors then compared normal and leukaemic progenitors. In this comparison, 362 genes were up regulated and 252 genes down regulated in CML LSC with respect to normal HSC. Again, the major functional groups highlighted were cell cycle, adhesion, transcriptional regulation and stem cell fate. Notably adhesion factors like *CDH2* and *CXCR4* were significantly down regulated in this population (Bruns et al., 2009). Additionally, there was significant down-regulation of critical transcription factors such as *C/EBP β* , nuclear receptor 4 A2 and A3 (*NR4A2 & 3*) and *KLF2 & 11* and other molecules (e.g. *SOX4*, ectopic viral integration site [*EVI*] *2B*, *CD133*, *FLT3*, *PTEN* and *CD53*) with known involvement in primitive haemopoiesis (Bruns et al., 2009).

As discussed above, while TKI therapy can rapidly reduce populations of proliferating differentiated cells, a population of primitive quiescent (non-cycling - G_0) but entirely functional leukaemic cells persists. To more rigorously focus on this population Affer *et al* and Graham *et al* performed global gene expression profiling on normal and leukaemic quiescent and cycling $CD34^+$ selected cell

populations sorted by Hoechst 33342 and Pyronin Y staining using Affymetrix oligonucleotide gene expression arrays (HG-U133A) (Affer et al., 2011; Graham et al., 2007).

Graham *et al*/compared G₀ and cycling populations from mobilised CD34⁺ cells from 3 normal volunteers and from 5 patients with CP CML (Graham et al., 2007). This work indicated that the transcriptional difference between normal quiescent and dividing cells was greater (188 genes differentially expressed at >3fold) than the equivalent comparison in CP CML (37 genes differentially expressed at >3 fold) and highlighted a list of 168 genes differentially expressed at >3 fold between each G₀ population. Not unsurprisingly, one of the key differentially expressed groups in G₀ vs dividing cells in normal and CP CML was those related to cell cycle status (Graham et al., 2007). In normal haemopoiesis this was also associated with up regulation of genes related to DNA repair and oxygen transport; this was not the case in CP CML. Additionally, G₀ cells expressed various pro-inflammatory chemokine ligands including *CXCL 1, 2, 3* and *6* (Graham et al., 2007). Specifically, looking at the differences between the dividing CML and normal populations, there were relatively few differences. However, there was increased expression of mitotic spindle and DNA structure-related genes but relative down regulation of antigen presentation, chemotaxis and chemokine activity-related genes (Graham et al., 2007). Looking at the similar comparison between quiescent cells, CP CML G₀ CD34⁺ cells had enriched expression of various categories of genes involved in DNA replication, cell cycle, mitosis and spindle regulation as well as haemoglobin and oxygen transportation genes (Graham et al., 2007).

Affer *et al*/reported significant differences in the gene expression signature of CML G₀ cells compared to normal G₀ cells, although it must be noted that their comparison was between CD34⁺ G₀ cells from 4 normal BM samples and 8 PB CML samples (5 CP and 3 AP) (Affer et al., 2011). The authors found significant differential expression of genes involved in many of the functional groupings discussed in the previous arrays; that is perturbed cell cycle regulation and mitotic apparatus, apoptosis signalling and DNA replication in addition to stem cell related factors and key differentiation associated genes (Affer et al., 2011). With respect to cell cycle, expression of genes favouring cell cycle progression and inhibition was seen in approximately equal numbers. Similarly to Bruns *et al*, the authors found that the expression of many factors associated with an HSC phenotype in

normal haemopoiesis were significantly down regulated in the CML G₀ population compared to the normal G₀ population (Affer et al., 2011; Bruns et al., 2009). These included *CD133*, *NMYC*, *FLT3*, *HOXA3*, *HOXA5*, *HOXB3*, *HOXB6* and the Notch target *HES1*. Similarly, they saw down regulation of key members of the polycomb complexes PRC1 and PRC2. In contrast, up regulation of key differentiation associated genes was seen, including *KLF1*, *GATA1* and *CD36*. Similarly to the study of Diaz-Blanco *et al*, megakaryocytic and erythroid associated genes were preferentially expressed in CML G₀ cells. They concluded that the gene expression signature of the CML G₀ populations analysed was significantly more mature than that of the normal G₀ population (Affer et al., 2011).

Thus, the congruent findings of the gene expression studies thus performed are an up regulation of mediators and ultimate genetic targets of BCR-ABL signalling, in most cases, a general activation of cell cycle, DNA replication, mitosis and proliferation machinery accompanied by reduction in expression of elements of the DNA damage response apparatus. Most confirm a reduction in cellular adhesion and adhesion molecules. Some reported intriguing and unexpected findings such as the lower expression of genes involved in antigen presentation and cellular response to infection in CML. The picture with regard to HSC-associated molecules and drivers of stem cell fate and differentiation is more complicated with some studies reporting up regulation of HSC-associated factors, while others reporting a profound down regulation of HSC factors and up regulation of differentiation associated factors in the CML LSC populations analysed, suggesting a more mature transcriptional phenotype in these populations.

1.5 Aims

It was in this context that I sought to better define the key differences between normal and leukaemic HSC at a transcriptional level. In particular, I wished to determine whether there was evidence of deregulated self-renewal signalling - particularly through the Hh pathway - in CML LSCs or their progeny compared to normal haemopoiesis. Lastly I sought to establish whether a novel, clinically relevant inhibitor of Hh signalling influenced *in vitro* measures of self-renewal in CP CML.

1.5.1 Specific objectives

- (1) To establish the global gene expression programme in pure populations of the most rigorously defined stem and progenitor populations identified by surface immunophenotype in non-leukaemic patient samples and in CML through a combination of flow sorting and gene microarray analysis using modern Affymetrix oligonucleotide gene chips.
- (2) To establish whether Hh signalling is active in the CP CML CD34⁺ cell population.
- (3) To establish whether targeted inhibition of Hh signalling in CP CML CD34⁺ cells:
 - a. Resulted in measurable transcriptional differences.
 - b. Affected *in vitro* culture through measurement of apoptosis, proliferation and cell cycle status.
 - c. Resulted in reduced *in vitro* measures of self-renewal in CP CML CD34⁺ cells.

2 Materials and Methods

2.1 Materials

2.1.1 Tissue culture

2.1.1.1 Tissue culture plastics

Plastics	Supplier
Cryotubes	Fisher Scientific, Loughborough, UK
Falcon tubes (15, 50mL)	Fred Baker Scientific, Cheshire, UK
FACS tubes (sterile/unsterile)	BD Biosciences, Oxford, UK
Media filtration system	Scientific Lab supplies, Coatbridge, UK
Syringes (5, 10, 20, 50 mL)	Invitrogen, Paisley, UK
Syringe filters (22 micron)	Invitrogen, Paisley, UK
Blunt ended needles	Invitrogen, Paisley, UK
Eppendorf tubes (0.5 / 1.5ml)	Invitrogen, Paisley, UK
Non-adherent tissue culture dishes (35mm / 100mm)	Scientific Lab supplies, Coatbridge, UK
Non-adherent tissue culture flasks (75cm ³)	Scientific Lab supplies, Coatbridge, UK
Pipette tips (p10, p20, p100, p200, p1000)	Greiner Bio one, Gloucestershire, UK
Sterile tissue culture pipettes (5, 10, 25, 50mL)	Scientific Lab supplies, Coatbridge, UK
Flat bottomed culture plates (6, 12, 24, 48, 96 well)	Greiner Bio one, Gloucestershire, UK
Flat bottomed culture plates coated with collagen type 1 (6 and 24 well)	Scientific Lab supplies, Coatbridge, UK
White opaque culture plates (96-well)	Scientific Lab supplies, Coatbridge, UK
Tissue culture flasks (25, 75, 150 cm ³)	Greiner Bio one, Gloucestershire, UK
Haemocytometer	Hawksey, UK

2.1.1.2 Tissue culture reagents

Reagent	Supplier
β-mercaptoethanol	Invitrogen, Paisley, UK
Bovine serum albumin (BSA)	Sigma-Aldrich, Dorset, UK
Bovine serum albumin, Insulin/Transferrin (BIT)	Stem Cell Technologies, BC, Canada
BrdU incorporation proliferation assay kit	R&D systems, Abingdon, UK
Bright Glo Luciferase system	Promega, UK
CliniMACS CD34 ⁺ beads	Miltenyi Biotec, Auburn CA
CliniMACS PBS/EDTA buffer	Miltenyi Biotec, Auburn CA
CliniMACS tubing set and columns	Miltenyi Biotec, Auburn CA
Demecolcine (Colcemid; N-Deacetyl – N – Methylcolchicine)	Invitrogen, Paisley, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Dorset, UK
DNase I (Recombinant human DNase)	Stem Cell Technologies, BC, Canada
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Dorset, UK
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich, Dorset, UK
Ethanol 100%	Sigma-Aldrich, Dorset, UK

Foetal calf serum (FCS)	Invitrogen, Paisley, UK
G418 (1013-027)	Invitrogen, Paisley, UK
Ham's F12 / DMEM (F12/DMEM)	Invitrogen, Paisley, UK
Hanks Balanced Salt Solution (Ca ²⁺ Mg ²⁺ free) (HBSS-CMF)	Invitrogen, Paisley, UK
Horse serum (HS)	Invitrogen, Paisley, UK
Human serum albumin 20% (HSA 20%)	Baxter, NHS pharmacy
Human serum albumin 4.5% (HSA 4.5%)	Scottish National Blood Transfusion Service,
Hydrocortisone 21 – Hemisuccinate	Stem Cell Technologies, BC, Canada
Hygromycin	Invitrogen, Paisley, UK
Iscove's Modified Dulbecco's Media (IMDM)	Invitrogen, Paisley, UK
L-glutamine 200mM (100x)	Invitrogen, Paisley, UK
Low density lipoprotein 10mg/mL (LDL)	Sigma-Aldrich, Dorset, UK
Magnesium Chloride (1.25M)	Sigma-Aldrich, Dorset, UK
Methocult H4034	Stem Cell Technologies, BC, Canada
Mitomycin C	Invitrogen, Paisley, UK
Myelocult 5100	Stem Cell Technologies, BC, Canada
Penicillin (5000u)/ streptomycin (5mg/ml) solution (Pen/Strep)	Invitrogen, Paisley, UK
Porcine Gelatin (Sigma – 2500 – type A)	Sigma-Aldrich, Dorset, UK
Recombinant human erythropoietin (EPO)	NHS pharmacy
Recombinant human Flt-3 ligand (Flt3L)	Stem Cell Technologies, BC, Canada
Recombinant human GCSF (GCSF)	Chugai Pharma, London, UK
Recombinant human IL3 (IL3)	Stem Cell Technologies, BC, Canada
Recombinant human IL6 (IL6)	Stem Cell Technologies, BC, Canada
Recombinant human Indian Hedgehog Ligand N-Terminus C24II modified (IHH)	R&D systems, Abingdon, UK
Recombinant human SCF (SCF)	Stem Cell Technologies, BC, Canada
Recombinant human Sonic Hedgehog Ligand N-Terminus C24II modified (SHH)	R&D systems, Abingdon, UK
RPMI 1640 medium (RPMI)	Invitrogen, Paisley, UK
Sodium azide	Sigma-Aldrich, Dorset, UK
Sonic Hedgehog ELISA Kit	Abcam, UK
Sterile water (dH ₂ O)	Baxter Healthcare, Northampton, UK
Tri-sodium citrate (0.155M)	Sigma-Aldrich, Dorset, UK
Trypan blue solution (0.4%)	Sigma-Aldrich, Dorset, UK
Trypsin-EDTA (0.25%)	Sigma-Aldrich, Dorset, UK

2.1.1.3 Composition of tissue culture media

2.1.1.3.1 Medium for culture of K562 cell line (RPMI*)

Constituent	Volume
RPMI 1640	460mL
FCS	50mL
L-Glutamine	5mL
Penicillin / Streptomycin	5mL

2.1.1.3.2 Medium for culture of GLI-Luc TM3 cell line

Constituent	Volume
1:1 mix of Hank's F12 medium and DMEM with HEPES 15mM	440mL
FCS	50mL
Horse serum (HS)	5mL
Penicillin / Streptomycin	5mL
Add 200 μ L of G418 solution per 20mL media for selection	

2.1.1.3.3 Medium for culture of modified M210B4 cell line

Constituent	Volume
RPMI 1640	430mL
FCS	50mL
Penicillin / Streptomycin	10mL
L-Glutamine	10mL
Every 2 weeks add 160 μ L of G418 solution and 25 μ L of Hygromycin B per 20mL media	

2.1.1.3.4 Medium for culture of modified SL/SL cell line

Constituent	Volume
DMEM	430mL
FCS	50mL
Penicillin / Streptomycin	10mL
L-Glutamine	10mL
Every 2 weeks add 320 μ L of G418 solution and 50 μ L of Hygromycin B per 20mL media	

2.1.1.3.5 Serum Free Medium (SFM) for primary cell culture

Constituent	Volume
IMDM	97.25mL
BIT	25mL
L-Glutamine	1.25mL
Penicillin / Streptomycin	1.25mL
β -Mercaptoethanol (200mM)	250 μ L (400 μ M)
LDL (10mg/mL)	250 μ L (40 μ g/mL)
Filter sterilise through 0.22 μ m filter before use	

2.1.1.3.6 SFM with high concentration 5 growth factor cocktail (SFM+HGF)

Constituent	Volume	Final Concentration
SFM	10mL	
IL3 (50µg/mL)	4µL	20ng/mL
IL6 (50µg/mL)	4µL	20ng/mL
GCSF (50µg/mL)	10µL	20ng/mL
SCF (50µg/mL)	20µL	100ng/mL
Flt3L (50µg/mL)	20µL	100ng/mL
<i>Filter sterilise through 0.22µm filter before use</i>		

2.1.1.3.7 SFM with low concentration 5 growth factor cocktail (SFM+LGF)

Constituent	Volume	Final Concentration
SFM	50mL	
IL3 (50µg/mL)	1µL	1ng/mL
IL6 (50µg/mL)	1µL	1ng/mL
GCSF (50µg/mL)	0.5µL	1ng/mL
SCF (50µg/mL)	4µL	20ng/mL
Flt3L (50µg/mL)	4µL	20ng/mL

2.1.1.3.8 Methocult for colony forming cell assay and re-plating assays

Constituent	Volume	Composition
Methocult™ H4034	100mL	IMDM, Methylcellulose, BSA, FBS, 2-mercaptoethanol rh SCF, rh GM-CSF, rh G-CSF, rh IL-3, rh EPO

2.1.1.3.9 Myelocult supplemented with hydrocortisone for LTC-IC culture (long term myeloid culture medium - LTMCM)

Constituent	Volume	Final Concentration
Myelocult 5100	500mL	
Hydrocortisone hemisuccinate (1×10^{-4} M)	1mL	0.2×10^{-6} M

2.1.1.4 Composition of tissue culture solution

2.1.1.4.1 Thawing solution for cryopreserved primary cells (DAMP)

Constituent	Volume	Final Concentration
DPBS	418.75ml	
Tri-sodium citrate (0.155M)	53ml	8.2mM
HSA 20%	25ml	1%
DNase I (~2500U/1mL vial)	2ml	10U/mL
MgCl ₂ (1.0M)	1.25ml	2.5mM

2.1.1.4.2 PBS/2%FCS washing buffer

Constituent	Volume
DPBS	490mL
FCS	10mL

2.1.1.4.3 PBS/20%FCS quenching solution

Constituent	Volume
DPBS	400mL
FCS	100mL

2.1.1.4.4 Cryopreservation solution for primary cells (2x final concentration)

Constituent	Volume
4.5% HSA	80mL
DMSO	20mL

2.1.1.4.5 Cryopreservation solution for cell lines (2x final concentration)

Constituent	Volume
90% FCS	80mL
DMSO	20mL

2.1.1.4.6 LTC-IC harvest washing buffer

Constituent	Volume
IMDM	500mL
FCS	2mL

2.1.1.4.7 Annexin buffer

Constituent	Volume
Annexin 10x buffer	1ml
IMDM	9ml

2.1.1.4.8 Cell culture equipment

Equipment	Supplier
Inverted microscope with camera	Nikon
Spectramax M5 plate reader	Molecular Devices

2.1.1.4.9 Drugs

Reagent	Supplier
LDE225	Novartis Pharmaceuticals, Basel, Switzerland
LEQ506	Novartis Pharmaceuticals, Basel, Switzerland
nilotinib	Novartis Pharmaceuticals, Basel, Switzerland
purmorphamine	Santa Cruz Biotechnology Inc, Santa Cruz, California, USA

2.1.2 Flow Cytometry

2.1.2.1 Flow cytometry reagents

Reagent	Supplier
7 Amino Actinomycin D (7AAD, Viaprobe solution)	BD Biosciences, Oxford, UK
Annexin V – FITC or Annexin V - APC	BD Biosciences, Oxford, UK
Annexin V 10x Buffer	BD Biosciences, Oxford, UK
Anti-mouse IgG2a FITC	BD Biosciences, Oxford, UK
Anti-rabbit IgG FITC or PE	Sigma-Aldrich, Dorset, UK
Anti-rabbit IgG PE	BD Biosciences, Oxford, UK
Anti-mouse IgG2a FITC	BD Biosciences, Oxford, UK
Anti-goat FITC	Santa Cruz Biotechnology Inc
CFSE	Invitrogen, Paisley, UK
FACS flow solution	Becton Dickinson, Oxford, UK
FACS clean solution	Becton Dickinson, Oxford, UK
Fix and Perm Kit	Invitrogen, Paisley, UK
Propidium Iodide (PI)	BD Biosciences, Oxford, UK

2.1.2.2 Flow cytometry antibodies

Reagent	Supplier
Mouse anti-human IgG	Cell Signaling Technology, Inc. Boston
Rabbit anti-human IgG	Cell Signaling Technology, Inc. Boston
Goat anti-human IgG	Santa Cruz Biotechnology Inc, California
Mouse anti-human-CD34 FITC / PE / APC antibodies	BD Biosciences, Oxford, UK
Mouse anti-human-lin cocktail -FITC	BD Biosciences, Oxford, UK
Mouse anti-human-CD123–APC antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD34 PERCP antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD45 FITC antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD45RA Pacific blue antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD90-PE antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD133-PE antibody	BD Biosciences, Oxford, UK
Mouse anti-human-CD38-PECy7	BD Biosciences, Oxford, UK
Mouse anti-human-CD133-PE antibody	BD Biosciences, Oxford, UK
Mouse IgG1a FITC	BD Biosciences, Oxford, UK
Mouse IgG1a PE	BD Biosciences, Oxford, UK
Mouse IgG1a PERCP	BD Biosciences, Oxford, UK
Mouse IgG1a APC	BD Biosciences, Oxford, UK
Rabbit anti-human-Ki67 FITC antibody	Cell Signaling Technology, Inc. Boston
Rabbit anti-human-Ki67 APC antibody	Cell Signaling Technology, Inc. Boston
Mouse anti-human-lineage cocktail (lin1), FITC (CD3, 4, 14, 16, 19, 20, 56)	BD Biosciences, Oxford, UK

2.1.2.3 Composition of flow cytometry solutions

2.1.2.3.1 Flow cytometry fixation solution

Constituent	Volume
DPBS	49.5mL
39% Formaldehyde solution	0.5mL

2.1.2.3.2 Flow cytometry permeabilisation solution

Constituent	Volume
DPBS	49.9mL
Triton-X-100	0.1mL

2.1.2.3.3 FACS Equipment

Constituent	Volume
FACS Aria	BD Biosystems
FACS Canto	BD Biosystems

2.1.3 Molecular Biology

2.1.3.1 Molecular biology reagents

Equipment	Supplier
RNA easy mini kit	Qiagen, Crawley, UK
RNA easy micro kit	Qiagen, Crawley, UK
RNA later	Qiagen, Crawley, UK
DNase 1	Qiagen, Crawley, UK
Agilent Bioanalyser kit	Agilent Technologies, UK Ltd Berkshire
High capacity reverse transcription kit	Applied Biosystems, Warrington, UK
Pre-amplification master mix	Applied Biosystems, Warrington, UK
Gene expression master mix	Applied Biosystems, Warrington, UK
Various Taqman [®] probe mixes	Applied Biosystems, Warrington, UK
TE buffer pH 8.0	Applied Biosystems, Warrington, UK
Molecular biology grade 100% Ethanol	Qiagen, Crawley, UK
Molecular biology grade H2O	Qiagen, Crawley, UK

2.1.3.2 Molecular biology equipment

Equipment	Supplier
Nanodrop 2100	Nanodrop technologies, Wilmington USA
Agilent Bioanalyzer	Agilent Technologies, UK Ltd Berkshire
Taqman [®] 7900 machine	Applied Biosystems, Warrington, UK
Fluidigm Biomark Analyser	Fluidigm, San Francisco, USA
Nucleotide free tips eppendorfs and PCR tubes	Applied Biosystems, Warrington, UK

2.1.3.3 Taqman® probesets

Probe	Applied Biosystems identifier	Probe	Applied Biosystems identifier
AURKA	Hs01582072_m1	HOXA3	Hs00601076_m1
BIK	Hs00154189_m1	HOXA9	Hs00365956_m1
BIRC5	Hs04194392_s1	HPRT1	Hs01003267_m1
BMI1	Hs00995536_m1	IGF1	Hs01547656_m1
BMP4	Hs00370078_m1	IGF1R	Hs00609566_m1
BRCA1	Hs01556193_m1	IGFBP2	Hs00236877_m1
CADPS2	Hs00604528_m1	IHH	Hs00745531_s1
CCNA2	Hs00996788_m1	KIF11	Hs00189698_m1
CCNB1	Hs01030097_m1	KIF23	Hs00370852_m1
CCNB2	Hs00270424_m1	KLF4	Hs00358836_m1
CCND1	Hs00765553_m1	LEF1	Hs01547250_m1
CCND2	Hs00153380_m1	MECOM	Hs00602795_m1
CDC25C	Hs00156411_m1	NUMB	Hs01105433_m1
CENPA	Hs00156455_m1	PLAG1	Hs04186040_m1
CHEK2	Hs00200485_m1	PLK1	Hs00153444_m1
CPA3	Hs00157019_m1	PRC1	Hs00187740_m1
CRIP2	Hs00373842_g1	PTCH1	Hs00181117_m1
CXCL1	Hs00236937_m1	PTCH2	Hs0018404_m1
CXCL12	Hs00171022_m1	RAD51	Hs00153418_m1
CXCR4	Hs00607978_s1	RASSF6	Hs00698249_m1
DACH1	Hs00974297_m1	RUNX2	Hs00231692_m1
DHH	Hs00368306_m1	SELP	Hs00174583_m1
EBF	Hs00395513_m1	SERPINB4	Hs00741313_g1
ESPL1	Hs00202246_m1	SETBP1	Hs01098447_m1
EZH2	Hs01016789_m1	SHCBP1	Hs00226915_m1
FAM38B	Hs00401026_m1	SHH	Hs00179843_m1
FANCD1	Hs00609073_m1	SMO	Hs01090242_m1
FOS	Hs00170630_m1	SOX5	Hs00753050_s1
FOXM1	Hs01073586_m1	SPINT1	Hs00173678_m1
GAPDH	Hs02758991_g1	ST14	Hs01058386_m1
GARS	Hs00157653_m1	STIL	Hs00161700_m1
GAS2	Hs01086684_m1	SUFU	Hs00171981_m1
GATA1	Hs01085823_m1	TCF4	Hs00162613_m1
GATA2	Hs00231119_m1	TCF7	Hs00175273_m1
GATA3	Hs00231122_m1	TMTTC1	Hs00230491_m1
GLI1	Hs01110766_m1	TOP2A	Hs00172214_m1
GLI2	Hs01119974_m1	TSHZ3	Hs01583885_m1
GLI3	Hs00609233_m1	WEE1	Hs01119384_g1
GUSB	Hs00939627_m1	TOP2A	Hs00172214_m1
HES1	Hs00172878_m1	TSHZ3	Hs01583885_m1
HES5	Hs01387463_g1	WEE1	Hs01119384_g1
HHIP	Hs01011015_m1	YWHAE	Hs00356749_g1
* BCR-ABL probe and primers	ENP541F-MGB; 6FAM-CCCTTCAGCGGCCAGT ENF501: TCCGCTGACCATCAAYAAGGA ENR561: CACTCAGACCCTGAGGCTCAA		

2.1.4 Immunofluorescence

2.1.4.1 Immunofluorescence reagents

Equipment	Supplier
Fix and Perm Reagents A/B	Invitrogen, Paisley, UK
DAPI	Invitrogen, Paisley, UK
Poly L Lysine	Invitrogen, Paisley, UK
LS1 BCR-ABL Dual Colour FISH probe set	Abbott Diagnostics, Maidenhead, UK
Rubber cement	Halfords, Braehead, UK
SSC	Invitrogen, Paisley, UK
Formamide	Invitrogen, Paisley, UK
Nail polish	Various suppliers
100% Ethanol	University of Glasgow,
100% Methanol	University of Glasgow,
KCl 0.75M	University of Glasgow,
Acetic acid	University of Glasgow,

2.1.4.2 Composition of fluorescence in situ hybridisation (FISH) solutions

2.1.4.2.1 Hypotonic KCl solution

Reagent	Quantity
KCl 0.75M	1mL
Sterile H ₂ O	10mL

2.1.4.2.2 Fixative

Reagent	Quantity
Acetic acid	3mL
Methanol	9mL
<i>Make fresh</i>	

2.1.4.2.3 Stock SSC buffer (x20 concentration) (20xSSC)

Reagent	Quantity
SSC	132g
dH ₂ O	500mL
Pure HCl	Titrate to pH 5.3

2.1.4.2.4 FISH wash buffer 1 (2xSSC 0.1% NP40)

Reagent	Quantity
20xSSC	100mL
dH ₂ O	899mL
NP40	1mL
Pure HCl	Titrate to pH 7

2.1.4.2.5 FISH wash buffer 2 (0.4xSSC 0.3% NP40)

Reagent	Quantity
20xSSC	10mL
dH ₂ O	493.5mL
NP40	1.5mL
Pure HCl	Titrate to pH 7

2.1.4.2.6 FISH denaturation buffer

Reagent	Quantity
Formamide 70%	49mL
SSCx20	7mL
dH ₂ O	14mL
Pure HCl	Titrate to pH 7

2.1.4.2.7 FISH hybridisation mixture (for 4 spots on a multisport slide)

Reagent	Quantity
Hybridisation buffer	7uL
Probe mix	1uL
H ₂ O	2uL

2.1.4.3 FISH Equipment

Reagent	Quantity
Poly L Lysine coated slides	Invitrogen, Paisley, UK
Water baths	N/A
Mercury thermometer	N/A
Humidified hybridisation box	N/A

2.1.5 Microarray

Equipment	Supplier
Affymetrix ST1.0 Gene Expression DNA Chip	Affymetrix
PARTEK GS	Partek
Ingenuity Pathway Analysis	Ingenuity

2.2 Methods

2.2.1 Drugs and reagents

LDE225 (FW: 681.51), LEQ506 (FW: 423.5) and nilotinib (FW: 566.0) were kindly provided by (Novartis Pharma, Basel, Switzerland). Purmorphamine (FW: 520.6), cyclopamine (FW: 411.6) and GANT61 (FW: 429.6) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Ten millimolar (mM) stock solutions were prepared in DMSO and stored at -20°C. Dilutions of these stock solutions were freshly prepared for each experiment in appropriate cell culture media. Growth factors were reconstituted according to the manufacturer's instructions and stored in single use aliquots at -80°C.

2.2.2 Standard cell culture conditions

All cell culture procedures were performed using sterile technique in a laminar air flow hood. All cell culture was in a humidified incubator at 37°C in 5% CO₂.

2.2.3 Patient samples

Leukapheresis or PB specimens were obtained with written informed consent, in accordance with the declaration of Helsinki and with Greater Glasgow and Clyde NHS Trust Ethics Committee approval from patients newly diagnosed with CML. Each sample was determined to be Ph⁺ by FISH and BCR-ABL⁺ by PCR. Samples were processed by Alan Hair and others with responsibility for cell banking. Samples were assessed for viability and proportion of CD34⁺ cells. If suitable for further processing, small volume samples (50mL) were purified using Histopaque 1077 to enrich for MNC followed by CD34⁺ enrichment using CD34⁺ selection columns and larger volume samples (apheresis samples) were purified directly through the CliniMACS system. Both are immuno-magnetic beads systems which positively select CD34⁺ cells using super-paramagnetic beads coupled with a suitable CD34⁺ monoclonal antibody. Briefly, CD34⁺ beads were added to the primary cell sample and the resulting suspension passed through a magnetic separation column which resulted in the retention of the magnetic beads as the sample passed through. These beads, along with the attached CD34⁺ cells, can be recovered by simply removing the magnetic field. Following recovery,

purity was assessed by flow cytometry to identify the CD34⁺ fraction prior to cryopreservation. All samples were >95% CD34⁺ following purification.

Non-CML CD34⁺ samples were obtained from the PB of patients with lymphoma or multiple myeloma undergoing G-CSF mobilisation following chemotherapy. These samples were processed and stored as described above.

2.2.4 Cryopreservation of cells

Primary cells were re-suspended in 5% HSA and mixed in a 1:1 ratio with 20% DMSO / 5% HSA solution and transferred to 2mL cryo-tubes in aliquots of $4 \times 10^6/2\text{mL}$ or $20 \times 10^6/2\text{mL}$. Cell lines were re-suspended in 10% DMSO / 90% FCS and transferred to cryo-tubes in aliquots of $1-10 \times 10^6$ cells/2mL. These cryo-tubes were then placed in a 5100 Cryo -1°C cell freezing container to enable controlled cooling at -1°C per minute until they were at -80°C at which point they were transferred to the vapour phase of liquid nitrogen until required.

2.2.5 Cell recovery post cryopreservation

CD34⁺ primary cells were removed from liquid nitrogen and immediately thawed at 37°C in a water bath. Following this, 10mL of thawing solution (DAMP) was gradually added to the cells over 10 minutes (min) with constant gentle agitation to prevent clumping. The cells were then centrifuged at 1000rpm for 10 min and washed a further twice in 10mL DAMP. Finally the cell pellet was re-suspended in SFM+HGF; cell number and viability was assessed by trypan blue dye exclusion and the cells cultured overnight at 37°C with 5% CO₂ in a non-adherent tissue culture flask at a maximum density of $1 \times 10^6/\text{mL}$. Culture in SFM+HGF maximises cell recovery post thaw. The following day these cells were washed twice in DPBS / 2%FCS, re-suspended in fresh SFM±GF supplementation and a viable cell count performed; in general a recovery of approximately 25% of the cryopreserved cell number was expected.

Cell lines were removed from liquid nitrogen and immediately thawed at 37°C in a water bath. Then they were washed once in their relevant media, re-suspended in media, viable cells counted and seeded in standard tissue culture flasks at the appropriate density.

2.2.6 Cell counting and viability assessment

All cell counts were performed using a haemocytometer. Cell viability was assessed by vital dye exclusion. Trypan blue stock solution (8mM) was diluted 1:10 with DPBS to give a working solution of 0.8mM. Cells were diluted in the trypan blue working solution and added to a haemocytometer counting chamber. Cell count was performed on an inverted microscope using the x10 objective. Given that the cover-slide is held 0.1mm above the counting surface, each 1mm² square has a volume of 0.1µL. Non-viable cells absorb trypan blue whereas viable cells do not. Therefore the absolute viable cell count per mL was the number of unstained cells per mm² multiplied by the trypan blue dilution factor and then by 10⁴.

2.2.7 Primary CD34⁺ cell culture experiments

In most instances CD34⁺ CML cells following recovery were cultured in SFM alone or supplemented with low GFs. Non-CML cells were grown in SFM+LGF supplementation. For certain cell tracking experiments it was considered important to drive cell expansion and these were therefore carried out with SFM+HGF supplementation. Cells in culture with the agents under investigation were harvested at 1d-12d post exposure. In the case of longer exposure fresh media and drug were added at d4 and d8.

2.2.8 Cell line culture

A variety of cell lines were utilised and are listed below. All cell lines were passaged according to American Type Culture Collection (ATCC) or Leibniz - Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) instructions.

2.2.8.1 Cell lines

Cells	Species / Type	Information	Supplier / Reference
K562	Human CML BC	Established from the pleural effusion of a 53 year old woman with CML in BC in 1970. BCR-ABL ⁺ (b3a2) Media used: RPMI* Split ~1:5 every 3d. Seeded at ~ 1x10 ⁵ /mL Maximum density: 1.5x10 ⁶ /mL	DSMZ /ACC10 (Drexler, 1994; Lozzio and Lozzio, 1977; Lozzio and Lozzio, 1975)
TM3 (GLI Luc)	Murine	Mouse leydig cell line engineered to stably express a GLI responsive luciferase reporter element (8xGLI-Luc) Media used: TM3 media Split ~1:10-20 every 3-4d. Seeded at ~ 1x10 ⁵ /mL Requires selection with G418 100ul/10mL	Gift from Marian Dorsch, Novartis Pharma (Pan et al., 2010) (Miller-Moslin et al., 2009) (Frank-Kamenetsky et al., 2002)
M210B4	Murine Stromal	A clone derived from bone marrow stromal cells from a (C57BL/6J X C3H/HeJ) F1 mouse. These cells were engineered to stably express and secrete human IL3 and human GCSF. These cells will support human myelopoiesis in long term culture. Media used: RPMI* Split: ~1:5 every 3d	Gift from C Eaves / Stem Cell Technologies (Sutherland et al., 1991)
SL/SL	Murine Stromal	A clone derived from BM of the SL/SL mouse. These cells have been engineered to produce human IL3 and SCF. These cells will support human myelopoiesis in long term culture. Media used: DMEM 10% FCS Split: ~1:5 every 3d	Gift from C Eaves / Stem Cell Technologies (Sutherland et al., 1991)

2.2.9 Flow Cytometry

All flow cytometry was performed on the BD FACS Canto II flow cytometer, data was acquired using BD FACS Diva (BD Biosciences) software, and analysed using FACS Diva or FlowJo (Tree Star, Inc.) software.

2.2.9.1 General aspects of flow cytometry

Flow cytometry is a method for simultaneously counting and measuring various properties of individual particles e.g. cells as they are passed, suspended in a stream of fluid, through an array of electronic detectors. This technology can be used to measure physical properties e.g. size, shape and granularity or can be combined with fluorescent dyes or fluorochrome conjugated antibodies raised against particular surface or intracellular epitopes. The combination of different antibodies conjugated to fluorochromes with different emission spectra permits the measurement of several different cell surface antigens simultaneously. In contemporary flow cytometry, using modern fluorochromes with emission wavelengths spanning the detectable spectra and utilising mathematical

compensation algorithms, it is possible to combine multiple fluorochromes to measure several different parameters simultaneously.

Flow cytometry requires that the cellular sample under investigation is carried in a stream of fluid that is hydrodynamically focused in order to cause the cells in question to be in single file as they pass across the path of one or more lasers of differing wavelengths focused onto the fluid stream. The laser light is scattered as it contacts the passing cell. Forward scatter (scatter in the original direction of travel of the laser) is proportionate to the size of the cell and side scatter (scatter perpendicular to the original direction of travel of the laser) is proportionate to the granularity of the cell. Additionally as the laser contacts the cell, it may excite fluorescence from certain associated fluorochromes e.g. from fluorochrome conjugated antibodies raised to a specific epitope. A series of filters and detectors are arranged in order to pick up and quantify these fluorescent emissions which can be mapped to the known spectral emission qualities of the fluorochromes used. Therefore it is possible to measure the physical properties of a cell, the presence of any fluorescent dye or, using conjugated antibodies, the level of expression of any suitable cell surface or intracellular target on individual cells simultaneously and both isolate a particular population of cell within a heterogeneous cell mixture and measure the relative abundance of various surface and intracellular proteins.

2.2.9.2 Measuring surface antigen expression by flow cytometry

Cells were harvested, washed and re-suspended in DPBS / 2% FCS. Fluorochrome conjugated antibodies were added at the specified concentrations and well mixed prior to incubation for 15min at room temperature (RT) in the dark. Cells were then washed twice in DPBS / 2% FCS, re-suspended and analysed immediately. Appropriate isotype controls and an unstained control were utilised to set the voltages and compensation when required. The viable cell population was gated using FSC and SSC characteristics. For targets where no fluorochrome conjugated antibody was available, I incubated the cells for 30min with an unconjugated antibody or the same concentration of a non-specific control prior to washing and re-suspending in DPBS / 2% FCS. A secondary fluorochrome conjugated antibody raised against the immunoglobulin species of the primary antibody was then added to the resultant cell suspension, thoroughly mixed and incubated for a further hour. A secondary only control was included in order to

determine non-specific secondary antibody binding. The cells were then washed twice in DPBS / 2% FCS, re-suspended and analysed.

2.2.9.3 Assessment of apoptosis by flow cytometry

As cells enter apoptosis, one of the earliest events is the translocation of negatively charged phosphatidylserine from the inner (cytosolic) to the outer (external) surface of the phospholipid bilayer that comprises the plasma membrane (Koopman et al., 1994; Martin et al., 1995). Annexin V is a phospholipid binding protein with a high affinity for phosphatidylserine in the presence of physiological concentrations of Ca^{2+} . Phosphatidylserine is also accessible to annexin V during necrosis due to disruption of plasma membrane integrity. In this case, although annexin V staining still occurs, the cells will also stain with a vital dye, in this case 7-amino-actinomycin D (7AAD; Viaprobe solution). The principle of these experiments was to measure the proportion of viable, early apoptotic and late apoptotic cells following exposure to the test drug combinations. Primary CD34^+ CP CML cells were assessed for apoptosis by flow cytometry using fluorochrome conjugated annexin V (annexin V-FITC) and 7AAD. Thus viable cells would be negative for both annexin V and 7AAD; cells in early apoptosis would be annexin V positive but 7AAD negative and cells in late apoptosis would be positive for both annexin V and 7AAD. Primary CD34^+ CP CML cells were cultured in SFM in the presence of the various combinations of test drugs or over the course of 72 hours (h). At each time point these cells were harvested, washed twice in DPBS / 2% FCS, re-suspended in 100 μL annexin V binding buffer and stained with 5 μL annexin V-FITC and 10 μL 7AAD for 15min. Prior to analysis a further 400 μL binding buffer was added to the samples which were then immediately analysed. Annexin V staining was measured on channel FL1 and 7AAD staining was measured on channel FL3. Unstained and single fluorochrome stained control cells were used to set voltages and compensation (Figure 2-1).

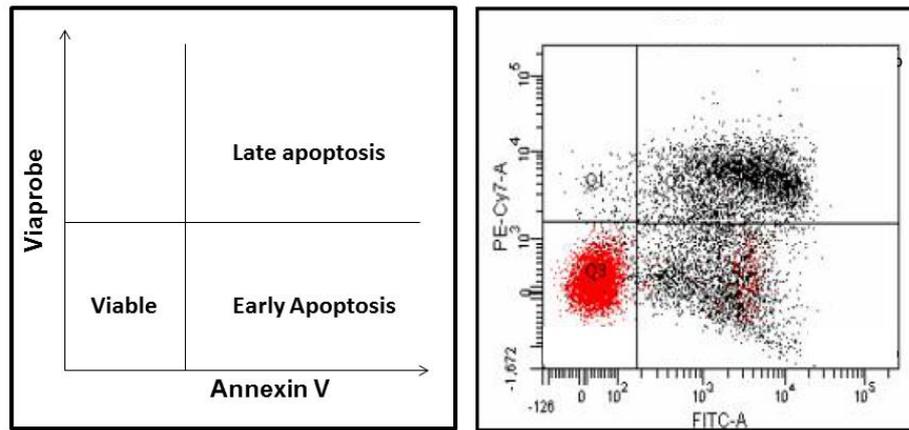


Figure 2-1: Annexin V / Viaprobe apoptosis assay.

On the left is a schematic map indicating the position of the flow cytometry populations in the annexin V / 7AAD apoptosis assay. On the right is a representative flow cytometry plot from an apoptosis assay.

2.2.9.4 High resolution cell cycle analysis by flow cytometry

The series of events that occur in a eukaryotic cell as it prepares for and undergoes division are termed the cell cycle. This can be divided into two distinct periods; interphase - representing the period when the cell is growing and duplicating its genetic material; and mitosis where the cell splits its cytoplasmic and nuclear material to form two distinct cells. Interphase can be further divided into 3 phases; G_1 from the end of the previous mitosis to the beginning of DNA synthesis, when there is considerable biosynthetic activity, growth and preparation for DNA synthesis; S in which there is duplication of the cell's genetic material and G_2 where there is further biosynthetic activity and preparation for mitosis. G_0 denotes cells that are quiescent - i.e. not in cycle. It is possible to use flow cytometry to measure progress through the cell cycle. As cells cycle the amount of DNA that they have increases from diploid (normal) to tetraploid (just before mitosis). Fluorescent dyes that bind in a stoichiometric manner to double stranded DNA (e.g. 7AAD) can be utilised to measure relative quantity of DNA per cell by flow cytometry. This will not easily allow differentiate between G_0 and G_1 as in both cases the quantity of DNA present will be similar. Jordan *et al*/published a refinement that allows the high resolution analysis of G_0 and G_1 phases of the cell cycle through measuring DNA content using 7AAD and the addition of a further parameter - the measurement of the intracellular protein; Ki67 (Jordan et al., 1996). This is a nuclear protein and is involved in cellular proliferation and ribosomal RNA transcription. It is expressed in all active phases of the cell cycle

but is absent in quiescent cells (G_0). Thus by combining both parameters it is possible to discern the proportion of cells in G_0 ($Ki67^-/7AAD^{lo}$); G_1 ($Ki67^+/7AAD^-$) and $G_2/S/M$ ($Ki67^+/7AAD^+$)

In these experiments primary $CD34^+$ CP CML cells were cultured in SFM in the presence of the various combinations of test drugs or ligands over 72h. At each time point these cells were harvested, washed twice in DPBS / 2% FCS and then re-suspended in 500 μ L DPBS/0.4% formaldehyde and incubated on ice for 30min to fix the cells. Following this, the cells were washed a further time with DPBS / 2% FCS and then re-suspended in PBS / 0.2% Triton-X-100. These cells were incubated overnight at 4 $^{\circ}$ C. The following day the cells were again washed and re-suspended in 200 μ L of DPBS. Eight μ L of Ki67-FITC or Ki67-APC conjugated antibody were added to the cell suspension which was mixed thoroughly prior to incubation in the dark at RT for 30min. After a further wash the cells were re-suspended in DPBS 200 μ L / 2% FCS and incubated with 1 μ L of 7AAD (final concentration: 1 μ g/mL) for at least 6h at 4 $^{\circ}$ C. The appropriate isotype and unstained controls were used to set the flow cytometer voltages, set gates and where necessary to adjust compensation. The viable cell population was gated using forward and side scatter characteristics. Ki67 was assessed using a log scale on FL1 (FITC) or FL4 (APC). 7AAD staining was assessed on a linear scale on channel FL3. Gates were set around the G_0 , G_1 and $G_2/S/M$ populations allowing the calculation of the relative proportions of cells in each of these phases to be recorded from a minimum of 10,000 events (Figure 2-2).

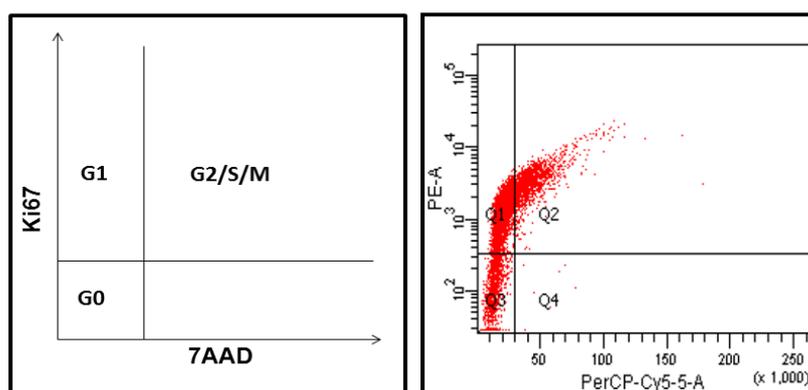


Figure 2-2: High resolution cell cycle analysis.

On the left is a schematic map indicating the positions of the respective cell cycle populations in high resolution cell cycle analysis. On the right is a representative flow cytometry plot (note Ki67 is in log scale whereas 7AAD is represented on a linear scale).

2.2.9.5 Cell division tracking by flow cytometry

CFSE is an intracellular dye which is widely used to follow cell division in heterogeneous and asynchronous cell populations. The principle was first described by Lyons and Parish in 1994 when this technique was used to track lymphocyte subset divisions (Lyons and Parish, 1994). It has subsequently been applied to many different cell types and is of particular relevance to this work to monitor haemopoietic progenitor cell division following GF exposure and drug treatment (Graham et al., 2002; Holyoake et al., 1999).

CFSE readily crosses the plasma membrane of cells. Once inside, intracellular esterases cleave the acetate groups to yield the fluorescent carboxyfluorescein molecule and the succinimidyl ester group reacts with primary amines on intracellular proteins, cross-linking the dye and fixing it within the cytoplasm of the cell (Lyons and Parish, 1994). When a cell divides, the CFSE molecules are shared equally between daughter cells. This can be represented as a successive halving of the measured CFSE fluorescence intensity as related to a similarly treated undividing control population and the number of divisions estimated by the fold reduction of CFSE fluorescence intensity. CFSE fluorescence is measured in channel FL1 although analogous substances can be measured in other flow cytometer channels.

In these experiments CD34⁺ CP CML cells were recovered from liquid nitrogen, washed counted and re-suspended in DPBS / 2% FCS as previously described. Stock CFSE solution (5mM CFSE in DMSO) was diluted at a ratio of 1:10 with DPBS / 2% FCS and the resultant working solution added to the cell suspension to a final concentration of 1 μ M. This cellular suspension was then incubated in a water bath at 37°C for exactly 10min prior to quenching with 10 x volume of ice cold PBS / 20% FCS. These cells were washed twice in DPBS / 2% FCS prior to re-suspension in SFM+HGF and overnight culture in non-adherent 75cm² tissue culture flasks at a maximum density of 1x10⁶/ml. In addition to the CFSE stained sample, about 10% of the original sample was retained and cultured in SFM+HGF as a CFSE⁻ unstained control.

The following day the CFSE⁺ and CFSE⁻ samples were harvested, washed in DPBS / 2% FCS, re-suspended in SFM + LGF and viable cell count determined. An aliquot of cells was removed and assessed by flow cytometry to determine the fluorescence of the undivided CFSE_{max} cells. The following day cells were seeded

at a concentration of 1×10^5 /ml in SFM+LGF with incremental concentrations of Hh inhibitor, nilotinib, Hh ligand or combinations thereof. For each experiment a non-dividing control was set up using CFSE stained cells treated with 100ng/mL demecolcine (Colcemid). This facilitated identification of the CFSE_{max} peak at each time point. Stained and unstained controls were included to allow for unstained, single stained and isotype controls at each stage of FACS analysis.

In each experiment, the wells were thoroughly mixed and an aliquot of cells was taken from each experimental arm at d4, d8 and d12. The cells were washed in DPBS / 2% FCS and divided into aliquots for viable cell count, flow cytometric analysis and reseeded. Viable cell count was assessed by trypan blue exclusion as described previously. In different experiments I also assessed the CD34⁺ cell fraction and the CD133⁺ cell fraction. For CD34 and CD133 I added 5 μ L of CD34-APC and/or CD133-PE to the cell suspension, mixed, and incubated for 15min at RT in the dark. These cells were then washed twice in DPBS / 2% FCS, re-suspended in 500 μ L DPBS / 2% FCS prior to FACS analysis. In each case, unstained cells and single stained isotype controls were included (Figure 2-3). Lastly, in the case of d4 and d8, the remaining cells were re-seeded in fresh media and drug at approximately the original cellular concentration for the next time point.

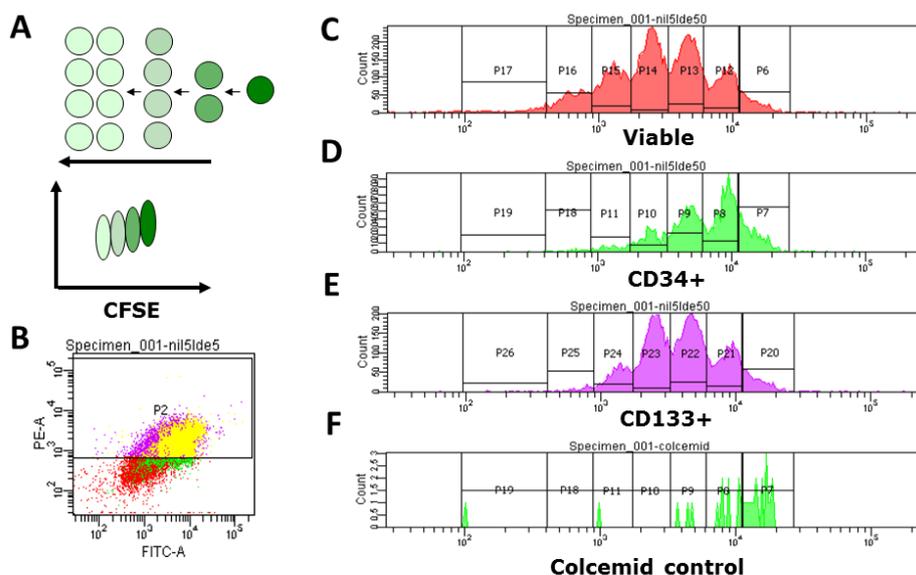


Figure 2-3: Cell division tracking by dye dilution.

(A) Schematic demonstrating the effect of successive dilution on CFSE fluorescence in a cell population. (B) Representative flow cytometry dot plot with CFSE treated primary CP CML cells cultured for 4d in SFM+HGF with nilotinib and LDE225. (C) – (E) Histograms at the same time point in the same sample tracking the division history of total viable cells, CD34⁺ cells and CD133⁺ cells. (F) Colcemid control to determine the level of CFSE_{max} (the undivided population).

2.2.9.6 *Flow cytometry considerations and calculation of cell numbers*

At each time point the viable cell count was obtained by trypan blue exclusion prior to flow cytometry analysis. Unstained cells and single stained isotype controls were included to assist the setting of voltages and compensation where required. The viable cell population was identified through FSC / SSC properties.

On this population I used a histogram to visualise number of events across the spectrum of CFSE in channel FL1 fluorescence. The colcemid treated control allowed gating of the undivided population. Gates could then be set using successive 2 fold diminutions of the geometric mean of FL1 fluorescence, encompassing cell populations within the sample that had similar levels of FL1 fluorescence and thus had divided a similar number of times.

Comparing fluorescence from CD34-APC or CD133-PE as surface markers, in addition to CFSE, I could then simultaneously measure proportion of cells in each cell division that expressed CD34-APC and CD133-PE.

In order to compare the effect of the drug and ligand treatments on the quiescent primitive fraction and on the proliferation behaviour of the CP CML CD34⁺ cells, the percentage recovery of viable CD34⁺ cells in the undivided fraction was calculated at each time point for each experimental condition. In essence, through looking at the number of viable and CD34⁺ cells in each division, the percentage of the original cells that could be accounted for in each arm was sought. The total number of viable cells, as well as the percentage of CD34⁺ cells, (and where relevant the percentage of CD133⁺ cells) in each division for each condition at each time point were recorded. For example, percentage recovery of CD34⁺ input cells in division x was calculated by multiplying the % CD34⁺ cells in division x by the total number of viable cells and dividing by the number of input cells.

2.2.9.7 Fluorescence activated cell sorting

A refinement of flow cytometry allows the physical sorting of a heterogeneous mixture of cells into different containers according to their physical size, granularity and fluorescence profile. The cell suspension of interest is hydrodynamically focused such that cells pass through the detectors in single file in the centre of a rapidly flowing fluid stream. These cells are comparatively far apart therefore a vibrating mechanism can break the stream into individual droplets with most droplets containing only one cell event. These droplets can be given an electrical charge and then diverted into specific containers in accordance with that charge as they pass through an electrostatic deflection system. The charge that the droplets acquire can be dictated by the operator, dependent on the measured physical or fluorescence characteristics of the cell.

The cell sorts were performed by Dr Mhairi Copland. Enriched CD34⁺ samples were thawed, viable cell count performed and cultured as described above. The following day the cells were washed twice in DPBS / 2% FCS and a viable cell count performed. The cells were simultaneously stained with lineage (lin) cocktail-FITC, CD90-PE, CD34PerCP, CD38PE-Cy7, CD123-APC and CD45RA-Pacific Blue for 30min at RT in the dark. They were then washed twice in DPBS / 2% FCS prior to re-suspension in multiple FACS tubes placed on ice.

This allowed sorting of the cells into HSC CD34⁺38⁻lin⁻CD45RA⁻CD90⁺; MPP CD34⁺38⁻lin⁻CD45RA⁻CD90⁻; CMP CD34⁺38⁺lin⁻CD123⁺ CD45RA⁻; [GMP] CD34⁺38⁺lin⁻CD123⁺ CD45RA⁺ and MEP CD34⁺38⁺lin⁻CD123⁻CD45RA⁻ sub-

populations on a FACS Aria cell sorter. This machine allows for the concurrent sorting of up to 4 populations, the fifth population (MPP) was swapped in when sufficient cells for one of the other populations had been collected (Majeti et al., 2007).

Following sorting the cells were counted and divided into aliquots either for cell culture (progenitor and proliferation assays) or for RNA purification using the RNEasy mini or micro kit. Cell culture assays and RNA purification were performed by Dr Copland. Isolated RNA was subsequently checked for purity and integrity as described below. All samples were subject to FISH analysis to confirm the presence and the proportion of cells carrying BCR-ABL in each sample in each sub-population. RNA processing and microarray analysis were carried out at the Sir Henry Wellcome Functional Genomics Facility (SHWFGF, University of Glasgow) using the Affymetrix GeneChip Human Gene 1.0 ST arrays according to the manufacturer's instructions. The raw data .CEL files were analysed as described in the next chapter. Quantitative RTPCR was performed on various genes to validate the microarray findings and investigate other gene expression patterns of interest. This sorting process required many cells and could only be performed a limited number of times and additionally was limited by the number of samples available in sufficient quantity to allow the necessary sorting. We were able to utilise 3 non CML samples, 6 CP CML samples, 4 AP CML samples and 2 BC CML samples.

2.2.10 Short term cellular techniques

2.2.10.1 GLI responsive luciferase assay

The TM3^(GLI-Luc) cells (a murine Leydig cell line with intact Hh signalling characteristics) previously transfected with a GLI-responsive luciferase reporter gene such that luciferase production was dependent on GLI activation through Hh signalling was obtained. These cells were cultured as described above in a 0.1% gelatine coated tissue culture flask under G418 selection. When required the TM3^(GLI-Luc) cells were harvested using 2mL trypsin 0.25% under standard conditions, washed twice and re-suspended in standard media prior to seeding at 1×10^5 cells per well in an opaque white 96 well tissue culture plate. In the first set of experiments, the cells were stimulated with escalating concentrations of Hh ligand (50-500ng/mL; SHH) or the Hh agonist purmorphamine (0.5 - 5 μ g/mL).

Luciferase activity was assessed at 24, 48 and 72h using the Bright-Glo system and a plate reading luminometer. All measurements were performed in triplicate.

In the second set of experiments the TM3^(GLI-Luc) cells were plated as before. The following day escalating concentrations of the Hh inhibitors were added and 6h later the cells were stimulated with the minimum concentration of purmorphamine that yielded maximum luciferase production as derived from the first experiment. Again, luciferase activity was assessed at 24, 48 and 72h using the Bright-Glo system and a plate reading luminometer and all measurements were performed in triplicate.

2.2.10.2 Measuring cell proliferation using BrdU incorporation

A measure of cellular proliferation can be gained through quantifying DNA synthesis. A convenient way to measure DNA synthesis is to measure the quantity of labelled DNA precursors incorporated into the genomic DNA of a cell over a set labelling period, the quantity of incorporated label is then proportionate to the rate of DNA synthesis and hence cell division in the sample.

In these experiments, 5×10^4 CD34⁺ CP CML cells were cultured in 96 well plates in 100 μ L SFM or appropriate cell line culture media in the presence of escalating concentrations of Hh inhibitor (1-1000nM LDE225; 1-50 μ M cyclopamine) for between 24 and 96h. Assays were performed in a minimum of triplicates. At each time point the thymidine analogue BrdU was added to the cell culture (final concentration: 10 μ M BrdU) and re-incubated for a further 24h. At this stage the culture plate was centrifuged at 300g for 10min to fix the cells to the bottom of the plate and the media plus labelling solution removed by inverting and tapping the plate. After this the cells were dried using a hairdryer for 15min and stored at 4°C for up to 4d. Analysis was by colorometric immunoassay. Briefly, the cells were fixed and denatured by the addition of 200 μ L fix-denat solution at RT for 30min. Following this the solution was removed by inverting and tapping the plate. Anti-BrdU antibody peroxidase conjugate solution was then added to each well and the plate incubated at RT for 90min after which the antibody conjugate was removed and the wells washed 3 times in 200 μ L DPBS. Following the final wash, the washing solution was removed from the wells and 100 μ L of substrate solution (tetramethyl benzidine; TMB) added and the plate incubated at RT for 15min. The reaction was quantified by absorbance measured at 370nm

(reference wavelength: 450nm) on a scanning plate reader. Both blank (no cells) and background controls (no BrdU) were performed for each plate.

2.2.10.3 Ligand expression by enzyme-linked immunosorbant assay

In order to determine the amount of Hh ligand produced by various cell types, we collected cell conditioned supernatants from cultured cells under standard conditions. CD34⁺ enriched CP CML and non CML cells were cultured in SFM+LGF at a concentration of 0.5×10^6 /mL for 24h. Stromal cell lines and primary human BM MSCs cultured from healthy individuals, patients with CP CML and an immortalised embryonic MSC (EMSC) line (<passage 2-6) were plated at the cell concentrations used for supporting haemopoiesis (M210B4 & SL/SL: 1×10^5 cells/mL and primary and EMSCs: 4×10^4 mL in 1mL relevant media for 24h.) Following culture the cell suspension was harvested, centrifuged at 1200rpm for 10min then the supernatant was collected, filtered and stored at -20°C until required.

I used the ab100639 Human SHH (SHH N Terminus) enzyme linked immunosorbant assay (ELISA) kit. An antibody specific to the N terminus of the human SHH had been immobilised in the wells of a 96 well plate. Standards and samples were added to the wells (100µL of undiluted sample or standard) and any SHH present in the sample binds to the antibody on the plate. The wells were then washed thoroughly and a biotinylated anti-human SHH antibody was added. The wells were washed; HRP-streptavidin was added, incubated for 30min and then washed out thoroughly. Finally TMB substrate solution was added to the wells and colour developed proportionate to the quantity of SHH originally present in the sample. Finally stop solution was added. The reaction was quantified by absorbance measured at 370nm (reference wavelength: 450nm) on a scanning plate reader and the quantity of SHH present could be deduced from the standard curve.

2.2.11 Functional Assessment of Haemopoietic Progenitors

Functional assessment of progenitor and stem cell characteristics can be achieved by using a number of different *in vitro* and *in vivo* assays. While the reconstitution of a haemopoietic system in xenotransplantation experiments remains the gold standard, *in vitro* examination of stem cell numbers and function are important. As time has progressed *in vitro* clonogenic assays have become

more reproducible due to advances in assay systems and the availability of recombinant standard GF combinations and now provide a comparatively cheap, fast and reproducible means of investigating the effect of a drug on stem or progenitor cell function. These assays can be divided into long and short term assays based on the length of time and number of divisions that are required to produce a detectable colony. Thus committed progenitor assays will run over approximately 2-3 weeks and assays for more primitive cells e.g. LTC-IC require significantly longer >5 weeks.

2.2.11.1 Colony forming cell assay

Colony forming cell (CFC) assays are clonal progenitor assays which measure the proliferation capacity and multipotency of haemopoietic progenitors. Cells of interest are inoculated into a semisolid culture medium containing a standard GF cocktail. The cells and their progeny will therefore be kept separate from neighbouring cells. Following culture for 2 - 3 weeks all progenitor cells with colony forming potential in that specific GF context will have proliferated and differentiated fully. The resultant colonies can then be assessed for size (broadly reflective of original proliferative capacity) and constituent cell types from which can be inferred the degree of lineage commitment and hence the maturity of the originator cell. Thus in the case of myeloid CFCs, the distribution and maturity of the progenitor cells assayed can be deduced from the number and relative frequency of single cell myeloid colonies.

Methylcellulose supplemented with GFs (Methocult H4034) was thawed at 4°C overnight, well mixed and divided into 2.6mL tubes. These aliquots could then be stored at -20°C until required. Following recovery, CD34⁺ CML cells were cultured for 72h in either SFM alone, escalating concentrations of LDE225, nilotinib 5µM or a combination of both treatments. The cells were then harvested, washed in DPBS, re-suspended in fresh SFM and a viable cell count performed. Each of the different cell suspensions was inoculated into a single tube of Methocult to give a final concentration of 4x10³ cells/mL and the final volume of inoculum made up with SFM to equal 10% of the original volume of Methocult. These tubes were thoroughly mixed, left to settle at RT and then 1.1mL of resultant single cell suspension plated in duplicate into 35mm culture dishes. The duplicate assays were placed in a 100mm culture dish with a further 35mm dish containing 3mL sterile H₂O to maximise humidity. The cells were cultured under standard

conditions for 14-18d prior to colony assessment using an inverted microscope and a counting grid.

2.2.11.2 Long term culture initiating cell assay

While the CFC assay measures a relatively mature and lineage committed progenitor population, it is not capable of detecting more primitive progenitors. These cells have differing optimal GF requirements and require a longer time and more cell divisions to give rise to differentiated progeny than can be achieved in the CFC system. Extended culture over 5 to 8 weeks is required for the assessment of more primitive progenitors. This time allows the immature cell to complete its differentiation and excludes contribution to the resultant colony formation from residual CFC. Additionally, prolonged *in vitro* culture of HSC will result in differentiation and irrevocable loss of their stem cell characteristics. However if HSC are cultured in the presence of a suitable supportive stromal environment e.g. BM MSCs or similar immortalised stromal cell lines, human HSC can be maintained *in vitro* for at least 8-10 weeks (Cashman et al., 1985; Coulombel et al., 1984; Coulombel et al., 1983; Dexter et al., 1977; Eaves and Eaves, 1988; Eaves et al., 1985a, b; Hogge et al., 1996; Humphries et al., 1981; Sutherland et al., 1989; Sutherland et al., 1990).

LTC-IC is a well-established technique for the enumeration and functional assessment of the primitive progenitor population in haemopoietic samples. Following prolonged stromal-supported culture in conditions favouring myeloid differentiation, the resultant cells are harvested and inoculated into myeloid colony supporting CFC assays. The presence of LTC-IC can then be inferred from the colony growth in the CFC assay (Hogge et al., 1996; Sutherland et al., 1989; Sutherland et al., 1993b; Sutherland et al., 1990). Variations in LTC-IC numbers between treatment arms in an experiment would suggest a difference in stem cell fate and self-renewal.

In these LTC-IC experiments I utilised two immortalised and genetically modified murine bone marrow fibroblast cell lines; SL/SL and M210B4 (Hogge et al., 1996; Sutherland et al., 1991). The SL/SL cell line had been previously genetically modified to stably express human SCF and IL3 and the M210B4 cell line to stably express human IL3 and GCSF. This combination of supportive stroma and GFs has been shown to optimally support myeloid haemopoiesis (Hogge et al, 1996). Both cell lines were cultured and maintained under selection

pressure as described above. Prior to initiating the LTC-IC, the fibroblast cell lines were trypsinised, counted and re-suspended in fresh media in a new tissue culture flask with a non-breathable lid. In order to prevent further proliferation these cells were irradiated under a cobalt γ -ray source for a total of 80Gy. Following irradiation, the cells were trypsinised, washed in DPBS and then re-suspended in optimised long term culture media. A viable cell count was performed and the concentration of each cell type adjusted to 1.5×10^5 /mL. The resultant cell suspensions could then be mixed in a 1:1 ratio and 1mL added to each central well of a 24 well type 1 collagen coated plate (8 central wells) to create a fully confluent stromal layer. Sterile water (2mL) was used to fill all peripheral wells to maximise humidity and the stromal layers were placed in an incubator until required (plates could be kept for up to a week prior to use). Long term myeloid culture medium (LTMCM) was prepared by supplementing Myelocult with hydrocortisone succinate (FW: 484.52) to a final concentration of $1 \mu\text{M}$. This media was stored at 4°C in aliquots and used for all subsequent LTC-IC culture.

In the first set of experiments, following recovery, CD34^+ CP CML cells or non CML CD34^+ were cultured for 72h in SFM (or in the case of non CML CD34^+ cells; SFM+LGF) in the treatment conditions described in the relevant results sections. Following this the cells were harvested, viable cell count assessed, washed in DPBS / 2%FCS and re-suspended in LTMCM. Myelocult was carefully aspirated from the pre-prepared stromal layer wells and equal quantities of treated cells in LTMCM were added to duplicate LTC-ICs. The final volume was made up to 1mL and the plates cultured under standard conditions for 5 weeks. Each week $500 \mu\text{L}$ of media was carefully removed without disturbing the stromal layers and replaced with fresh media. After 5 weeks culture, the entire contents of each well were harvested and inoculated into CFC clonal progenitor assays to assess the presence of LTC-IC. Firstly, the supernatant from each well was collected into a 15mL falcon harvest tube then the wells were washed twice with 1mL HBSS with all liquid added to the harvest tubes. The stromal layer and any adherent cells were removed through the addition of $500 \mu\text{L}$ 0.25% trypsin and incubation at 37°C with intermittent gentle pipetting. Following trypsinisation, $25 \mu\text{L}$ of FCS was added to each well to inactivate the trypsin and the entire contents collected to the harvest tube. The wells were washed in 1mL IMDM / 2% FCS twice with the liquid collected to the harvest tube. The harvest tubes were then centrifuged for 10min at 1000 rpm. Following this the supernatant was carefully poured off and the cells

re-suspended in the remaining volume. This volume was recorded and viable haemopoietic cells counted. Each of the different cell suspensions was inoculated into CFC assays as described in section 2.2.11.1. This allowed comparison of the resultant CFCs generated from the input cells in each treatment arm (Figure 2-4).

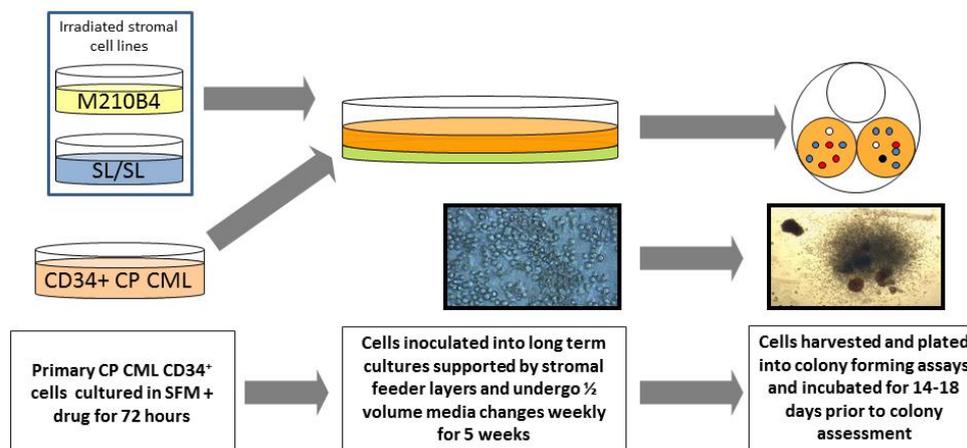


Figure 2-4: Experimental schema for the LTC-IC assay.

Supportive stromal layers were prepared from a 1:1 mixture of irradiated (80Gy) M210B4 and SL/SL cell lines. Primary cells were thawed as discussed above and treated with the test compounds. Following treatment the cells were washed and cultured over supportive stroma in LTMCM for 5 weeks. Stem cells formed cobble stone areas under the stroma as shown above (central image). Following 5 weeks culture the entire contents of the wells were harvested, washed and inoculated into CFC assays. Colony formation was assessed after 14-18d.

A second set of experiments was undertaken to more closely examine the potential role of stromal interactions in the context of the test drugs. Briefly, CD34⁺ CP CML cells were inoculated into duplicate LTC-IC wells without prior culture with test drugs. Instead, the LTMCM in each LTC-IC well was supplemented directly with the relevant concentration of the drug. Assays for LTC-IC progressed as described above except that 80% of the LTMCM was removed and replaced with fresh media and drug each week. Therefore, both the supportive stroma and haemopoietic cells were both exposed to the drug continuously over the 5 week culture period. Following the 5 week culture period the LTC-IC wells were harvested as described above and inoculated into CFC assays for assessment as above.

2.2.11.3 Re-plating assay

While differences in stem cell fate can be inferred from the results of prolonged supported culture experiments, another approach to measuring the

stem cell property harks back to the original experiments performed by Till and McCulloch in 1961 where HSCs in murine BM were found to form colonies in the spleens of irradiated mice and that the cells from these colonies form similar colonies following secondary transplantation (Till and McCulloch, 1961, 1963; Wu et al., 1967). Re-plating colonies from CFC assays with or without supportive stromal support and under various GF conditions is a well-established technique for estimating self-renewal behaviour of cell populations *in vitro*. Essentially each colony formed in a CFC is the progeny of a single cell with a given proliferative potential. Following 14d culture proliferation potential of that cell and its progeny will be extinguished. If this colony is then re-dispersed in fresh media the resultant colony formation will represent the number of daughter cells in the primary colony that have residual proliferative capacity, the existence of cells with secondary colony formation capacity suggests the presence of primitive progenitors / HSC in the primary colony and can be used to approximate self-renewal activity in the sample. This technique has been widely employed in the investigation of HSC properties of normal and leukaemic cells (Carow et al., 1993; Gordon et al., 1998; Humphries et al., 1981; Lu et al., 1993b; Marley and Gordon, 2005; Marley et al., 2001). Here CFC assays were prepared as described above, after 14d culture colonies were assessed and 20-40 non-erythroid colonies were plucked from the Methocult using a pipette and an inverted microscope prior to re-dispersal in 100 μ L fresh Methocult in a 96 well plate ensuring no air bubbles were introduced and that there were no cell clumps. The plates were incubated at 37 $^{\circ}$ C in a humidified atmosphere with a row of empty wells around the edges filled with sterile H₂O. After 7d the wells were assessed for secondary colony formation. The total contents of the wells were then re-dispersed in a further 100 μ L Methocult and tertiary colony formation was assessed a further 7d later as shown in Figure 2-5.

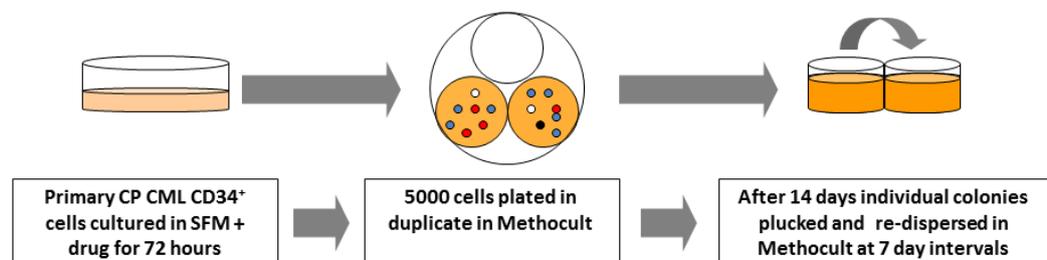


Figure 2-5: Schematic for CFC re-plating assay.

2.2.11.4 Fluorescent in situ hybridisation

Fluorescent in situ Hybridisation (FISH) is a cytogenetic technique that can be used to demonstrate the presence of a particular DNA sequence in the genetic material of a given cell. This technique relies on the use of one or more fluorescent DNA probes designed to be complementary to a highly specific sequence within the gene target(s). Generally speaking the cell sample is treated in hypotonic salt solution to swell the cells and then fixed onto a glass slide prior to dehydration, prolonged hybridisation with the probe and then a series of washes to remove the non-specific signal. The slide preparation can then be visualised by fluorescence microscopy.

In these experiments, the Vysis LSI t(9;22)(q34;q11.2) dual colour, dual fusion translocation probe set produced by Abbott was utilised. Dual colour, dual fusion probes increase the specificity of the FISH signal, reducing the likelihood of false positives. This probe comprises an orange ABL probe and a green BCR probe. The ABL probe binds a 650kb region of chromosome 9 extending from beyond the 5' end of the arginosuccinate synthase gene which is 5' of the ABL gene to beyond the 3' end of the ABL gene. The BCR probe spans a 600kb region of chromosome 22, across the variable segments of the IGL locus and across the entire BCR gene, misses a 300kb region directly 3' of the BCR gene and then spans another 600kb region telomeric of the BCR gene. Generally speaking cells that are BCR-ABL negative would be expected to have 2 green signals representing the two copies of BCR that they possess - one for each chromosome 22 and 2 orange signals representing 2 copies of ABL - one for each chromosome 9. The expected signal from a BCR-ABL⁺ cell would be to have one orange signal (normal chromosome 9) one green signal (normal chromosome 22) and two yellow fusion signals (one for the BCR-ABL fusion on the shortened chromosome 22; the Ph chromosome, and one from the reciprocal translocation on chromosome 9). Although there are other possible patterns in more complex translocations the dual fusion BCR-ABL FISH probe increases specificity by about 5 fold.

Sorted cell subpopulations were re-suspended in 100µL of pre-warmed (37°C) 0.075M KCl and carefully added to Poly L Lysine pre-treated and pre-labelled microspot slides. The cells were incubated at RT for a minimum of 20min or until dry. Freshly made fixative was added to each microspot and allowed to dry. Fixing was repeated 3 times and for the final fixation the slides were immersed in a coplin jar containing fresh fixative for 10min. The slides were then

dried overnight and stored in parafilm at -20°C . For clonogenic assays and LTC-IC, colonies were plucked from the Methocult and processed individually or in pools. The cells were repeatedly washed in DPBS in order to remove the Methocult and then processed as above.

To perform hybridisation, the slides were warmed to RT. They were then placed in a coplin jar containing $2\times\text{SSC} / 0.5\%\text{NP40}$ pre-warmed to 37°C and then subjected to alcohol dehydration (3 coplin jars were prepared with 70%, 85% and 100% ethanol in DH_2O ; the slides were placed in the ethanol, starting at the lowest concentration and moving towards the highest for 5min each). Following dehydration the slides were incubated in formamide denaturation buffer which had been pre-warmed to exactly $73^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for 5min, following which they were again dehydrated by alcohol immersion (5 min in 70, 85 then 100% ethanol). The slides were then air dried and warmed to $40\text{-}50^{\circ}\text{C}$. The probe and hybridisation buffer were mixed according to the manufacturer's instructions, denatured at $73^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the water bath for 5min and $2.5\mu\text{L}$ of the resulting solution applied per microspot. The slides were sealed with rubber cement and a coverslip prior to hybridisation in a humid environment for 16-24h. Following this the coverslip and the rubber cement were removed, the slides were then washed with $2\times\text{SSC} / 0.1\% \text{NP40}$ at RT in a foil covered coplin jar for 1min followed by washing in $0.4\times\text{SSC} / 0.3\% \text{NP40}$ pre-heated to $73^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a foil covered coplin jar for 3min and then in $2\times\text{SSC} / 0.1\%\text{NP40}$ at RT in a foil covered coplin jar for 1min. The slides were dried by capillary action in the dark, DAPI and mounting medium added and sealed by coverslip and nail varnish. The resulting slides could then be examined by fluorescent microscopy and scored as positive, negative or unclear according to the signal seen. In most cases all available cells were examined. Scoring was not blinded, however to minimise interpretational bias, some results were compared with those of a blinded second observer.

2.2.12 Molecular Biology Techniques

2.2.12.1 Extraction of RNA

Following thaw, culture and treatment or FACS, cells were collected, washed in DPBS / 2% FCS, and pelleted in a 1.5ml eppendorf. RNA was either extracted immediately or the pellets were snap-frozen in dry ice prior to storage at -80°C until use. Total RNA was isolated from pellets using the RNeasy mini or micro kits available from Qiagen according to the manufacturer's instructions. The

resulting RNA was quantitated using the Nanodrop 2100 spectrophotometer with absorbance measured at 260nm and the 260/280 ratio determined to ascertain purity. Additionally, RNA integrity was assessed using the Agilent 2100 Bioanalyser platform.

2.2.12.2 Preparation of cDNA

Reverse transcription was performed using the high capacity cDNA kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. In each experiment the same quantity of RNA was processed in each experimental arm to minimise inter-arm variation and in order that the same concentration of cDNA was obtained.

2.2.12.3 Quantitative RTPCR

PCR allows the isolation and amplification of short specific regions of DNA provided at least part of the sequence is known. Briefly, two short oligonucleotide primers are designed, one to be complementary to an area on each of the DNA strands comprising the DNA double helix at opposite ends of the sequence to be amplified (forward and reverse primers). These act as primers for a heat stable DNA polymerase (Taq polymerase). In the presence of these oligonucleotide primers, excess nucleotides and the correct salt balance, heating causes the double stranded DNA to separate - each strand thereby able to act as a template for DNA polymerisation. Cooling allows the primers to anneal to their complementary sequences. The DNA sequence 3' to the primer can then be copied by Taq polymerase forming 2 new complete double stranded DNA molecules extending from forward to reverse primer sequences and incorporating the region of interest between. Successive rounds of heating and cooling will therefore geometrically amplify the sequence of interest for as long as the DNA polymerase works efficiently and primer and nucleotide concentrations in the reaction mixture are not limiting. To investigate gene expression, mRNA species in the cells of interest can be reverse transcribed to cDNA to allow subsequent PCR amplification. Quantitative RTPCR is a well-established technique for measuring gene expression levels in different tissues and is more accurate and precise than standard PCR as measurement occurs after each cycle as the reaction is proceeding and allows assessment of amplicon levels at the exponential phase of the reaction. While there are several qRTPCR strategies available, I utilised the Taqman[®] system. Here, in addition to the two unlabelled

specific PCR primers on either side of the area of interest there is a specific dual-labelled probe for an area within the target sequence. This probe has a 5' reporter fluorophore (e.g. 6-carboxyfluorescein - FAM) covalently bound to the 5' end and a quencher dye (e.g. tetramethylrhodamine -TAMRA) covalently bound to the 3' end. Due to fluorescent resonance energy transfer (FRET) - a distance-dependent phenomenon whereby electronic excitation of a high energy fluorescent molecule is transferred to a nearby low energy acceptor molecule instead of being emitted, as long as both dyes are in close proximity laser excitation will not cause reporter fluorescence. However, when the dye and quencher are separated reporter fluorescence will occur. During annealing, all three complementary sequences bind to the target DNA increasing the specificity of the reaction over conventional PCR and, during the DNA synthesis stage, the 5' - 3' exo-nuclease activity of the Taq polymerase cleaves the probe releasing the reporter fluorophore and causing a measurable increase in reporter signal that is directly proportionate to the amount of target product produced (Figure 2-6).

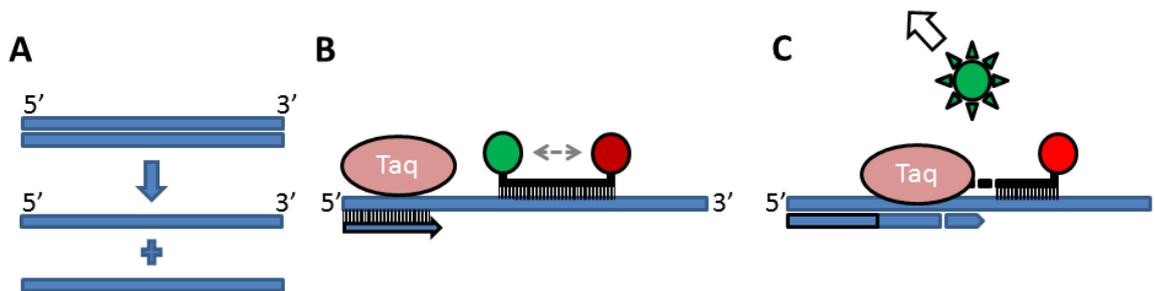


Figure 2-6: Schematic diagram of Taqman[®] chemistry

Step 1 (A) - Denaturation: The DNA template is heated and the strands of the double helix separate. Step 2 (B) -Annealing: The specific primers and the dual labelled probe bind to their complementary sequences. Taq polymerase can begin to initiate nascent strand elongation and the fluorescent reporter label is quenched by FRET due to its very close proximity to the quencher dye. Step 3 (C) – Elongation: Taq polymerase completes DNA synthesis, and 5'-3' exo-nuclease activity degrades the dual labelled probe releasing the reporter dye which fluoresces under the light source of the machine. These steps are cycled repeatedly and the quantity of reporter fluorescence increases in line with the quantity of amplified product which is determined by the quantity of template initially present.

The amount of fluorescence is measured at every cycle and is usually plotted on a logarithmic scale. The exponential phase of amplification can be readily seen and the point at which fluorescence increases above background is the cycle threshold (C_t). Comparison of C_t values provides information about the

abundance of target species and when compared to the expression level of an endogenous control gene chosen because of its stable expression between samples, allows the relative levels of target expression between different samples to be considered (Figure 2-7). The difference between the C_t of the target gene and the endogenous control can be expressed as;

$$\Delta C_t = C_t (\text{target}) - C_t (\text{control})$$

Furthermore the relative change in expression between e.g. treated and untreated cells can be ascertained by calculating the difference in ΔC_t between the cell types;

$$\Delta\Delta C_t = \Delta C_t (\text{treated}) - \Delta C_t (\text{untreated})$$

Thus, as each cycle represents a doubling, the fold difference in expression is approximated by the calculation below, assuming that the relative amplification efficiencies are similar between the comparators.

$$\text{Fold difference} = 2^{-\Delta\Delta C_t}$$

I specifically utilised Taqman[®] probe-sets as they are rigorously quality controlled, optimised mixtures of target-specific forward and reverse primers and dual fluorescence labelled probes. The majority are exon spanning to eliminate genomic DNA amplification and each one has been proven to have a minimum of 90% amplification efficiency.

In this work, three manifestations of Taqman[®] technology were utilised; individual reactions in 384-well PCR plates, Taqman[®] Low Density Arrays (TLDA) and Fluidigm qRT-PCR arrays. The former 2 manifestations were run on the Taqman[®] 7900 platform and the latter using the Fluidigm Biomark platform.

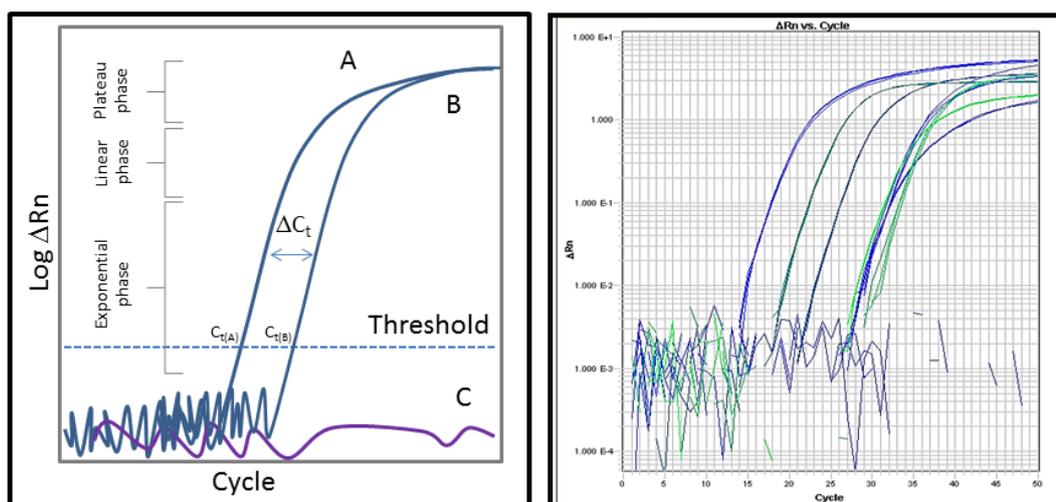


Figure 2-7: Schematic and representative example of Taqman[®] qRT-PCR

The left panel indicates the significant features of a Taqman[®] qRT-PCR reaction where A and B are two different target genes that are amplified at different rates (A representing a high abundance gene target and B a low abundance target gene). C is a target that is not amplified. The threshold line, cycle thresholds for each target gene and the difference between the two (ΔC_t) are indicated. The right panel shows a representative qRT-PCR tracing following a set of qRT-PCR reactions.

2.2.12.4 Selecting an endogenous control gene

Suitable endogenous control genes were selected by comparing the relative stability of 16 common candidate genes across three different CD34⁺ primary CP CML specimens at baseline and following culture in the presence or absence of LDE225 100nM and nilotinib 5 μ M for up to 72h. Isolation of RNA, sample quality control and reverse transcription to cDNA were performed as described above. Each gene expression assay was performed in triplicate. The cDNA synthesised from 100ng RNA from each specimen was mixed in a 1:1 ratio with Applied Biosystems universal master mix, the resultant mixture was loaded into the ports of an Applied Biosystems endogenous control array TLDA card, spun at 1200rpm twice for 1min each, sealed and loaded and run on the Applied Biosystems prism 7900HT real time PCR machine at the standard recommended settings. Variability was assessed by calculating the standard deviation of the C_t values for each candidate endogenous control gene across all samples and by utilising the geNorm program of Vandesompele *et al* to calculate the average expression stability values for the candidate control genes (Vandesompele et al., 2002). For our samples I found *ACTB*, *PPIA*, *HRPT1*, *RPLP0* and *GAPDH* to have the most

stable expression and I utilised *GAPDH* or *HRPT1* as endogenous control genes in all subsequent PCR experiments.

2.2.12.5 Standard individual well qRTPCR

Standard qRTPCR was performed in 384 well PCR plates. All reactions were performed in triplicate 10 μ L reactions. Equal quantities of cDNA template for each sample were diluted with sufficient nuclease free H₂O, thoroughly mixed and 4.5 μ L of the resulting mixture added to each relevant well. Mixtures of 5 μ L gene expression master mix and 0.5 μ L of the required Applied Biosystems gene expression probe set per well (with a minimum of 10% overage) were prepared, well mixed and 5.5 μ L added to each reaction well. No template controls were included in each run. The plates were then sealed, spun at 1200rpm and carefully inspected to ensure that there were no bubbles prior to being loaded onto the Applied Biosystems Prism 7900HT real time PCR machine on standard 384 well settings (50°C for 2 min, 95°C for 10 min followed by 40-50 cycles of 95°C for 15 seconds and 60°C for 1min). The resultant fluorescence was measured, recorded and interpreted by the native software (SDS 3.0 and RQ manager) prior to being exported to Microsoft Excel files for further analysis. Relative quantification was performed as described above.

2.2.12.6 Fluidigm Biomark

The Fluidigm Biomark system is a high throughput, microfluidic dynamic array qRTPCR platform. It utilises integrated fluidic circuits comprised of reservoirs for samples and assays at either side (48 or 96) connected to a network of microfluidic channels, miniscule (10nL) mixing chambers and valves that function automatically to mix the templates and reagents during the PCR reaction. This reduces pipetting steps, and the quantity of reagents required and permits the simultaneous performance of many different qRTPCR reactions on a single sample. Most importantly, the amount of initial starting material can be very small. All samples require to be pre-amplified prior to use.

Briefly, 200ng of RNA from each sorted subpopulation was reverse transcribed as previously described. A 5 μ L aliquot of each sample was pre-amplified using the Applied Biosystems pre-amplification kit and Applied Biosystems Taqman[®] probe sets. Following pre-amplification the resultant amplified products were loaded in triplicate onto 2 primed 48x48 Fluidigm

microfluidic dynamic arrays. The desired Taqman[®] probe sets were loaded onto the chip. The assays and samples were automatically combined on chip prior to thermal cycling and fluorescence detection. This allows the comparison of 48 assays across 15 samples in triplicate and 1 in duplicate. I included a common sample across chips to ensure that inter chip variation was minimal. Amplification curves were produced for each reaction, and expression levels calculated from the mean cycle threshold (C_t) of each triplicate reaction. I utilised GAPDH as an endogenous control gene, calculating the ΔC_t as the C_t of the target - the expression of GAPDH. I expressed the fold change as 2 to the power of the negative difference between the ΔC_t of the target sample and the mean of all the non CML samples i.e. $(2^{-(\Delta C_t [\text{CML sample}] - (\text{mean } \Delta C_t [\text{Non CML samples}]})$).

2.2.12.7 Pre-amplification of cDNA

As primary cell numbers were limited and the gene targets of interest not abundant, in some experiments it was found necessary to amplify the cDNA product prior to quantitative PCR. This was performed using the Taqman[®] Pre-amp master mix kit. This protocol enables the unbiased amplification of up to 100 specific gene targets from extremely small quantities of cDNA (1-250ng). Before pre-amplification, the probe sets of interest were pooled according to the manufacturer's instructions. Each sample was amplified over 14 cycles in a 25 μ L reaction comprising 12.5 μ L of optimised pre-amplification master mix, 6.25 μ L of pooled assay mix and 6.25 μ L of cDNA template. Following amplification, the product could be diluted 1:20 in TE buffer and stored in aliquots at -80°C until required. In order to ensure non biased amplification of Hh mediators and targets, parallel qRT-PCR reactions were performed using amplified and non-amplified cDNA templates on test cDNA according to the manufacturer's protocol. Here, triplicate 10 μ L qRT-PCR reactions were set up comprising of 5 μ L Taqman[®] gene expression master mix, 0.5 μ L Taqman[®] gene expression assay, 2 μ L nuclease free water and either 2.5 μ L of the pre-diluted (1:20) pre-amplified cDNA product or 2.5 μ L of the original cDNA template at a final concentration of 0.3ng/ μ L (assuming 1:1 RNA to cDNA conversion). Uniformity of amplification could be checked by subtracting the average C_t of the endogenous control from the average C_t of the target genes to provide the ΔC_t for each gene in the non-amplified and amplified samples and then subtracting the ΔC_t (unamplified) from the ΔC_t (amplified) to yield the $\Delta\Delta C_t$; a value close to 0 indicated amplification uniformity - I found that all but one probe set (*GLI2*) gave values that were satisfactory ($\Delta\Delta C_t = -0 \pm 1.5$).

2.2.12.8 Statistical Analysis

Statistical analyses of results were performed using Microsoft Excel 2010 or Graphpad Prism v5.0 software to perform Students' T-test or ANOVA as appropriate.

3 Gene Expression Analysis of CML Stem and Progenitor Cells

3.1 Introduction

CML represents an excellent model to investigate the transcriptional changes that occur in oncogenesis and progression of a stem cell driven malignancy. There is also a powerful clinical impetus to better understand the mechanisms that underlie the apparent resistance of CML LSC to current optimal therapy with a view to developing novel therapeutic strategies to target resistant cells. This need has become all the more pressing with the accumulating evidence that CML LSCs persist and remain functional for many years in optimally responding patients. Therefore the majority of patients with robust molecular responses to TKI still relapse following treatment withdrawal. Furthermore, CML LSCs are not dependent on BCR-ABL signalling for survival suggesting that alternative approaches will be required to eradicate this population (Bhatia et al., 2003; Chomel and Turhan, 2011; Chu et al., 2011; Hamilton et al., 2012; Mahon et al., 2010; Ross et al., 2010).

The rapid progress in microarray technology and the bioinformatics that are required to deal with the vast quantities of data produced, now affords the researcher an extremely comprehensive approach to the analysis of gene expression in various tissues. Since the gene expression data that is produced from a given population is ultimately an average of the gene expression activity in every cell therein, in the study of rare cell types, such as HSCs, it is important to begin with as homogenous a population as possible. Recent data refining our hierarchical models of normal and malignant haemopoiesis afford the capacity to purify increasingly enriched HSC populations (Majeti et al., 2007). This affords the opportunity to more clearly establish the transcriptional profile of HSCs and their progenitors, in addition to the changes that occur during differentiation.

As was discussed in detail in chapter 1 (section 1.4.5), several groups have made use of microarray analysis in CML and a wealth of information exists regarding response to treatment, progression to advanced phases of the disease and most relevant to the work undertaken here, defining the differences between various normal populations and the relevant BCR-ABL⁺ equivalents.

The first microarray comparison between diseased and normal haemopoietic cells was in the highly heterogeneous BM MNC populations (Nowicki et al., 2004). Two subsequent studies employed cell fractionation utilising CD34⁺ as a marker for primitive haemopoietic populations in CML and normal tissue

(Kronenwett et al., 2005) (Diaz-Blanco et al., 2007). While CD34⁺ identifies the population containing the vast majority of cells with stem cell characteristics, these are relatively rare cells within this population which is largely comprised of committed mature progenitors whose abundance is different in CML compared to normal samples (Kronenwett et al., 2005) (Diaz-Blanco et al., 2007). An alternative approach utilising hoescht and pyronin to identify quiescent and cycling haemopoietic cells has been employed by another two groups (Affer et al., 2011; Graham et al., 2007). Lastly Bruns *et al*/performed gene expression analysis on the more primitive CD34⁺38⁻lin⁻ cell population and progenitor populations from CP CML and normal volunteers (Bruns et al., 2009). These studies have yielded some insights regarding the transcriptional landscape in CML compared to normal populations but have also provided differing and non-coherent results - particularly with regard to the expression of genes involved in early haemopoiesis and stem cell function. This may be due to the difficulty in comparing these disparate studies as a consequence of differences between microarray platforms used and the purity/method of sorting the populations analysed.

Additionally the above studies investigated transcriptional behaviour in populations in which the transcriptional activity of the HSC population may be obscured by the much greater progenitor population. In order to gain greater insight into the transcription regulation that is required for the maintenance of the HSC population in CML greater resolution is necessary.

One possible refinement could be the expression of CD90 (see detailed discussion in chapter 1, section 1.1.2.4) CD34⁺90⁺ cells are known to harbour an enriched HSC population that is transplantable in mice and in humans (Michallet et al., 2000; Murray et al., 1995). Seminal work by Majeti *et al*/and Notta *et al*/ recently led to significant refinements in our model of early human haemopoiesis. Both groups phenotypically defined human populations with substantially increased HSC activity in CB and BM. Majeti *et al*/defined the population CD34⁺38⁻45RA⁻90⁺ as having the greatest HSC activity in human CB (1:10) and suggested that the population CD34⁺38⁻45RA⁻90⁻ might represent the human equivalent of the murine MPP population although this population was not entirely functionally distinct from the HSC population CD34⁺38⁻45RA⁻90⁺ (Majeti et al., 2007). Notta *et al*/very recently went even further and found that expression of CD49f (integrin α 5) in CD34⁺38⁻45RA⁻90^{+/-} cells was associated with highly enriched HSC activity (Notta et al., 2011). Thus in normal haemopoiesis, at least,

refinement of phenotypic sorting strategies has resulted in increasingly pure HSC populations.

The relevance of such populations in the context of CML is not yet known. While CD34⁺38⁻45RA⁻90⁺ cells have been well characterised in normal human CB and BM this population has not been adequately functionally defined in CML. Brendel *et al*/reported that the majority of CD34⁺/90⁺ cells from patients with CML were BCR-ABL positive (Brendel et al., 1999). Similarly Janssen *et al*/recently reported the presence of a long term colony forming CD34⁺ population with high expression of CD90 in CML and furthermore suggested that the long term CFC within the co-existing residual normal haemopoietic population had a much lower expression of CD90 (Janssen et al., 2012). Likewise the CD34⁺38⁻45RA⁻90⁻ “MPP” population has not been characterised in CML haemopoiesis. However more rigorous phenotypic selection along these lines followed by transcriptional and functional analysis might yield significant insights into the genetic and cellular behaviour of the LSC population in CML, in particular a greater understanding of the survival/proliferation mechanisms and transcriptional programmes that drive LSC fate decisions in CML and, most importantly, how they are altered from the normal state.

Thus, I sought to better define the key differences between normal and leukaemic HSC/LSC by performing global gene expression analysis using new generation Affymetrix whole transcript microarrays on the purest obtainable populations of rigorously defined stem and progenitor populations identified by surface immunophenotype in non-leukaemic patient samples and equivalent immunophenotypic populations in CML.

3.2 Cell Preparation, FACS and Preparation of RNA

Cell processing and sorting were performed by Dr M Copland. Approximately 6-8x10⁷ cryogenically preserved CD34⁺ cells from normal volunteers or patients with CML were thawed and cultured overnight in SFM supplemented with HGF. While it is appreciated that the transcriptome will inevitably change following overnight culture, all samples were treated similarly and this method is consistent with previous methodology from this laboratory and allows the removal of non-viable and apoptotic cells which would otherwise contaminate the transcriptional landscape of the populations under analysis. The following day the cells were thoroughly washed, suspended in PBS with 2% FCS

and stained with antibodies to cell surface proteins. Sorting was performed to isolate the rigorously defined populations described by Majeti *et al* and Manz et al (Majeti et al., 2007, Manz et al. 2002) (see Figure 3-1 and chapter 2 section 2.2.9.7). Briefly, the cells were simultaneously stained with lineage (lin) cocktail-FITC, CD90-PE, CD34PerCP, CD38PE-Cy7, CD123-APC and CD45RA-Pacific Blue. This allowed sorting of the cells into HSC CD34⁺38⁻lin⁻CD45RA⁻CD90⁺; MPP CD34⁺38⁻lin⁻CD45RA⁻CD90⁻; CMP CD34⁺38⁺lin⁻CD123⁺ CD45RA⁻; [GMP] CD34⁺38⁺lin⁻CD123⁺ CD45RA⁺ and MEP CD34⁺38⁺lin⁻CD123⁻CD45RA⁻ sub-populations. Concurrent sorting of up to 4 populations was possible with the fifth population (MPP) swapped in when sufficient cells for one of the other populations had been collected (Majeti et al., 2007) unfortunately CP CML MPP were not able to be collected. Table 3-1 and Figure 3-1 below summarise the sub-populations and samples used. Following sorting, cell populations were confirmed to be highly enriched for BCR-ABL by FISH and RNA extraction was performed. A table providing sorted cell numbers and RNA quantity for each sub-population in each sample is provided in Appendix 1 and the FISH results are shown in Table 3-2 below. This sorting process required many cells and could only be performed a limited number of times and additionally was limited by the number of samples available in sufficient quantity to allow the necessary sorting. Three non CML samples, 6 CP CML samples, 4 AP CML samples and 2 BC CML samples were utilised yielding a total of 67 specimens for analysis.

Sub-population	Immunophenotype	Number of biological samples			
		Normal	CP CML	AP CML	BC CML
HSC	CD34 ⁺ 38 ⁻ lin ⁻ CD45RA ⁻ CD90 ⁺	3	6	4	2
MPP	CD34 ⁺ 38 ⁻ lin ⁻ CD45RA ⁻ CD90 ⁻	3	0	2	2
CMP	CD34 ⁺ 38 ⁺ lin ⁻ CD123 ⁺ CD45RA ⁻	3	6	4	2
GMP	CD34 ⁺ 38 ⁺ lin ⁻ CD123 ⁺ CD45RA ⁺	3	6	4	2
MEP	CD34 ⁺ 38 ⁺ lin ⁻ CD123 ⁻ CD45RA ⁻	3	6	4	2

Table 3-1: Table indicating the immunophenotype and number of samples in each of the progenitor populations analysed.

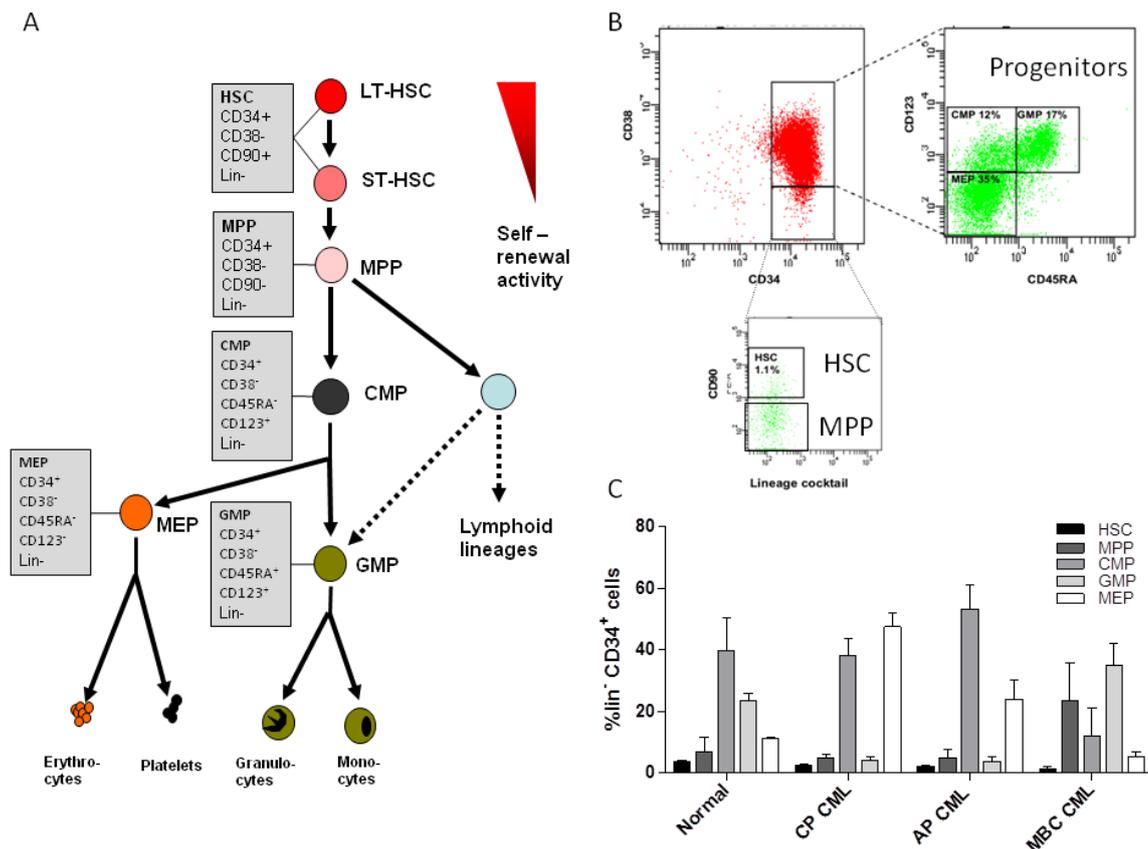


Figure 3-1: Haemopoietic hierarchy and cell sorting strategy.

Panel A shows a simplified view of the haemopoietic hierarchy adapted from Majeti *et al* indicating the cell surface phenotype associated with each population. Panel B shows a representative flow cytometry plots indicating the cell sorting strategy employed. Panel C shows the relative proportions of progenitor sub-populations obtained from sorting CD34⁺ lin⁻ cells from CP, AP and myeloid BC CML or normal cells. Panels B and C are courtesy of Dr Copland

3.3 FISH Analysis of Sorted Populations

Each subpopulation in each sample was analysed by dual fusion FISH (D-FISH) for the presence of the Ph chromosome and scored (see section 2.2.11.4). Virtually all cells of all subpopulations were Ph⁺ by D-FISH analysis. There was no significant difference between subpopulations from individual patients or between subpopulations derived from samples from differing stages of the disease. Greater than 90% of the LSCs in all patients were Ph⁺ confirming that the translocation arises in the most primitive haemopoietic cells that can be isolated by surface phenotype and is the dominant clone in all myeloid progenitor subpopulations (Table 3-2).

Patient	% BCR-ABL FISH positive				
	HSC	MPP	CMP	GMP	MEP
CP CML 1	98	-	100	100	99
CP CML 2	89	-	90	89	90
CP CML 3	-	-	-	-	-
CP CML 4	100	-	91	98	100
CP CML 5	98	-	97	100	100
CP CML 6	87	-	94	91	97
AP CML 1	98	-	97	100	100
AP CML 2	92	-	98	95	98
AP CML 3	100	100	100	100	100
AP CML 4	100	92	100	100	100
BP CML 1	100	92	100	100	100
BC CML 2	98	98	100	98	100

Table 3-2: FISH for BCR-ABL translocation in samples utilised for gene expression studies.

Percentage of cells Ph⁺ by FISH analysis in each sorted population across CP, AP and BC samples. FISH was performed by David Irvine and Heather Morrison.

3.4 Affymetrix Gene Expression Microarray

Affymetrix gene expression arrays are economical in comparison to high throughput sequencing and represent a relatively mature and robust platform for global gene expression analysis. Advances in technology have led to both increased density of probesets, increased signal to noise ratio (enabling rarer transcripts to be detected with greater sensitivity), and a reduced requirement of starting material. We elected to run our samples on the Affymetrix GeneChip Human Gene 1.0ST array system. This is a modern whole transcript gene expression array that represents a significant improvement from prior manifestations of this technology. It is designed to interrogate 28,869 well annotated transcript targets with 764,885 distinct 25-mer oligonucleotide probes. The RNA samples were provided to the Sir Henry Wellcome Functional Genomics Facility (SHWFGF) at the University of Glasgow who performed sample quality control, processing, labelling, application of the samples to the microarray chips and post hybridisation quality control (QC). The data was returned in the form of .CEL files (recording fluorescence intensities for each genetic target in the array). The process is briefly described below. RNA samples were quantitated and contamination measured by spectrophotometry using the Nanodrop system and

RNA integrity assessed by Agilent Bioanalyser microfluidics system at the SHWFGF.

Sample preparation was according to the manufacturer's instructions for the Ambion WT Expression system. Labelled, fragmented cDNA was prepared from total RNA through a series of steps which minimise ribosomal RNA contamination while amplifying and maximising recovery of target sequences. The resulting samples were hybridised to the microarray chips, washed and then scanned. In the Affymetrix GeneChip Human Gene 1.0ST array the hybridisation reaction requires terminally labelled cDNA prepared from the target RNA to anti-sense probes. Here, perfect match only probe-sets are used, but there are internal background assessment probes in addition to labelling and hybridisation control probe-sets enabling both initial QC and background normalisation. Each interrogated gene has probe-set targets in every exon throughout the transcript (a median 26 probes per gene). Thus these microarrays have increased sensitivity, less bias, should correctly register all splice variants of each gene and can be analysed at either exon or gene level. For each microarray a .DAT file is produced (raw pixelated image of the scanned array) and following comparison with the probe library and grid structure of the array a single fluorescence intensity value is provided for each feature (.CEL) file which is ready for normalisation, summarisation and down-stream analysis.

3.5 Validation of Microarray Findings using qRT-PCR and the Fluidigm Platform

Despite the substantial improvements in technology discussed above, microarray analysis remains only the first step in analyses of the molecular events that underpin cellular behaviour. Gene expression at the RNA level may not correspond to variations in protein levels. Validation is required using a different platform and preferably one would wish to confirm, not only that the gene expression data produced is replicable, but that there are commensurate changes in the effector molecules, usually proteins. This can represent a challenge in stem cell biology as cell numbers are limiting and protein studies therefore extremely difficult to perform. Here qRT-PCR using the Fluidigm Biomark platform was chosen to validate the microarray findings (see methods section 2.2.11.4). Several groups of genes differentially regulated in the microarray dataset were selected

and qRT-PCR performed to determine their relative expression. The qRT-PCR are presented for comparison alongside the microarray data as it is discussed.

3.6 Results

Both the raw CEL files from this work and the normalised expression data derived from my analysis have been uploaded onto the public GEO database (<http://www.ncbi.nlm.nih.gov/geo>) and can be accessed under accession number GSE 47927.

3.6.1 Global gene expression analysis

The annotated raw fluorescence intensity (.CEL) files were uploaded into Partek Genomic Suite (Partek GS), a third party bioinformatics platform. Quality control metrics were assessed and no outliers or problematic arrays were identified; therefore all were included in subsequent analyses. Background correction and normalisation was performed using Partek GS which implements RMA - a well-recognised multichip normalisation method (Shah et al., 2004) in which the background is corrected for by subtraction of the background values from the positive match (PM) probes, followed by quintile normalisation across all the arrays, log₂ transformation of the values and final summarisation using the median polish procedure (a robust summarisation that reduces the effect of outliers on the median value). Summarisation of probe intensities to gene level targets was then performed. The uploaded, summarised and normalised dataset was subject to global analysis and clustering to investigate broad differences in expression. I performed PCA on the entire data set. This is a statistical technique that allows very complex multi-dimensional variability in a data-set to be represented by a smaller (e.g. 3) number of uncorrelated components (principal components) (Peterson, 2003). This analysis is particularly good for graphical representation of the variation within data sets and groups as differences can be visualised as distances along axes representing the various principal components. The results of this analysis are presented in Figure 3-2.

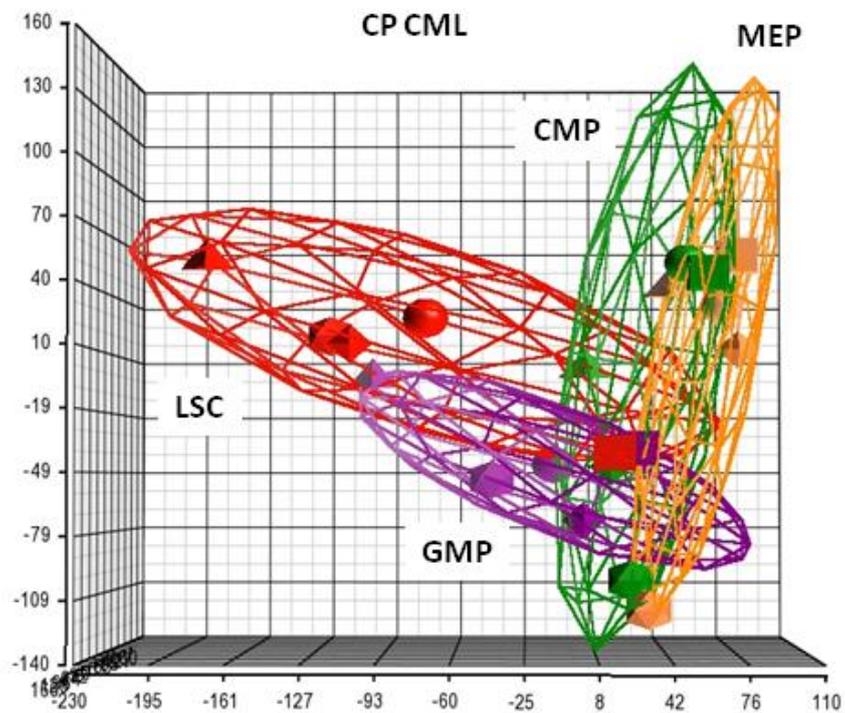
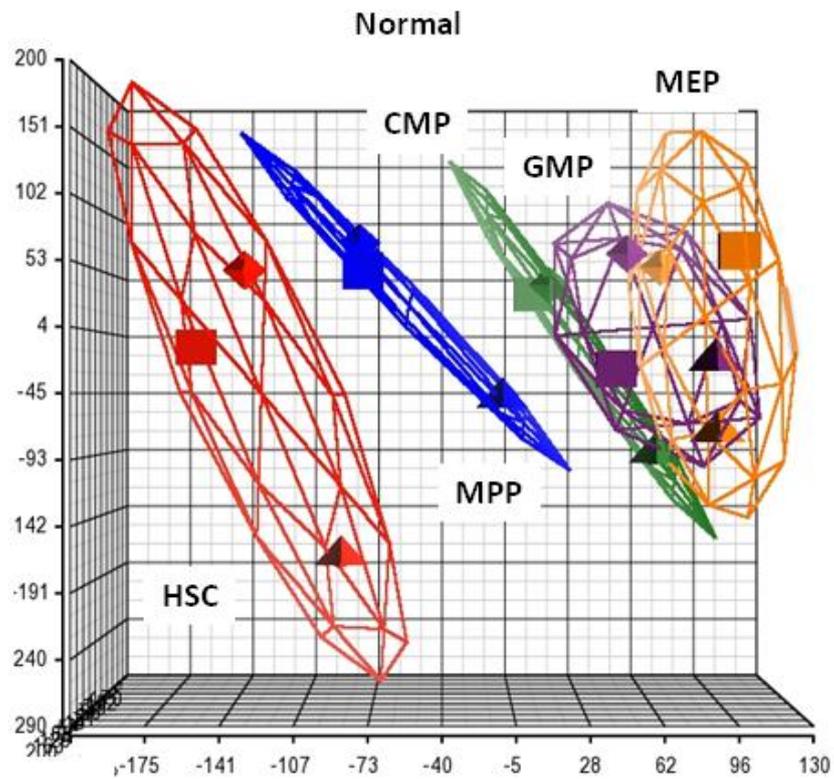


Figure 3-2: PCA of non CML and CP CML populations.

Each axis represents a principal component of the variability in the normal and CP samples described in Table 3-1. Each microarray is represented by an individual point within the axes and each sub-population grouping by a different colour as shown (red; HSC/LSC, blue; MPP, green; CMP, purple; GMP, and orange; MEP). An ellipse is positioned at 2 standard deviations (2SD) around each population.

In the normal samples it is immediately apparent that the variation within each sub-population group is small compared to the CP CML samples and that each cellular sub-group represents a distinct entity at the transcriptional level. Furthermore, there is a striking progression from primitive to relatively mature sub-populations in terms of general similarity in global gene expression. In CP CML this progression appears to be significantly disordered; in particular the LSC grouping significantly overlaps the progenitor groupings indicating similarity in global gene expression. Furthermore when both normal and CP CML sub-populations are plotted together it is clear that LSC from patients with CP CML have a global gene expression signature that appears significantly more mature than healthy HSC, and is positioned between the normal MPP and other progenitor sub-populations in the PCA plot in Figure 3-3.

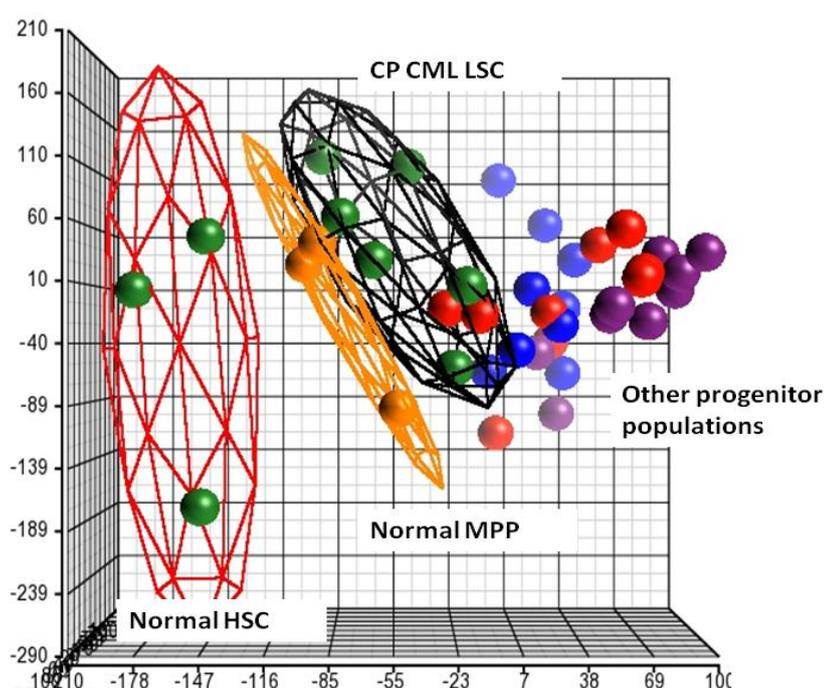


Figure 3-3: PCA of non CML and CP CML HSC/LSC and MPP populations.

Here the PCA analyses of CP CML HSC and non CML HSC / MPP are superimposed on the same axes. Again each microarray is represented by an individual point and each sub-population grouping of interest is differently coloured (green for HSC and orange for MPP). An ellipse is positioned at 2SD around the non CML HSC (red), non CML MPP (orange) and CP CML LSC (black). The other progenitor populations are shown but not circumscribed by ellipses.

In advanced CML there is increased overlap between the sub-populations indicative of increasing variation both between different biological specimens and within the different sub-populations in each specimen. This is reflective of increasingly disordered gene expression and loss of the gene expression patterns

seen in normal haemopoiesis (Figure 3-4). The two BC specimens analysed were found to have quite different gene expression profiles demonstrating significant biological variability in advanced disease.

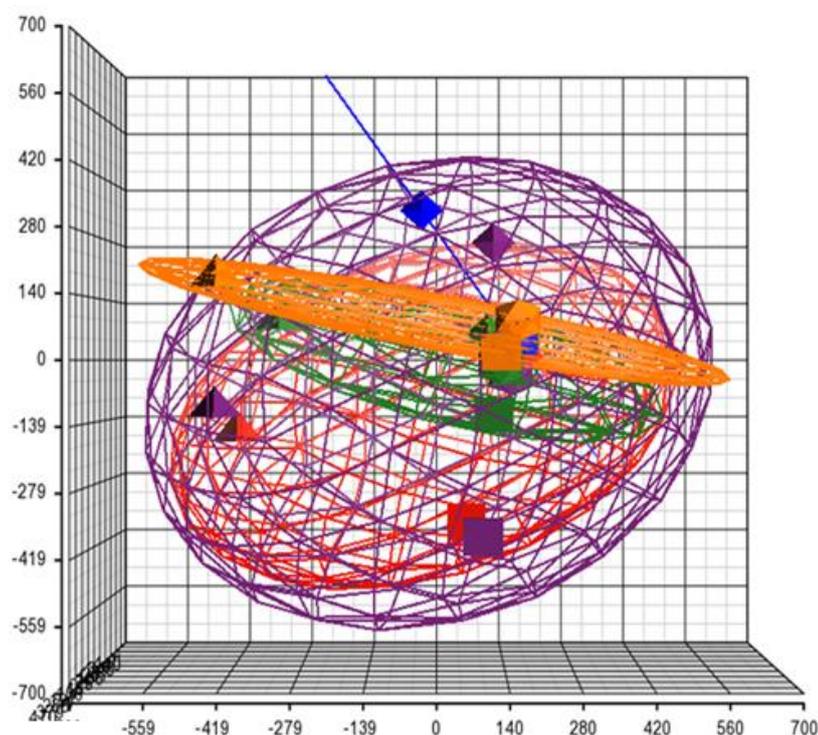


Figure 3-4: Principal components analysis of AP CML populations.

Each axis represents a principal component of the variability in the samples. Each microarray is represented by an individual point within the axes and each sub-population grouping by a different colour as shown. An ellipse is positioned at 2 standard deviations (2SD) around each population. The different populations are as before: red; HSC, blue; MPP, green; CMP, purple; GMP and orange; MEP.

3.6.2 Contrasts and comparative gene expression analysis

In order to look specifically at gene expression differences between our populations, the raw data was imported into Partek GS which is an ANOVA based bioinformatics system designed for analysis of microarray and similar data. Essentially ANOVA is a model based system that allows the variability within a dataset and the contribution of various experimental factors to that variance to be assessed jointly. Partek GS implements a sophisticated and flexible ANOVA model allowing balanced or unbalanced experiments, random and fixed effects with any number of factors and linear contrasts (comparisons between one group and another group) (Ayroles and Gibson, 2006; Churchill, 2004). Following log transformation, normalisation and summarisation, which presents the data in a more evenly distributed manner, I performed one way ANOVA using Partek GS,

sequentially contrasting every sub-population with every other sub-population in our data set. Figure 3-5 indicates the possible contrasts between microarray samples. As a result of these analyses lists of differentially expressed genes with fold change and p-value data for each possible comparison were generated and then ranked in descending order of significance.

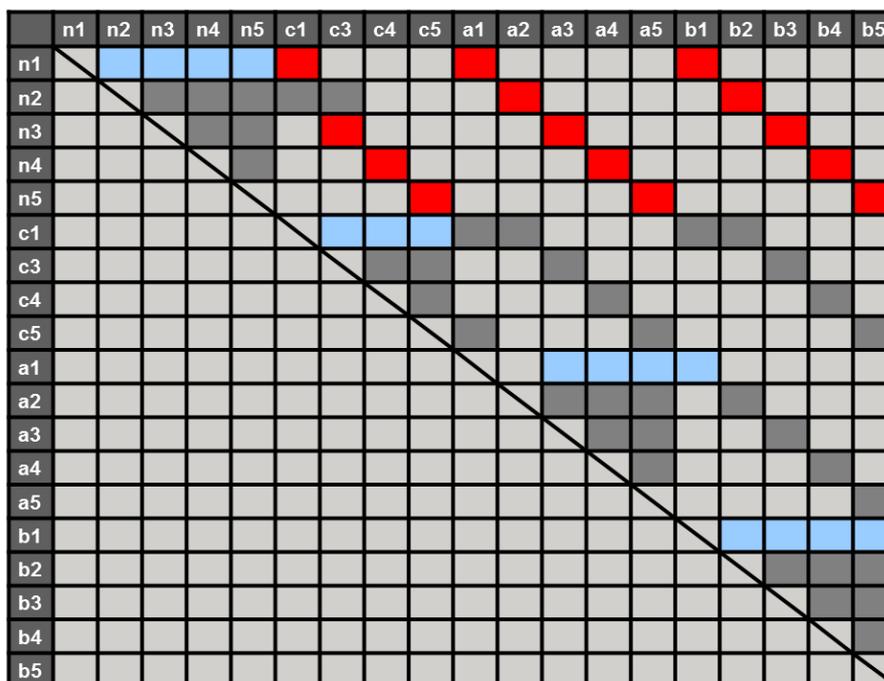


Figure 3-5: Diagram showing potential microarray contrasts.

Here the ANOVA contrasts performed are represented. Population groups are noted across the top and left hand side of the table. Contrasts can be made between mean gene expression levels in each grouping. Each box above the diagonal represents a potential comparison. Contrasts between disease states are represented in red whereas contrasts between sub-populations within a particular disease state are represented in blue. Population abbreviations are as follows (N; normal, C; CP CML, A; AP CML, B; BC CML, 1; HSC/LSC, 2; MPP, 3; CMP, 4; GMP, 5; MEP).

Due to the volume of data and the difficulties involved in the analysis of multiple cell populations across multiple disease states, I elected to focus our subsequent investigations largely on the differences between CP CML and normal haemopoietic tissue and given our findings from our global analyses above I was particularly interested in the gene expression differences that appeared to exist between normal and malignant HSC and their progeny. Multiple comparisons were controlled by utilising the FDR as described by Benjamini and Hochberg electing a threshold of 0.05 (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003). I analysed differential gene expression by comparing each CP CML sub-population with its normal counterpart and also by comparing differentially expressed genes between primitive and mature sub-populations in normal and CP

CML populations. Lists of differentially expressed genes were prepared as illustrated in Figure 3-6.

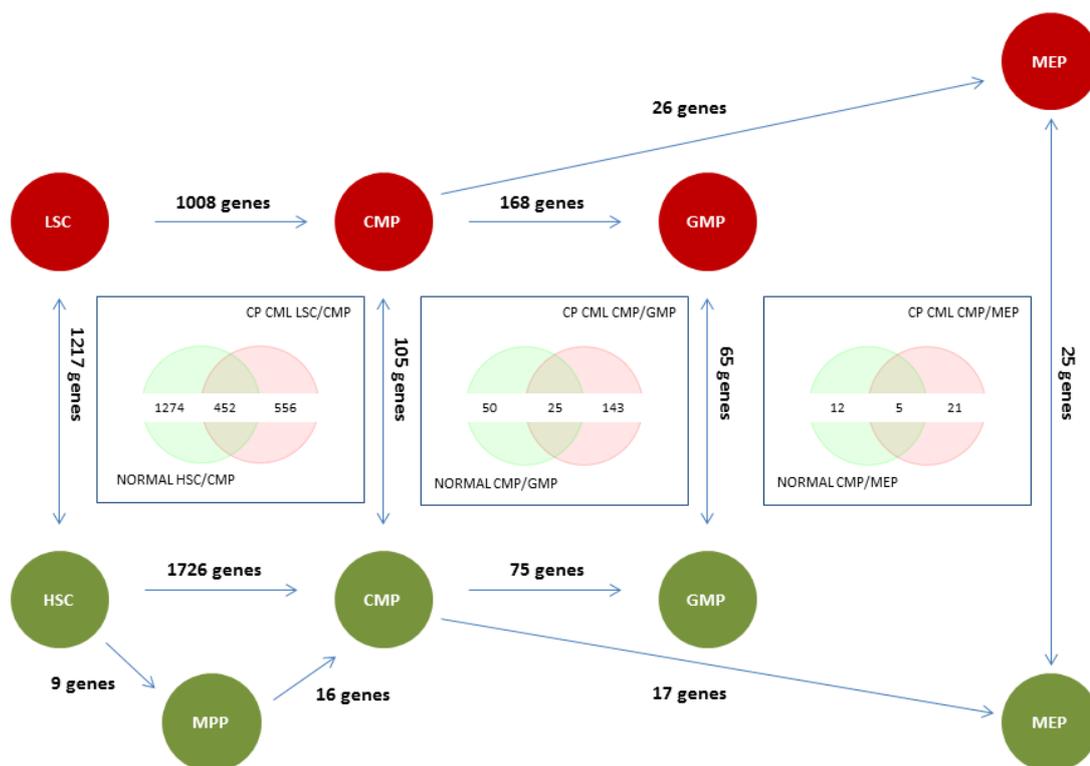


Figure 3-6: Comparison of gene expression along each haemopoietic hierarchy and between each corresponding sub-population isolated from normal and CP CML patients.

The numbers next to the arrows indicate the number of differentially expressed genes between the two populations specified. CP CML is presented in red and normal haemopoiesis is presented in green. Additionally, Venn diagrams illustrate the differentially expressed genes in each transition unique to CP CML (red), normal haemopoiesis (green) and those in common (intersection). Thus for example in the HSC to CMP transition, 1274 genes were differentially regulated between normal HSC and CMP whereas 556 were differentially regulated between CP CML LSC and CMP and 452 were differentially regulated in both normal and CP CML.

An initial appraisal indicates that there are many significantly deregulated genes between CML and normal subpopulations and that the relative difference between sub-populations both along the differentiation path and between normal and malignant tissue becomes progressively smaller with maturity. Following creation of these lists I utilised gene ontology classification, gene ontology (GO) ANOVA in Partek GS and IPA software to place the data in a functional context to better understand the biological differences between CML and normal HSC and progenitors.

GO ANOVA utilises the gene ontology database and has the advantage of being able to be applied directly to the data rather than requiring post processing

and filtration. I performed GO ANOVA on the signal intensity imported normalised data sheet using the standard settings. Partek GS maps the genes to functional groupings. The ANOVA model was configured to compare the different sub-populations and disease states in order to determine the significance and average fold change of each GO category across all the comparator groups. These results were visualised using dot plots where the average expression of all genes in that functional group is represented as a single dot. The average expression is plotted on a log₂ scale on the y axis - and each subpopulation in each disease state on the x axis.

3.6.2.1 *Genes expressed at different stages of maturity*

The patterns of gene expression in normal and malignant haemopoiesis were compared as transiting occurred from the HSC population to the more mature progenitor populations. It was anticipated that this would provide evidence that the sorting strategy had isolated defined populations at the gene expression level in both sample groups. The median expression of CD34, CD38 and CD90 was as expected both in normal and malignant haemopoiesis (Figure 3-7).

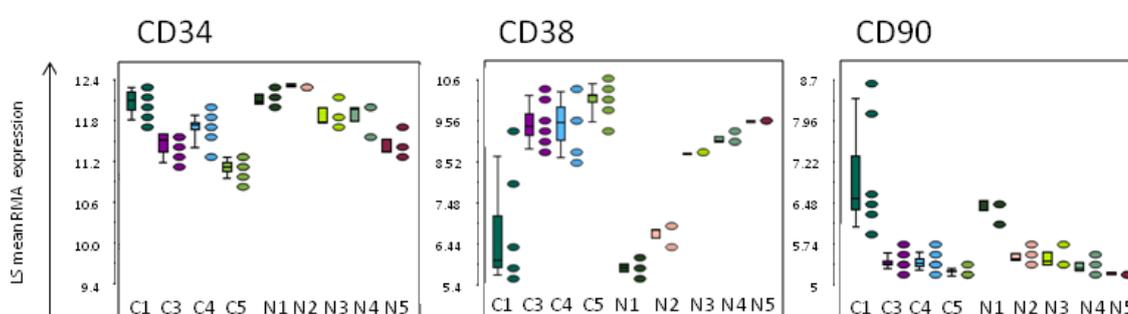


Figure 3-7: Relative expression of the primary cell surface antigens used to sort the HSC populations.

Here each point represents a single sample. The box describes median and quartile values, whiskers represent 10th and 90th percentiles. Population abbreviations are as follows (N: normal, C: CP CML, 1: HSC, 2: MPP, 3: CMP, 4: GMP, 5: MEP).

Further confirmation of the segregation of these populations was obtained by the demonstration of increased expression of both granulocytic/monocytic and megakaryocytic/erythroid specific genes in the CMP population but differential expression of genes related to erythroid/megakaryocytic function and development in the MEP population and genes related to monocytic/granulocytic development and function in the GMP population in both normal and malignant tissue although the expression differences are less marked in CP CML (Figure 3-8).



Figure 3-8: Heat map demonstrating expression of haemopoiesis related genes across HSC and progenitor populations from normal and CP CML samples.

Mean expression levels between normal control and CP CML sub-populations from the microarray experiment described above and shown table 3-1. Expression are ordered with respect to the normal MEP population and expressed relative to the normal HSC population (blue denotes down regulation and red denotes up regulation). Genes were selected from GO classifications from Partek GS and IPA to reflect different haemopoietic cell signatures e.g. erythroid, megakaryocytic, granulocytic and HSC cell function. The majority of megakaryocytic genes localise to the top as they are most highly expressed in the normal MEP population, several granulocytic/monocytic genes are present in the middle of the heat map and the majority of genes with a role in primitive haemopoiesis localise to the bottom of the heat map. Population abbreviations are as follows (N: normal, C: CP CML, 1: HSC, 2: MPP, 3: CMP, 4: GMP, 5: MEP). Expression levels are the mean of the biological replicates described in section (N: n=3; C: n=6).

3.6.2.2 Comparison of normal MPP and HSC population

Comparing normal HSC with MPP it was surprising to find that only 9 gene targets appeared to be differentially regulated when the significance criteria were applied (Figure 3-6). These included down regulation of Ca²⁺-dependent secretion activator 1 (*CADPS2*), a plasma membrane protein normally involved in exocytosis of neurotransmitter vesicles and transmembrane and tetratricopeptide repeat containing 1 (*TMTC1*), a cytoplasmic protein with poorly defined role in mitochondrial function, as well as up regulation of non-SMC condensin I complex, subunit H (*NCAPH*), a regulatory protein involved in chromatin condensation, dermokine (*DMKN*), a developmental cytokine, solute carrier family 7, amino acid transporter light chain, L system member 5, (*SLC7A5*), critical for the transport of neutral amino acids into cells, and *LMNB1*, a nuclear laminar protein providing nuclear stability and interacting with chromatin and other nuclear proteins notably HOXA9, cAMP responsive element binding protein (CREB) and MEIS1 to influence gene expression. The remaining three targets were unclassified. The small numbers probably reflects the stringency of our criteria as applied to two populations which are comparatively similar - i.e. the global expression difference may be comprised of relatively small changes in abundance of transcripts that are not reliably detectible in this system due to biological variability and the lack of sufficient replicates or that small differences were obscured by the microarray normalisation procedure (Table 3-3).

Symbol	Gene	p- value	FC
<i>LOC1720</i>	-	1.22E-05	-4.4
<i>NCAPH</i>	non-SMC condensin II complex, subunit H2	7.24E-06	-4.4
<i>LMNB1</i>	lamin B1	1.24E-05	-2.5
<i>SLC7A5</i>	solute carrier family 7	1.12E-05	-2.0
<i>TMTC1</i>	transmembrane and tetratricopeptide repeat containing 1	2.34E-07	1.6
<i>DMKN</i>	Dermokine	7.35E-06	1.7
<i>CADPS2</i>	Ca ²⁺ dependent secretion activator 2	1.28E-15	1.9

Table 3-3: Differentially expressed genes between normal HSC and MPP.

Positive values indicate increased expression in HSC. Expression levels are the mean of several biological replicates as described in table 3-1 (n=3).

3.6.2.3 Comparison of HSC and CMP

When the HSC populations were compared to their equivalent CMP population in normal haemopoiesis or CP CML, 1726 and 1008 differentially expressed genes were identified between the primitive and mature populations respectively (Figure 3-6). These genes were further analysed in IPA. The

predominant functions were related to cell cycle control and cell assembly, haemopoiesis, development, differentiation and DNA replication and repair reflecting the transit to the more proliferative but increasingly lineage restricted state expected of the progenitor population.

A direct comparison between CMP from CP CML patients and CMP from our normal volunteers was performed. In total 105 differentially expressed genes met significance criteria. These were subjected to gene ontology and pathway analysis in IPA and Partek GS. The most differentially regulated functional classification was related to immunosurveillance and antigen presentation. Significantly reduced expression of class 1 and class 2 histocompatibility complex and related genes were noted in CP CML CMP. Figure 3-9 and Table 3-4 indicate expression of key antigen presentation genes identified in Partek GS gene GO classifications for antigen presentation. Interestingly, in this category there was up regulation of 2 genes *ATP7A* and *RGS6* in CP CML; *ATP7A* is an important mediator of copper homeostasis and *RGS6* is a G protein associated protein.

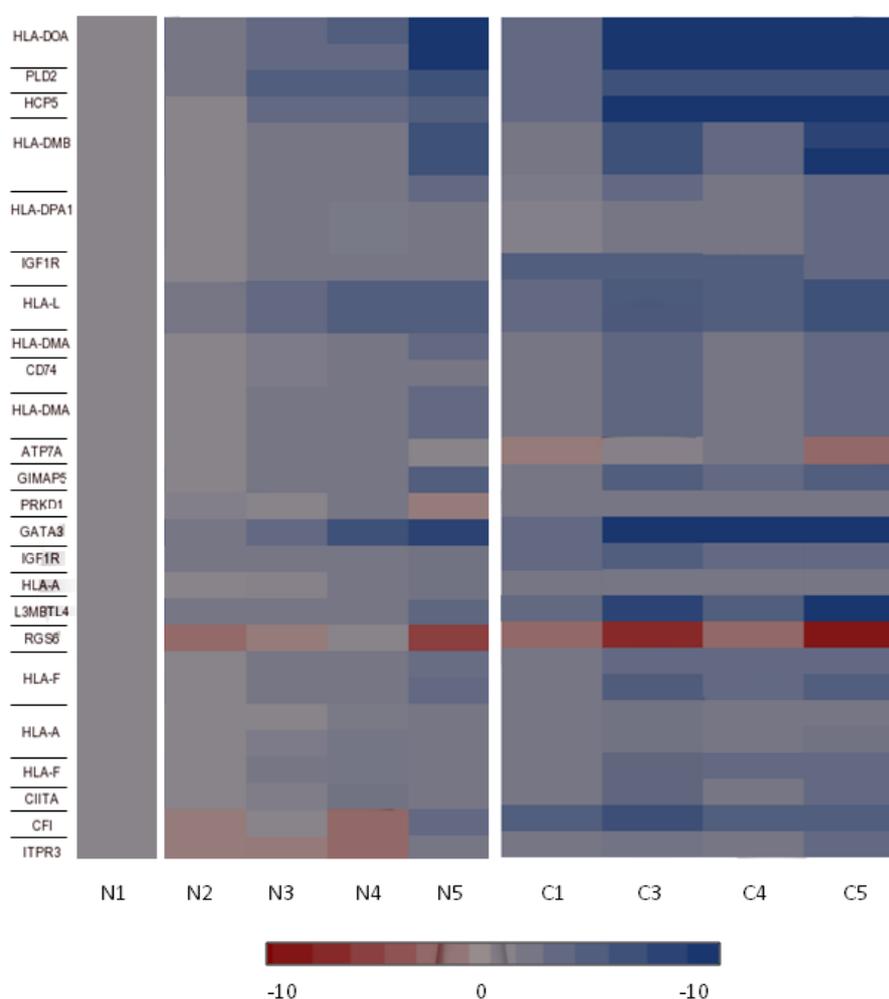


Figure 3-9: Heat map representing the expression level of various genes associated with antigen presentation in normal haemopoiesis and in CP CML.

All gene expression was expressed relative to the normal HSC population with blue representing reduced expression and red increased expression. Gene symbols are summarised in the list of abbreviations. Population abbreviations are as follows (N: normal, C: CP CML, 1: HSC, 2: MPP, 3: CMP, 4: GMP, 5: MEP). Expression levels are the mean of the biological replicates described the preceding sections and table 3-1 (N: n=3; C; n=6).

Symbol	Gene	p- value	FC
<i>HLA-DMA</i>	major histocompatibility complex, class II, DM alpha	2.51E-07	-1.8
<i>HLA-DPA1</i>	major histocompatibility complex, class II, DP alpha 1	1.11E-07	-1.8
<i>CD74</i>	CD74 molecule, major histocompatibility complex, class II invariant chain	1.47E-07	-1.8
<i>HLA-F</i>	major histocompatibility complex, class I, F	1.20E-04	-2.0
<i>HLA-A</i>	major histocompatibility complex, class I, A		
<i>HLA-DQA1</i>	major histocompatibility complex, class II, DQ alpha 1	2.68E-03	-2.1
<i>CIITA</i>	class II, major histocompatibility complex, transactivator	8.70E-04	-2.1
<i>HLA-DRA</i>	major histocompatibility complex, class II, DR alpha	3.85E-07	-2.1
<i>HLA-DPB1</i>	major histocompatibility complex, class II, DP beta 1	6.74E-05	-2.3
<i>HLA-DQB2</i>	major histocompatibility complex, class II, DQ beta 2	9.10E-05	-2.4

Table 3-4: Expression of selected antigen presentation genes in normal versus CP CML CMP.

Negative fold change represents reduced expression in CP CML CMP.

Several other classes of genes were also differentially expressed between CMP in normal and CP CML samples. I noted significantly increased expression of several genes associated with erythroid development and function such as ankyrin 1 (*ANK1*), spectrin B (*SPTNB*) and blood group Kell (*KEL*). This has been previously reported in CML CD34⁺ cells and may represent an MEP bias in developing CML progenitors (Bruns et al., 2009; Diaz-Blanco et al., 2007; Graham et al., 2007). Additionally several genes involved in cell-cell interaction and adhesion were significantly reduced in CML, these included epithelial cell adhesion molecule (*EPCAM*), N-cadherin (*CDH2*) an important constituent of niche signalling, fibulin 5 (*FBLN5*) and *CD96*. Interestingly, in CP CML, I found down regulation of several genes with a role in cell fate determination including insulin-like growth factor receptor 1 (*IGFR1*) - which has a role in oncogenesis and cell survival (Shimon and Shpilberg, 1995), and brain and acute leukaemia, cytoplasmic (*BAALC*) which interacts with the HOX transcription factors and has a role in differentiation inhibition and leukaemogenesis (Heuser et al., 2012). Additionally there was down regulation of the self-renewal modulator and candidate oncogene SET binding protein 1 (*SETBP1*) which is discussed in greater detail in subsequent paragraphs. Thus CP CML CMP have down regulated antigen presentation machinery and exhibit a relatively mature transcriptional profile that does not include an excess of stem cell or self-renewal associated genes.

3.6.2.4 Comparison between the GMP and MEP progenitor populations

The relatively mature GMP and MEP populations were more similar, with few differentially expressed genes between normal and CML haemopoiesis, reflecting the fact that as haemopoiesis progresses normal differentiation programmes are established and functional progenitors and effector cells are produced in both normal and CP CML.

3.6.3 Comparison between normal and leukaemic HSC

While the above contrasts yielded interesting information about potential differences in gene expression between normal and CP CML at the committed progenitor level, it is the comparison between the HSC/LSC populations that was felt to be most interesting. There were two reasons for this; firstly, no prior microarray analyses has been performed comparing normal and CP CML LSC at

this resolution and secondly, from the analyses above it is clear that the largest differences at the gene expression level are between the HSC/LSC populations. At an FDR level of 0.05 over 1200 genes were differentially expressed between the HSC/LSC populations. These genes were subject to ontological and pathway analysis using Partek GS and IPA to place these differences in a functional context. The key functional differences between these populations were related to cell cycle progression, DNA replication, recombination and repair, cellular organisation, maintenance and movement and energy production and nucleic acid metabolism. Many of these entities mapped to functional categories associated with cancer signalling and haematological disease or to physiological processes related to embryonic or adult tissue development and differentiation e.g. haemopoietic myeloid and lymphoid tissue development (Figure 3-10 and Figure 3-11).

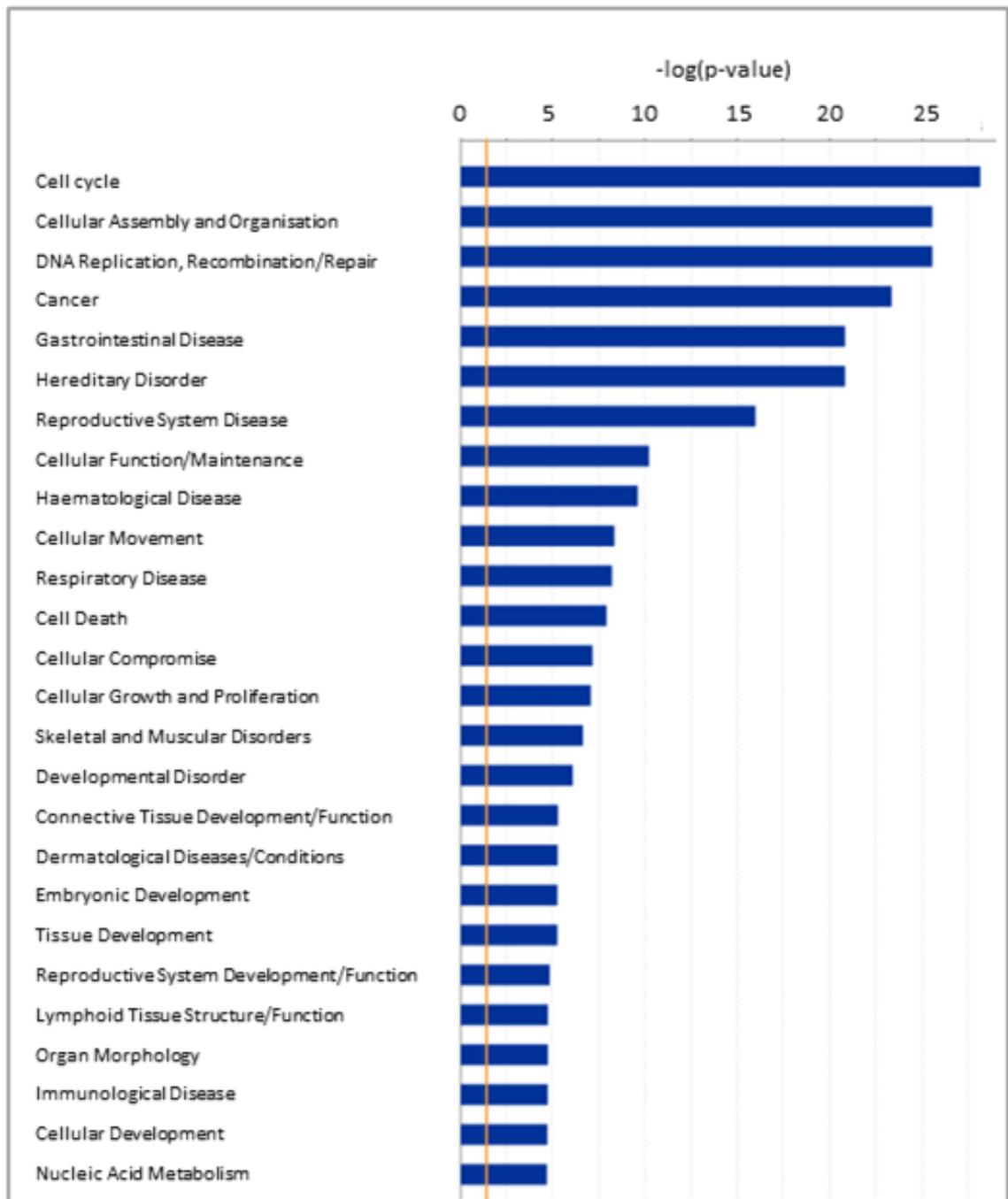


Figure 3-10: Ingenuity® pathway analysis comparing normal HSC and CP CML LSC.

Differentially expressed genes are assigned to broad functional groupings or to canonical pathways according to their known function and interactions. This graph shows the most significantly enriched functional groupings in CML HSC compared to normal HSC i.e. C1 compared with N1. Significance is presented as the negative log of the p-value of the group overlapping with the functional category and is measured using Fishers exact test. A significance threshold ($p=0.05$ or $-\log 1.3$) is shown by the orange line.

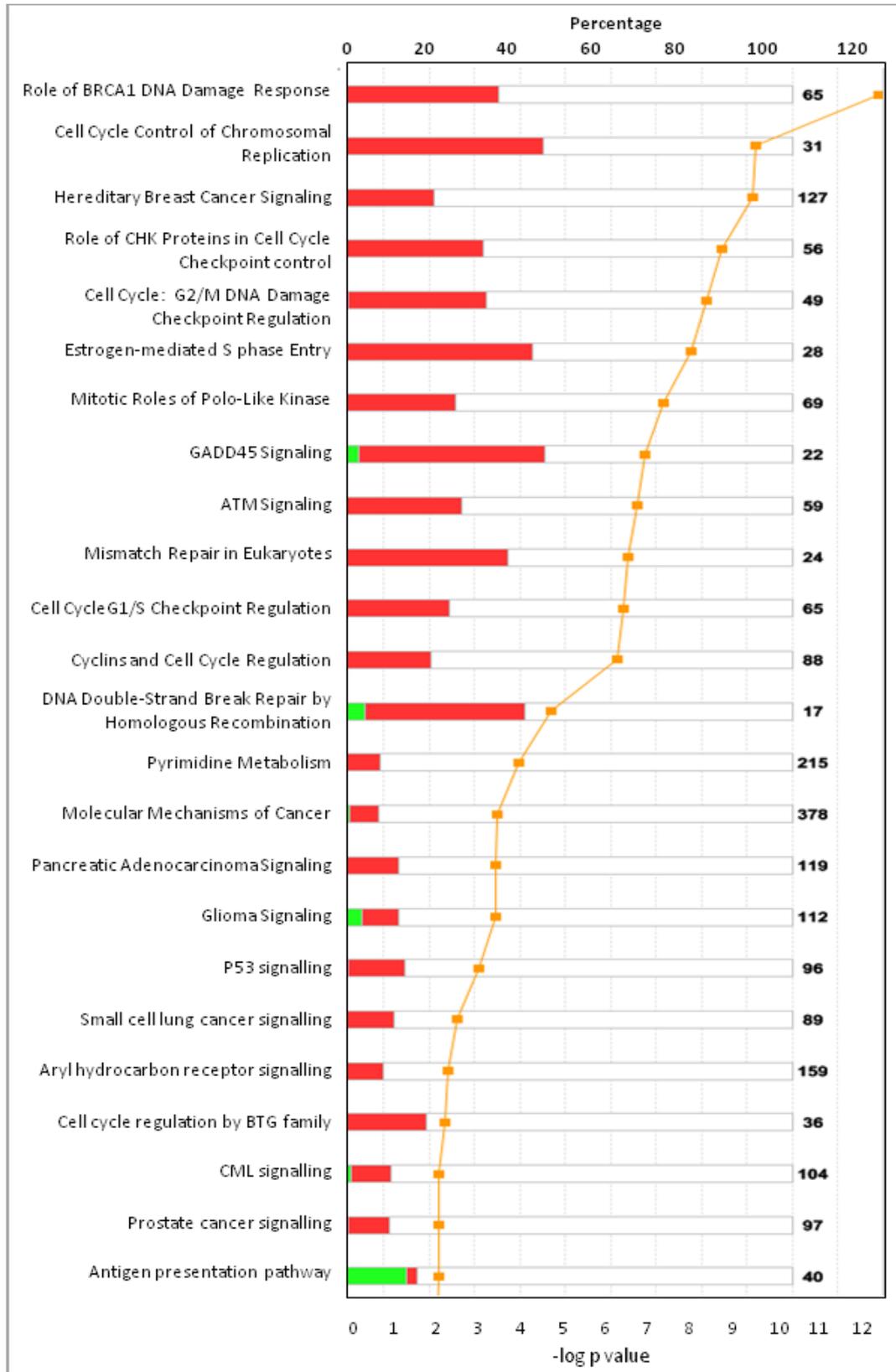


Figure 3-11: Ingenuity® pathway analysis comparing normal HSC and CP CML LSC showing the most significantly differentially expressed canonical pathways.

Data is presented as a stacked bar chart where red represents up regulation in CP CML and green down regulation. The total number of genes included in the pathway is presented at the head of the bars and the proportion of differentially regulated genes represented by the bars as a percentage of that total. Orange line denotes significance level (-log p value).

3.6.3.1 Cell cycle, growth and proliferation

There was evidence of profound up regulation of the cell cycle machinery in CP CML LSC compared with non CML HSCs. In total 209 genes mapped to GO classifications across all aspects of the cell cycle were differentially expressed; these included significant up regulation of genes associated with all aspects of cell cycle progression, segregation of chromosomes, spindle formation and mitosis. Additionally, 132 genes mapping to cellular assembly and organisation in processes primarily related to organisation, segregation and re-alignment of chromosomes and of nuclear material during and post mitosis had altered expression in CP CML LSC. Deeper analysis suggested that while the vast majority of differentially expressed genes favoured positive regulation of cell cycle progression i.e. progression through all stages of the cell cycle, mitosis and proliferation in the CML LSC as compared to normal HSC, a minority of the differentially expressed genes were predicted to act by inhibiting cell cycle progression. The relative expression of a selected group of cell cycle genes are shown in Figure 3-12. Downstream analysis was also performed in IPA. This is a statistical test that seeks to predict the net effect of differences in gene expression on particular biological functions by relating the expression levels of the constituents of a functional group to what is known about their cellular function. Thus, if the majority of a functional group of genes is up regulated and its function is known to be advancement through the cell cycle then this would result in a prediction that cell cycle progression would be increased (if the gene function is not clear or variable, no prediction is made), a z score is provided measuring the strength of the prediction; >2 is considered significant. Thus in CP CML, IPA predicted that centrosome cycling, S phase progression, interphase progression, M phase progression and mitosis were all likely to be increased. Accordingly in CML, there was significant up regulation of several cyclins (CCN) (CCNA2, CCNB 1 and 2, CCND3, CCNE1 and 2 and CCNF); several cyclin dependent kinases (CDK) (CDK1,2 and 4), several E2F transcription factors (E2F1,3 and 7), the FOXM1 proto-oncogene, in addition to the aurora kinases (AURK) (AURK-A and B) and polo like kinases (PLK) (PLK1 and 4). There was also significant up regulation of genes involved in the orchestration of cellular, nuclear and chromosomal architecture through mitosis. These included broad increase in expression of most of the kinesin family, critical in mitotic spindle dynamics and separation of the centrosomes during mitosis; broad up regulation of the centrosomal associated proteins (CENP) including CENPA involved in kinetochore

formation, mitotic progression and chromosomal segregation and various other genes whose products have significant roles in these processes, for example centrosomal protein 55 (CEP55), budding uninhibited by benzimidazoles 1 homolog (BUB1), ZW10 interactors ZWINT and ZWILCH; mitotic arrest deficient-like (MAD) 2 binding protein (MAD2L) and the HAUS augmin like complex constituents (HAUS6 and 7). On the other hand there was also comparative up regulation of several genes with a predominant cell cycle inhibitory function in CP CML, for example, protein kinase, membrane associated tyrosine/threonine 1 (PKMYT1) which inhibits the action of CDC2 and prevents progression from G2 to M phase; pituitary tumour-transforming 1 (PTTG1) which has an inhibitory effect on mitosis; the DNA replication inhibitor geminin (GMNN) and the cell cycle dependent kinase inhibitor CDKN3 although no significant difference in the cell cycle inhibitors p15,16,18,21 or 57 was noted. Interestingly there was up regulation of MYBL2, retinoblastoma like 1 (RBL1), retinoblastoma binding protein (RBBP4) 4, transcription factor DP (TFDP) 1 and several Lin homologue genes including Lin52 and Lin54. These molecules are constituents of the LINC complex which exists in quiescent cells and inhibits expression of CDK genes, thereby negatively regulating the cell cycle (Litovchick et al., 2007). See Table 3-5, Figure 3-12 and Figure 3-13 below for selected expression data relating to genes derived from Ingenuity® IPA categories related to cell cycle, growth and proliferation. In order to validate the microarray results, qRTPCR was performed to independently quantify selected differentially regulated genes in pre-amplified cDNA from specimens utilised in the initial microarray analysis, the results are presented in Figure 3-13 where broad up regulation of selected cell cycle genes can be seen in CP CML HSC compared with normal HSC.

Symbol	Gene	p- value	FC
<i>CCNB2</i>	cyclin B2	3.70E-11	14.1
<i>TOP2A</i>	topoisomerase (DNA) II alpha 170kDa	8.75E-12	11.7
<i>PLK1</i>	polo-like kinase 1 (Drosophila)	3.90E-12	11.6
<i>CDK1</i>	cyclin-dependent kinase 1	9.10E-09	8.6
<i>FOXM1</i>	forkhead box M1	2.80E-10	8.5
<i>KIF20A</i>	kinesin family member 20A	4.30E-07	7.5
<i>CEP55</i>	centrosomal protein 55kDa	3.60E-08	7.4
<i>KIF15</i>	kinesin family member 15	4.20E-07	7.2
<i>MYBL2</i>	v-myb myeloblastosis viral oncogene homolog like 2	3.30E-07	6.9
<i>CCNA2</i>	cyclin A2	1.60E-08	6.5
<i>KIF11</i>	kinesin family member 11	3.10E-07	6.4
<i>KIF23</i>	kinesin family member 23	5.90E-09	6.3
<i>BUB1</i>	budding uninhibited by benzimidazoles 1 homolog (yeast)	1.40E-08	5.8
<i>PKMYT1</i>	protein kinase, membrane associated tyrosine	2.40E-06	5.7
<i>CENPI</i>	centromere protein I	1.40E-06	5.6
<i>PLK4</i>	polo-like kinase 4 (Drosophila)	3.90E-07	5.6
<i>KIF4A</i>	kinesin family member 4A	6.80E-07	5.5
<i>KIF2C</i>	kinesin family member 2C	3.50E-08	5.3
<i>BIRC5</i>	baculoviral IAP repeat-containing 5	1.81E-08	5.0
<i>STIL</i>	STIL (SCL interrupting Locus)	4.25E-11	4.7
<i>AURKB</i>	aurora kinase B	6.50E-09	4.6
<i>ZWINT</i>	ZW10 interactor	3.20E-07	4.5
<i>CENPM</i>	centromere protein M	1.00E-08	4.2
<i>AURKA</i>	aurora kinase A	1.70E-07	4.0
<i>CCNB1</i>	cyclin B1	1.20E-05	3.9
<i>CCNE2</i>	cyclin E2	1.90E-04	3.8
<i>E2F8</i>	E2F transcription factor 8	1.80E-05	3.7
<i>E2F7</i>	E2F transcription factor 7	3.20E-06	3.6
<i>CDC25C</i>	cell division cycle 25 homolog C	7.79E-07	3.4
<i>CCNE1</i>	cyclin E1	2.70E-04	3.4
<i>CENPA</i>	centromere protein A	1.00E-11	3.4
<i>CDKN3</i>	cyclin-dependent kinase inhibitor 3	2.50E-05	3.3
<i>CCNF</i>	cyclin F	6.10E-06	3.2
<i>E2F1</i>	E2F transcription factor 1	1.00E-06	3.1
<i>CENPK</i>	centromere protein K	1.70E-05	3.0
<i>KIF18B</i>	kinesin family member 18B	6.40E-05	3.0
<i>PTTG1</i>	pituitary tumour-transforming 1	1.30E-05	2.7
<i>CENPO</i>	centromere protein O	2.50E-07	2.6
<i>MAD2L1</i>	MAD2 mitotic arrest deficient-like 1 (yeast)	2.30E-05	2.6
<i>WEE1</i>	WEE1 homolog (S. pombe)	8.20E-04	2.6
<i>CENPN</i>	centromere protein N	1.90E-05	2.5
<i>GMNN</i>	geminin, DNA replication inhibitor	4.40E-04	2.4
<i>HAUS6</i>	HAUS augmin-like complex, subunit 6	1.10E-06	2.3
<i>RBL1</i>	retinoblastoma-like 1 (p107)	4.60E-04	2.1
<i>CCND3</i>	cyclin D3	4.70E-04	2.0
<i>CDK2</i>	cyclin-dependent kinase 2	5.50E-04	1.9
<i>KIF4B</i>	kinesin family member 4B	3.70E-05	1.9
<i>ZWILCH</i>	Zwilch, kinetochore associated, homolog (Drosophila)	3.30E-06	1.9
<i>TFDP1</i>	transcription factor Dp-1	1.10E-03	1.8
<i>CDK4</i>	cyclin-dependent kinase 4	1.90E-03	1.6
<i>E2F3</i>	E2F transcription factor 3	6.60E-05	1.6
<i>HAUS7</i>	HAUS augmin-like complex, subunit 7	5.50E-03	1.5
<i>RBBP7</i>	retinoblastoma binding protein 7	1.65E-05	1.4
<i>LIN52</i>	lin-52 homolog (C. elegans)	6.80E-04	1.4

Table 3-5: Differential expression of selected cell cycle / proliferation genes in CP CML LSC compared to normal HSC.

FC values indicate fold change in CML LSC over normal HSC.

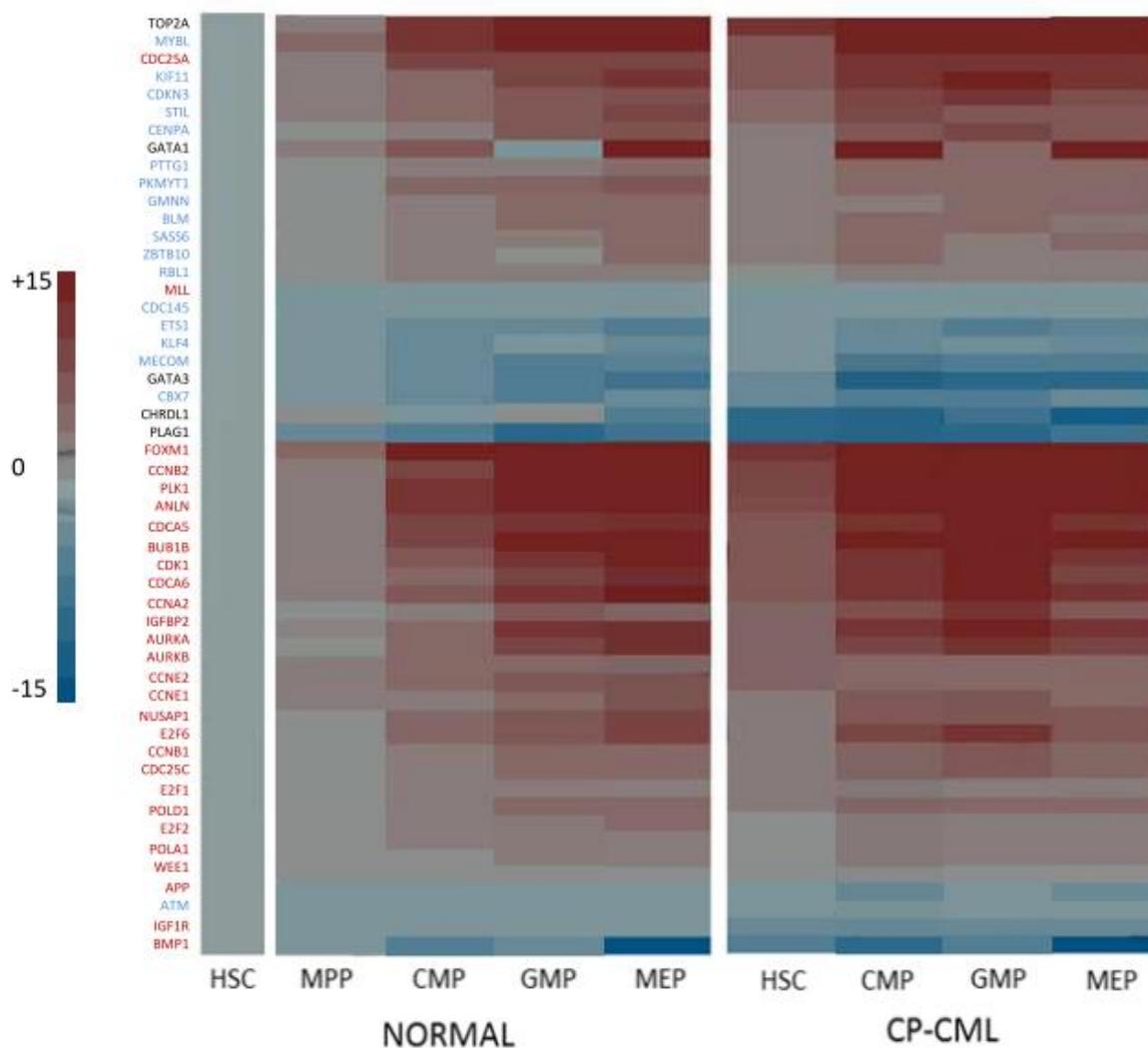


Figure 3-12: Selected cell cycle active genes that were differently expressed between normal and CP CML LSC.

The mean relative expression of a selection of cell cycle active genes across all sub-populations between normal and CP CML (normal n=3, CP CML n=6, see Table 3-1) normalised to the normal HSC population. The positive regulators of cell cycle progression are denoted in red whereas primarily negative regulators are in blue. In the heat map blue indicates reduced expression and red increased expression relative to the normal HSC population.

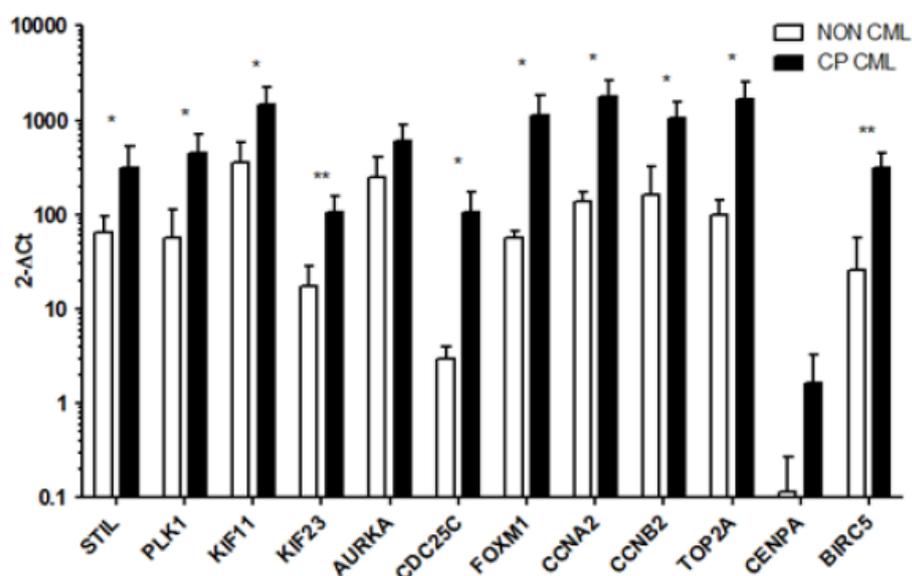


Figure 3-13: Validation of microarray expression levels of selected cell cycle active genes by qRT-PCR.

The relative expression levels of a selection of deregulated cell cycle active genes in qRT-PCR validation experiments comparing the normal HSC and LSC populations from several biological replicates (normal n=3, CP CML n=5-6). Error bars indicate the SEM. Expression expressed as $2^{-\Delta C_t}$. Significance values as follows * p<0.05; ** p<0.01 (unpaired T-test).

3.6.3.2 DNA replication, recombination and repair

Another highly deregulated functional gene set was the DNA replication, recombination and repair grouping. Maintaining genomic integrity is critical throughout the process of cellular replication. Here I noted significant deregulation of various systems involved in the correct regulation of DNA replication with significant over expression of origin recognition complex (ORC) subunits (*ORC1,3* and *6*); many of the minichromosome maintenance complex (MCM) components (*MCM 2-7*); chromatin licensing factors e.g. *CDT1* and the cell division cycle 45 homolog (*CDC45*). All these factors are critical in the initiation of DNA replication.

Genomic instability or loss of fidelity of DNA replication or DNA damage would normally result in activation of DNA repair pathways at checkpoints at various phases of the cell cycle (at G₁/S within S and at G₂/M). It is recognised that CML LSC have an unusual genetic instability. There is accumulating evidence that suggests that BCR-ABL causes this directly through induction of ROS inducing DNA breaks requiring repair and by interacting directly with the repair machinery to inhibit its performance (Sallmyr et al., 2008). It has also been shown that BCR-ABL directly drives expression of certain members of the DNA repair

pathways e.g. *RAD51* and the over abundance of these molecules results in increased DNA instability (Sallmyr et al., 2008). In this study, a total of 217 genes assigned to GO categories related to DNA replication, recombination and repair were shown to be differently regulated. The expression levels of selected molecules are shown in Table 3-6. I saw consistent and significant down regulation of the master regulator ataxia telangiectasia mutated (*ATM*) and up regulation of many genes involved in DNA repair, either by homologous recombination or by mismatch repair. Regarding mismatch repair of DNA, significant up regulation of exonuclease 1 (*EXO1*), various members of the mutS homolog family (MSH) (*MSH1, 2* and *6*) and the replication factor C family (*RFC2-5*) was present. Up regulation of several factors involved in DNA repair by homologous recombination was noted. Consistent with previous reports I saw strong up regulation of *RAD51* in CML LSC (Slupianek et al., 2001). Additionally there was up regulation of the breast cancer genes (*BRCA1*) and associated molecules e.g. BRCA1 associated RING domain (*BARD1*) and BRCA1 interacting protein (*BRIP1*), various members of the Fanconi anaemia complementation group genes (FANC) (*FANCA, B, C, D2, G*), the checkpoint kinase genes (*CHEK1,2*) and E2F transcription factors 1-4. Expression levels of selected genes are presented in Table 3-6 and Figure 3-15. Again, I selected several genes of particular interest and quantified their expression in the original microarray samples by qRT-PCR to ensure that similar results were obtained (Figure 3-14).

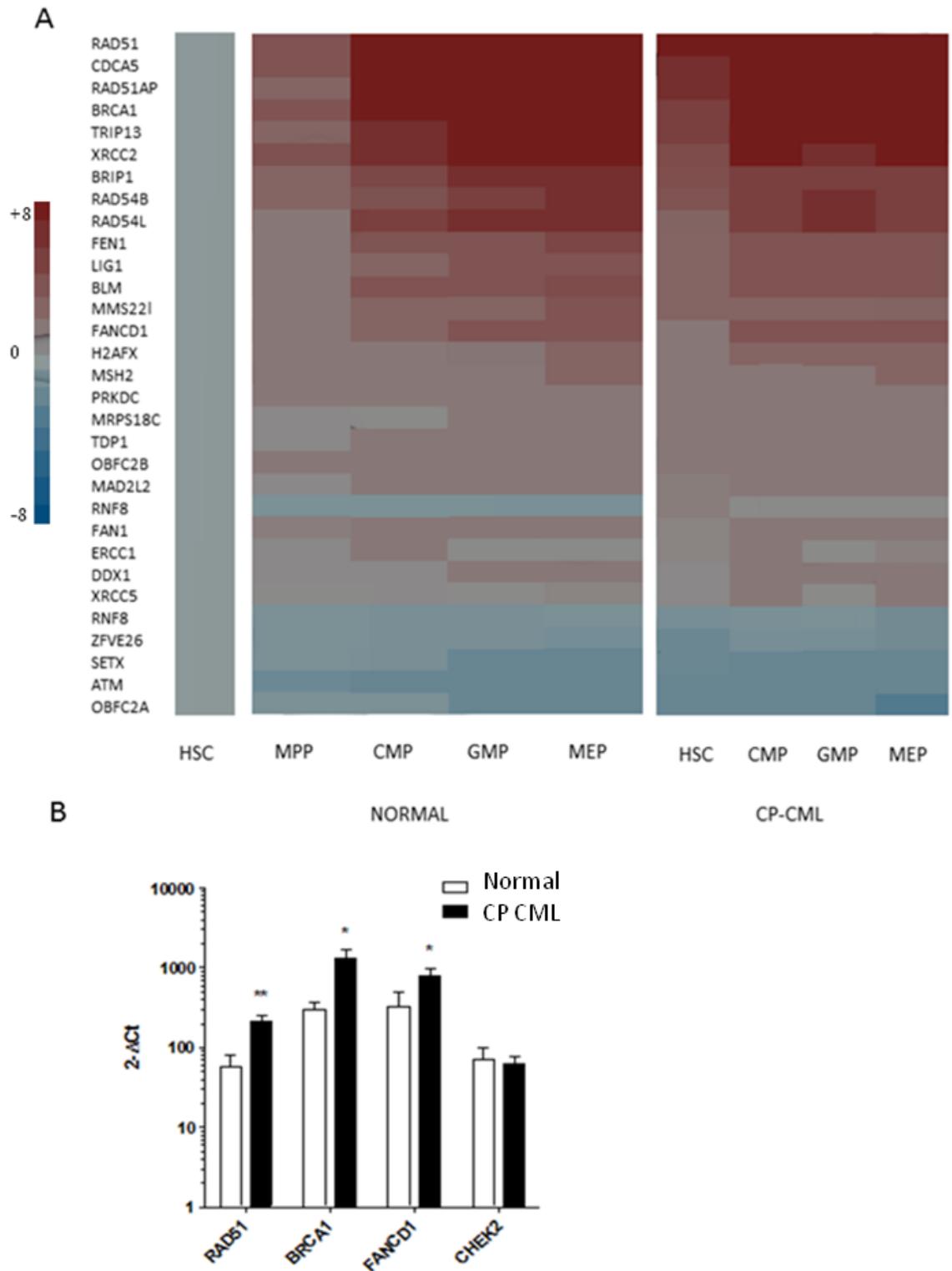


Figure 3-14: Selected genes with a role in DNA recombination and repair.

Panel A indicates the mean relative expression of a selection of genes across all sub-populations between normal and CP CML sub-populations (see Table 3-1) all normalised to the normal HSC population. Blue indicates reduced expression and red increased expression. Panel B shows the relative expression levels of a selection of these genes in qRT-PCR validation experiments comparing the normal HSC and LSC from several biological replicates (non CML n=3, CP CML n=5-6). Expression values are expressed as $2^{-\Delta Ct}$. Error bars indicate the SEM. Significance values as follows * p<0.05; ** p<0.01 (unpaired T-test).

Symbol	Gene	p value	FC
<i>CDC45</i>	cell division cycle 45 homolog (<i>S. cerevisiae</i>)	9.31E-09	9.0
<i>MCM10</i>	minichromosome maintenance complex component 10	2.80E-07	8.6
<i>EXO1</i>	exonuclease 1	3.84E-07	7.9
<i>CDC20</i>	cell division cycle 20 homolog (<i>S. cerevisiae</i>)	2.27E-07	7.6
<i>RAD51</i>	RAD51 homolog (RecA homolog, <i>E. coli</i>) (<i>S. cerevisiae</i>)	4.63E-09	7.6
<i>CDCA5</i>	cell division cycle associated 5	7.46E-09	7.0
<i>RAD51AP1</i>	RAD51 associated protein 1	1.81E-07	5.6
<i>MCM4</i>	minichromosome maintenance complex component 4	1.59E-06	5.6
<i>CDC25A</i>	cell division cycle 25 homolog A (<i>S. pombe</i>)	1.75E-07	5.5
<i>BRCA1</i>	breast cancer 1, early onset	1.03E-06	5.4
<i>ORC1L</i>	origin recognition complex, subunit 1-like (yeast)	2.21E-07	5.3
<i>CDCA2</i>	cell division cycle associated 2	5.55E-08	4.8
<i>BRIP1</i>	BRCA1 interacting protein C-terminal helicase 1	1.13E-05	4.8
<i>FANCD2</i>	Fanconi anaemia, complementation group D2	1.21E-08	4.7
<i>FANCI</i>	Fanconi anaemia, complementation group I	1.69E-08	4.5
<i>MCM2</i>	minichromosome maintenance complex component 2	1.01E-06	4.3
<i>CHEK1</i>	CHK1 checkpoint homolog (<i>S. pombe</i>)	3.44E-08	3.9
<i>CDT1</i>	chromatin licensing and DNA replication factor 1	1.35E-08	3.7
<i>RFC3</i>	replication factor C (activator 1) 3, 38kDa	7.34E-05	3.7
<i>FANCB</i>	Fanconi anaemia, complementation group B	4.45E-06	3.5
<i>CDC25C</i>	cell division cycle 25 homolog C (<i>S. pombe</i>)	8.25E-07	3.3
<i>ORC6L</i>	origin recognition complex, subunit 6 like (yeast)	2.86E-06	3.0
<i>RFC4</i>	replication factor C (activator 1) 4, 37kDa	1.93E-06	3.0
<i>MCM6</i>	minichromosome maintenance complex component 6	1.24E-04	2.9
<i>MCM5</i>	minichromosome maintenance complex component 5	2.14E-05	2.9
<i>MCM8</i>	minichromosome maintenance complex component 8	3.62E-04	2.8
<i>FANCA</i>	Fanconi anaemia, complementation group A	6.82E-05	2.8
<i>BRCA2</i>	breast cancer 2, early onset (FANCD1)	5.19E-06	2.7
<i>BRI3BP</i>	BRI3 binding protein	5.15E-04	2.5
<i>CHEK2</i>	CHK2 checkpoint homolog (<i>S. pombe</i>)	7.71E-04	2.4
<i>MCM3</i>	minichromosome maintenance complex component 3	4.71E-05	2.4
<i>RFC5</i>	replication factor C (activator 1) 5, 36.5kDa	3.09E-05	2.3
<i>MSH6</i>	mutS homolog 6 (<i>E. coli</i>)	6.64E-04	2.2
<i>RFC2</i>	replication factor C (activator 1) 2, 40kDa	1.63E-04	2.0
<i>FANCM</i>	Fanconi anaemia, complementation group M	3.13E-03	1.7
<i>ORC3L</i>	origin recognition complex, subunit 3-like (yeast)	9.13E-04	1.5
<i>ATM</i>	ataxia telangiectasia mutated	5.01E-03	-1.7
<i>CDC23</i>	cell division cycle 23 homolog (<i>S. cerevisiae</i>)	1.97E-08	-1.6
<i>CDC14B</i>	CDC14 cell division cycle 14 homolog B (<i>S. cerevisiae</i>)	5.00E-08	-2.8
<i>MCM7</i>	minichromosome maintenance complex component 7	9.20E-04	-2.9

Table 3-6: Differential expression of selected DNA replication, recombination and repair genes (CP CML LSC compared with normal HSC).

Negative values of fold change indicate reduced expression in CML.

3.6.3.3 Antigen presentation in HSC

As with the CMP comparison above, a significant reduction in expression of several of the type 1 and 2 histocompatibility molecules and associated entities were noted in the CP CML LSC as compared to normal HSC and this reduction is maintained and deepened throughout maturation; see Table 3-7

Symbol	Gene	p value	FC
<i>HLA-E</i>	major histocompatibility complex, class I, E	8.77E-03	-1.6
<i>HLA-A</i>	major histocompatibility complex, class I, A	2.42E-06	-1.6
<i>HLA-DQB2</i>	major histocompatibility complex, class II, DQ beta 2	3.72E-03	-1.8
<i>HLA-B</i>	major histocompatibility complex, class I, B	1.26E-03	-1.8
<i>HLA-DOB</i>	major histocompatibility complex, class II, DO beta	1.94E-03	-2.2
<i>HLA-DOA</i>	major histocompatibility complex, class II, DO alpha	4.01E-05	-2.8
<i>HLA-F</i>	major histocompatibility complex, class I, F	1.00E-05	-4.2

Table 3-7: Differential expression of selected antigen presentation genes (CP CML LSC compared with normal HSC).

Negative values of fold change indicate reduced expression in CML.

3.6.3.4 *Haemopoietic cell development and stem cell function*

Chronic myeloid leukaemia is a paradigm for the CSC hypothesis and the first malignancy in which a clonal stem cell origin was demonstrated (Fialkow et al., 1977). I wished to determine whether there were fundamental differences in expression of factors that influence stem cell fate decisions between CP CML and normal HSC. An analysis of our data set was performed to determine whether there was differential regulation in factors that are proposed to influence this process in HSC. A comprehensive list of all genes with GO functions relating to haemopoietic differentiation and self-renewal were collated from IPA, this yielded 1407 non-duplicated entities. This list was then used to filter our dataset. Our significance criteria were then applied yielding a list of 116 significantly differentially expressed genes with functions related to haemopoiesis and stem cell function, thus in our data set the vast majority of genes purported to regulate stem cell quiescence, activity, self-renewal and differentiation were not significantly differentially expressed between normal and CP CML HSC/LSC. Of those differentially expressed, the majority were in fact down regulated in CP CML. For example it was noted that expression of the HOX transcription factors (*HOXA3*, *A4*, *A7*, *A9*, *B3* and *B4*) were significantly reduced. These transcription factors all have well established roles in haemopoiesis, HSC function and leukaemogenesis.

Other factors with roles in HSC fate were also down regulated including MDS1 and EVI1 complex locus (*MECOM*), fms-related tyrosine kinase 3 (*FLT3*), mixed-lineage leukaemia translocated to 3 (*MLLT3*), runt related transcription factor (*RUNX2*), Kruppel like factor (*KLF*) 4, transducin-like enhancer of split (*TLE*) 4, transcription factor 4 (*TCF4*), SRY (sex determining region Y)-box (*SOX*) 5 and pleomorphic adenoma gene (*PLAG*)1. *FLT3* and *MECOM* are expressed in primitive HSC and play an important role in both normal haemopoiesis and leukaemogenesis, in addition to having prognostic relevance in a clinical context

(Goyama and Kurokawa, 2010; Groschel et al., 2010; Parcels et al., 2006; Steinleitner et al., 2012). *RUNX2* has a role in development and differentiation of bone and other tissues and interacts with various developmental signalling pathways e.g. TGF β and BMP signalling (Cohen, 2009). KLF4 is a transcription factor with a role in the maintenance of pluripotency and directing ES cell fate (Bernhardt et al., 2012; Pei, 2009). TCF4 is a transcription factor effector of Wnt signalling, whereas TLE4 is a transcriptional repressor of β -catenin / TCF signalling involved in cell determination in multiple tissues, tumour suppressor activity and appears to inhibit proliferation and survival of myeloid leukaemic progenitors through interaction with RUNX1 (Dayyani et al., 2008; Hanson et al., 2012; Yao et al., 1998). SOX5 belongs to a large, highly conserved family of transcription factors with a role in regulation of embryonic development and in the determination of the cell fate (Lefebvre, 2010). PLAG1 is a developmentally regulated Zn²⁺ finger transcription factor which is up regulated in various tumours, including pleomorphic adenomas and lipoblastomas. It is active in haemopoietic tissue, has been shown to expand primitive myeloid progenitor numbers and enhance self-renewal capacity in addition to cooperating with CBF translocations to cause leukaemia in murine models (Landrette et al., 2005).

As previously mentioned, the gene *SETBP1* was significantly down regulated in CP CML HSC and CMP. This is intriguing as the SET protein is up regulated by BCR-ABL signalling in CML, contributing to leukaemogenesis through inhibition of protein phosphatase 2A (PP2A) (Neviani et al., 2005). SETBP1 binds SET and may stabilise it by protecting it from proteolytic cleavage thereby enhancing its effect (Cristobal et al., 2010; Minakuchi et al., 2001). SETBP1 appears to have a role in regulation of HSC function promoting self-renewal (Du et al., 2005). Co-expression of *SETBP1* with *BCR-ABL* into myeloid progenitors confers self-renewal activity and results in aggressive myeloid leukaemia (Oakley et al., 2012). Additionally, over expression of *SETBP1* is seen in AML where it is a poor prognostic factor and in CML where it may be associated with progression (Cristobal et al., 2010; Neviani et al., 2005). Deregulation of SETBP1 is also seen in chronic myelomonocytic leukaemia (CMML) and atypical AML (Damm et al., 2013; Piazza et al., 2013). Recent studies have demonstrated a role for SETBP1 in the maintenance of self-renewal activity through interaction with HOXA9 and A10 in murine myeloid progenitors (Oakley et al., 2012).

I also noted deregulation of members of the polycomb repressive complexes. There was differential regulation of several members of both PRC1 and PRC2 complexes (both up and down regulation of components) between CML LSC and normal HSC. Perhaps most interestingly, there was a significant and consistent up regulation of *EZH2* in CML LSC. *EZH2* is cell cycle regulated but is also a constituent of PRC2 and is important in HSC maintenance and self-renewal. It also has a well established role in myeloid malignancies (Martin-Perez et al., 2010). There was also significant up regulation of another component of PRC2 - *SUZ12*, which is purported to have a role in stem cell maintenance and also has been implicated in haemopoietic malignancies particularly lymphoma (Majewski et al., 2008; Martin-Perez et al., 2010). In this study *BMI1* and *EED* were not found to be significantly differently expressed between CML LSC and normal HSC populations. Significant deregulation of members of the PRC1 complex was also noted. This included comparative down regulation of *CBX7*, differential expression of the polycomb group ring finger protein (PCGF) family with *PCGF5* down regulated but *PCGF6* up regulated in CML.

These findings are interesting because polycomb complexes are evolutionarily conserved mechanisms which maintain the transcriptional profile of cells through the regulation of chromatin structure. They are broad suppressors of transcriptional activity and are critical to normal embryonic development, maintenance of tissue homeostasis and stem cell function. Polycomb complexes are thought to regulate stem cell function by repression of genetic programmes favouring differentiation and play a vital role in regulating normal haemopoietic function. Furthermore, aberrant polycomb function is a feature of many disparate haematologic and solid human malignancies (Martin-Perez et al., 2010). Differential expression of components of these complexes may have a significant effect on their overall function (Gao et al., 2012). For example, over expression of *EZH2* increases HSC self-renewal potential, prevents exhaustion and cooperates with other lesions in leukaemogenesis (Kamminga et al., 2006) whereas heterozygosity for *Suz12* appeared to enhance HSC activity in a murine model (Majewski et al., 2008) and *CBX7* has a critical role in the maintenance of pluripotency in ES cells and has tumour suppressor activity (Forzati et al., 2012; Morey and Helin, 2010).

Additionally, differential regulation of the GATA transcription factors was noted. GATA transcription factors have key roles in haemopoiesis. GATA1 is

associated with megakaryocytic and erythroid differentiation, GATA2 has a role in HSC function and GATA3 is primarily involved in lymphoid, particularly T cell, specification and differentiation (Bresnick et al., 2012). In this study, *GATA3* was down regulated and *GATA1* was up regulated in CML LSC. Notably, expression of *GATA2* was not significantly different between the two populations.

Several other intriguing differences were noted. Multiple components of the IGF signalling pathway were differentially expressed e.g. there was down regulation of *IGF1* and its receptor (*IGF1R*) which are involved in cellular proliferation and survival and may have a role in maintaining self-renewal properties in leukaemic HSC (Medyouf et al., 2011) but up regulation of IGF binding protein (*IGFBP2*) - which has been shown to expand human CB HSC in culture (Yilmaz et al., 2006). Additionally there was down regulation members of the RAS associated factor family (RASSF) especially *RASSF6* in CML LSC. This family has a tumour suppressor role and have been found to be epigenetically inactivated in some malignancies, including ALL. Their cellular function is to modulate the mitogenic effects of RAS signalling (Hesson et al., 2009; Richter et al., 2009).

Lastly I noted differential expression of several adhesion molecules including down regulation of cadherin (CDH) 9, protocadherin 17 and also intriguingly *CDH2* which plays a role in osteoblastic niche signalling and may be involved in protecting CML LSC from TKI-mediated apoptosis (Calvi et al., 2003; Zhang et al., 2013). Several other molecules with roles in cell-cell interaction and adhesion were also differentially regulated. These included reduction in expression of several lectin domain family members, particularly C type lectin domain molecules (CLEC) (*CLEC2B*, *4A*, *7A*). Many of those down regulated are expressed in nerve tissue and have roles in normal neuronal development (neuronal cell adhesion molecule 2 [*NCAM1*], contactin1 [*CNTN1*], cell adhesion molecule with homology to L1CAM [*CHL1*]). In contrast CP CML LSC over expressed several integrin molecules e.g. *ITGA9* (part of the receptor for VCAM1 and osteopontin - a potent mediator of quiescence (Nilsson et al., 2005)), *ITGA2B* and *ITGB3*. Additionally *CXCR4* expression has been reported to be down regulated in BCR-ABL expressing cells (Geay et al., 2005); however, in this work, *CXCR4* expression was variable and therefore no significant difference in expression was detected between CP CML and normal LSC/HSC in our dataset. See Table 3-8, Figure 3-15 and Figure 3-16 for expression levels of a selection of

haemopoiesis and stem cell associated genes. In order to confirm the microarray findings expression levels of genes of particular interest were independently assessed in the original specimens by qRT-PCR using the Fluidigm platform. The results obtained are presented for comparison in Figure 3-16. Of those tested the majority followed the expression pattern described by the microarray, however in the case of *GATA1*, *EZH2*, *SOX5* and *KLF4*, the difference in expression did not reach statistical significance (see Figure 3-16).

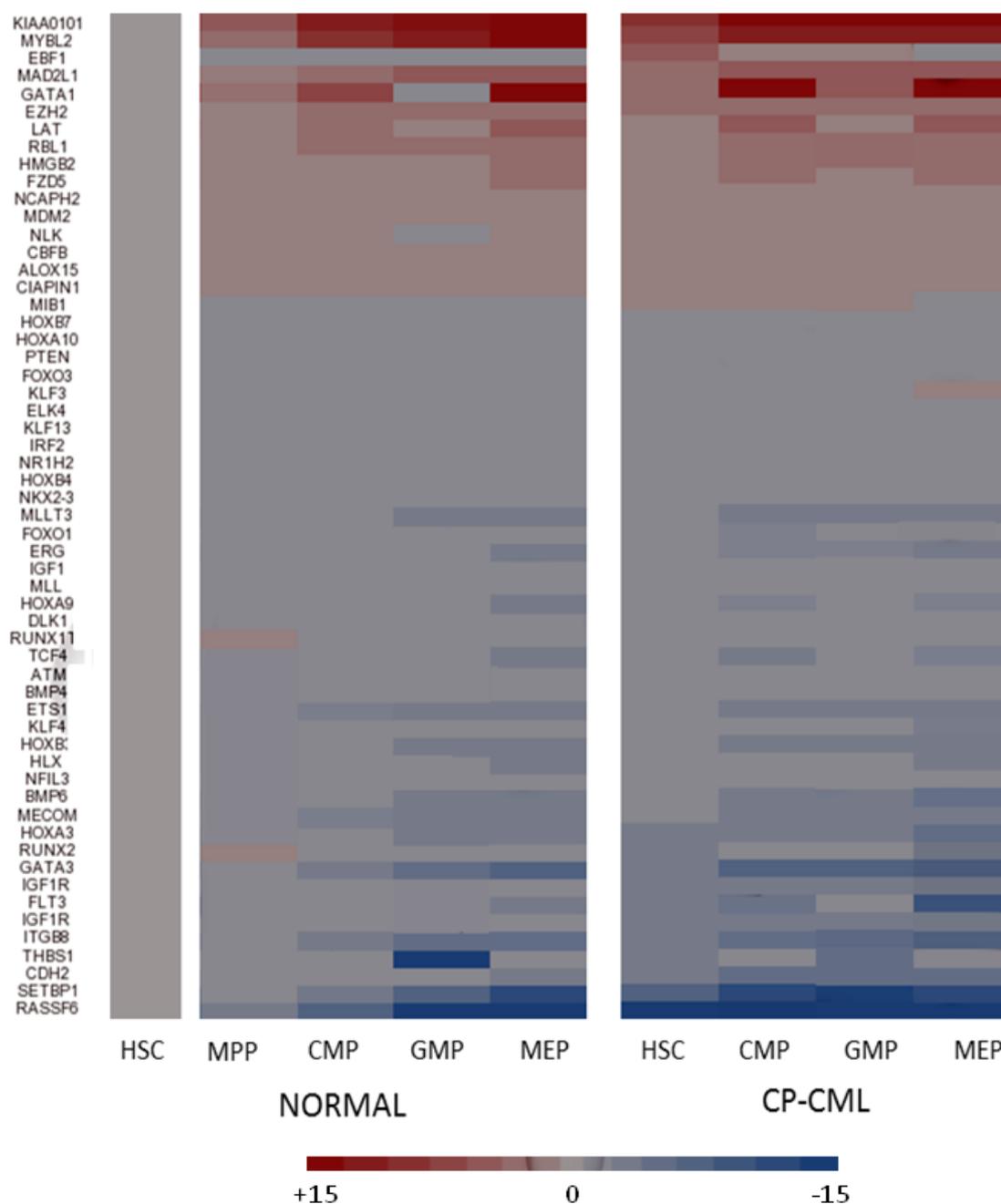


Figure 3-15: Selected genes with roles in stem cell fate, differentiation and self-renewal that were differentially regulated in the microarray comparison.

The relative expression of a selection of genes across all sub-populations between normal and CP CML (see table 3-1) all normalised to the normal HSC population is shown. Blue indicates reduced expression and red increased expression.

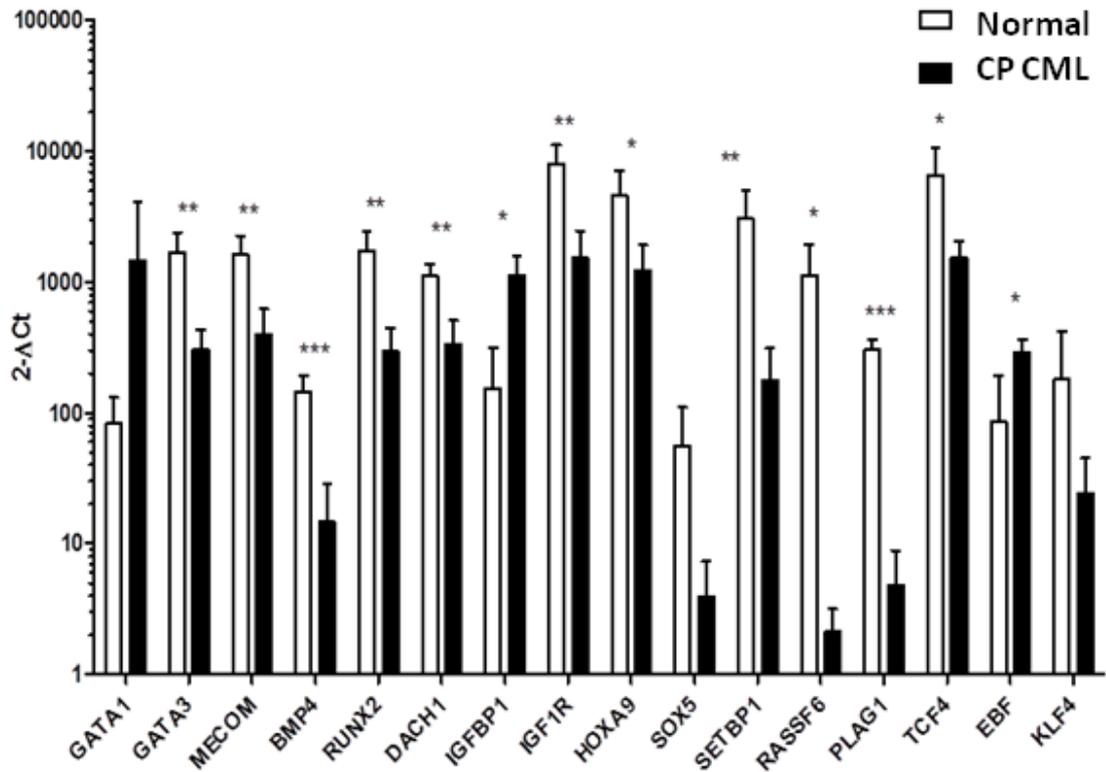


Figure 3-16: Validation of microarray expression levels of selected stem cell fate, differentiation and self-renewal genes by qRT-PCR.

The relative expression levels of a selection of deregulated genes in qRT-PCR validation experiments comparing the normal HSC and LSC from several biological replicates (non CML n=3, CP CML n=5-6) is shown. Error bars indicate the SEM. Expression values are expressed as $2^{-\Delta C_t}$. Significance values as follows * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ (unpaired T- test).

Symbol	Gene	p value	FC
<i>RASSF6</i>	Ras association domain family member 6	2.30E-13	-30.1
<i>PLAG1</i>	pleiomorphic adenoma gene 1	5.45E-23	-12.1
<i>CHRD1</i>	chordin-like 1	3.28E-09	-10.3
<i>CDH2</i>	cadherin 2, type 1, N-cadherin (neuronal)	2.17E-10	-7.8
<i>SETBP1</i>	SET binding protein 1	2.47E-10	-7.7
<i>CDH9</i>	cadherin 9, type 2 (T1-cadherin)	3.25E-05	-7.6
<i>CLEC4A</i>	C-type lectin domain family 4, member A	2.21E-05	-5.4
<i>TLE4</i>	transducin-like enhancer of split 4	2.73E-03	-4.7
<i>PCDH17</i>	protocadherin 17	1.91E-11	-4.4
<i>IGF1R</i>	insulin-like growth factor 1 receptor	1.55E-07	-4.1
<i>HOXA4</i>	homeobox A4	1.12E-03	-3.4
<i>FLT3</i>	fms-related tyrosine kinase 3	7.60E-04	-3.3
<i>SOX5</i>	SRY (sex determining region Y)-box 5	4.69E-06	-3.2
<i>TSHZ3</i>	teashirt zinc finger homeobox 3	1.58E-12	-2.9
<i>CLEC2B</i>	C-type lectin domain family 2, member B	8.07E-04	-2.8
<i>SMAD1</i>	SMAD family member 1	2.29E-05	-2.8
<i>RUNX2</i>	runt-related transcription factor 2	1.09E-03	-2.7
<i>HOXA3</i>	homeobox A3	9.67E-04	-2.6
<i>IGF1</i>	insulin-like growth factor 1	3.61E-04	-2.6
<i>RASSF9</i>	Ras association domain family member 9	2.07E-06	-2.6
<i>DLK1</i>	delta-like 1 homolog	5.79E-05	-2.5
<i>GATA3</i>	GATA binding protein 3	5.58E-04	-2.4
<i>MAML2</i>	mastermind-like 2	6.65E-03	-2.4
<i>BMP4</i>	bone morphogenetic protein 4	1.23E-09	-2.3
<i>CBX7</i>	chromobox homolog 7	7.35E-05	-2.3
<i>MECOM</i>	MDS1 and EVI1 complex locus	8.76E-08	-2.3
<i>NCAM2</i>	neural cell adhesion molecule 2	5.39E-05	-2.2
<i>HOXB3</i>	homeobox B3	7.98E-03	-2.1
<i>CLEC7A</i>	C-type lectin domain family 7, member A	3.65E-03	-2
<i>DACH1</i>	dachshund homolog 1	6.82E-03	-1.9
<i>EZH1</i>	enhancer of zeste homolog 1	1.09E-04	-1.9
<i>KLF4</i>	Kruppel-like factor 4	3.12E-05	-1.9
<i>MLLT3</i>	myeloid/lymphoid or mixed-lineage leukaemia	4.83E-05	-1.8
<i>CHL1</i>	cell adhesion molecule homology (L1CAM/VCAM1)	2.71E-03	-1.7
<i>CLEC3B</i>	C-type lectin domain family 3, member B	5.62E-04	-1.7
<i>GLI3</i>	GLI family zinc finger 3	5.00E-03	-1.7
<i>HOXA9</i>	homeobox A9	3.68E-03	-1.7
<i>TCF4</i>	transcription factor 4	3.72E-04	-1.7
<i>HOXA7</i>	homeobox A7	7.93E-03	-1.5
<i>HOXB4</i>	homeobox B4	4.81E-05	-1.5
<i>SUZ12</i>	suppressor of zeste 12 homolog	1.35E-04	-1.3
<i>FZD5</i>	frizzled homolog 5	4.77E-04	1.9
<i>ITGA9</i>	integrin, alpha 9	5.62E-04	1.9
<i>PCGF6</i>	polycomb group ring finger 6	3.06E-04	1.9
<i>GATA1</i>	GATA binding protein 1 (globin transcription factor 1)	1.84E-03	2.6
<i>EZH2</i>	enhancer of zeste homolog 2 (Drosophila)	1.77E-07	2.9
<i>ITGA2B</i>	integrin, alpha 2b	1.10E-04	3.8
<i>IGFBP2</i>	insulin-like growth factor binding protein 2	1.50E-04	6.4
<i>ITGB3</i>	integrin, beta 3 (CD61)	4.25E-04	8.3

Table 3-8: Differential expression of selected genes with roles in stem cell fate, differentiation and self-renewal (CP CML HSC compared with normal HSC).

Negative values of fold change indicate reduced expression in CP CML.

3.6.3.5 Embryonic signalling pathways

Self-renewal behaviour is modulated by micro-environmental signalling and there is a wealth of data implicating embryonic signalling pathways as key signals governing HSC fate. I was keen to establish whether constituents and targets of the embryonic morphogenic pathways e.g. BMP/TGF β , Hh, Notch and Wnt were differently expressed in CP CML as compared to normal haemopoiesis in our data. In our initial analysis these pathways were not highly represented in the lists of differentially expressed genes indicating broadly consistent expression between sample groups. However differential expression of several constituents and downstream targets of these pathways was noted. Most strikingly there was significant reduction in the expression of *BMP4*, the inhibitor of BMP signalling, chordin-like (*CHRD1*), as well as the target *SMAD1* in CP CML LSC. I confirmed *BMP4* expression by qRT-PCR (see Figure 3-15, Figure 3-16 and Table 3-8). This is interesting because BMP signalling plays an important role in the haemopoietic niche in normal haemopoiesis, can expand the stem and progenitor cell pool and appears to act downstream of Hh signalling in haemopoiesis (Bhardwaj et al., 2001; Sadlon et al., 2004).

Regarding canonical Wnt signalling, I discussed down regulation of *TCF4* and *TLE4* in the paragraph above, however I also noted up regulation of frizzled receptor *FZD5* in CP CML LSC (Table 3-8). No significant difference in expression was noted in the other main constituents of canonical Wnt signalling in this comparison.

With regard to Notch signalling, reduced expression of delta like homologue (*DLK*) 1 and marginal reduction in mastermind like (*MAML*) genes were demonstrated (Table 3-8). No significant difference in expression of other components of the pathway or of the downstream targets hairy enhancer of split (*HES*) 1 or 5 was noted.

Finally, the data was interrogated to demonstrate whether constituents and targets of the Hh pathway were differentially expressed. I did not note significant differences in expression of the core elements of Hh signalling (e.g. Hh ligands, *PTCH*, *SMO* or the positive *GLI* transcription factors) between the sample groups. I did demonstrate significant up regulation of other members and targets of the pathway including the B type cyclins, *FOXM1* and the cell cycle regulator *STIL*, although these may have alternative stimulae and I noted a decrease in

expression of the Hh repressive transcription factor *GLI3*. A more detailed review of Hh signalling is the subject of the next chapter.

During data analysis it was apparent that differential expression in these pathways was either subtle or variable between biological replicates, thus our studies may not have had the sensitivity or the power to detect variations in expression of pathway elements or evidence of their relative activation.

3.6.3.6 *The relative position of the CML LSC*

Given that in both overall and specific microarray analyses, CP CML LSC appeared to have a transcriptional signature that was significantly more mature than normal HSC and, in PCA, most closely resembled the normal MPP or CMP population, a cross-wise comparison between CP CML LSC and both these normal populations was performed. The results are shown in Figure 3-17. Specific analysis of the differentially expressed genes between normal MPP and CP CML LSC indicated that CP CML LSC had up regulation of genes related to cell cycle progression, organisation of mitosis and DNA replication or repair, even compared to the MPP population (see Table 3-9). Notably the differences seen in the HSC populations were retained in many instances; thus *EZH2* was up regulated in CML LSC. Several of the previously mentioned adhesion molecules e.g. *CDH2* and *9* were reduced compared to MPP. Additionally, the tumour suppressor *RASSF6*, *SETBP1*, *IGF1R* and *PLAG1* remained significantly down regulated. Also the BMP signalling target *SMAD1* and the BMP4 inhibitor *CHRD1* were also down regulated in CP CML. Thus it appears that in some respects, the CML LSC population has a more mature and proliferative transcriptional profile than normal MPP.

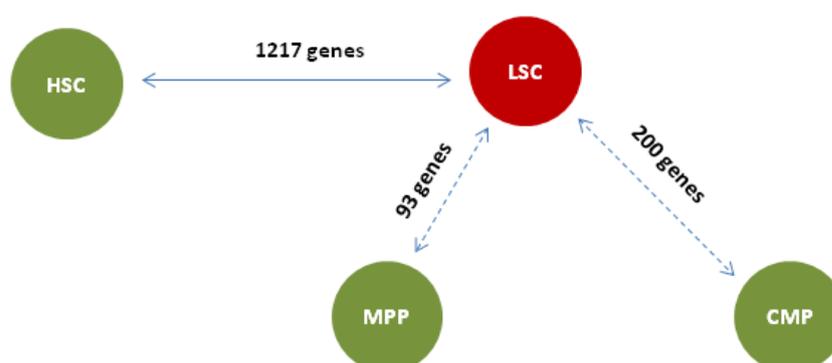


Figure 3-17: Relative similarity of CP CML LSC and normal HSC and progenitors.

Symbol	Gene	p value	FC
<i>CHRD1</i>	chordin-like 1	4.53E-08	-9.3
<i>CDH9</i>	cadherin 9, type 2 (T1-cadherin)	2.32E-04	-6.6
<i>RASSF6</i>	Ras association (RalGDS/AF-6) domain family member 6	5.26E-08	-6.2
<i>PLAG1</i>	pleiomorphic adenoma gene 1	6.24E-12	-4.1
<i>SETBP1</i>	SET binding protein 1	9.98E-08	-3.8
<i>CDH2</i>	cadherin 2, type 1, N-cadherin (neuronal)	8.45E-07	-3.1
<i>IGF1R</i>	insulin-like growth factor 1 receptor	1.92E-07	-2.8
<i>SMAD1</i>	SMAD family member 1	7.72E-05	-1.9
<i>WNT10B</i>	wingless-type MMTV integration site family, member 10B	8.42E-05	1.5
<i>CD109</i>	CD109 molecule	2.46E-04	1.5
<i>EZH2</i>	enhancer of zeste homolog 2 (Drosophila)	2.35E-04	1.7
<i>CDC25C</i>	cell division cycle 25 homolog C (S. pombe)	2.21E-04	2.3
<i>PTTG1</i>	pituitary tumour-transforming 1	3.02E-05	2.3
<i>BIRC5</i>	baculoviral IAP repeat containing 5	1.09E-04	2.4
<i>CCNB1</i>	cyclin B1	1.45E-04	2.5
<i>LEPR</i>	leptin receptor	1.75E-04	2.6
<i>THY1</i>	Thy-1 cell surface antigen	8.03E-06	2.8
<i>CENPA</i>	centromere protein A	1.94E-05	2.9
<i>AURKB</i>	aurora kinase B	2.08E-05	3.1
<i>PLK1</i>	polo-like kinase 1	8.19E-05	3.1
<i>CCNB2</i>	cyclin B2	1.29E-04	3.1
<i>KIF20A</i>	kinesin family member 20A	1.78E-04	3.9
<i>EBF1</i>	early B-cell factor 1	2.19E-04	6.0

Table 3-9: Differential expression of selected genes in CP CML LSC compared with normal MPP.

Negative values of fold change indicate reduced expression in CP CML LSC.

3.7 Summary and Conclusions

Microarray assessment of gene expression is an extremely powerful tool allowing the researcher to obtain a snapshot of the expression, not just of a gene, pathway or set of genes, but of the entire transcriptional landscape of the specimens under analysis. This has the capacity to identify, for example, new targets for pharmacological intervention or allow interrogation of the interrelated processes and networks that underlie the biological behaviour of a cell. CML represents a paradigm of a stem cell driven malignancy, however transcriptional analysis of the LSC activity at its root has been hampered by the lack of sufficiently pure populations of HSC. In this study, the transcriptional differences between normal and CP CML CD34⁺38⁻45RA⁻Lin⁻90⁺ cells and their progeny have been characterised. This population represents a highly refined HSC population in CB where 1:10 cells have HSC activity and, it was reasoned, was likely to represent a similarly enriched population in adult PB. Given that CML is thought to generate from a malignant HSC, HSC activity may reside in a phenotypically similar population. Therefore a comparison between these populations in CML would allow a better appreciation of the transcriptional differences between the two

states. The consequence of increasing stringency in terms of defining a cell population for analysis is reduced cell numbers for analysis. We addressed this through sorting large numbers of pre-enriched CD34⁺ cells to obtain sufficient HSC material to proceed to the microarray analysis. Our work represents the only gene expression comparison between these extremely small cell populations to date. These studies demonstrate that, at a transcriptional level, mature progenitors from CML and normal haemopoiesis are very similar. As CML, as a clinical entity, is characterised by accumulation of functionally terminally differentiated myeloid effector cells, this is not unexpected, however it is interesting to note that expression of BCR-ABL in the more mature sub-populations (MEP or GMP) did not have a significant impact on the expression levels of many genes. As more primitive populations are considered, I saw increasing differences in gene expression between the normal and malignant haemopoietic populations that we isolated. The difference is largest between the normal HSC and CP CML LSC and, interestingly, CML LSC would be much more akin, in terms of overall gene expression, to normal MPP or CMP cells. These results are supported by the results of PCA analysis of the dataset. Similar findings have been reported in less refined populations by Bruns *et al*/who concluded that the CD34⁺38⁻ population was transcriptionally similar to normal CMP and also by Graham *et al*/and Affer *et al*/who reported that the G₀ CD34⁺ population utilised in their gene expression studies exhibited transcriptional similarity to dividing cells rather than the similarly isolated population in normal cells (Affer *et al.*, 2011; Bruns *et al.*, 2009; Graham *et al.*, 2007). Thus, the further phenotypic selection that was performed in this study did not reveal a CML population with an HSC transcriptional signature; rather it suggests that even this population of rigorously sorted CP CML LSC are, at a transcriptional level at least, behaving more like progenitor cells than their normal counterparts.

Following general analyses of the dataset as a whole, a number of analyses were performed with the intention of ascribing biological meaning to the data produced. Here Partek GS, a powerful bioinformatics platform was used to compare relative expression between samples and classify the data produced into functional groupings while pathway analysis was performed using IPA. These processes allowed lists of differentially expressed genes to be defined according to our criteria and determine their relevance in a biological context. Broad up regulation of many aspects of cellular machinery required for proliferation, DNA replication and repair and segregation and mitosis was demonstrated in CML LSC

in comparison to the similar normal population. These findings are strikingly similar to other published microarray studies in CML, for example Norwicki *et al*, Kronenwett *et al*, Diaz-Blanco *et al* and Bruns *et al*, all reported up regulation in various mediators of proliferation, cell cycle advancement and mitosis, with up regulation of several cyclins e.g. *CCND/B/A*, cyclin dependent kinases e.g. *CDK4* and up regulation of factors involved in DNA tertiary structure and those mediating mitosis e.g. *BUB1*, *MAD2L* and *TOP2A* in concordance with the data presented here (Bruns *et al.*, 2009; Diaz-Blanco *et al.*, 2007; Kronenwett *et al.*, 2005; Nowicki *et al.*, 2003). Indeed, even in populations sorted according to cell cycle (G_0) status there is an apparent up regulation of genes involved in DNA replication or mitosis. Thus even when the CP CML HSC population is not dividing, there is evidence of a capacity to rapidly do so. Likewise, previously performed analysis of gene expression in CML highlighted DNA replication and repair systems as being highly deregulated in CML compared to normal haemopoiesis. One explanation for this might be the ubiquitous expression of BCR-ABL in all of the populations analysed. BCR-ABL is associated with promiscuous activation of intracellular mitogenic pathways therefore its expression might be expected to result in a transcriptional activity akin to that described. Thus the proliferative profile seen across all the CML populations analysed in this way (from MNC to the rigorously sorted population described here) may represent active BCR-ABL signalling irrespective of the phenotypic population isolated.

This study demonstrated comparative down regulation of genes involved in adhesion. In particular, there was reduction in expression of *CDH2* and *9* and differential expression of several integrins and other adhesion molecules. Both Bruns *et al* and Diaz Blanco *et al* reported similar findings (Bruns *et al.*, 2009; Diaz-Blanco *et al.*, 2007). One of the most striking findings, however, was the significant and consistent reduction in many genes known to be expressed in primitive haemopoietic cells, in particular, those with potential roles in directing stem cell fate. There was consistently lower expression of many of the HOX genes e.g. *HOXA3*, *A4*, *A7*, *A9*, *B3* and *B4*, reduction in *FLT3*, *SETBP1*, *GATA3*, *RUNX2*, and *MECOM*. There was also differential regulation in PRC complex components notably up regulation of *EZH2* and down regulation of *SUZ12* and *CBX7*. Previous expression studies have produced disparate results; Kronenwett *et al* found increased expression of several self-renewal determinants in $CD34^+$ CP CML cells (e.g. *GATA2* & *C/EBP α*) and suggested that CML HSC may in fact have greater self-renewal capacity than similarly defined non CML HSC (Kronenwett *et al.*,

2005). Similarly Diaz-Blanco *et al*/found up regulation of several HSC, quiescence and self-renewal associated genes in their CP CML population (e.g. *ALDH1A1*, *GATA2*, *HOXA10*, *HOXA9*, *MEIS1*, *PBX3* and *PCGF4*). They also concluded that CP CML CD34⁺ cells had enhanced self-renewal activity (Diaz-Blanco *et al.*, 2007). In contrast to the above, Bruns *et al*/found reduced levels of expression of genes with purported roles in directing stem cell fate (e.g. *FLT3*, *PTEN*, *CD133*, *CD53* and *ZFP64*) and suggested reduction of self-renewal capacity in CP-CML HSC (Bruns *et al.*, 2009). Finally, Affer *et al*, found reduced expression of various stem cell factors in CP CML G₀ CD34⁺ cells (e.g. *CD133*, *FLT3*, MUSASHI-2 (*MSI2*), *HOXA3*, *A5*, *B3*, *B6* as well as several members of the PRC complexes including *CBX7* and *PCGF3*) and concluded that CML LSC are more mature than their normal equivalents (Affer *et al.*, 2011). Our results are more consistent with the latter view. However the picture is not straightforward, *EZH2* was found to be up regulated in CML and *SUZ12* down regulated. These entities have roles in controlling HSC fate and some haemopoietic malignancies but have not been described in CML.

In this study I also report significant differential regulation of several novel factors not previously uncovered by gene expression analysis; for example down regulation of genes with tumour suppressor activity e.g. *TLE4* and the RASSF family of RAS inhibitors as well as down regulation of *PLAG1*, a foetal transcription factor and a potential oncogene. Recent proteomic work has suggested that RASSF factors and *PLAG1* may interact within a complex to modulate *TGFβ1* signalling (Bhaskaran and Souchelnytskyi, 2008). Lastly, there was broad reduction in the expression of molecules important in antigen presentation in CML LSC and progenitor cells. Thus, the picture of the CP CML LSC that emerges from these studies is again very much of a cell with a far more mature phenotype than the equivalent normal HSC population. It is also more mature, in many respects, than the normal MPP population, a population that has the capacity to more readily enter and progress through cell cycle, more readily enter myeloid differentiation and which generally has lower expression of genes associated with an on-going capacity for self-renewal. Furthermore CP CML LSC and their progeny would appear from this study, to have significant deficiencies in molecules that would allow interaction with the immune system and present antigen for immunosurveillance; this has potential ramifications for therapeutic intervention with immuno-modulatory approaches. Of note, Nowicki *et al*/also noted a

reduction in expression of various genes related to cellular response to infection in CML MNC (Nowicki et al., 2003).

This transcriptional landscape is consistent with functional observations. Although there is a relatively quiescent population within the broader CD34⁺ population in CML, the majority of cells are in cycle (Eaves et al., 1998b). Additionally *in vitro* and *in vivo* measures of self-renewal indicate that CML LSC have significantly impaired stem cell activity (Jiang et al., 2002; Udomsakdi et al., 1992b; Wisniewski et al., 2011).

A second question relates to the populations isolated. Although more stringently defined than other similar studies, our HSC population is still heterogeneous with true HSC residing within a mixed population. Again these proportions may be variable between CML and normal populations; if the proportion of Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ cells with HSC activity amidst the committed progenitors were generally less in CML this might explain the transcriptional differences in self-renewal and stem cell associated genes.

In the work by Majeti *et al*/Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ and CD90⁻ cells could both contribute to long term haemopoiesis but CD90⁻ did so with less frequency and with lesser capacity for secondary transplantation (Majeti et al., 2007). It is possible that the efficient ordered process of normal haemopoiesis is significantly disrupted in CML and that overlap occurs between expression of pro-cycling and differentiation elements and some pro-stem cell maintenance and self-renewal elements in this population. When I compared the CML LSC population to the normal MPP population, I demonstrated that the HSC population still had comparatively high levels of expression of cell cycle related genes coupled with down regulation of various cell adhesion and self-renewal associated genes. This is concordant with a recent study that demonstrated significantly reduced engraftment potential of Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ from CML patients compared to normal PB and CB samples (Wisniewski et al., 2011). Thus, the distinction between Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ and CD90⁻ populations may be less relevant in CML compared to normal haemopoiesis. This is important as the immunophenotypic populations under discussion in this work were resolved and functionally defined through analysis of human CB and adult BM and may not be directly applicable in the context of adult CP CML (Manz et al., 2002, Majeti et al., 2007). Functional analysis of these stem and progenitor populations in CP CML is underway in this laboratory.

One of the main objectives of this study was to identify potential gene expression differences between CML and normal haemopoiesis at the stem cell level with a view to the development of a targeted therapy that would facilitate the eradication of the malignant clone. A wealth of information has been obtained from these studies so far and its analysis is indeed on-going. However it is fascinating to speculate that self-renewal behaviour may be more tenuous in CP CML HSC, possibly less due to intrinsic cellular properties, with less capacity to reside in the niche and greater likelihood of division resulting in differentiation. Under these circumstances abrogation of externally generated cues signalling for self-renewal might, in time, result in preferential clonal extinction.

4 Expression of Components and Targets of the Hh Signalling Pathway in CML

4.1 Introduction

As experience with TKI therapy against the BCR-ABL oncogene has grown, it has become clear that, for the majority of patients at least, TKI therapy alone will not eradicate the disease. Investigative efforts of various groups have turned towards defining other potential pharmacological targets in the LSC that might individually, or in combination with conventional treatment, target this resistant population. Extending from the CSC hypothesis, one attractive target might be the processes that govern the maintenance and self-renewal behaviour of the CSC themselves as if these factors could be altered to diminish or abrogate self-renewal, extinction of the malignant clone would result.

As discussed in chapter 1, section 1.3.4, there is convincing evidence that BCR-ABL does not, of itself, have the capacity to convey self-renewal and that BCR-ABL expressing cells have impaired self-renewal capacity. This in turn has re-focused attention on the mechanisms governing LSC fate and self-renewal activity in CML LSC and led to consideration as to whether these signalling pathways may constitute valid LSC specific targets.

Several different pathways have been analysed and found to be implicated in HSC/LSC expansion and survival (discussed in depth in chapter 1, sections 1.2 and 1.3.4), however one of the major pathways influencing stem cell self-renewal is the Hh signalling pathway. This pathway has a critical role in haemopoietic development and embryonic morphogenesis but remains active in adult tissue where it contributes to tissue homeostasis, regeneration and healing through control of HSC function. Aberrant Hh signalling is implicated in human oncogenesis and has been proposed to have an important role in CSC activity. Hh signalling mechanics, its function in normal haemopoiesis, not to mention its role in oncogenesis, CSC maintenance and CML are discussed in detail in chapter 1 (sections 1.2.6, 1.2.8.1, 1.2.9.5 and 1.3.4.3) however a summary diagram is shown below for reference.

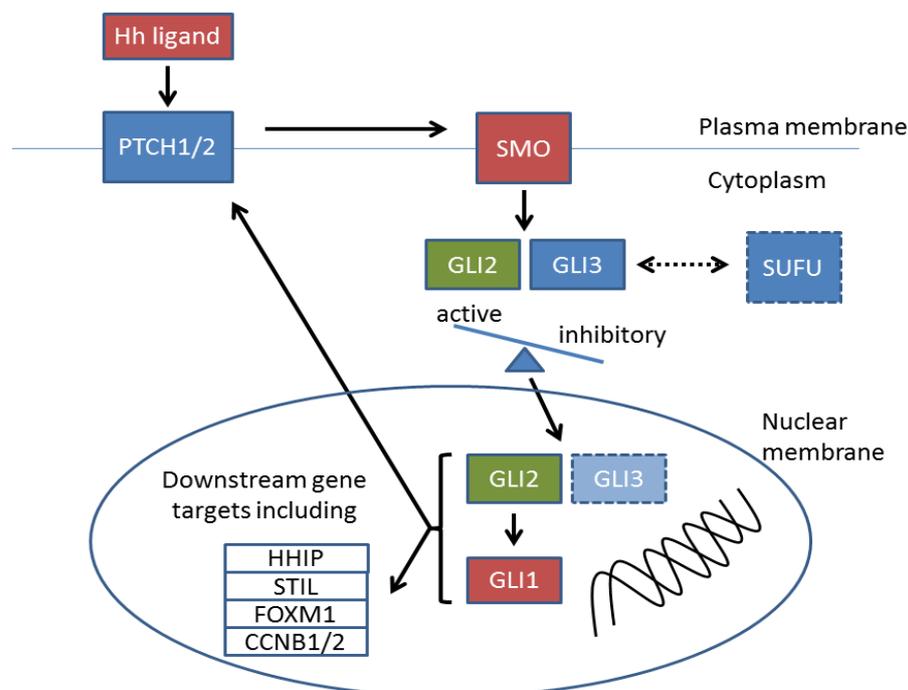


Figure 4-1: Key components of Hh signalling.

Canonical Hh signalling is ligand-dependent and comprises a series of repressive reactions which are released following receptor activation. In vertebrates there are three related ligands DHH, SHH, and IHH which bind to two 12-span trans-membrane receptors PTCH 1 and 2 (Pathi et al., 2001; Stone et al., 1996). In the inactive state, PTCH1 exerts an inhibitory effect on SMO - a G-protein like 7-span trans-membrane protein. When Hh signalling occurs, this inhibition is removed allowing active SMO accumulation on the plasma membrane. The effect of Hh signalling through SMO is to alter the balance of active versus repressive forms of the GLI family of Zn^{2+} finger transcription factors (GLI1, GLI2 and GLI3) mediated through alteration of their capacity to interact with an inhibitory complex in the cytoplasm including SUFU (Hui and Angers, 2011; Ruiz i Altaba et al., 2007). This activates the Hh signalling program which includes cell cycle regulators, inhibitors of apoptosis and positive and negative feedback loops through expression of *GLI1* and *PTCH1* respectively (Dierks et al., 2007; Duman-Scheel et al., 2002; Sasaki et al., 1999). Here the predominant function of the component is colour coded; positive/activators – red; negative regulators/repressors - blue and GLI2 which has significant positive and negative roles – green.

Mounting evidence, albeit largely from gene expression studies in advanced CML primary cells and from murine modelling, suggest a potential role for Hh signalling in CML (Dierks et al., 2008; Sengupta et al., 2007; Zhao et al., 2009). Two studies have directly related Hh signalling to CML pathogenesis using different murine models (Dierks et al., 2008; Zhao et al., 2009). Both indicated that *Smo* deletion reduced LSC numbers and reduced the incidence of leukaemia, with prolonged latency in primary transplantation and greatly reduced capacity to recrudescence disease in secondary hosts. Both groups also performed pharmacological inhibitor studies, demonstrating prolonged survival in diseased

mice, reduced *LSC* population, and lower functional activity *in vivo* and *in vitro* after exposure to the non-clinical grade SMO inhibitor cyclopamine (Dierks et al., 2008; Zhao et al., 2009). Therefore Hh signalling is active in CML and appears to be critical to the maintenance and expansion of the disease clone in primary human CML samples and in murine models of CML.

Whether Hh signalling is also active and important in CP CML is not clear as both studies were largely conducted on primary human BC samples and murine models that most closely resemble advanced phase CML (Koschmieder and Schemionek, 2011). This is critically important as the vast majority of CML patients are in CP and any stem cell-directed therapy might arguably be more effectively employed at early stages of the disease.

In chapter 3, a global gene expression approach was adopted to investigate the transcriptional differences between CML and normal LSC/HSC or progenitor cells. Here a more focused, hypothesis driven approach is employed specifically to investigate whether Hh signalling mediators and targets were differentially regulated in CML (particularly in CP CML but also in advanced phase CML stem and progenitor cells) compared with normal haemopoiesis. Three simultaneous investigative lines were adopted;

- (1) Gene expression was measured by qRT-PCR on primary CD34⁺ cell populations from patients with CP CML and compared with mobilised non CML CD34⁺ cells.
- (2) Quantitative RT-PCR to measure relative gene expression of constituents and targets of the Hh pathway in primary stem and progenitor populations from patients with CP, AP and BC CML compared with normal haemopoietic sub-populations and in relation to *BCR-ABL* expression.
- (3) Global gene expression microarray data interrogation to determine the level of Hh related gene expression within the global gene expression analysis.

4.2 Results

4.2.1 Expression of Hh constituents and targets in primary CP CML CD34⁺ cells in comparison to normal CD34⁺ cells

Quantitative RT-PCR was used to assess the relative gene expression of components of the Hh signalling pathway in CD34⁺ cells from patients with CP (n=7-9) CML compared with normal CD34⁺ haemopoietic cells (n=6-7). RNA was extracted from CD34⁺ cells from normal donors or patients with CML in CP, similar quantities were reverse transcribed prior to qRT-PCR analysis using Applied Biosystems validated and optimised probe and primer sets (see methods section 2.2.12). As the comparison was between two populations the data is presented as $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008) and is shown in Figure 4-2. When performing comparisons between different patient samples, the inherent biological heterogeneity often results in far greater variability of gene expression when compared with cell line based experiments. This can make it difficult to demonstrate statistically significant differences in primary cell samples. There was variably increased expression of several downstream Hh target genes including *HHIP* (CP CML vs normal: 3.3 fold; p=0.02), *GLI1* (CP CML vs normal: 2.4 fold; p=0.08), *PTCH1* (CP CML vs normal: 2 fold; p=0.28) and *GLI2* (CP CML vs normal: 3.5 fold; p=0.07). In contrast to Dierks *et al*/who had previously demonstrated up regulation of *SMO* at the gene and protein level, no difference in *SMO* gene expression was noted (Dierks et al., 2008). Interestingly, *SUFU*, a repressor of the Hh pathway was significantly more abundant at the mRNA level in normal than in CP CML CD34⁺ cells (1.6 fold; p=0.02). Furthermore *GLI3*, the main repressor of Hh signalling, was down regulated in CP CML compared with healthy CD34⁺ specimens (3 fold; p = 0.21). Hh ligands were expressed at very low levels, however both *IHH* and *SHH* expression were higher in CML than in healthy CD34⁺ cells (6 fold; p=0.3 and 5 fold; p=0.05 respectively).

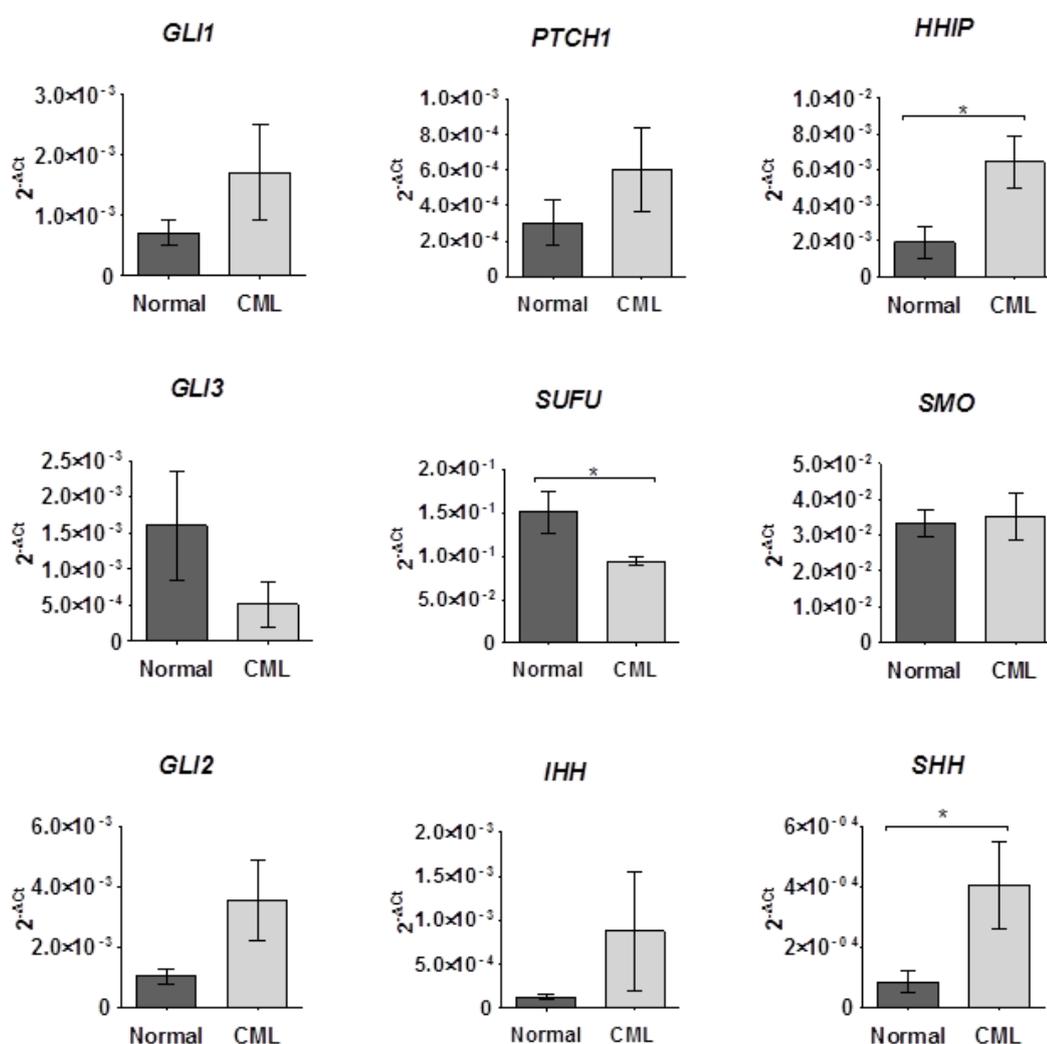


Figure 4-2: Comparison of Hh pathway related gene expression in PB CD34⁺ cells in CP CML versus normal haemopoiesis.

Expression of *GLI1*, *GLI2*, *GLI3*, *PTCH1*, *SMO*, *SHH*, *SUFU*, *HHIP* and *IHH* in PB CD34⁺ cells derived from patients with CP CML at diagnosis compared with normal CD34⁺ cells. Purification of CD34⁺ cells is described in section 2.2.3. Results shown represent the mean \pm SEM for a minimum of 6 samples normalised to GAPDH and expressed as 2^{-ΔCt}. The reader's attention is drawn to the fact that y-axis scale vary between the differing gene targets due to their variable abundance. Significance values; * p<0.05. n≥6 (unpaired T-test).

Thus, while constituents and targets of Hh signalling are expressed in CML cells, their expression is low level, rather variable amongst the primary specimens analysed and the differences in expression small. As a result, few of the comparisons generated statistically significant differences. Nonetheless, we see an intriguing pattern whereby Hh ligands and downstream targets (*PTCH1*, *GLI1* and *HHIP*) are up regulated and predominantly inhibitory molecules (*GLI3* and *SUFU*) are comparatively down regulated in CP CML.

4.2.2 Gene expression analysis of Hh signalling molecules in CML compared with normal stem and progenitor populations

The previous experiments established that Hh signalling molecules were expressed in primary CML CD34⁺ cells and moreover suggested that there may be differential expression of key targets and pathway regulators between normal and malignant primary CD34⁺ cells however the results were not conclusive. The key problems identified were that expression of Hh components was, generally speaking, low in the samples analysed and there was a significant degree of variation in expression between different biological samples, particularly between the primary CP CML CD34⁺ cells analysed. The CD34⁺ population spans the entire stem and progenitor pool with expression values being determined by the net expression of all cells within this grouping whereas self-renewal activity in normal haemopoiesis and CP CML is largely confined to the most primitive cells within this group. Thus it will be difficult to compare levels of expression of these key mediators of self-renewal in populations comprised largely of non self-renewing committed haemopoietic cells and variable numbers of HSC.

To better understand alterations in Hh signalling in CML stem and progenitor cell populations I assessed gene expression of Hh pathway mediators and downstream targets in pure populations of LSC/HSC, CMP, GMP and MEP subpopulations in CP and advanced phase CML versus normal haemopoiesis. These experiments were performed in parallel with and utilising the same sorted samples as the microarray experiments detailed in chapter 3. Gene expression analysis was performed by qRT-PCR utilising the Fluidigm Biomark system with validated Applied Biosystems probe and primer sets following pre-amplification as detailed in chapter 2, section 2.2.12. Note the $2^{-\Delta Ct}$ values are larger than in Figure 3-2 due to the fact that in the Fluidigm analysis pre-amplification of targets but not *GAPDH* was performed.

4.2.3 Analysis of key Hh signalling mediators and targets

I observed that the most primitive populations had the highest expression of Hh mediators and targets and that expression typically reduced with degree of maturation (see Figure 4-3). For example, expression of the key downstream target of Hh signalling, *GLI1* is down regulated with maturity in normal haemopoiesis. This pattern is less clear in CML, with larger variation in expression between the biological replicates and expression was noted to be slightly

increased in the CMP progenitor population in CP CML. In advanced phases even more disruption is evident. Other key downstream targets of Hh signalling, *PTCH1* and *2*, are also comparatively reduced in expression in mature subpopulations, particularly in CMP and GMP subpopulations and may increase in the MEP population. A similar pattern albeit with greater variability is seen in *PTCH2* expression. Additionally, it appears that expression of *PTCH1* in mature populations may increase with disease phase whereas this pattern is not evident with *PTCH2*. Comparing overall expression of *GLI1*, *PTCH1* and *PTCH2* between normal populations and their relevant equivalent in CP, AP and BC CML, the only difference that reaches significance is *PTCH2* expression between normal and CP GMP populations. There was a trend towards a slight increase in *GLI1* expression in CP CML LSC and CMP over normal HSC and CMP and an increase in *GLI1* expression in the BC CML samples. For *PTCH1* there was a tendency for expression to increase with worsening disease severity in CML.

In contrast to *GLI1* and *PTCH1/2* I noted a significant reduction in expression of the key Hh pathway inhibitors *GLI3* and *SUFU* in CML stem and progenitor cells when compared with normal haemopoiesis. In normal haemopoietic maturation, there was a progressive down regulation of *GLI3* from HSC with maturity. This pattern was not evident in CML, where *GLI3* was reduced in LSC and remained similarly low in CMP and GMP populations. Likewise *SUFU* expression was comparatively high in normal HSC reduced with maturity whereas in CML *SUFU* was reduced in LSC and similarly expressed across the sorted sub-populations. Again, there was no alteration in expression of *SMO* in the various HSC/LSC populations analysed however there was a tendency for *SMO* expression levels to be increased in the CMP population in comparison to the HSC/LSC population which reached significance only in the advanced phase comparisons.

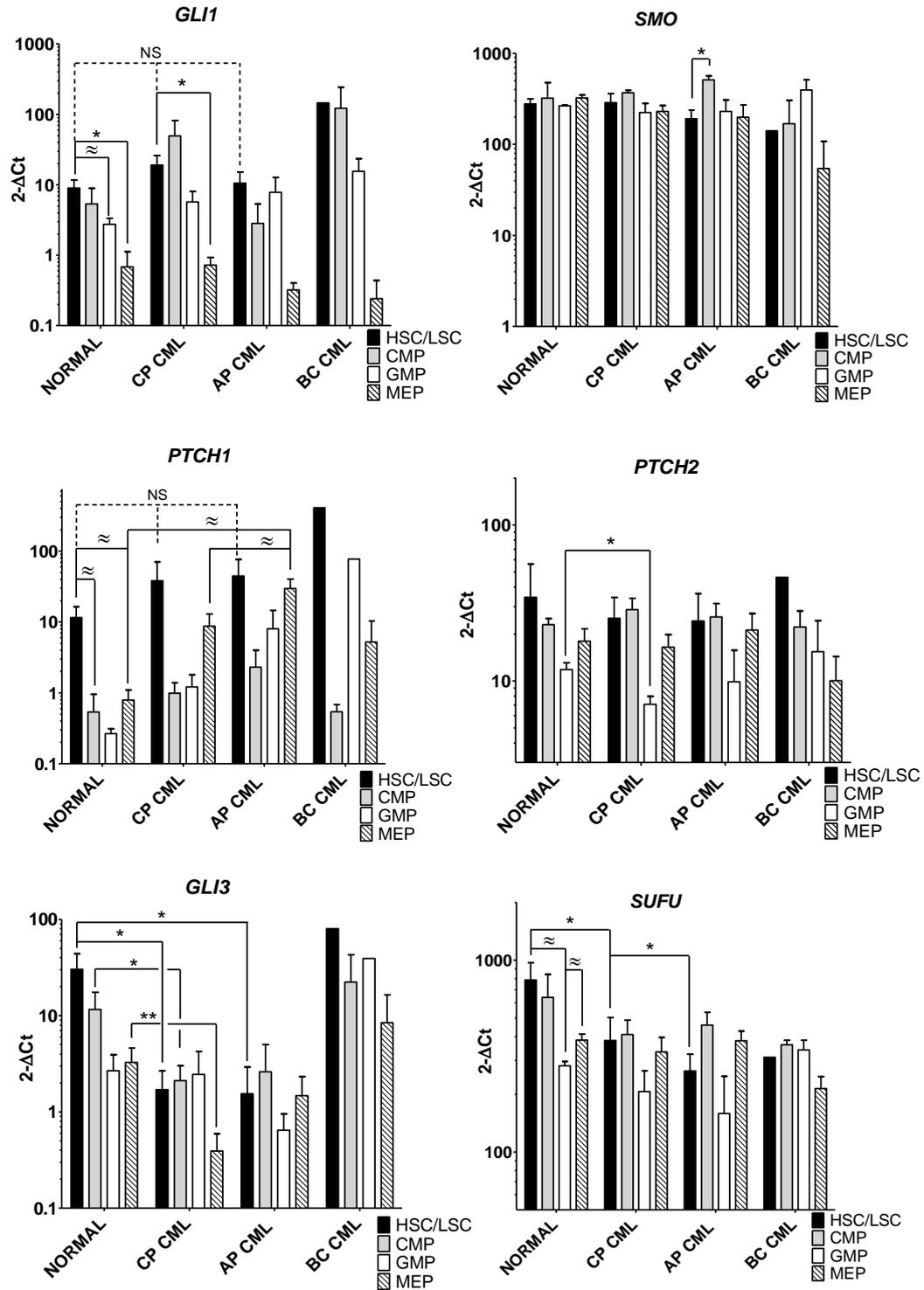


Figure 4-3: Relative expression of key targets and modulators of Hh signalling in sorted sub-populations (HSC, CMP, GMP and MEP) from PB CD34⁺ cells derived from patients with CP, AP or BC CML at diagnosis compared with normal sub-populations.

Results are presented as the mean \pm SEM for multiple samples normalised to GAPDH and are expressed as $2^{-\Delta Ct}$. Significance values (unpaired T-test); NS, not significant, \approx borderline $p < 0.1$, *, $p < 0.05$; **, $p < 0.01$; CP CML, $n = 6$; normal, $n = 3$, AP CML, $n = 4$, BC CML $n = 2$ (note BC CML LSC is a single sample and statistical analysis is not presented for BC CML comparisons)

Furthermore I analysed the expression levels of the downstream targets of Hh signalling, *CCNB1*, *CCNB2*, *FOXM1* and *STIL* (Figure 4-4).

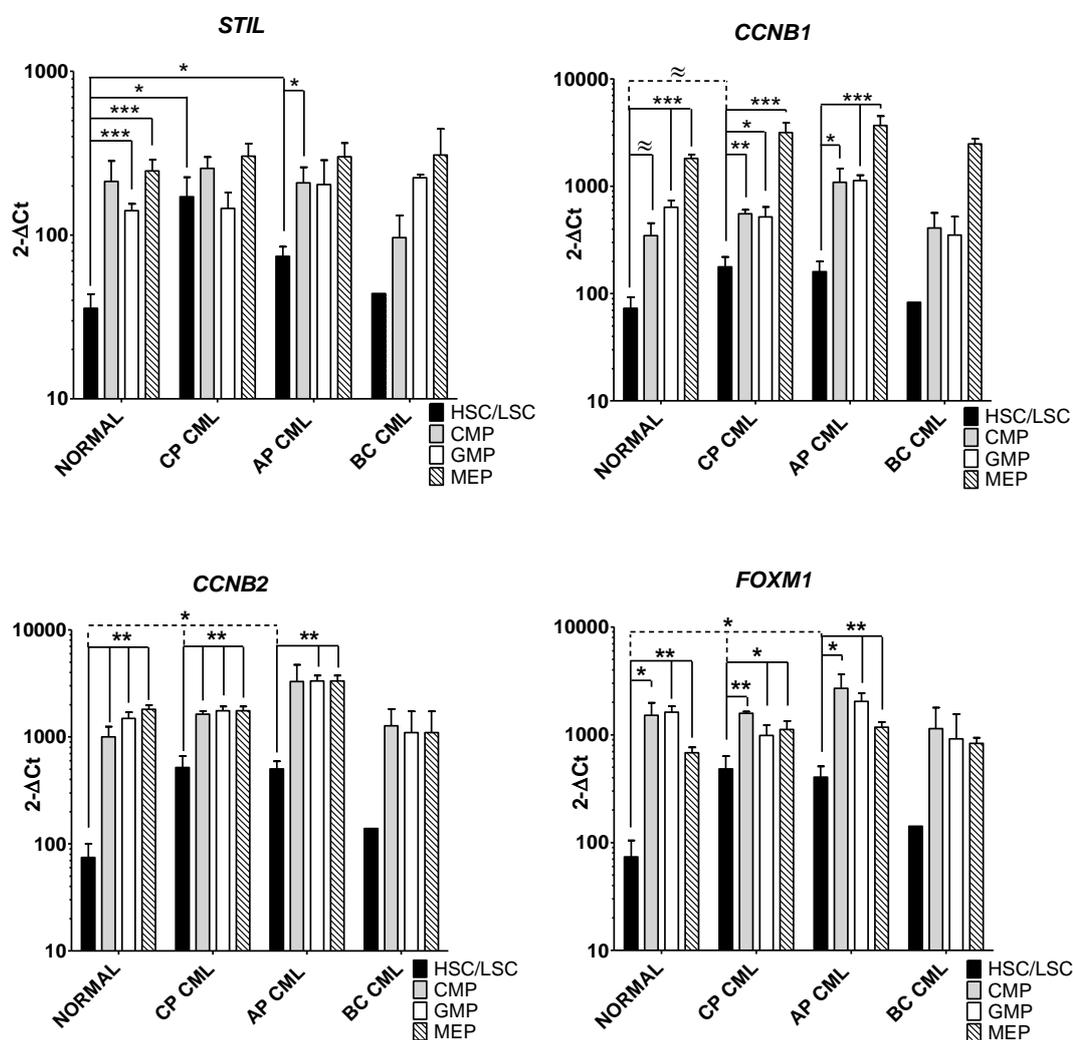


Figure 4-4: Relative expression of additional targets of Hh signalling across sub-populations in normal, CP, AP and BC CML haemopoiesis.

Gene expression of HSC, CMP, GMP and MEP sub-populations from PB CD34⁺ cells derived from patients with CML at diagnosis compared with normal sub-populations. Results are presented as the mean \pm SEM for multiple samples normalised to GAPDH and are expressed as $2^{-\Delta Ct}$. Significance values (unpaired T-test); \approx borderline $p < 0.1$, *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.005$; CP CML, $n = 6$; normal, $n = 3$, AP CML, $n = 4$, BC CML $n = 2$ (note BC CML LSC is a single sample). Statistical analysis is not presented for BC CML samples.

In these analyses markedly increased expression of *FOXM1*, *CCNB1* and *2* and *STIL* was noted in CML compared with normal HSC and in the progenitor populations compared to HSC in both normal and malignant haemopoiesis. No difference in expression was noted between CP and advanced phase disease or

between different progenitor populations within or between different disease states.

4.2.4 Analysis of hedgehog ligand expression

The expression of Hh ligands in CML and normal haemopoietic stem and progenitor cells was analysed. The results of these analyses confirmed that expression levels are extremely low in haemopoietic cells despite pre-amplification. Consistent expression results were not obtained for *DHH* and *IHH*. However *SHH* was not expressed by normal HSC, CMP and GMP populations but interestingly was expressed in CP through advanced phase CML, see Figure 4-5.

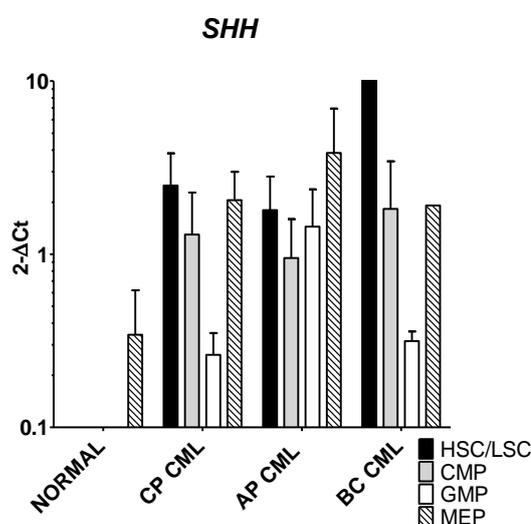


Figure 4-5: Relative expression of *SHH* ligand across sub-populations in normal, CP, AP and BC CML haemopoiesis.

Relative gene expression of *SHH* in sorted sub-populations (HSC, CMP, GMP and MEP), from PB CD34⁺ cells derived from patients with CML at diagnosis compared with normal sub-populations. Results are presented as the mean \pm SEM for multiple samples normalised to GAPDH and are expressed as $2^{-\Delta Ct}$ CP CML, n=6; normal, n=3, AP, n=4, BC n=2 (note BC CML LSC is a single sample and statistical analyses are not presented for BC CML samples).

Given the expression results above, I attempted to determine whether SHH ligand was actively secreted by primary normal and CML cell populations. To achieve this I performed SHH-specific ELISA as described in chapter 2, section 2.2.10.3. Briefly, equal quantities of cells (0.5×10^6 cells/mL) were cultured for 24h after which the supernatant was collected and equal quantities added as appropriate to the pre-prepared ELISA plates. Sonic HH production between normal and CP CML MNC and CD34⁺ cells was compared. Media obtained from culture of similar quantities of various stromal cell lines and primary MSC and

relevant fresh media was also included in the analysis. The culture and collection of conditioned media was performed by Jennifer Richmond. No difference was demonstrated between supernatants obtained from either the CD34⁺-enriched fractions or the MNC fractions between CML and normal haemopoietic cells, in fact no difference in optical density was noted above baseline in either supernatant suggesting that SHH was not expressed at appreciable levels by either cell type (at least down to 10ng/mL - the lower range of the SHH ELISA kit). Low levels of SHH were detected in supernatants obtained from the human stromal line HS-5 and in some primary MSC cultures.

4.2.5 *BMP4* expression

Assessment of *BMP4* expression levels was performed as *BMP4* is known to co-operate with Hh signalling in determining HSC fate both in embryonic and adult haemopoiesis (Bhardwaj et al., 2001). It was surprising and intriguing to find that *BMP4* is abundantly expressed in normal HSC but is down regulated significantly with maturation. However in CP and advanced CML a significant reduction in *BMP4* expression was demonstrated at the HSC and progenitor (CMP) level, see Figure 4-6.

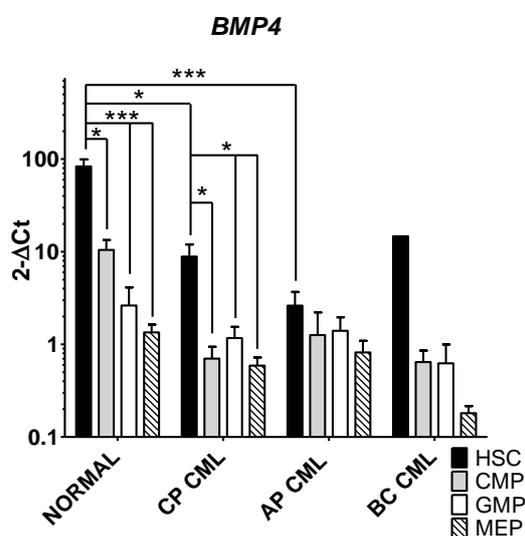


Figure 4-6: Relative expression of *BMP4* across sub-populations in normal, CP, AP and BC CML haemopoiesis.

Relative gene expression of *BMP4* in sorted sub-populations (HSC, CMP, GMP and MEP), from PB CD34⁺ cells derived from patients with CML at diagnosis compared with normal sub-populations. Results are presented as the mean \pm SEM for multiple samples normalised to GAPDH and are expressed as 2^{-ΔCt}. Significance values (unpaired T-test): *, p < 0.05; **, p < 0.01, *** p < 0.005; CP CML, n=6; normal, n=3, AP CML, n=4, BC n=2 (note BC CML LSC is a single sample and statistical analyses are not presented for BC CML samples)

The significance of this finding is uncertain, however it may be that autocrine and/or paracrine *BMP4* signalling from HSC to HSC and haemopoietic cell to niche is a further mechanism important in determining stem cell fate and could co-operate with Hh signalling. Reduced expression in CML LSC may be reflective of the fact that CML LSC populations comprise a greater number of committed cells or may suggest fundamental differences in cellular signalling between normal and CML cells at the HSC fate determination level. It is possible that lack of autocrine *BMP4* may ultimately render CML LSC more dependent on other signalling pathways e.g. Hh, to control HSC fate decisions.

4.2.6 Gene expression analysis of Hh signalling molecules in CML compared with normal haemopoiesis from gene expression microarray

As I briefly discussed in chapter 4, global microarray analysis failed to identify the majority of Hh constituents as being differentially expressed in CP CML LSC compared with normal HSC. However, there are a number of factors that made further analysis desirable. Firstly standard microarray normalisation has the effect of compressing differences between genes, thus differences in expression are generally less marked and may be less accurate and more variable in gene products with low abundance (Bemmo et al., 2008; Canales et al., 2006). Secondly, in global analysis, multiple comparisons must be borne in mind; however this is less relevant when the analysis is performed with a specific hypothesis. In this section, I present the expression data from a specific analysis of the Hh pathway in order to provide a comparison with the data above. The results are presented below in the form of a heat map and comparison of selected expression intensity plots both generated from Partek GS. From the results presented in Figure 4-7, differences in expression are small and the variability between different biological specimens is comparatively large (especially in advanced CML) for some genes (e.g. *GLI1* in comparison with *GLI3* or *STIL*).

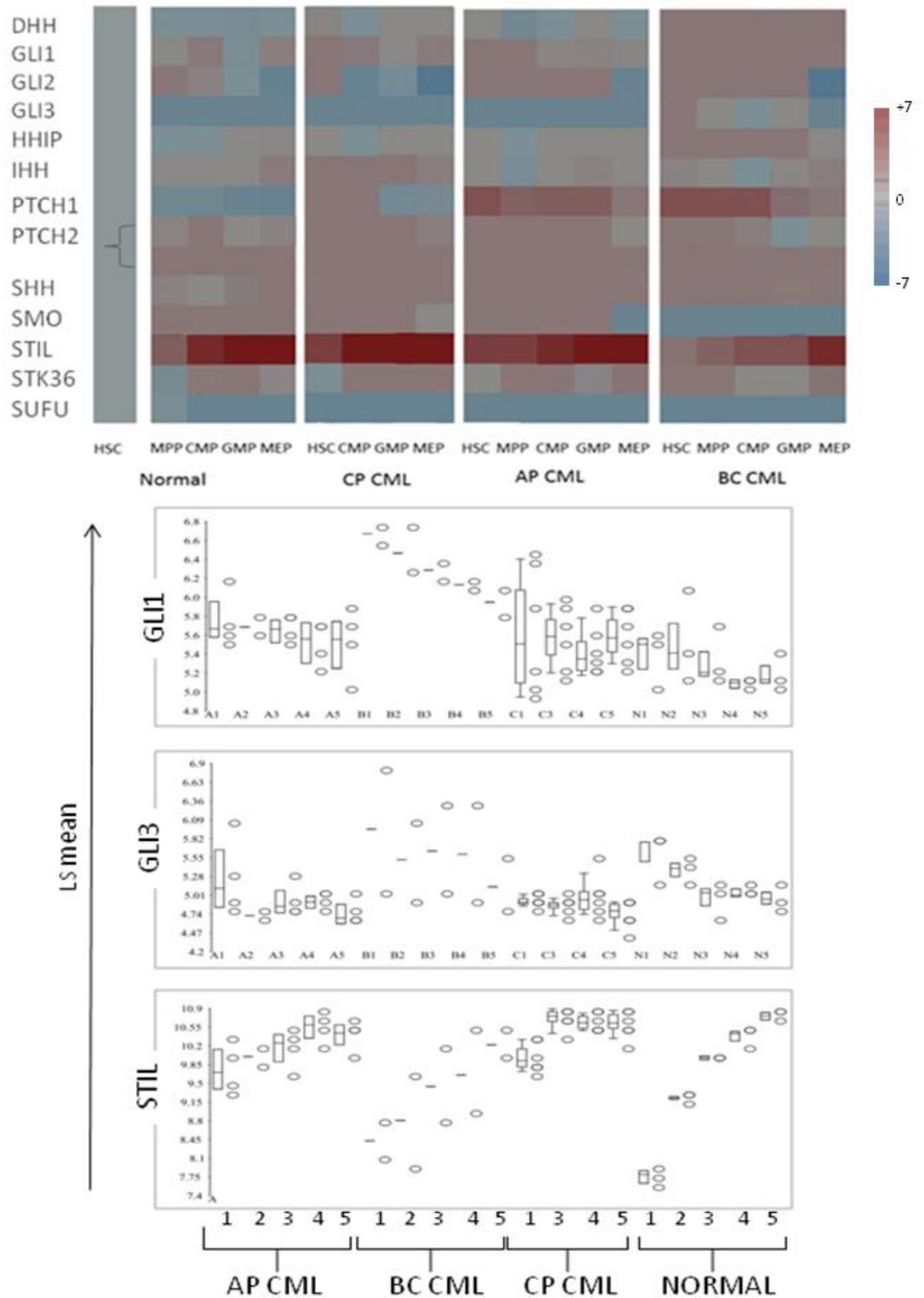


Figure 4-7: Microarray comparison of selected Hh related genes.

In (A) I present a heat map created in Partek GS representing the expression levels of various genes associated with Hh signalling in normal and CML haemopoiesis. All gene expression was relative to the normal HSC population with blue representing reduced expression and red increased expression. In (B) the relative expression levels for various Hh related genes are shown microarray by microarray. Samples are grouped by disease stage and each column represents the mean expression level for each cell population, individual microarrays are represented by a hollow dot. Samples denoted as follows; 1: HSC/LSC, 2: MPP, 3: CMP, 4: GMP, 5: MEP.

4.2.7 *BCR-ABL* expression in CML LSC and progenitor sub-populations

All CML progenitor cells harbour the *BCR-ABL* mutation, and as a consequence of their proliferative rate and mobilisation into the PB, will constitute the vast majority of circulating progenitors. The proportion of stem cells expressing *BCR-ABL* has been noted to be lower as this will reflect the proportion of remaining normal HSC compared with the number of LSC in primary BM samples. Our samples were all derived from PB and as such might be expected to have a higher proportion of LSC than BM. It was felt important to determine the frequency of *BCR-ABL* positive cells and the relative levels of expression of *BCR-ABL* in each sample and sub-population of CP and advanced phase CML.

Each sub-population in each sample was analysed by dual fusion FISH (D-FISH) as discussed in chapter 3, with results shown in Table 3-2. I also assessed *BCR-ABL* gene expression in the different stem and progenitor cell populations in CP, AP and BC CML by qRT-PCR. Although all LSC populations had substantially increased levels of *BCR-ABL* RNA, quantities were variable and the difference between the means in the different sub-populations in CP, AP or BC CML did not reach statistical significance by unpaired T-test; see Figure 4-8.

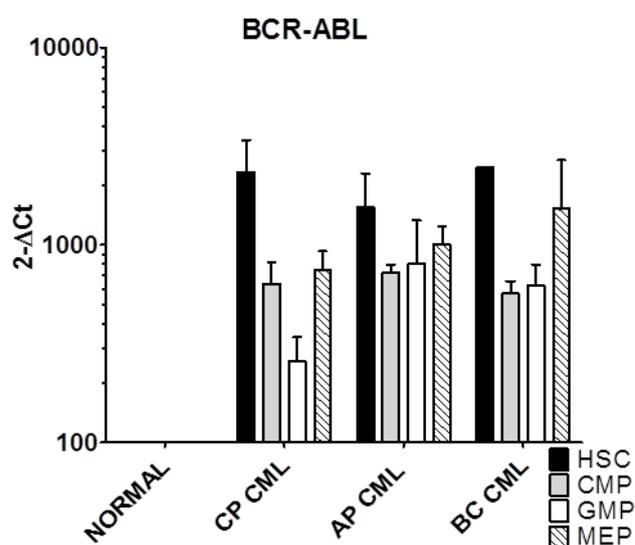


Figure 4-8: *BCR-ABL* expression in CP and advanced CML stem and progenitor cells.

Panel A shows relative gene expression *BCR-ABL* in sorted sub-populations, (HSC, CMP, GMP and MEP), from PB CD34⁺ cells derived from patients with CML at diagnosis. Results are presented as the mean \pm SEM for multiple samples normalised to GAPDH and are expressed as 2^{-ΔCt}. (CP CML, n=6; normal, n=3, AP CML, n=4, BC CML n=2; note BC CML LSC is a single sample).

4.3 Discussion

In this chapter I have presented the results of gene expression analysis for constituents and targets of the Hh morphogenic pathway in primary cells derived from CML in various phases and from normal controls in selected CD34⁺ cells in total and also across the haemopoietic stem and progenitor sub-populations that comprise this CD34⁺ population.

I assessed *BCR-ABL* gene expression in the different stem and progenitor cell populations using qRT-PCR and demonstrated expression in all sub-populations. I was intrigued to discover whether *BCR-ABL* expression was comparatively up regulated or down regulated in the leukaemic HSC over other sub-populations as this population (CD34⁺38⁻45RA⁻Lin⁻90⁺) has not been previously studied. Most previous reports have concluded that *BCR-ABL* expression is increased in CD34⁺38⁻ CML cells compared to progenitors (Copland et al., 2006; Jamieson et al., 2004; Jiang et al., 2007b), however, two early reports suggested that *BCR-ABL* might be transcriptionally repressed in primitive CML cells (Bedi et al., 1993; Keating et al., 1994). In all of the CML samples analysed, there was variably increased *BCR-ABL* expression in the LSC populations compared to the progenitor populations. Given that I had already determined that the prevalence of *BCR-ABL*⁺ cells was largely equivalent in each sub-population this suggests that *BCR-ABL* transcription appears to be increased in CML LSCs. It is not clear however whether higher expression of *BCR-ABL* is clinically relevant as recently Modi *et al*/demonstrated that cells with high levels of *BCR-ABL* expression were more sensitive to TKI therapy in a human cell model of CML and Kumari *et al*/reported that persistent *BCR-ABL*⁺ cells following TKI therapy had low levels of *BCR-ABL* expression compared to the initial diagnostic samples (Kumari et al., 2012; Modi et al., 2007).

Interesting differences were seen between the expression of key elements of Hh signalling across the progenitor populations. Quantitative RT-PCR demonstrated that expression of the key targets of Hh signalling *GLI1* and *PTCH1* is highest in the HSC population in normal haemopoiesis but tends to decline with maturity. In CML, *GLI1* expression is seen in the leukaemic HSC but is retained at a higher level in some progenitor populations (CMP, GMP) whereas *PTCH1* expression falls with maturity (Figure 4-3). Both of these genes are widely utilised as reporters of Hh activity as both are regulated at the gene expression level and expression is responsive to Hh signalling. The presented results also indicate that

the main negative regulators of Hh signalling, *GLI3* and *SUFU* are also down regulated with degree of maturity in normal haemopoiesis, but stably expressed at a low level in CML. I saw no difference in expression of the key positive regulator of Hh signalling *SMO* across sub-populations.

Whether Hh signalling is differentially expressed in CML compared to normal haemopoiesis is less clear. In our initial analysis of CD34⁺ cell populations between normal and CP CML I found that the expression of key Hh mediators and targets was variable between biological samples. I noted a tendency for increased expression of targets *GLI1*, *PTCH1* and *HHIP* over normal CD34⁺ cells but this did not reach statistical significance. I also noted that, while *SMO* did not appear to be differentially expressed, *GLI3* and *SUFU*, both negative regulators of Hh signalling, were significantly down regulated in CML. This was confirmed by the results obtained from careful dissection of the transcriptional profile of the constituent sub-populations in CML and normal samples. Here I demonstrated a striking reduction in expression of the inhibitory molecules (*SUFU* and *GLI3*) in CML, both in CP and AP. Additionally, a tendency for higher levels of expression of *GLI1* and *PTCH1* in CML LSC, possibly rising with disease progression was noted, but no differential expression of *SMO* was seen. These are intriguing results; the lack of difference in expression of the key positive regulator of Hh signalling *SMO* is in contrast with Dierks *et al*/who demonstrated that *Smo* expression was up regulated at the RNA and protein level in murine *BCR-ABL*⁺ cells (Dierks *et al.*, 2008). This provided a possible mechanism for Hh over activity in CML cells. Our results hint toward an alternative mechanism of activation relating to reduced expression of key inhibitory elements. If *SUFU* and *GLI3* are down regulated, the result would likely be to make a cell more sensitive to Hh signalling and the production of a stronger and more prolonged response (Ruiz i Altaba *et al.*, 2007). This would require further work to confirm differential expression at the protein level to take forward but is an area of great mechanistic interest.

Expression of several other targets of Hh signalling are differentially expressed between normal and CML HSC. These include *FOXM1*, *CCNB1/2* and *STIL*. While these targets are all significantly up regulated here, other signalling pathways converge on these molecules and expression may also be driven by other factors. For example *FOXM1* is a transcription factor with a role in cell proliferation and DNA repair, and in addition to Hh signalling, is regulated by input from various other paths at a transcriptional level e.g. *FOXO3A* (Delpuech *et al.*,

2007), p53 and E2F1 (Millour et al., 2011). STIL is involved in haemopoiesis and is regulated by various factors including GATA transcription factors (Bockamp et al., 1997).

Additionally, I measured expression of Hh ligands in CML compared to normal volunteers. I found extremely low levels of expression in both CD34⁺ bulk population and in sorted HSC and progenitor populations. Intriguingly, I noted up regulation of *SHH* in CML, however this did not translate into detectable levels of *SHH* protein following culture. This may be due to lack of sensitivity of our ELISA or instability of the protein. Longer culture of the cells prior to harvesting the supernatant or concentration of the supernatant might improve sensitivity.

I compared the results of our qRT-PCR with those of the global expression microarray described in chapter 3. No significant differences in expression levels were noted after adjustment for multiple comparisons. One reason for this is the variability between biological replicates evident, however another contributory factor may be that the normalisation procedures involved in microarray analysis are known to compress differences and may result in smaller differences between populations than would be seen by other techniques. This is most evident in low abundance targets (Bemmo et al., 2008; Canales et al., 2006). It is therefore perhaps not surprising that Hh signalling (or for that matter, other low abundance targets) were not highlighted in the initial microarray analysis. Further replicates would have been beneficial but were precluded by the cost and resource implications.

During this study I was fortunate to be able to develop collaboration with Professor Ravi Bhatia at the City of Hope, Duarte, California. In a set of parallel qRT-PCR expression experiments in the *Scf-tTa-BCR-ABL* murine model of CML (Koschmieder et al., 2005), Professor Bhatia and his team demonstrated increased expression of *Gli1*, *Ptch1*, *CcnB1* and *Ptch2* in CML LT-HSC compared to control cells, and a significant difference in *Gli3* was noted in CML BM compared to normal BM and are presented in Appendix 2.

Previous studies have investigated whether Hh signalling components are up regulated in CML. These studies have largely, but not exclusively, been confined to advanced phase disease and/or murine models of CML and have been discussed in chapter 1. More recently other studies have reported variably increased expression of Hh mediators and targets in patients with CML. Long *et al* and Su *et al* have performed semi-quantitative PCR on MNC from patients with

CML at diagnosis and during treatment. Both groups found up regulation of *GLI1*, *PTCH1* and *SMO*, the key positive components of Hh signalling but did not investigate the expression of the negative regulators of the pathway. In these studies it is not clear to what extent their findings reflected the likely heterogeneous composition of the samples analysed (Long et al., 2011; Su et al., 2012). While several studies (noted above) have investigated the expression of Hh mediators and targets at the gene expression level in CML, none have convincingly approached the expression of these entities at the protein level. This is most probably due to the difficulty inherent in performing techniques such as Western blotting for low abundance proteins in highly purified and hence highly limited cell populations, not to mention the paucity of available human reactive Hh antibodies. Proteomic analysis would certainly be desirable, particularly for investigating the interactions between GLI2, GLI3 and other cytoplasmic proteins that have both positive and negative roles in Hh signal propagation depending on cytoplasmic protein complex associations, phosphorylation and ubiquitination and partial truncation. Dierks *et al*/performed immunohistochemistry on human and murine BM confirming expression of SMO and GLI1 at the protein level. Additionally they saw elevation of SMO expression by flow cytometry in cells obtained from their murine model (*pMSCV/Bcr-Abl/GFP*) (Dierks et al., 2008). Western blot analyses in primary cells were not attempted as this would have required a prohibitively large number of cells. Attempts to measure SMO and GLI1 by flow cytometry in primary cells in our studies were not successful.

Lastly I noted the associated signalling molecule *BMP4* was highly expressed in the normal HSC population but down regulated rapidly with maturity; however *BMP4* was significantly reduced in all CML LSC populations. *BMP4* is a member of the TGF β family of growth factors and acts via cell surface heteromeric receptors to activate intracellular SMAD signalling through phosphorylation of SMAD1/5/8 which interact with SMAD4, translocate to the nucleus and activate a transcriptional programme that includes GATA1/2, SCL and LMO2 (Sadlon et al., 2004). *BMP4* has an important role in developmental haemopoiesis and cooperates with IHH to specify haemopoietic development (Sadlon et al., 2004). *In vitro* studies of adult haemopoiesis have demonstrated potential dose-dependent roles for *BMP4* in the maintenance or differentiation of HSC populations in culture (Bhatia et al., 1999). *BMP4* signalling also interacts with Hh signalling in this context, functioning downstream of Hh and cooperating with it to maintain or expand the HSC population *in vitro* (Bhardwaj et al., 2001). *In vivo*, reduced

microenvironmental expression of BMP4 resulted in reduced maintenance and transplantability of normal HSC (Goldman et al., 2009). BMP4 signalling is an important component of microenvironmental signalling and may affect HSC function directly or indirectly through affecting the cells that comprise the niche (Goldman et al., 2009). There is evidence that osteoblasts and endothelial cells are responsive to BMP4 (Suzuki et al., 2008; Zhang et al., 2003). Therefore the fact that BMP4 is reduced in CML LSC might have functional consequences through CML LSC to LSC autocrine/paracrine signalling or indirectly by influencing stromal cells and consequently the signalling milieu in which the CML LSCs reside. It might also be the case that as CML LSC are less likely to be resident in the BM niche due to reduced adhesion and chemotaxis, that autocrine/paracrine signalling is comparatively more important. Certainly, particular aspects of extramedullary haemopoiesis in the mouse, e.g. development of stress erythroid progenitors in the spleen, require interaction of BMP and Hh signalling (Perry et al., 2009).

In summary I have confirmed expression of the key mediators of Hh signalling in CML and in normal haemopoiesis. Active signalling is suggested by the expression of the key targets *GLI1* and *PTCH1* amongst others, and this activity is largely confined to the more primitive HSC populations in normal haemopoiesis. In CML, expression of these targets tended to be increased in the LSCs and, in the case of *GLI1*, the CMP population as well. There was consistent reduction in expression of the key negative regulators *SUFU* and *GLI3*, which may result in increased magnitude and duration of response to received signal. Therefore HH signalling may be considered a possible stem cell target in CML, although its utility would be dependent on there being a therapeutic window of effect between normal and malignant haemopoiesis.

5 Effect of Specific Inhibitors of Hh Signalling on CP CML in Short Term *in vitro* Culture

5.1 Introduction

In chapter 1 we discussed the critical role of Hh signalling during embryogenesis and in early haemopoiesis, in contrast to its function in adult organisms, where it has a potential role in regeneration, directing stem cell fate and potentially as a driving force in malignancy. While the Hh signalling pathway is one of many with overlapping functions in the complex processes that govern self-renewal behaviour, development of Hh inhibitors have had a head start compared with other self-renewal pathways. In the first instance, a prototype SMO inhibitor is available from nature; the steroidal alkaloid cyclopamine has been studied extensively, both in terms of its pharmacological and biochemical effect and in terms of functional outcome in models of Hh-dependent tumours. Cyclopamine acts by binding directly to the hepta-helical bundle of SMO, preventing the conformational change that would normally occur when PTCH repression is removed (in response to Hh ligand) and causing an alteration in its sub-cellular distribution (figure 1-4, chapter 1) (Chen et al., 2002). The effect of this is to prevent downstream activation of the GLI transcription factors and therefore implementation of the Hh signalling transcriptional programme (Chen et al., 2002; Taipale, 2000).

Cyclopamine is unsuitable for clinical use due to instability, significant off-target effects and toxicities (Heretsch et al., 2010). However, it has provided the impetus to develop more clinically relevant compounds, including LDE225 (Erismodegib) and its derivative LEQ506. LDE225 was identified as a highly selective SMO inhibitor in a cell-based high throughput screen looking for compounds with highly selective Hh inhibitor activity. Mechanistically, LDE225 was shown to displace BODIPY-cyclopamine from human and murine SMO in competitive assays (Pan et al., 2010). Thus, LDE225 appears to interact directly with SMO in a similar way to cyclopamine. Functionally, LDE225 has been shown to reduce expression of downstream targets of Hh signalling in various cell lines and clinical models of Hh-driven malignancy (Pan et al., 2010). Unlike cyclopamine, LDE225 represents a clinical - grade SMO inhibitor with real translational potential and is currently under clinical evaluation in solid malignancies.

5.1.1 Measuring the activity of Hh signalling in cells

In order to assess the effects of LDE225 on the Hh pathway *in vivo* and *in vitro*, biomarkers of Hh activity are required. The measurement of Hh activity is most readily achieved through assessing its effect on gene expression (measured by qRT-PCR) of immediate downstream targets. The most widely accepted direct targets of Hh signalling are *GLI1* and *PTCH1/2* (Marigo and Tabin, 1996; Pearse et al., 2001; Shahi et al., 2010; Vokes et al., 2007; Vokes et al., 2008; Yang et al., 2011; Yang et al., 2010; Yoon et al., 2002; Zhu and Lo, 2010). While *PTCH1/2* exhibit basal expression that increases following Hh signalling, *GLI1* is principally controlled by activated *GLI2* itself, influenced by upstream Hh signalling, and is not normally expressed (Dai, 1999; Hui and Angers, 2011).

5.1.2 Hh modulation of cell cycle and proliferation

As discussed in chapter 1, Hh signalling exerts a cell type and context specific influence on cell cycle progression and cell proliferation (Yoon et al., 2002). Potential downstream targets of the Hh pathway include elements acting at various stages in the cell cycle including several cyclins e.g. *CCNB1/2*, *CCND1/2* and *CCNE* in addition to other cell cycle active proteins, such as *SCL/TAL*, *FOXA2*, *FOXM1* and *p57^{Kip2}* (Barnes et al., 2001; Duman-Scheel et al., 2002; Hao et al., 2006; Hochman et al., 2006; Kenney and Rowitch, 2000; Roy and Ingham, 2002; Shahi et al., 2010; Teh et al., 2002; Trowbridge et al., 2006; Yoon et al., 2002) (see chapter 1 table 1-4). Hh signalling generally has a stimulatory effect on cell cycle and proliferation, driving proliferation of sensitive tissues and Hh-dependent tumours (Berman et al., 2002; Berman et al., 2003; Cayuso et al., 2006; Duman-Scheel et al., 2002; Roy and Ingham, 2002; Stecca et al., 2007).

In normal haemopoiesis, as discussed in chapter 1 section 1.2.6.1, Hh signalling drives the expansion of haemopoietic precursors. Bhardwaj *et al* reported that exogenous Hh ligands caused primitive haemopoietic cell proliferation and expansion of cell numbers in *in vitro* culture that could be abrogated by specific antibody or pharmacological inhibition (Bhardwaj et al., 2001; Sengupta et al., 2007). Trowbridge *et al* reported expansion of primitive haemopoietic cells in *Ptch*^{+/-} heterozygotic mice, a model of Hh activation, associated with modulation of several cell cycle mediators including *Ccnd1* prior to HSC exhaustion (Trowbridge et al., 2006). Additionally, Merchant *et al* reported a reduction in proliferation associated with reduced expression of *Ccnd1* in HSCs

and myeloid precursors derived from a murine model lacking expression of *Gli1* (Merchant et al., 2010). However in the Smo deletion models reported by Gao *et al.* and Hofmann *et al.*, no evidence of reduction of the HSC or progenitor populations was found nor was there a difference in cell cycle distribution in these cells (Gao et al., 2009; Hofmann et al., 2009).

5.1.3 Hh as a survival signal

Hedgehog signalling can also influence apoptosis and survival. The anti-apoptosis regulator BCL2 has been shown to be a downstream target of Hh signalling. (Abe et al., 2008; Bigelow et al., 2004; Cayuso et al., 2006; Regl et al., 2004). Additionally, Hh signalling affects sensitivity to p53-mediated apoptosis through down regulation of expression and up regulation of MDM2-mediated ubiquitination and clearance in breast cancer (Abe et al., 2008). Inhibition of Hh signalling results in reduced viability and increased apoptosis in disparate tumours e.g. models of pancreatic or colon cancer and diffuse large B cell lymphoma (Berman et al., 2003; Dierks et al., 2007; Guo et al., 2009; Singh et al., 2010).

5.1.4 Measuring Hh activity and its effect in CML haemopoiesis

Studies in CML have utilised these markers to demonstrate Hh activity. Dierks *et al.* demonstrated modulation of *GLI1* expression in response to genetic or pharmacological SMO inhibition in their murine model but the murine models of Zhao *et al.* did not directly look at downstream transcriptional effects of Smo inhibition either in *Smo* deleted or cyclopamine treated cells (Dierks et al., 2008; Zhao et al., 2009). Neither study directly assessed downstream transcriptional effects of Hh inhibition in primary CML samples. The only study to do so was by Sengupta *et al.*, who witnessed a 2-fold induction of *GLI1* in response to 24h culture with SHH in CP CML CD34⁺ cells, but no effect of *CCND1* or *PTCH1* expression (Sengupta et al., 2007). Reduction of *GLI1* and *PTCH1* transcription was seen following exposure to forskolin. However the dose of SHH used was supra-physiological (3000ng/mL) and inhibition was indirect as forskolin acts via PKA activation through adenylyl cyclase leading to reduced GLI activation (Tiecke et al., 2007). Additionally, it is now appreciated that, particularly in malignancy, alternative activation pathways converge on GLI reflecting the complex network of interactions between various converging signalling pathways that determine cell fate (Blotta et al., 2012; Katoh, 2007; Lauth and Toftgard, 2007; Li et al., 2007a; Maeda et al., 2006; Nolan-Stevaux et al., 2009; Sengupta et al., 2007). Thus the

question of the optimal biomarker for Hh activity in CML has not been comprehensively studied and requires further investigation, particularly in the context of *in vitro* treatment of primary CML samples. In CML, contrasting with normal haemopoiesis, Dierks *et al*/reported both significant levels of apoptosis and alteration in cell cycle distribution in BCR-ABL⁺ cells in their murine model following short term culture with 2 μ M or 5 μ M cyclopamine. However, these may be non-specific, as in their study, Zhao *et al*/utilised 1 μ M cyclopamine and demonstrated off target effects with 3 μ M cyclopamine (toxicity in *Smo*^{-/-} cells) (Dierks et al., 2008; Zhao et al., 2009).

Thus, from the above discussion, a key question relates to whether more specific inhibition of Hh signalling with LDE225 can be measured through assessment of downstream transcriptional targets in CML cells in *in vitro* culture and whether such inhibition would affect cell cycle status, proliferation and apoptosis in primitive primary CP CML cells.

In this chapter, I sought to: (1) determine if LDE225 inhibited transduction of Hh signalling, through inhibition of transcription of its immediate downstream targets thereby identifying a biomarker for SMO inhibition in CP CML; and (2) characterise the effect of LDE225 alone and in combination with conventional treatment with nilotinib, a potent TKI against BCR-ABL, on primary CP CML CD34⁺ cells in short term *in vitro* culture.

Specific objectives covered in this chapter are described below;

- (1) Examination of the effect of LDE225 on downstream targets of Hh signalling:
 - a. In a GLI-driven luciferase reporter assay;
 - b. In the K562 BC CML cell line;
 - c. In primary CP CML CD34⁺ cells.
- (2) Examination of the effect of inhibition of SMO with LDE225 alone and in combination with nilotinib on:
 - a. CD34⁺ CP CML cell viability and apoptosis measured by vital dye exclusion and annexin V / 7AAD apoptosis assays;
 - b. CD34⁺ CP CML cell proliferation and cell cycle status;
 - c. CP CML cell division history and expression of primitive cell surface markers.

5.2 Results

5.2.1 LDE225 and LEQ506 cause dose dependent reduction in luminescence in a GLI responsive luciferase reporter assay

In order to measure the concentration of SMO inhibitor required to inhibit expression of downstream targets, TM3^(GLI-Luc) cells were first obtained from Marion Dorsch, Novartis. This is a murine Leydig tumour line with intact Hh signalling machinery, stably transfected with a retroviral vector selectable by G418 sensitivity, carrying 8 GLI-responsive elements and a luciferase gene, such that luciferase expression is dependent on GLI activation. Therefore, increased Hh signalling will result in increased luciferase expression (Pan et al., 2010). These cells were cultured in TM3 media under G418 selection. In a first set of experiments, the optimal concentration of agonist was determined. The TM3 cells were seeded at 10,000 cells per well in an opaque white 96 well culture plate and exposed to incremental concentrations of the direct SMO agonist purmorphamine. Luciferase activity could then be assessed in triplicate, at 24, 48 and 72h. The results of this experiment indicate that maximal luciferase activity was demonstrated following 48h exposure to 2.5µg /mL purmorphamine, as shown in Figure 5-1. In a second set of experiments, the TM3 cells were again seeded as above but this time with incremental concentrations of the SMO inhibitors under analysis. After 6h the optimal dose of purmorphamine indicated by the first experiment was added and luciferase activity measured at 48h. Two clinical grade SMO inhibitors were assessed in this way; LDE225 and LEQ506. The results of this experiment indicate that LDE225 has an IC50 for inhibition of GLI activity of approximately 8nM and LEQ506 has an IC50 for inhibition of GLI activity of approximately 2nM Figure 5-1. Despite the higher potency of LEQ506, further experiments were performed using LDE225 as material transfer agreements with Novartis prevented publication of data pertaining to LEQ506.

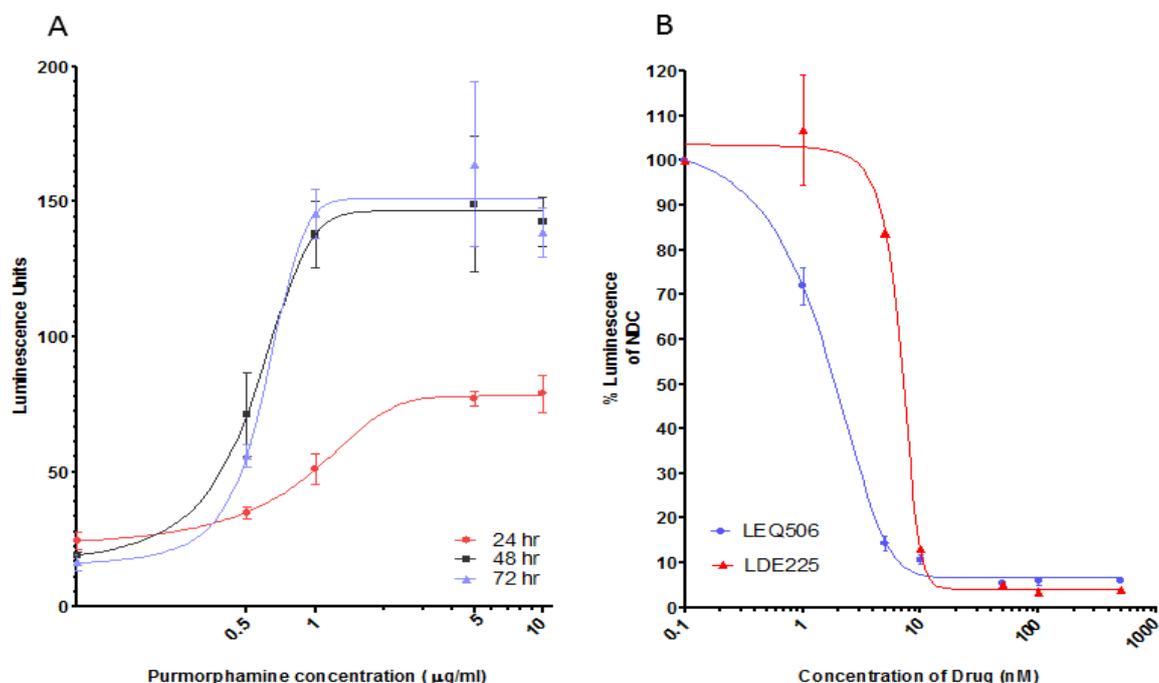


Figure 5-1: SMO activation and inhibition in Hh responsive TM3 reporter cell line.

Panel A indicates the luciferase activity following culture of TM3^{GLI-Luc} cells in TM3 media supplemented with incremental doses of the known direct SMO inhibitor purmorphamine at 24h (red), 48h (black) and 72h (blue). Maximal luciferase activity was demonstrated after 48h culture with 1µg/mL purmorphamine. Panel B demonstrates the relative activity of two small molecule SMO inhibitors in TM3^{GLI-Luc} cells. Cells were exposed to incremental doses of SMO inhibitor and stimulated by 2.5µg /mL purmorphamine. Results are expressed as a percentage of the luminescence of the untreated control (stimulated, but no SMO inhibitor). The point represents the mean of at least 3 replicates and error bars indicate the SEM. NDC, no drug control.

5.2.2 SMO inhibition with LDE225 reduces expression of downstream Hh target genes in K562 cells

In the previous section, I demonstrated the effect of LDE225 on *GLI1*, a key downstream target of Hh signalling in a non-haemopoietic luciferase reporter system. It would be desirable to demonstrate whether the effect of LDE225 could be similarly measured in a more suitable system. An early culture and expression experiment using the BC CML cell line, K562, demonstrated that K562 cells expressed *GLI1* and *PTCH1* and *CCND1* and that following 16h exposure to high concentrations of LDE225 there was a reduction in *GLI1* and *CCND1* expression but not *PTCH1* (Figure 5-2).

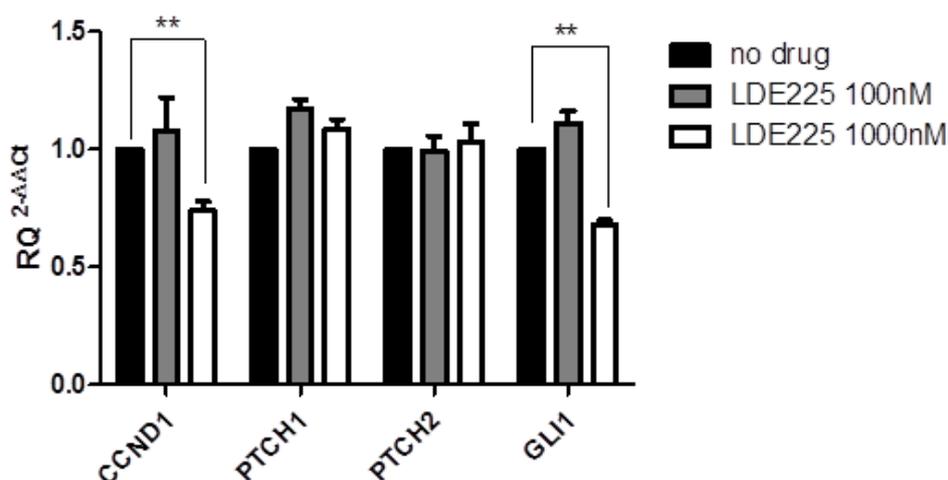


Figure 5-2: Gene expression analysis of the BC CML cell line K562 following exposure to LDE225.

K562 cells were cultured in standard media with LDE225 at the stated concentrations over 16h. Extraction of RNA, conversion to cDNA and amplification were performed as indicated in the methods section. Expression levels are shown as fold change ($2^{-\Delta\Delta Ct}$) relative to the untreated control using *GAPDH* as an endogenous control. The mean of three reactions in a single experiment are presented with error bars indicating SEM and significance $p < 0.01$ represented by ** (paired T-test).

5.2.3 SMO inhibition with LDE225 reduces expression of GLI1 in CD34⁺ CP CML cells

While a number of CML-derived cell lines exist and offer a plentiful supply of cellular material for investigation, these cell lines, including K562, are derived from advanced phase disease, have become immortal and consequently are likely to have radically altered self-renewal behaviour and machinery. Primary CD34⁺ CP CML cell populations offer a more relevant model in which to study potential biomarkers of Hh activity following SMO inhibition as they retain cells with stem cell activity derived from the stage of disease that was of primary interest.

In order to define a potential biomarker for LDE225 activity in CP CML, CD34⁺ cells were cultured in SFM in the presence of incremental concentrations of LDE225 (10-1000nM) over 6, 24 and 72h. Following culture the cells were harvested for expression analysis of downstream targets and constituents of the Hh pathway by qRT-PCR as described in detail in the methods section (section 2.2.12). The results are presented in Figure 5-3 and are discussed below. No significant alteration in expression of target genes *GLI1*, *CCND1*, *CCND2*, *PTCH1* or *PTCH2* was detected at 6 or 24h following exposure to up to 1000nM LDE225 (Figure 5-3A; 24h; 6h data not shown). At 72h, a small down regulation of *GLI1*

expression was noted following exposure to LDE225 (LDE225 10nM, 0.7 fold, LDE225 100nM 0.73 fold; Figure 5-3B). Cells treated with LDE225 at 1000nM concentration showed a non-significant reduction in *GLI1* expression (0.84 fold). A non-significant reduction in *PTCH1* expression was also noted (LDE225 1000nM, 0.72 fold). As would be anticipated, no alteration in the expression of *GLI2* or *SMO* was seen at any time point following treatment with LDE225 (they are components but not direct targets of Hh signalling).

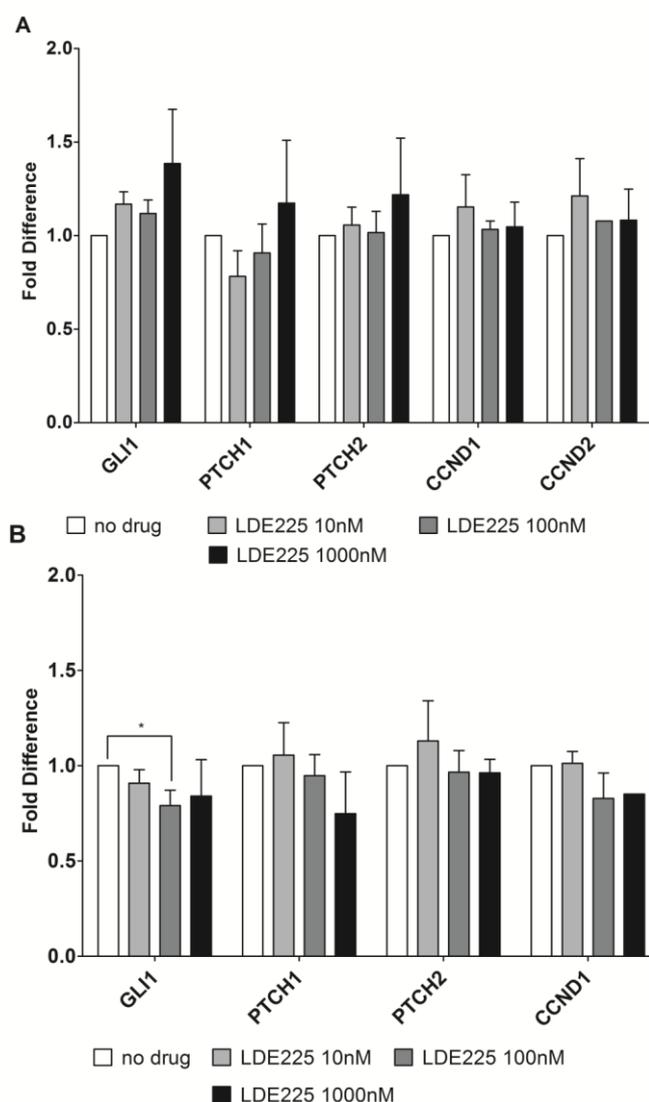


Figure 5-3: Quantitative RT-PCR measuring expression of several constituents and targets of Hh signalling in CD34⁺ CP CML cells after (A) 24h and (B) 72h culture in SFM in the presence of LDE225.

Primary CD34⁺ cells were cultured in SFM in the presence of LDE225 10, 100 or 1000nM as stated for 24 or 72h, prior to qRT-PCR analysis. Expression levels are shown as fold change ($2^{-\Delta\Delta C_t}$) relative to the untreated control with *GAPDH* as an endogenous control. The mean of 5-8 independent samples are presented with error bars indicating SEM and significance $p < 0.05$ represented by * (paired T-test). Data for non-targets *SMO* and *GLI2* is not shown.

Closer analysis of the distribution of response to LDE225 between different samples demonstrated significant biological variability in LDE225-mediated *GLI1* response (Figure 5-4). From the samples tested, several respond to exposure to LDE225 by reducing expression of *GLI1* whereas others do not, suggesting that susceptibility to Hh inhibition may be variable or dependent on as yet unknown cooperating factors. It would be of great interest to test a significantly larger cohort of samples in order to validate this observation and investigate potential causes.

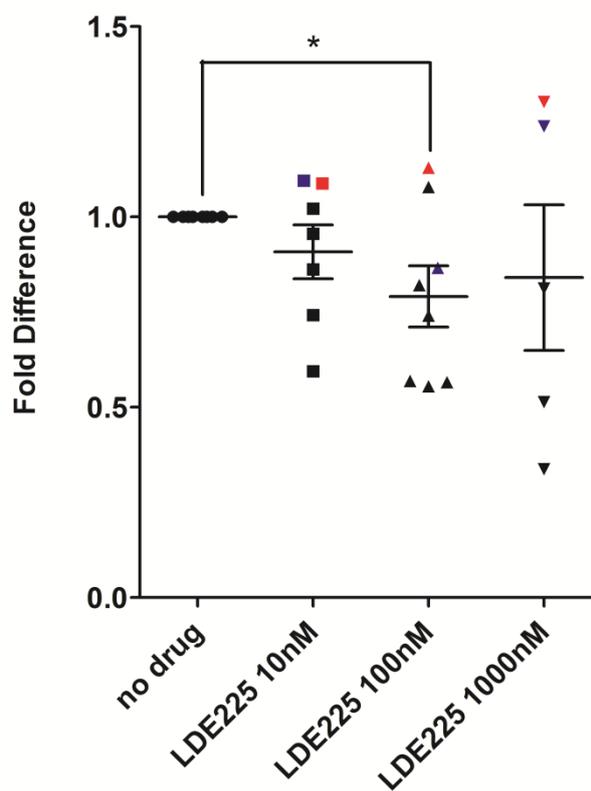


Figure 5-4: Quantitative RTPCR measuring expression of *GLI1* in CD34⁺ CP CML cells after 72h culture in SFM in the presence of LDE225.

Individual sample values plotted by fold difference in *GLI1* expression indicating the variability between independent biological samples following exposure to LDE225 in culture over 72h as above. Expression levels are shown as fold change ($2^{-\Delta\Delta Ct}$) relative to the untreated control with *GAPDH* as endogenous control. The mean of 5-8 reactions are presented with error bars indicating SEM and significance $p < 0.05$ represented by * (paired T-test). Non-responding samples are shown as follows - 339; red and 274; blue.

Additionally, in an attempt to enhance baseline Hh signalling, several CP CML CD34⁺ samples were also cultured in the presence of rSHH (at up to 500ng/mL) over 24 and 72h. Up regulation of downstream targets was noted as shown below, however the response to rSHH was variable and the differences seen did not reach statistical significance (Figure 5-5). This is in contrast to work published by Sengupta *et al*/who did note a 2-fold up regulation of *GLI1* and a 1.5

fold up regulation of *PTCH1* in the presence of rSHH. One possible explanation for this difference is that Sengupta *et al*/used considerably higher doses of rSHH (3000ng/mL) (Sengupta et al., 2007).

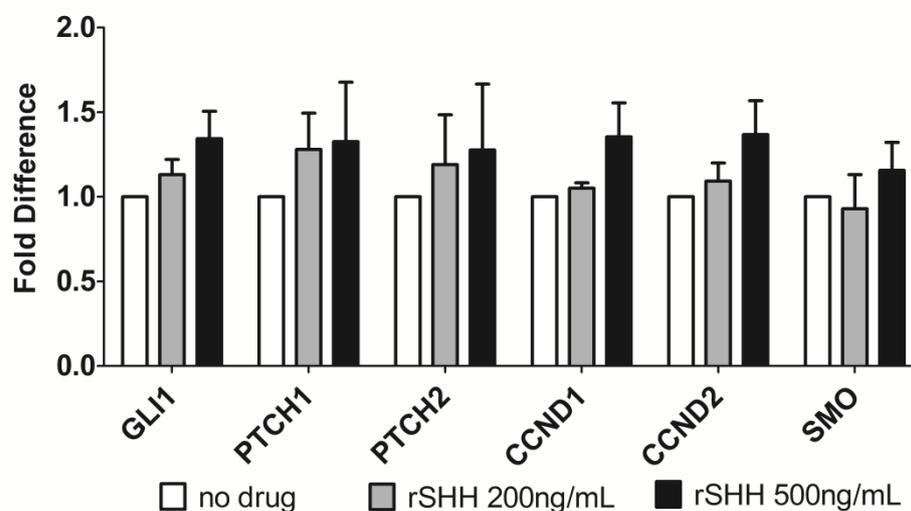


Figure 5-5: Quantitative RTPCR measuring expression of *GLI1* and other downstream targets of Hh signalling in CD34⁺ CP CML cells after 24h culture with rSHH.

Primary CD34⁺ cells were cultured in SFM in the presence of rSHH as stated for 24h, prior to qRTPCR analysis. Expression levels are shown as fold change ($2^{-\Delta\Delta Ct}$) relative to the untreated control with *GAPDH* as endogenous control. The mean of 3 independent samples are presented. Error bars represent the SEM.

5.2.4 SMO inhibition with LDE225 alone and in combination with nilotinib does not influence viability in CP CML CD34⁺ cells in short term *in vitro* culture

Primary CD34⁺ CP CML cells were cultured in SFM in incremental concentrations of LDE225 (up to 1000nM LDE225) alone or in combination with 5 μ M nilotinib for 24, 48 and 72h. Viable cell counts were performed using trypan blue exclusion and the results are presented in Figure 5.6 below. Additionally, separate cultures were performed with CD34⁺ CP CML cells in SFM+HGF and in the presence of LDE225 (5-50nM) and/or nilotinib (5 μ M). These were extended over 12d with re-seeding in fresh media and drug every fourth day. No difference in viable cell count was seen compared to the untreated control in SFM alone at up to 72h following drug exposure as assessed by trypan blue dye exclusion. Furthermore while nilotinib, as expected, reduced viable cell counts after 4d, addition of LDE225 at up to 50nM did not enhance the effect of nilotinib in culture at up to 12d duration (Figure 5-6).

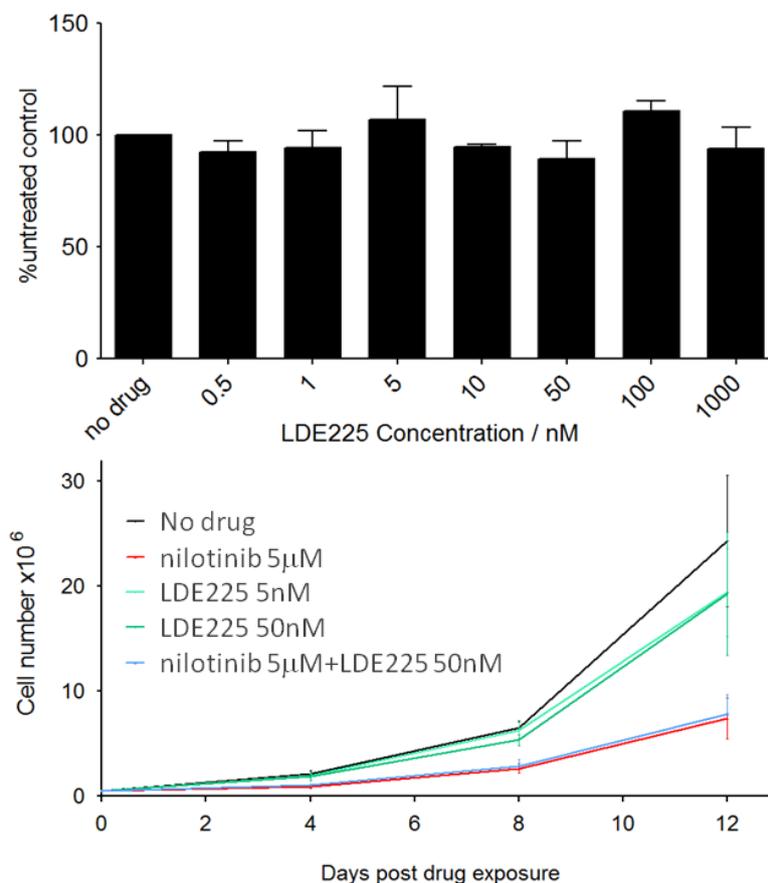


Figure 5-6: Viable CD34⁺ CP CML cells following exposure to LDE225 +/- nilotinib.

The top panel indicates the number of viable cells at 72h following culture in SFM with incremental concentrations of LDE225 (n=3-6). The bottom panel indicates the number of viable cells at 4, 8 and 12d following culture with LDE225 +/- nilotinib at the stated concentrations in SFM +HGF. Error bars represent the SEM (n=4).

5.2.5 SMO inhibition with LDE225, alone and in combination with nilotinib, does not influence proliferation of CP CML CD34⁺ cells in short term *in vitro* culture

While incorporation of vital dye provides some indication of comparative proliferative rate and viability, there are limitations to its usefulness e.g. counting accuracy, homogeneity of cell solution, the fact that staining increases over time and that the recognition of viable cells by membrane integrity will not count cells as non-viable until fairly late in apoptosis. Therefore apoptosis was also measured by annexin V / 7AAD staining and proliferation by BrDU incorporation (see methods section 2.2.9.3 and 2.2.10.2). Chronic phase CML CD34⁺ cells were cultured in SFM in the presence of incremental concentrations of LDE225 over 24-72h. To measure proliferation, BrDU was added to the culture wells 16h prior to harvest, the cells were then washed, fixed and the quantity of BrDU incorporation was measured by colourimetric change according to the manufacturer's instructions.

No difference in overall proliferation rate was seen by BrDU incorporation at up to 1000nM LDE225 for up to 72h (Figure 5-7).

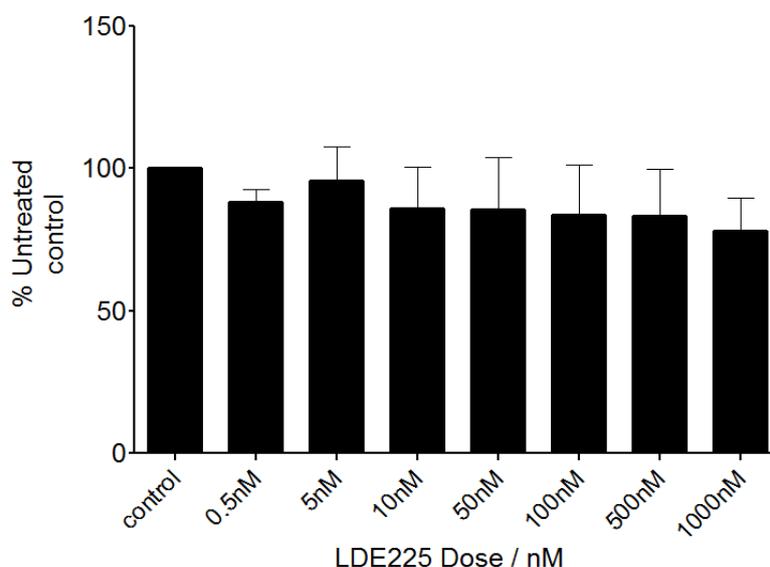


Figure 5-7: Chronic phase CML CD34⁺ cell proliferation in presence of LDE225 at 72h.

The mean proliferation of 3 independent CD34⁺ CP CML samples as measured by BrDU incorporation at 72h is shown above. Values are presented as a percentage of untreated control. Error bars indicate the SEM.

5.2.6 SMO inhibition with LDE225, alone and in combination with nilotinib, does not cause apoptosis in CP CML CD34⁺ cells in short term *in vitro* culture

To measure apoptosis, CD34⁺ CP CML cells were cultured in SFM in the presence of LDE225 (5-500nM) and/or nilotinib 0.5 μ M-5 μ M. Apoptosis was assessed at 24h intervals for 72h by annexin V and 7AAD incorporation, measured by flow cytometry. As expected, nilotinib caused increased apoptosis in CP CML CD34⁺ cells. LDE225 neither caused apoptosis nor accentuated the apoptotic effect of nilotinib (Figure 5-8).

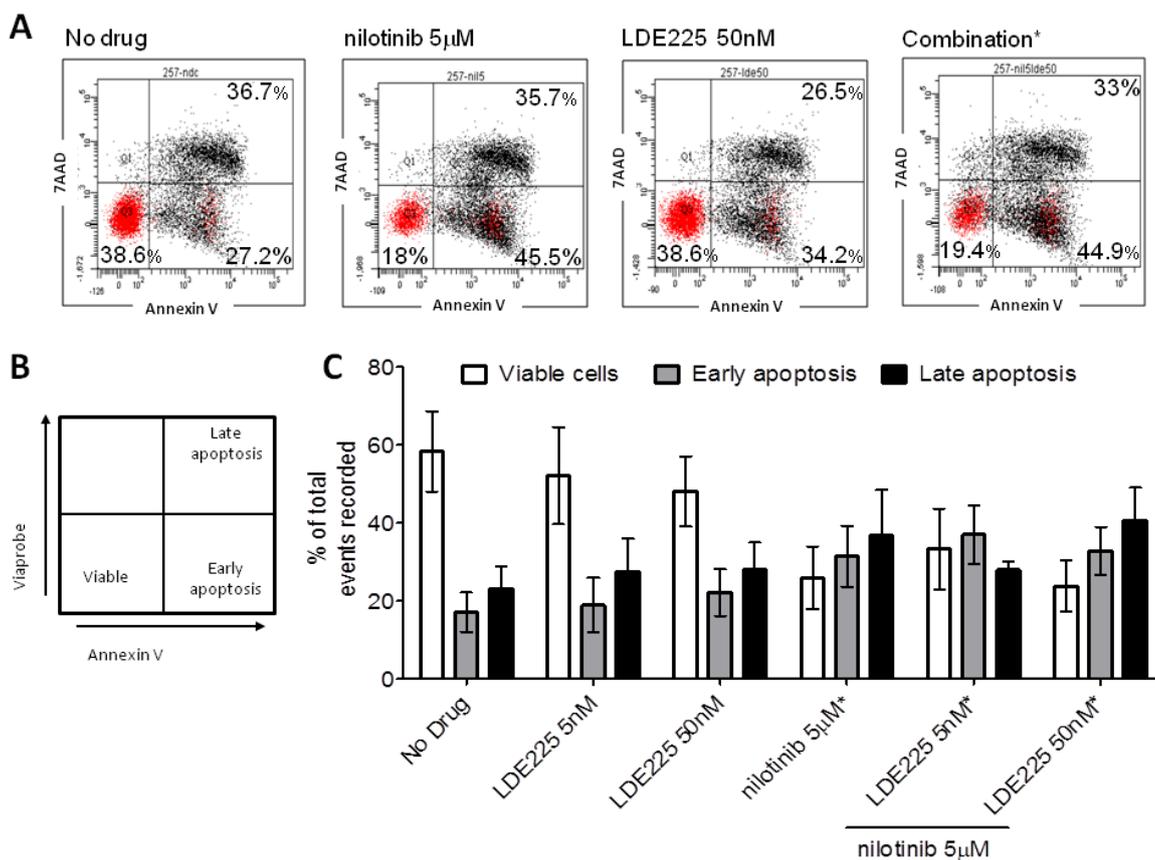


Figure 5-8: Apoptosis assays in CP CML CD34⁺ cells exposed to LDE225 and/or nilotinib.

Panel A shows representative FACS plots following exposure to LDE225, nilotinib or the combination over 72h. The CD34⁺ cells were stained with annexin V and 7AAD prior to analysis by flow cytometry. The percentages shown indicate the proportion of total events that fell within the respective gates. The combination shown is LDE225 50nM and nilotinib 5µM. Panel B provides the schema for interpretation of the flow cytometry plots. Panel C indicates the proportion of viable cells, those in early apoptosis and those in late apoptosis in 3 independent CD34⁺ cell samples following 72h culture with LDE225 and or nilotinib at the concentration shown. Error bars represent the SEM.

5.2.7 SMO inhibition with LDE225, alone and in combination with nilotinib, does not influence cycle distribution of CP CML CD34⁺ cells in short term *in vitro* culture

To assess cell cycle status CD34⁺ CP CML cells were cultured in SFM in the presence of LDE225 at 5 or 50nM +/- nilotinib at 5µM. Cell cycle status was assessed by flow cytometry using the high resolution cell cycle analysis employed by Jordan *et al.* (Jordan et al., 1996) and described fully in methods section 2.2.9.5. Briefly, cells under analysis were stained with Ki67 and 7AAD. The resultant stained cells could then be assessed by flow cytometry which allowed identification of cells in G₀ (Ki67⁻/7AAD^{lo}), G₁ (Ki67⁺/7AAD^{lo}) and G₂/S/M (Ki67⁺/7AAD⁺). While, as expected, nilotinib caused accumulation of cells in G₀, previously reported by

Jorgensen *et al*, LDE225 neither affected the cell cycle distribution of CD34⁺ CP CML cells at up to 72h exposure and up to 50nM concentration or accentuated the effect of nilotinib (Figure 5-9) (Jorgensen et al., 2007).

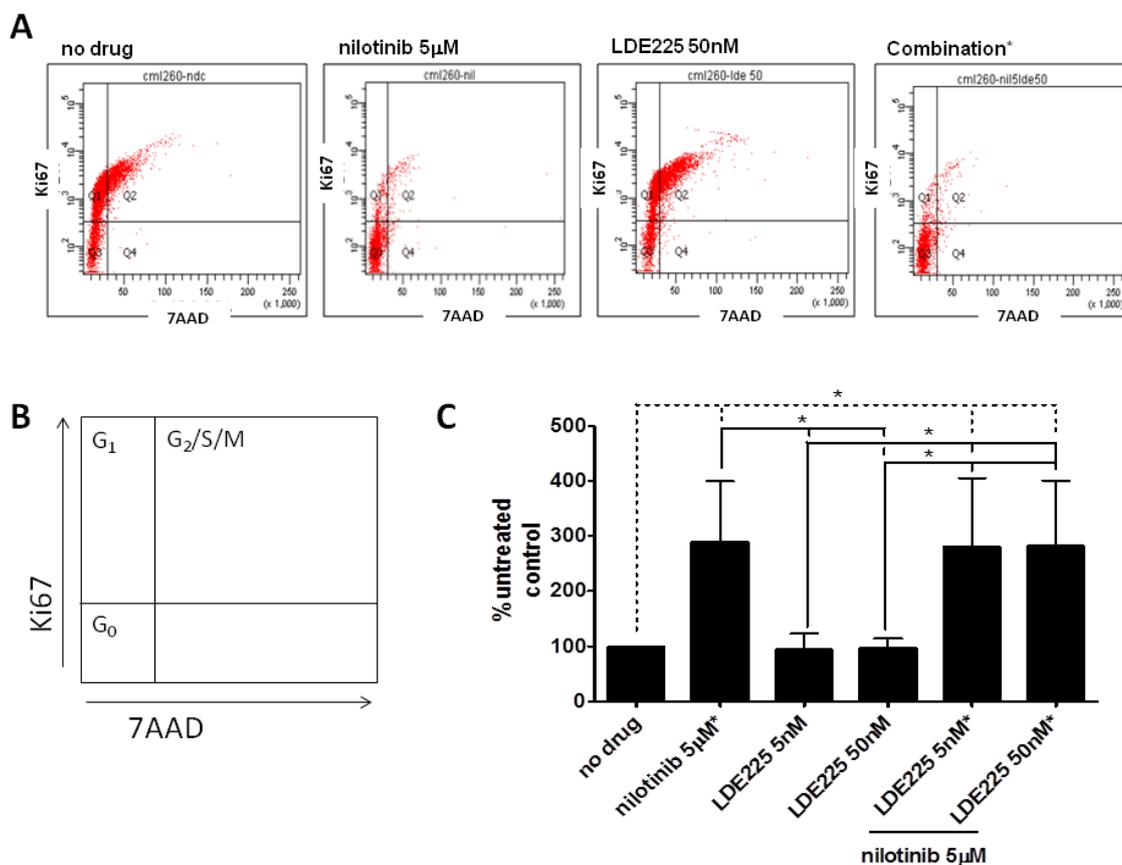


Figure 5-9: High resolution cell cycle analysis in CP CML CD34⁺ cells exposed to LDE225 and/or nilotinib.

Panel A shows representative FACS plots following exposure to LDE225, nilotinib or the combination over 72h. The CD34⁺ cells were stained with Ki67 and 7AAD prior to flow cytometry analysis. The combination shown is LDE225 50nM and nilotinib 5µM. The flow cytometry plots are represented on log/linear axes with Ki67-PE staining represented on the logarithmic y axis and 7AAD detected in FL-3 on the linear x axis. Panel B provides the schema for interpretation of the flow cytometry plots. Panel C indicates the proportion of cells residing in G₀ in 3 independent CD34⁺ cell samples following 72h culture with LDE225 and or nilotinib at the concentration shown, the asterisk indicates the presence of nilotinib 5µM. Error bars represent the SEM, significance p<0.05 represented by *(paired T-test).

5.2.8 SMO inhibition with LDE225, alone and in combination with nilotinib does not influence cell division kinetics or retention of primitive cell surface markers on CP CML CD34⁺ cells

If Hh inhibition was influencing self-renewal behaviour of HSC it might be expected to have a demonstrable effect on the division kinetics of primitive cells

and the expression of surface markers associated with immaturity. Additionally, previous studies have demonstrated the existence of a quiescent fraction within the CD34⁺ population that are seen not to divide in CFSE tracking experiments and that this population increases following treatment with TKIs (Holyoake et al., 1999; Jorgensen et al., 2007). Therefore, it is possible that the combination of LDE225 with TKI might mitigate this accumulation through inhibition of self-renewal.

If an agent was able to alter the balance between the number of self-renewing and commitment divisions that occur, there would be less primitive cells at each division gate due to the resultant increased differentiation which should be measurable through reduced expression of primitive surface phenotypic markers (e.g. CD34 and others) compared to untreated and TKI treated arms.

In order to investigate the effect of LDE225 alone or in combination with nilotinib on cell division kinetics of CD34⁺ cells, CFSE dye incorporation was used with subsequent flow cytometry as described in detail in the methods section 2.2.9.5. Briefly, CP CML CD34⁺ cells were thawed, stained with CFSE and cultured in the presence of incremental concentrations of LDE225 (0.5, 5 and 50nM) and/or nilotinib (0.5 or 5 μ M). At d4, 8 and 12 samples were harvested for viable cell count, flow cytometry analysis and re-seeding in fresh media and drug. Flow cytometry was performed as previously described; CD34⁺ expression was assessed with a monoclonal CD34⁺ antibody conjugated to the fluorescent dye APC. Antibody treated cells were passed through the flow cytometer, only events in the viable cell gate (set by FSC and SSC criteria) were included in the analysis. CFSE intensity (FL1) formed the x axis of the resultant plot with APC intensity (hence CD34⁺ expression) on the y axis. Gates were placed to isolate all viable cells and subsequently to identify all CD34⁺ expressing cells. These gates allowed the creation of two FL1 plots demonstrating, respectively, the relative CFSE fluorescence in total viable cells and purely in the CD34⁺ fraction. A series of gates extending from the CFSE_{max} population, encapsulating successive populations with 2-fold reductions in CFSE intensity, was generated. These gates therefore contained sub-populations having undergone a similar number of cell divisions (see Figure 5-10).

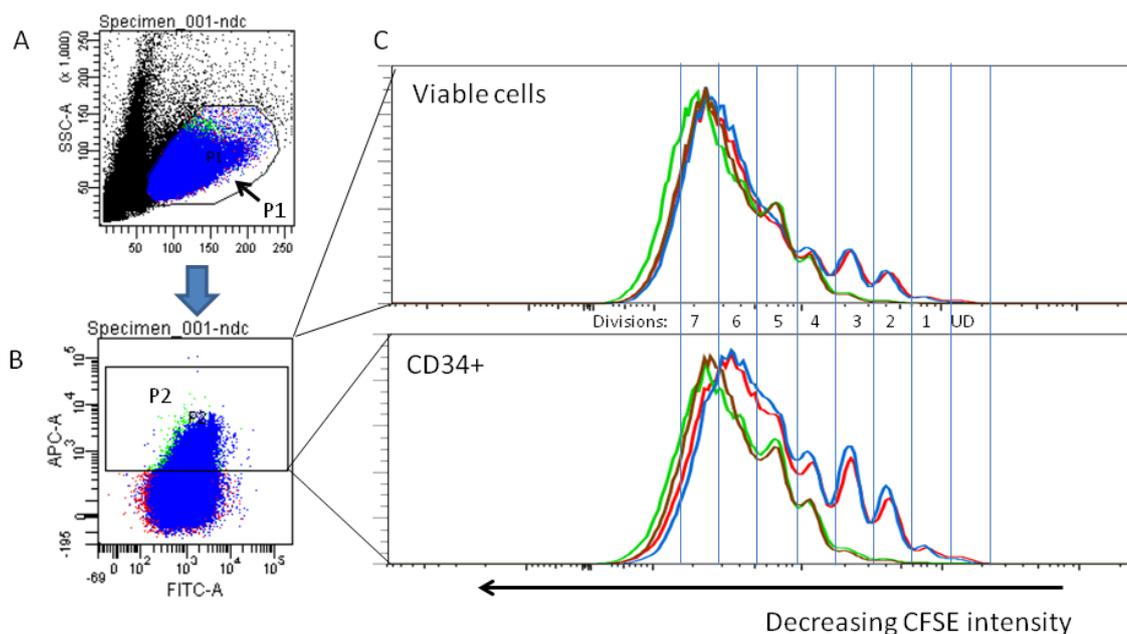


Figure 5-10: Representative CFSE flow cytometry plot indicating the effect of LDE225 and nilotinib on CD34⁺ CML CP cells.

These cells were stained with CFSE and cultured in SFM+HGF in the presence or absence of nilotinib and/or LDE225 over 12d with analysis occurring on d4, d8 and d12. (A) Viable cells were gated according to FSC and SSC characteristics (P1). (B) CFSE intensity is demonstrable in FL1 (FITC) channel and CD34⁺ expression was assessed with CD34-APC conjugated monoclonal antibody FL4 / APC channel. P2 thus includes all CD34⁺ cells. (C) Event histograms were produced for total viable cells and for CD34⁺ stained cells for all experimental arms, shown here are untreated (brown), nilotinib 5 μM alone (blue), LDE225 50nM alone (green) and nilotinib 5 μM in combination with LDE225 50nM (red). Divisions (peaks) are seen passing from right (high CFSE fluorescence intensity) to left (low fluorescence intensity) as indicated.

After 4d culture, CD34⁺ cells had undergone several divisions. The proportion of undivided cells or cells with a low number of divisions reduced with prolonged (8d or 12d) culture. The nilotinib containing arms underwent fewer divisions in comparison to the other treatments as has been previously described (Jorgensen et al., 2007). LDE225 was not found to be anti-proliferative at up to 50nM; LDE225 single treatment arms overlaid the untreated control and LDE225 and nilotinib-containing arms overlaid the nilotinib single treatment arm.

Indeed no significant difference in the mean percentage of total viable cells in each division gate after 4, 8 or 12d culture was noted either between untreated and single agent LDE225 up to 50nM or between nilotinib 5 μM as a single agent or in combination with LDE225 up to 50nM (Figure 5-11).

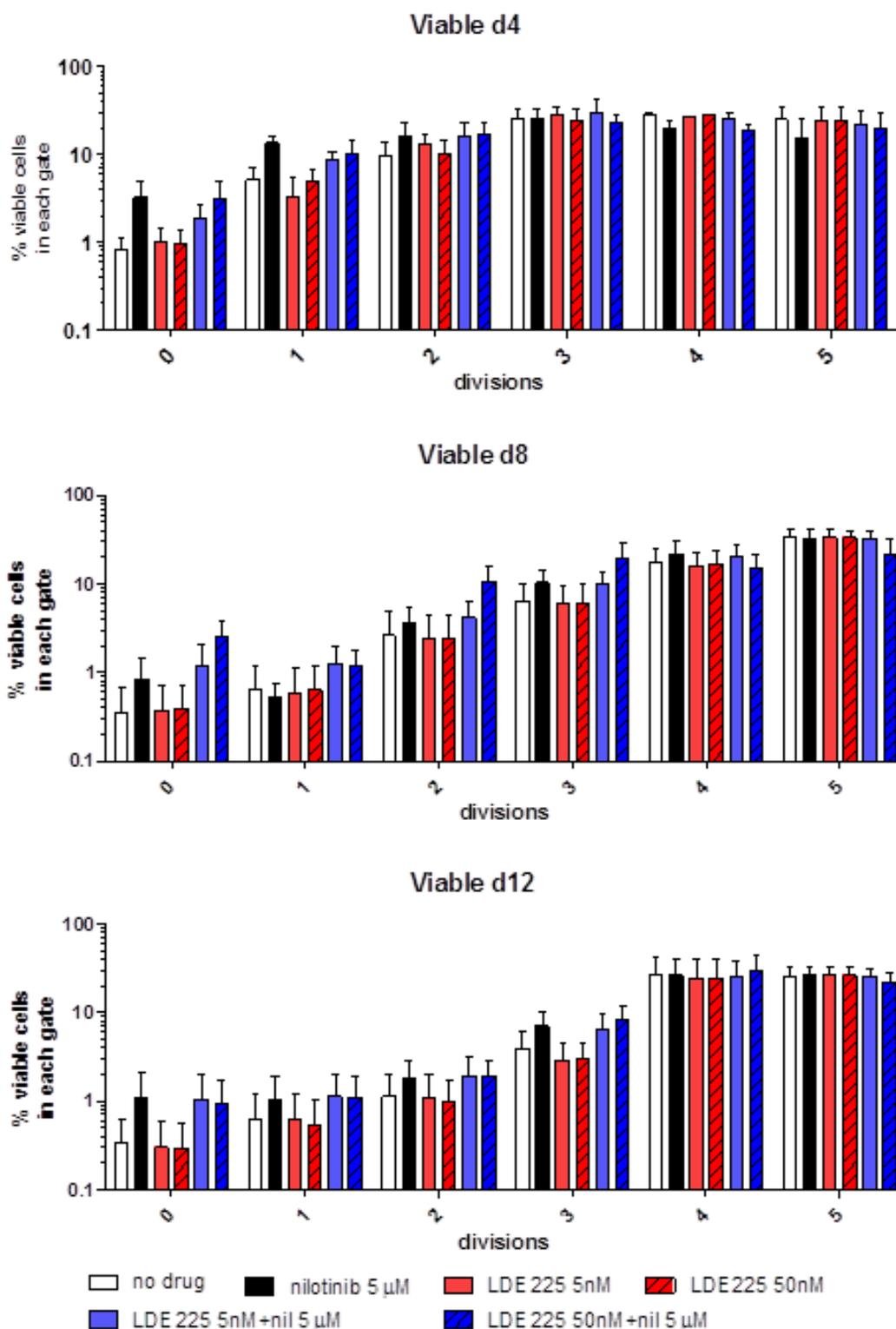


Figure 5-11: CFSE stained viable cells following culture for 4, 8 and 12d in SFM+HGF, untreated or with nilotinib and LDE225 alone and in combination.

Primary CD34⁺ CP CML cells were stained with CFSE and cultured in SFM+HGF with nilotinib 5 μ M and/or LDE225 5 or 50nM. The percentage of the total viable cells that reside in each division gate is shown for each time point. The bars represent the mean value from 4 primary samples, while the error bars represent SEM. Similar results were obtained with LDE225 0.5nM and nilotinib 0.5 μ M and are not presented here.

In order to more closely examine the effect of LDE225 ± nilotinib on CD34⁺ CP CML cell division, the recovery of viable input CD34⁺ cells was calculated for each division in each treatment arm at each time point as described in the methods section 2.2.9.6. This allows the assessment of the number of original input cells contributing to the population within each division gate. The results were normalised to the untreated arm at the given time point and division gate and were expressed as a percentage relative to the untreated control (100%). In the nilotinib-containing arms relative recovery of viable CD34⁺ cells that had undergone few divisions (0-3) was increased while recovery of viable CD34⁺ cells in later divisions (>3) was reduced compared to no drug control, confirming the anti-proliferative effect of nilotinib and is consistent with previous reports (Jorgensen et al., 2007). LDE225 with or without nilotinib had no significant effect on CD34⁺ cell recovery (Figure 5-12).

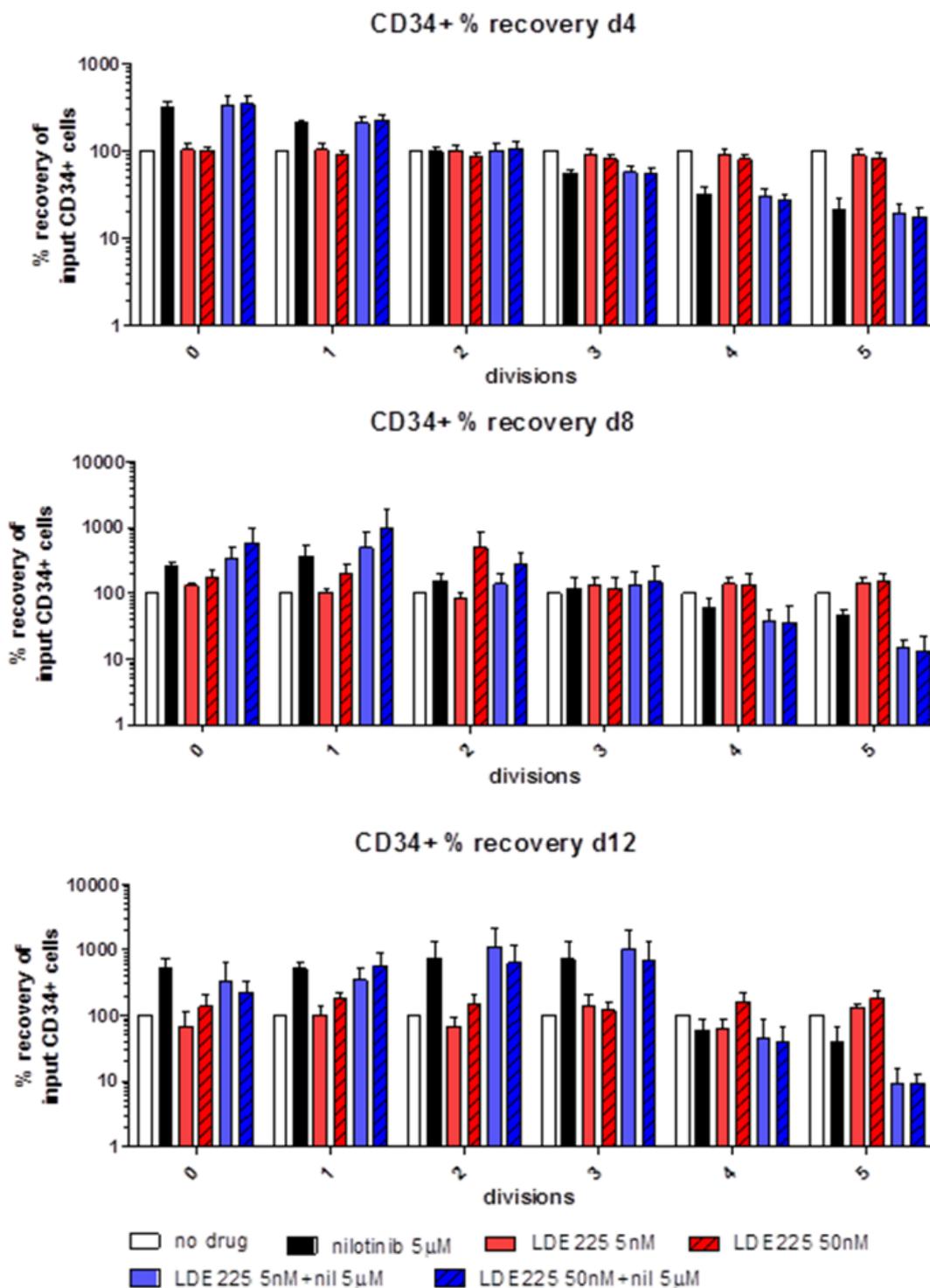


Figure 5-12: Percentage recovery of input cells following culture for 4, 8 and 12d in SFM+HGF untreated or with nilotinib and LDE225 alone and in combination.

Primary CP CML cells were stained with CFSE and cultured in SFM+HGF with nilotinib 5μM and/or LDE225 5 or 50nM. The percentage recovery of input CD34⁺ in each division from 4 independent primary samples is presented normalised to the untreated control (100%) from that time point and division gate. Error bars represent SEM. Significance p<0.05 is indicated by * (paired T-test).

Given that a self-renewal inhibitor may influence the type but not necessarily the number or frequency of cell divisions, it was intriguing to analyse the expression of cell surface markers that are associated with stem cell phenotype. It was reasoned that a reduction in self-renewal behaviour might lead to discernible reductions in the expression of these markers as commitment/differentiation choices are favoured and on the other hand, stimulation of self-renewal through exposure to rSHH might have the opposite effect. In order to test this hypothesis, a further set of CFSE experiments was performed as above with the following modifications; cells were cultured in SFM and a physiological growth factor cocktail (LGF), experimental arms containing rSHH at a dose of 100ng/mL were included and, prior to analysis, cells were stained with CD133-APC and CD34-PE. The results of two independent primary samples after 4d culture are presented in the figure below (Figure 5-13).

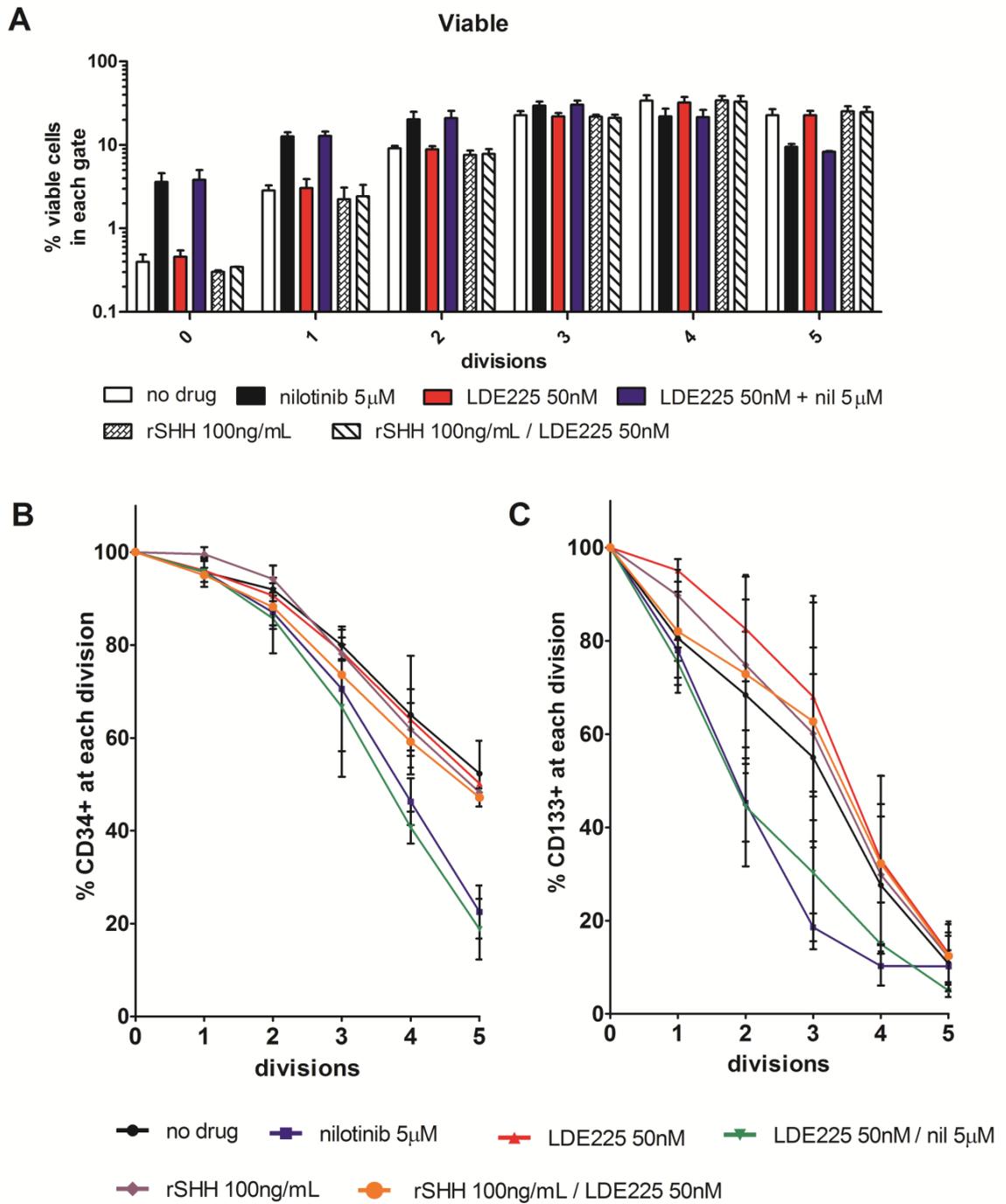


Figure 5-13: CFSE stained cells following culture for 4d in SFM+LGF untreated, with nilotinib, LDE225 or rSHH alone and in combination.

Primary CD34⁺ CP CML cells were stained with CFSE and cultured in SFM+LGF with nilotinib 5 μ M +/-LDE225 50nM +/- rSHH 100ng/mL. Cells were stained with CD34 and CD133. Panel A indicates the percentage of total cells that are CD34⁺ residing in each division gate is shown. Panel B indicates the percentage of CD34⁺ cells in each division gate relative to the undivided population. Panel C indicates the percentage of CD133⁺ cells in each division gate relative to the undivided population. The bars represent the mean value from 2 primary samples, while the error bars represent SEM.

Consistent with the previously discussed results, the anti-proliferative effect of nilotinib can be seen alone and in combination with LDE225. Neither LDE225 nor rSHH at a concentration of 100ng/mL materially affected cell division. There was a reduction of expression of CD34 and CD133 with cell division consistent with maturation. Cells treated with nilotinib alone or in combination with LDE225 lost expression of CD34 and CD133 in fewer divisions than other treatment arms or the control. Cells treated with rSHH retained CD34 over the first 2 divisions marginally better than other treatment arms and control. However, by division 3 this marginal difference had disappeared and additionally was not reflected in the expression of CD133.

5.3 Discussion

Having demonstrated the expression of components of the Hh signalling pathway in the most primitive CP CML LSC, I sought to determine whether Hh signalling could be inhibited in primary CML CD34⁺ cells using a specifically designed clinical grade small molecule SMO inhibitor (LDE225), whether a biomarker for this activity could be identified in these cells and whether inhibition affected cellular proliferation, survival or maturation in short term *in vitro* culture.

The compound LDE225 was tested in demonstrated activity in a non-haemopoietic Hh driven luciferase reporter cell line and reduced the expression of the Hh targets *GLI1* and *CCND1* but not *PTCH1* or 2 in the cell line K562. When CD34⁺ CP CML cells were cultured in the presence of LDE225 at up to 1000nM concentration, a significant reduction in expression of *GLI1* was noted, however this represented a comparatively small mean reduction which was only evident at the 72h time point. The suppression of *GLI1* expression is in keeping with the expected result following inhibition of Hh signalling. The magnitude of the difference is small, indicating that LDE225 is unable to fully suppress *GLI1* expression. One possible reason for this is that the doses utilised were insufficient to effect full inhibition, however, the IC₅₀ of LDE225 in our reporter system was less than 10nM and CD34⁺CP CML primary cells were exposed to between 10-1000nM LDE225 prior to analysis, therefore this seems unlikely. An alternative explanation is that *GLI1* expression is not solely driven by Hh activity in CP CML. Several recent publications suggest non-Hh driven mechanisms regulating *GLI1* expression in various malignancies (Blotta et al., 2012; Katoh, 2007; Lauth and Toftgard, 2007; Li et al., 2012a; Maeda et al., 2006; Nolan-Stevaux et al., 2009). Intriguingly, these include RAS/RAF/MEK, PI3K, TGFβ and CXCR4/SDF1

signalling pathways which have emerging roles in the pathogenesis of CML (Andreu et al., 2005; Miyazono, 2012; Naka et al., 2010b; Ptaszniak et al., 2002; Steelman et al., 2004).

The prolonged exposure to LDE225 that was required to discern any inhibitory effect in primary CML was somewhat unexpected and could indicate an indirect effect on *GLI1* expression. The *GLI* proteins have wide ranging effects and cooperative relationships in the nucleus influencing the transcription of a great number of genes. Many of these genes are involved in the further modification of gene expression or chromatin structure themselves. *GLI1* is itself part of a positive feedback system reinforcing the Hh signal. While *GLI1* is a downstream target in Hh signalling, its expression in primary CML cells is likely to be influenced by the whole programme of activity occurring in the nucleus and differences in expression might be initiated early but escalate over time. However, it is notable that even in our TM3 reporter system, optimal *GLI*-dependent luciferase expression in response to purmorphamine exposure did not occur until 48h. Furthermore, studies by Novartis also concluded that *GLI1* expression changes were optimally detected at 48h post drug exposure (personal communication; Paul Manley, Novartis). Thus, our results from primary patient material are in keeping with similar expression studies.

There was considerable biological variability between primary samples across all genes tested. In one sense, this is problematic as the gene expression studies performed thus far have not identified a robust biomarker for Hh signalling activity in CD34⁺ CP CML in an unselected population. These studies were performed in CD34⁺ CP CML cells and it is possible that similar studies in a more enriched stem cell population may have yielded more definitive results. However, reductions in *GLI1* expression and to a lesser extent, *PTCH1* expression were detectable in some of the patient samples analysed. It is intriguing to speculate that there may be two sub-populations; one responsive, the other not, as indicated by *GLI1*. If the non-responding samples are analysed separately then the magnitude of mean reduction in *GLI1* following LDE225 exposure is greater (0.54 fold; $p < 0.01$) and therefore may be potentially useful in responsive patients. It would be of great interest to analyse *GLI1* response in many further primary samples to determine if such populations truly exist.

The effect of LDE225 on short term *in vitro* culture was not marked. No differences in viability, apoptosis, proliferation or cell cycle status were noted

following up to 72h culture in SFM with single agent LDE225. Furthermore, there was no suggestion of additive activity with combination treatment with up to 50nM LDE225 and nilotinib 5 μ M over and above single agent nilotinib. These results demonstrate that LDE225 does not readily cause cell death and harbours no significant anti-proliferative effect in CML. However, agents that selectively influence stem cell fate would not necessarily be expected to affect survival, proliferation, apoptosis or cell cycle status over short culture periods and, therefore, these results may simply confirm the relative specificity of LDE225 and lack of non-specific toxicities at the concentrations used, in contrast to cyclopamine (Zhao et al., 2009). The lack of effect of LDE225 on cell division history is confirmed by the CFSE cell division tracking experiments. It is clear that LDE225 does not cause or abrogate accumulation of the quiescent fraction alone or in response to nilotinib and no difference in CD34⁺ recovery was noted at up to 12d culture. In very recent published work Okabe *et al*/investigated the effect of GDC-0449, another targeted SMO inhibitor in combination with dasatinib in Ph⁺ OM9;22 cells, K562 cells and primary CML samples grown on S9 stromal layers. They found that exposure to GDC-0449 or siRNA mediated KD of SMO reduced proliferation and augmented dasatinib induced apoptosis. This is in contrast to the findings presented here and while the effect on primary cells was modest these results reinforce the importance of microenvironmental context in Hh signalling (Okabe et al., 2012).

A significant shift in the balance of self-renewal or differentiation divisions may have altered the observed proportion of cells in each gate that exhibited primitive surface markers. In this work I utilised CD34⁺ and CD133⁺ as primitive immunophenotypic markers. No difference in surface expression of these markers was noted between experimental arms. It is likely that neither surface marker was sufficiently specific to allow resolution of these differences over the time, population nor number of divisions analysed. A more refined population of LSC and/or more specific markers defining either the primitive population or the self-renewing progeny would facilitate the measurement of these differences.

In summary I have demonstrated that LDE225 exerts a dose-dependent inhibition of Hh signalling in a GLI-driven luciferase reporter system and at higher doses modestly reduces expression of the key downstream target *GLI1* both in K562 cells and in responsive primary CP CML CD34⁺ cells. Short term exposure to LDE225, either alone or in combination with nilotinib, did not affect viability,

apoptosis or cell cycle distribution of CD34⁺ CP CML cells. Likewise detailed cell division tracking experiments demonstrated no anti-proliferative effect with LDE225, either alone or in combination with nilotinib, nor did LDE225 influence retention of primitive cell markers over successive divisions. Whether exposure to LDE225 affects LSC function in CML is the subject of the next chapter.

6 Effect of LDE225, a Specific Inhibitor of Hh Signalling, on Stem Cell Function of CP CML HSC

6.1 Introduction

In chapter 4, expression of key constituents and targets of Hh signalling were found to be expressed in the most primitive identifiable CP CML LSC population and in chapter 5, LDE225 was demonstrated to modestly reduce expression of *GLI1* in K562 and CD34⁺ CP CML populations albeit at higher doses and later time points. In chapter 5 no significant difference was seen following short term culture of CP CML CD34⁺ cells with LDE225 alone or in combination with nilotinib on measures of cell cycle activity, proliferation, viability or expression of primitive phenotypic markers. However if the influence of LDE225 was largely confined to LSCs, one would not necessarily expect short term alterations in any of these measures as any change within the LSC population would be greatly diluted by the much larger progenitor population. As Hh signalling is proposed to influence the cell fate decisions of self-renewing stem cells, the key question is whether its inhibition with LDE225 had demonstrable effects on functional assays of stem cell activity and self-renewal in CP CML.

6.2 Assessment of Stem Cell Numbers and Self-Renewal

Self-renewal activity and assays designed to assess stem cell numbers and function are discussed at length in the introduction and methods sections. In principal both *in vivo* and *in vitro* assays approximate stem cell activity in the original population by observing the behaviour of their progeny under various conditions. While possibly the most robust demonstration and quantification of self-renewal activity is from limited dilution xenograft transplantation, *in vitro* assays of surrogate measures of self-renewal activity or stem cell quantity are also frequently utilised. One approach is to perform re-plating of primary colonies from progenitor (CFC) assays. This allows quantification of the number of cells within the original colony that can give rise to secondary and tertiary colonies thereby assessing the proportion of original cells that gave rise to non terminally differentiated progeny with significant residual proliferation capacity and can be used to approximate self-renewal capacity in HSC and LSC populations (Carow et al., 1993; Gordon et al., 1998; Humphries et al., 1981; Lu et al., 1993b; Marley and Gordon, 2005; Marley et al., 2001). LTC-IC assays are another well established surrogate for stem cell function. Following extended culture over > 5 weeks in supportive stromal co-culture, cells with residual colony forming capacity (LTC-ICs) are assessed and their prevalence in the initial sample inferred. The LTC-IC assay

has been used to assess primitive cell function in normal and malignant haemopoiesis alone and in response to various drugs (Copland et al., 2008; Heaney et al., 2010; Holtz et al., 2002; Holyoake et al., 2000; Pettengell et al., 1994).

As previously discussed in chapter 1 section 1.3.4.3, existing studies in retroviral transduction models of CML and in primary CML (largely BC) have demonstrated that genetic knockdown of SMO or its pharmacological inhibition with cyclopamine resulted in reduced re-plating capacity, reduced LTC-IC and reduced capacity to transmit leukaemia to secondary transplanted murine hosts.

Therefore the aim of this chapter was to examine the effect of the clinical grade, specific SMO inhibitor LDE225 alone and in combination with nilotinib on primary CP CML LSC on *in vitro* surrogates of LSC number and function. Complementary studies interrogating the effect of this combination in *in vivo* murine models were performed by collaborators and are discussed in Appendix 2.

Specific objectives covered in this chapter are described below;

Examination of the effect of LDE225 alone and in combination with nilotinib in CD34⁺ CP CML *in vitro*:

- (1) On primary colony formation in CFC assays.
- (2) On secondary re-plating efficiency.
- (3) On LTC-IC frequency following exposure to drug.
- (4) On LTC-IC frequency following prolonged exposure to LDE225 in stromal co-culture.

6.3 Results

6.3.1 Inhibition of Hh signalling with LDE225 does not affect primary CFC formation but significantly reduces secondary re-plating efficiency

6.3.1.1 Primary CFC formation

Following recovery, CD34⁺ CML cells were cultured for 72h in either SFM alone, escalating concentrations of LDE225, nilotinib 5 μ M or a combination of both treatments. The cells were harvested, washed, and re-suspended in fresh SFM. Equal numbers of cells were inoculated into Methocult H4034 as described in the methods section 2.2.11.1. The CFC assays were cultured under standard conditions for 14d prior to colony assessment. All colony types (>50 cells) were counted. While nilotinib reduced colony formation as would be expected, increasing concentrations of LDE225 did not significantly alter primary CFC quantitatively or qualitatively and reflects the fact that primary colony formation in progenitor assays is largely driven by committed progenitor and not stem cell self-renewal behaviour (Figure 6-1).

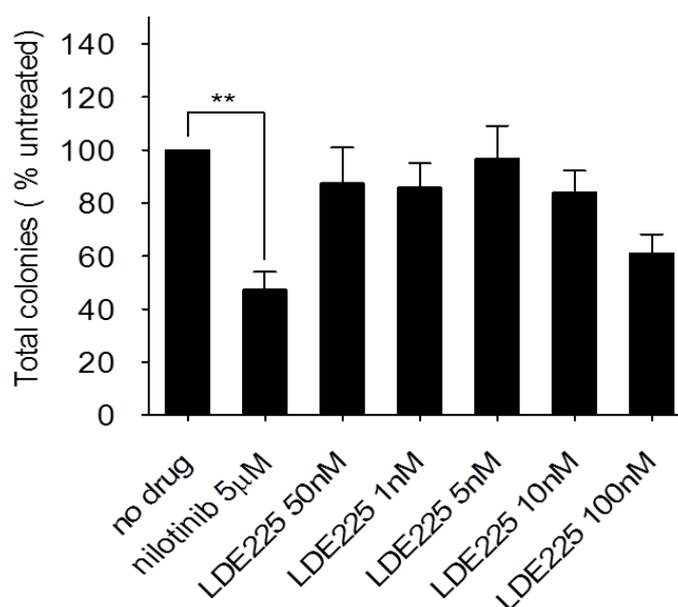


Figure 6-1: Relative frequency of total CFC in clonogenic colony assays.

CD34⁺ CP CML cells were cultured over 72h in SFM in the presence of incremental concentrations of LDE225 or nilotinib 5 μ M prior to inoculation into clonal progenitor assays. The graph indicates the mean total number of colony of 3-7 independent primary samples after 14d culture normalised to an untreated control. Error bars represent the SEM and ** indicates significance ($p < 0.01$) (paired T-test).

6.3.1.2 Secondary and tertiary re-plating capacity

To determine whether self-renewal behaviour had been altered by exposure to LDE225, the secondary re-plating capacity of these colonies was assessed as described in the methods section 2.2.11.3. Following their initial assessment, primary colonies were carefully plucked under an inverted microscope using a p10 pipette and carefully re-dispersed in 100 μ L Methocult H4034 in a 96 well plate prior further incubation. Between 20 and 40 non-erythroid colonies were plucked for each treatment condition. After 7d culture the secondary colonies were assessed and counted. A significant reduction in re-plating capacity with increasing concentrations of LDE225 (5, 10 and 50nM LDE225; $p < 0.01$) was noted (Figure 6-2). Wells bearing secondary colonies were re-dispersed in further Methocult and incubated for a further week prior to reassessment. There was a similar trend in tertiary colony formation, however due to the reduction in available colonies for tertiary re-plating, the results did not reach statistical significance (Figure 6-2).

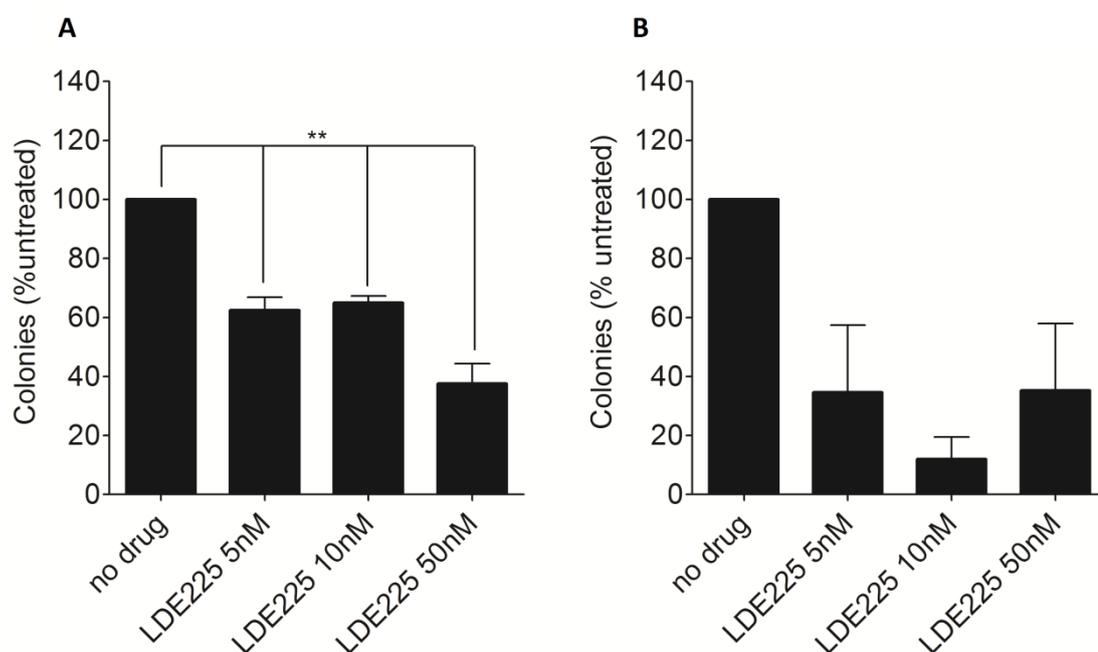


Figure 6-2: Secondary and tertiary colony formation following re-plating of primary colonies.

Primary colonies derived from CFC assays described in section 6.3.1.1 were plucked and re-dispersed into fresh Methocult. Further culture enabled the assessment of the total number of secondary colonies derived from the re-plated colonies at first re-plate (A) and second re-plate (B) in each of the treatment arms compared to an untreated control. (A) Indicates the total number of secondary colonies from 3 independent primary samples (20-40 colonies re-plated) and (B) indicates the total number of tertiary colonies from 2 independent primary samples normalised to an untreated control. Error bars represent the SEM and ** indicates significance ($p < 0.01$) (paired T-test).

A second set of re-plating experiments was performed to evaluate the effect of LDE225 in combination with nilotinib on primary CD34⁺ CP CML cells. These experiments were performed as described above and in the methods section 2.2.11.3. Again, no difference in primary colony formation was noted, however, here there was a relative increase in secondary colony formation by colonies derived from cells treated with nilotinib and a reduction from colonies derived from cells treated with LDE225. Notably there was a deeper reduction in secondary colony formation in cells that had been treated with the combination of nilotinib and LDE225 both with respect to the untreated control arm and the single agent nilotinib arm (Figure 6-3).

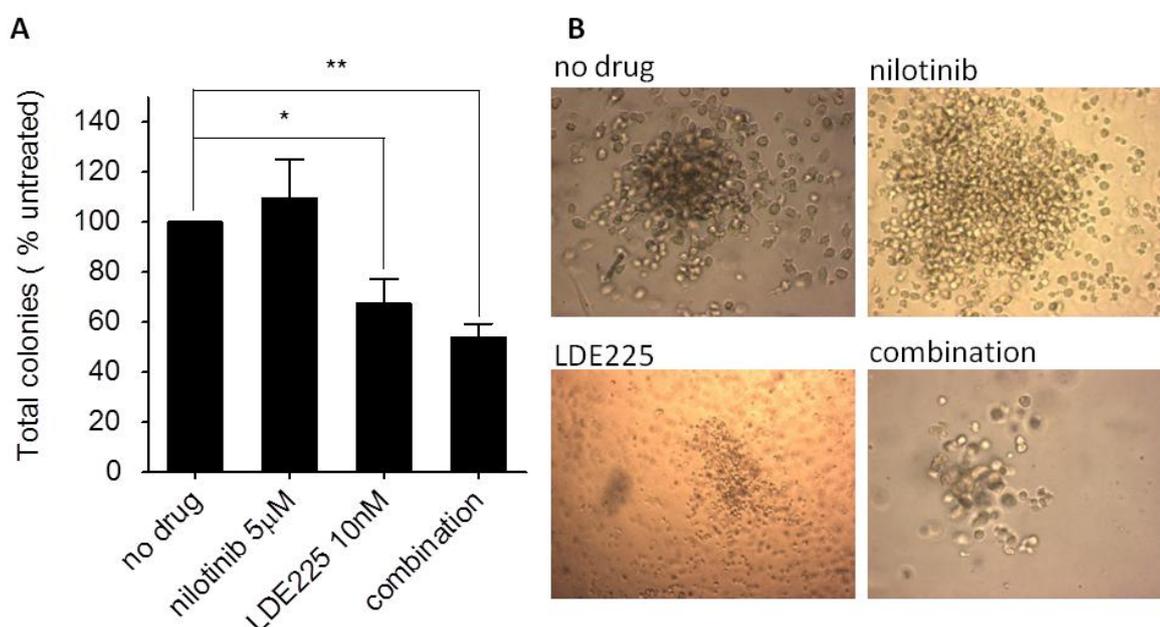


Figure 6-3: Secondary colony formation following re-plating of primary colonies derived from CP CML CD34⁺ cells exposed to LDE225 ± nilotinib over 72h.

Primary colonies derived from CFC assays were plucked and re-dispersed into fresh Methocult. Further culture enabled the assessment of the total number of secondary colonies derived from the re-plated colonies. (A) Indicates the total number of secondary colonies in at least 3 independent primary samples normalised to an untreated control. Error bars represent the SEM. * indicates significance of $p < 0.05$ and ** indicates significance of $p < 0.01$. (paired T-test) (B) Illustrative examples of secondary colonies after secondary re-plating.

6.3.2 LDE225 in combination with nilotinib significantly reduces LTC-IC frequency in CP CML

The LTC-IC assay is the most stringent *in vitro* surrogate measure of the functional activity of HSC. If LDE225 affected the self-renewal activity of LSCs then this would affect LSC abundance and be quantifiable by the LTC-IC assay.

Primary CD34⁺ CP CML cells were incubated in SFM with LDE225 10nM and/or nilotinib 5 μ M for 72h then washed, counted and inoculated into a pre-prepared stromal co-culture comprised of equal quantities of immortalised irradiated SL/SL and M210B4 modified murine fibroblast stromal layers optimised for the support of human myeloid haemopoiesis (Hogge et al., 1996) and cultured for 5 weeks with 50% media changes weekly. After 5 weeks, the contents of the wells were harvested and inoculated into clonal progenitor assays allowing comparison of the resultant CFCs generated from the input cells in each treatment arm (see methods section 2.2.11.2).

Compared with the untreated control, LDE225 resulted in a lower LTC-IC recovery although the magnitude was not large (38% reduction; $p < 0.03$). As previously shown with dasatinib, in prolonged primary CML culture, treatment with single agent nilotinib resulted in an increase in LTC-IC (Copland et al., 2008). Intriguingly, the combination of LDE225 with nilotinib resulted in a significant reduction of LTC-IC relative to nilotinib alone ($p < 0.05$) - see Figure 6-4.

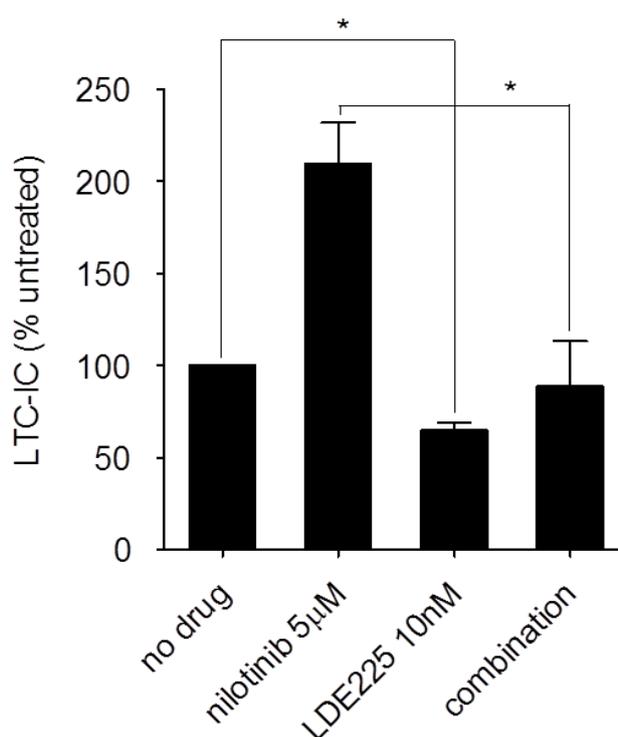


Figure 6-4: LTC-IC recovery from CP CML CD34⁺ cells exposed to LDE225 \pm nilotinib over 72h.

CD34⁺ CP CML cells were cultured over 72h in SFM in the presence of LDE225 10nM, nilotinib 5 μ M or both prior to inoculation into pre-prepared LTC-IC cultures. The figure indicates the total number of colonies produced as a proportion of the total input cells in each experimental arm from 5 primary samples, normalised to untreated control. Error bars represent the SEM. * indicates significance of $p < 0.05$ (paired T-test).

In order to determine whether LDE225, nilotinib or the combination of both influenced *in vitro* surrogate measures of HSC activity in normal haemopoiesis, a similar experiment was performed using normal CD34⁺ haemopoietic cells. Cryopreserved normal CD34⁺ haemopoietic cells were thawed and cultured in SFM + LGF in the presence of LDE225 10nM, nilotinib 5 μ M or both over 72h prior to inoculation into LTC-IC as described above. No significant alteration of LTC-IC abundance was noted between the LDE225, nilotinib or combination arm relative to the untreated control in 3 independent primary samples. There was a small increase in LTC-IC recovery from the nilotinib-containing arms. This may reflect the effect of nilotinib on other tyrosine kinase targets e.g. c-KIT also present in BCR-ABL negative cells (Weisberg et al., 2005) (Figure 6-5).

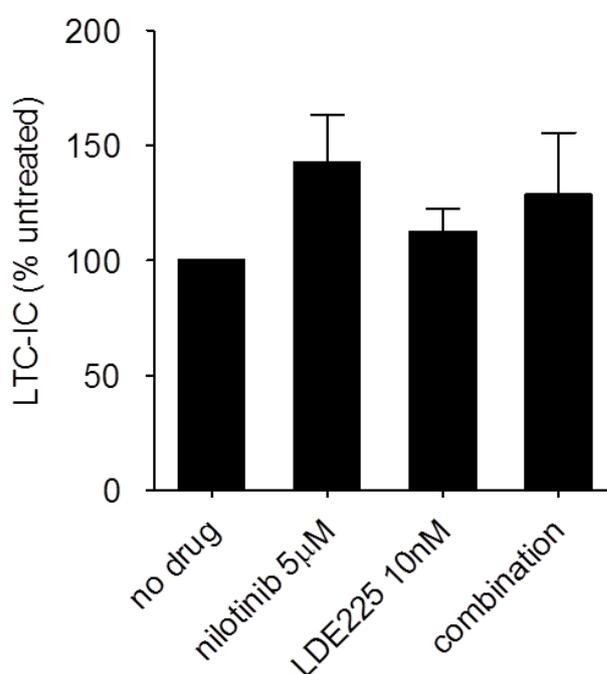


Figure 6-5: LTC-IC recovery from normal CD34⁺ cells exposed to LDE225 \pm nilotinib over 72h.

Normal CD34⁺ cells were cultured over 72h in SFM+LGF in the presence of LDE225 10nM, nilotinib 5 μ M or both, prior to inoculation into pre-prepared LTC-IC cultures. The figure indicates the total number of colonies produced as a proportion of the total input cells in each experimental arm from 3 primary samples, normalised to untreated control. Error bars represent the SEM.

6.3.3 LDE225 in combination with nilotinib significantly reduces LTC-IC frequency following prolonged treatment in stromal co-culture

Stem cell fate decisions are formulated in the context of complex micro-environmental conditions and signalling cascades (Zon, 2008). In the LTC-IC

experiments described so far, pure populations of CD34⁺ CML cells were exposed to LDE225 and/or nilotinib over a relatively short time frame prior to long term culture in the presence of stroma under standard conditions. Thus, any effects of the treatments under evaluation were on CD34⁺ primary cells in isolation during this time frame and are subsequently evaluated by the read-out from the LTC-IC. In order to add a further dimension to the analysis by investigating the effect of long term exposure to LDE225 ± nilotinib on CML within a more complex microenvironment, I performed a final set of *in vitro* stromal co-culture experiments in which CD34⁺ CP CML cells were not treated in isolation but were directly inoculated into the stromal co-cultures as described above and then cultured in incremental concentrations of LDE225 (10-1000nM) ± nilotinib 1µM over 5 weeks with media and drug changes every week. The stromal layer and haemopoietic cells were examined weekly. After 5 weeks the contents of the wells were harvested and inoculated into clonal progenitor assays as described above.

There was a significant reduction in LTC-IC numbers in all the LDE225 treated arms (LDE225 10nM: $p < 0.05$; LDE 225 10-100nM: $p < 0.05$; Figure 6-6). There was a significant reduction in colony output from the nilotinib arms reflecting the ongoing anti-proliferative effect of TKI exposure ($p < 0.01$). It was interesting to note that 5 weeks of continual exposure to nilotinib did not entirely deplete the LTC-IC population and a significant number of LTC-IC remained (28% of the untreated control). Lastly, in the experimental arms containing the combination of LDE225 and nilotinib, there was a trend to further reduction in the LTC-IC recovery over nilotinib alone, suggesting that LDE225 targets a population of cells in CML that are resistant to TKI (Figure 6-6).

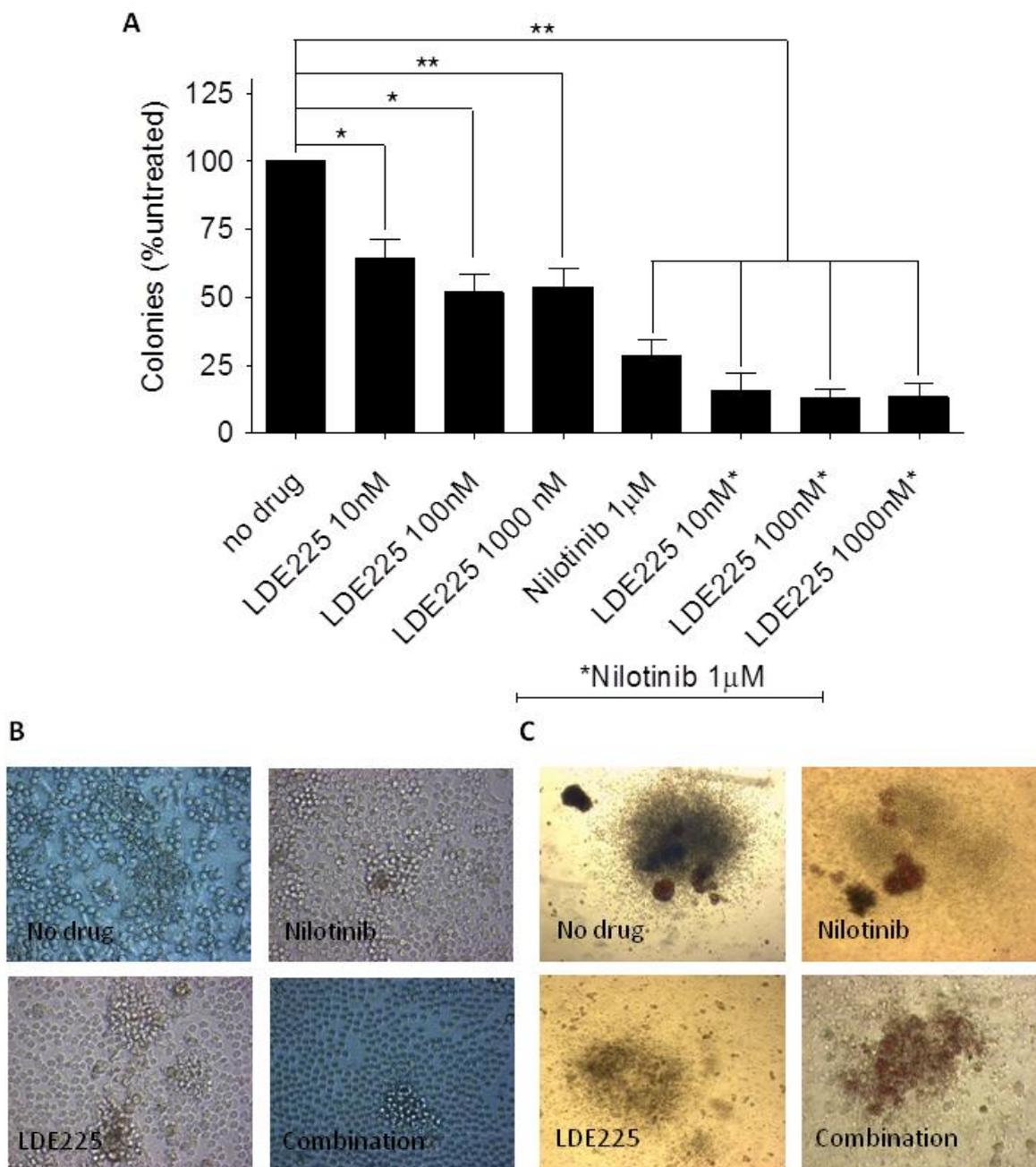


Figure 6-6: CFC recovery from CD34⁺ CP CML cells following prolonged stromal co-culture in the presence of LDE225 ± nilotinib.

CD34⁺ CP CML cells were thawed and inoculated directly into pre-prepared stromal co-culture and cultured over 5 weeks in the presence of incremental concentrations of LDE225, nilotinib or both. (A) Indicates the mean total number of resultant colonies as a proportion of the total input cells in each experimental arm from 4 primary samples, normalised to untreated control. Error bars represent the SEM. * indicates significance of $p < 0.05$ and ** indicates significance of $p < 0.01$ (paired T-test). (B) Representative photographs of CD34⁺ CP CML cells during stromal co-culture with drug, so called “cobble stone” areas of LSC expansion under the supporting stroma can clearly be seen. (C) Representative colonies derived from harvested cells in clonal progenitor assays.

6.4 Discussion

In this chapter I have described the effect of LDE225 alone and in combination with standard treatment with TKI (nilotinib) in normal or CP CML CD34⁺ cells in prolonged *in vitro* culture experiments. These experiments were designed to investigate the effect of these drugs alone and in combination on *in vitro* measures of HSC/LSC abundance and self-renewal behaviour. The results of these experiments are encouraging. The specific SMO inhibitor LDE225, alone or in combination with nilotinib, was found to reduce the primitive leukaemic cell population as measured by LTC-IC and reduces self-renewal activity as evidenced by re-plating capacity. These results suggest that SMO inhibition with LDE225 specifically targets the TKI-resistant primitive stem/progenitor cell population in CML but not normal HSC.

The clonal progenitor assays performed here demonstrated that SMO inhibition had no effect on primary colony formation, whereas TKI inhibition with nilotinib demonstrated marked reduction in progenitor output likely due to the combination of direct toxicity and potent anti-proliferative effect exerted by nilotinib and other TKIs on this population as has been extensively reported (Deininger et al., 1997; Druker et al., 1996; Konig et al., 2008a; Konig et al., 2008b).

This is in contrast to results reported by both Zhao *et al*/and Dierks *et al*/who found that cyclopamine reduced primary colony formation in BCR-ABL⁺ murine cells (Dierks et al., 2008; Zhao et al., 2009). This may reflect the non-specific toxicity of cyclopamine and be due to the presence of the drug in the clonal progenitor assay medium, or indeed the different biology of the cells from their respective retroviral models. Interestingly, Zhao *et al*/demonstrated a similarly significant reduction in colony formation in CFC assays performed on primary BC CML CD34⁺ cells (Zhao et al., 2009). Blast crisis CML cell populations are known to harbour progenitors that have re-acquired stem cell characteristics, thus it may be that they are more dependent on self-renewal signalling for survival/proliferation (Jamieson et al., 2004; Schairer et al., 2010).

While CFC assays measure progenitor expansion, re-plating capacity is a well established *in vitro* surrogate for self-renewal activity in normal and malignant haemopoiesis. In normal haemopoiesis, re-plating capacity is greater in primitive multi-lineage or mixed colonies and declines with maturity (Carow et al., 1991; Carow et al., 1993; Leary and Ogawa, 1987; Lu et al., 1993a; Marley et al., 2003).

Re-plating has been utilised previously as a tool in the study of CML, where it was found to be variable between different patients and related to patient characteristics like prognostic score (Gordon et al., 2003). Other groups have demonstrated modulation of re-plating capacity in CFCs derived from patient samples following activation or inhibition of WNT signalling, through forced expression of β -catenin, axin or misspliced GSK3 β (Abrahamsson et al., 2009; Jamieson et al., 2004; Zhao et al., 2007).

In these studies, it was demonstrated that while exposure to nilotinib modestly enhanced re-plating efficiency, LDE225 or the combination of both agents significantly reduced secondary colony formation. The former is most likely to be explained by the anti-proliferative effect of nilotinib preventing stem cell division during drug exposure, resulting in a population that was relatively enriched for more primitive cells compared to the untreated control (Copland et al., 2008). The reduction in secondary re-plating capacity demonstrated by LDE225 suggests reduction in the number of primitive cells with the capacity to form secondary colonies in these treatment arms. This is consistent with our hypothesis that SMO inhibition shifts the balance of stem cell self-renewal divisions in favour of differentiation (hence loss of capacity to self-renew) in the population analysed. The reduction relative to the untreated arm and the single treatment with nilotinib arm is intriguing as LDE225 appears to completely counteract the enrichment of cells with re-plating capacity seen with nilotinib. I previously demonstrated that LDE225 has no effect on cell cycle or apoptosis and therefore LDE225 cannot be directly neutralising the anti-proliferative effect of nilotinib or influencing cell survival. One explanation could be that SMO inhibition has already influenced the intracellular machinery controlling stem cell fate such that when the anti-proliferative stimulus is removed (through washing and inoculation into CFC) and the cells can divide, a lower proportion of self-renewal divisions occur, leading to a reduction in re-plating potential.

Long term culture-initiating cell assays were developed for quantitative assessment of HSC function and have been extensively used for this purpose (Hogge et al., 1996; Sutherland et al., 1989; Sutherland et al., 1993b; Sutherland et al., 1990). As CML haemopoiesis, particularly CP CML haemopoiesis, is not dissimilar (Sloma et al., 2010), these assays have also been extensively used to quantify CML LSCs at baseline and to measure the effect of various compounds

on the LSC population (Copland et al., 2008; Heaney et al., 2010; Pellicano et al., 2011).

In this work, the LTC-IC assay results corroborated the findings from the re-plating assays and, furthermore, demonstrated that normal CD34⁺ cells were unaffected by exposure to LDE225 or nilotinib. This is important as it indicates that there is a potential therapeutic window for the clinical use of LDE225 in CML.

These results are in keeping with and build on published data. Dierks *et al* demonstrated a marked increase in secondary re-plating capacity in *BCR-ABL*⁺ *Ptch*^{1^{-/-}} murine cells and a reduced re-plating capacity in *BCR-ABL*⁺ *Smo*^{+/-} and *Smo*^{-/-} cells *in vitro*. They also found that cyclopamine with or without nilotinib reduced LTC-IC recovery in primary BC CML samples but not in normal healthy CD34⁺ cells (Dierks et al., 2008).

While these *in vitro* assays have provided a wealth of information, they cannot hope to reflect the complex interactions present in the stem cell niche. In an attempt to better reflect this multi-directional signalling environment the LTC-IC protocol was modified to allow CP CML haemopoietic cells and supportive stroma to be continuously exposed to LDE225 and nilotinib. This is important, as although autocrine / paracrine Hh signalling between LSCs has been postulated, it is not clear at this juncture whether this is the dominant signalling mechanism. Examples from other malignancies have suggested that Hh signalling from CSC to stroma or vice versa may play a role (Dierks et al., 2007; Tian et al., 2009).

The results from these experiments demonstrate significant reduction of LTC-IC recovery at low doses of LDE225. They also suggest an additive effect with nilotinib, although the number of colonies surviving in the nilotinib-containing arms was relatively small and therefore the differences with LDE225 in combination did not reach significance.

As discussed in the introduction to this chapter the gold standard proof of HSC/LSC function extends from the reconstitution of a haemopoietic system in xenotransplantation experiments. Additionally, while the effect of treatment with LDE225 ± nilotinib on LSC in a supportive stromal microenvironment had been addressed *in vitro*, it was considered important to assess the effect of LDE225 and nilotinib treatment *in vivo* in a complete haemopoietic system.

In order to pursue these goals, collaboration with Professor Ravi Bhatia's team at City of Hope, San Diego, California was instigated. Professor Bhatia and

his team have considerable expertise in murine modelling of CML and have access to the severely immunocompromised NSG mice and the transgenic inducible *Scl-tTa-BCR-ABL-GFP* murine model of CML (Koschmieder et al., 2005; Shultz et al., 2005; Zhang et al., 2012a). These parallel studies have since been completed and provide a strong validation to the *in vitro* work discussed in this chapter.

Pre-treatment with the combination of LDE225 and nilotinib reduced the capacity of CP CML CD34⁺ cells to contribute to haemopoiesis in NSG mice at 6 weeks post engraftment but did not affect normal CB engraftment. Additionally there was a significant reduction in BCR-ABL⁺ cells recovered following engraftment suggesting a specific anti-leukaemic effect. Further validation was obtained from the results of *in vivo* treatment of the *Scl-tTa-BCR-ABL-GFP*⁺ inducible transgenic mouse model of CML (Koschmieder et al., 2005; Zhang et al., 2012a) demonstrating that LDE225 in combination with nilotinib reduced the frequency of LT-HSC in the spleen, reduced the leukaemogenicity of BM and splenic LT-HSC in secondary transplantation and prolonged survival of treated animals. A more in depth treatment of Professor Bhatia and Dr Zhang's results is presented in Appendix 2.

6.5 Conclusion

In this chapter, evidence is presented demonstrating that the SMO inhibitor LDE225 alone and in combination with nilotinib targets CP CML HSC, most probably through influencing LSC self-renewal activity. In *in vitro* measures of LSC number and function, LDE225 alone and in combination with nilotinib reduced CFC secondary re-plating capacity and LTC-IC recovery. These results are corroborated by murine modelling experiments by our collaborators demonstrating reduced engraftment of CD34⁺ CP CML cells in immunocompromised murine xenografts and by reduced splenic LT-HSC, improved survival and reduced capacity to recapitulate leukaemia in secondary hosts in the *Scl-tTa-BCR-ABL* murine model of leukaemia.

7 General Conclusion

7.1 Summary of Results

Presented here are the results of a detailed gene expression array, taking advantage of the modern improvements in microarray technology to identify expression differences between the most rigorously defined pure HSC and progenitor populations from CML and normal haemopoietic tissue to date. These studies demonstrate that, while there is an ordered progression in terms of transcriptional activity from the HSC to progenitor populations in normal haemopoiesis, this is substantively deregulated in CP CML particularly at the LSC level. There are marked transcriptional differences between HSC and the equivalent CP CML LSC population. Here the CML LSC are transcriptionally more mature, exhibiting increased expression of programmes associated with proliferation and differentiation, but reduced expression of many genes associated with HSC populations and self-renewal.

In parallel in *in vitro* expression studies on primary CP CML samples, positive regulators of the developmental signalling pathways were found to be up regulated and negative regulators down regulated in CML compared to normal haemopoiesis. Targeted inhibition of Hh signalling with LDE225 only minimally affected the transcription of downstream targets of the pathway, but nevertheless, alone or in combination with nilotinib led to reduction of *in vitro* surrogate measures of self-renewal in primary CP CML, but not normal CD34⁺ cells, corroborated by consistent results in two mutually supportive murine models. The effect both *in vitro* and *in vivo* is most marked in combination with nilotinib. Thus, from this and similar published work, Hh inhibition through SMO antagonism in combination with conventional TKI therapy appears to be a promising approach in dealing with the persisting LSC population in CML (Dierks et al., 2008; Zhao et al., 2009).

7.2 A Combinatorial Approach to Therapy

The data presented here and in other published work are consistent in that the effect of SMO inhibition, both *in vitro* and *in vivo*, is most marked in combination with TKI therapy. While this is welcome and strongly suggests that, therapeutically, LDE225 and other SMO inhibitors would be best employed in combination with conventional TKI treatment, it is perhaps surprising, given that CML LSC are reportedly resistant to TKI-mediated BCR-ABL kinase inhibition. The augmented effect seen in combination strongly suggests that SMO inhibition and TKI are working in concert in the CML LSC. This challenges the model where

TKI and SMO inhibition have discrete effects on progenitors and LSC respectively and suggests a mode of action more consistent with the induction of synthetic lethality in CML LSC (Kaelin, 2005). In this model, CSCs are dependent on multiple parallel pathways for maintenance and survival. They are not “addicted” to the input of any single pathway and, in all probability the input of one may completely or partially compensate for loss of another. In order for the target cell to be rendered sensitive to the effect of one drug, the compensatory or alternative survival pathways must also be inhibited. In the context of CML, there are multiple examples of this e.g. inhibition of autophagy in TKI treated cells leading to augmented apoptosis, co-inhibition of the mitogen activated protein kinase / extracellular receptor kinase (MAPK/ERK) and JAK2 signalling pathways with TKI. (Bellodi et al., 2009; Chen et al., 2013; Naka et al., 2010a; Packer et al., 2011). BCR-ABL kinase has a promiscuous effect in the cell, activating multiple different pathways and additionally while nilotinib is comparatively specific for BCR-ABL kinase, it also inhibits a number of other intracellular kinases and other targets including discoid domain receptor family member 1 (DDR1), NAD(P)H dehydrogenase quinone 2 (NQO2), Abelson related gene (ARG), KIT and PDGFR. Thus, the augmented effect seen in CML LSC in this and other studies might relate to inhibition of both BCR-ABL and non-BCR-ABL mediated kinase activity or other off target effects of nilotinib (Dierks et al., 2008; Zhao et al., 2009). Alternatively, it has been shown that the microenvironmental signalling of the stem cell niche provides a protective effect to CML LSC during treatment with TKI (Seke Etet et al., 2012; Zhang et al., 2013). In this work, I demonstrated that Hh ligands are expressed at low levels by CML LSC, so there remains a possibility of autocrine/paracrine signalling. I also demonstrated SHH secretion by some stromal cell lines which may contribute to LSC survival in the niche. This has also been reported in other contexts e.g. lymphoma (Dierks et al., 2007; Seke Etet et al., 2012). Niche signalling of this nature might be more relevant in the context of TKI therapy as niche homing and adhesion is rectified by the effect of TKI (Jin et al., 2008). Additionally SDF1 signalling through CXCR4 promotes SHH production and Hh signalling in pancreatic cancer, whereas Hh augmented CXCR4 surface localisation and signalling in medulloblastoma (Li et al., 2012a; Sengupta et al., 2012b; Singh et al., 2012). This is as yet unstudied in CML but may prove to be important as TKI therapy recovers CXCR4 expression and therefore SDF1 signal transduction (Jin et al., 2008).

While the precise mechanism of combinatorial action requires resolution, these pre-clinical studies of combinatorial treatment with SMO inhibitors and TKI have yielded promising results suggesting this therapeutic avenue should be explored in further *in vivo* and clinical studies. Since this work was commenced, and as a consequence of it and other pre-clinical studies, several phase 1 clinical trials and one phase 2 clinical trial of Hh inhibition in CML have been instigated (see Table 7-1).

Treatment	Sponsor	Commenced / Recruiting / No. pts	Phase	Patient population	Identifier
LDE225 + Nilotinib	Novartis	2012 Recruiting (36)	1b	CP CML Failure of prior CML therapy	NCT01456676
BMS833923 +/- Dasatinib	BMS	2011 Not recruiting (271)	2	Newly diagnosed CP CML (1y Dasatinib alone followed by 2y combination if suboptimal response)	NCT01357655
BMS833923 + Dasatinib	BMS	2011 Not recruiting (36)	1	CML (any) & Ph+ ALL Suboptimal response, resistance or progression	NCT01218477
PF04449913 + Dasatinib or Bosutinib	Pfizer	2010 Not recruiting (52)	1	Myeloid malignancies refractory resistant or intolerant to prior therapy	NCT00953758

Table 7-1: Clinical trials in CML utilising various small molecule SMO inhibitors in combination with conventional TKI therapy

7.3 Current and Future Directions in CML

Rarely has there been such astonishing progress in the treatment of a malignant disease. The application of our understanding of the pathophysiology of the disease has fundamentally changed our therapeutic approach to the condition and yielded truly remarkable improvements in the survival of patients diagnosed with CML over the last 15 years. Modern treatment with potent TKIs means that most patients newly diagnosed with CML can generally expect to achieve rapid disease control with complete cytogenetic resolution and in many cases deep molecular remission, which correlate with long term PFS (Bjorkholm et al., 2011; Jabbour and Kantarjian, 2012). In the case of resistance there is the opportunity for dose modification and switch to an alternative agent and with the advent of ponatinib this even extends to the highly resistant T315I mutation (O'Hare et al., 2009). Additionally, recent evidence suggests that, in some patients at least, TKI therapy can be safely withdrawn, suggesting possible TKI-mediated cure (Mahon et al., 2011).

While generally effective, TKI therapy has clinically significant limitations. As has been discussed in chapter 1, CML LSC are not sensitive to TKI therapy alone and therefore persist in the BM of optimally responding patients (Bhatia et al., 2003; Chu et al., 2011; Corbin et al., 2011; Hamilton et al., 2012; Jiang et al., 2007b). While their numbers may be low and the likelihood of progression in a responding patient reportedly slim there is strong evidence suggesting that discontinuation of TKI is likely to lead to molecular relapse (61% in the recently updated data from the French STIM study) (Mahon et al., 2011). Furthermore, even with up-front treatment with the potent second generation TKIs, a sizable minority of patients do not respond optimally (Kantarjian et al., 2011; Kantarjian et al., 2012). Consequently for most patients at least, TKI therapy is not curative meaning that patients are likely to require lifelong therapy in order to maintain disease control. This may become problematic from several angles. Firstly development of secondary resistance and progression are possible and may increase with time due to increasing numbers of patients treated with TKI (O'Hare et al., 2007; Quintas-Cardama et al., 2009). Furthermore, long term compliance with therapy is of increasing clinical concern as it directly relates to the risk of progression (Jabbour et al., 2012; Marin et al., 2010). Secondly, while generally well tolerated TKIs do have significant side effects and consequently a negative impact on the quality of life and health of CML patients (Hochhaus, 2011). Thirdly, the long term effects of TKI therapy are only now becoming evident and will become more important with time. These include class effects and specific problems with particular TKIs and are comprehensively reviewed by Steegmann *et al.* (Steegmann et al., 2012). Tyrosine kinase inhibitor therapy potentially impacts on many organ systems. Notable examples include immunomodulatory effects of uncertain long term significance, undesirable effects on bone, calcium and phosphate metabolism, glucose metabolism not to mention chronic oedema and fluid accumulation and vascular disease (Steegmann et al., 2012). There is also a question of health economics, with the advent of effective treatment, the prevalence of CML is increasing and with it the burden of TKI related healthcare costs (Chen et al., 2012; Huang et al., 2012).

While these problems do not detract from the utility of TKI therapy in comparison to previous alternatives, they do provide a clinical imperative to develop novel approaches that may be more effective and, in particular, to really closely examine whether an effective cure through eradication of the residual LSC may be coming within our reach.

7.4 The Potential Role of Hh Inhibitors in the Treatment of CML

To progress from pre-clinical indications of utility to an understanding of whether SMO inhibition might augment our therapeutic arsenal in CML, careful evaluation in the clinical trial setting is required.

While the success of current treatments in reducing disease burden and controlling CML is a major therapeutic triumph, it leads to difficulty in assessing response to novel treatments in a clinical trial context. This is compounded by the relative rarity of the resistant LSC within the leukaemic population. Additionally while molecular responses have been evaluated to determine response to TKI therapy at the very lowest detectable levels, the presence of residual genetic evidence of BCR-ABL is not necessarily associated with relapse (Bartley et al., 2010; Ross et al., 2010; Sobrinho-Simoes et al., 2010). Also relevant in measuring very low levels of residual disease is the observation of BCR-ABL mRNA in normal populations (Biernaux et al., 1995; Bose et al., 1998).

Additionally short term use of Hh inhibition in solid tumour trials has provided information regarding the potential side effects of such therapy in humans. While these reports suggest that the various SMO antagonists under trial are overall reasonably well tolerated and the adverse events generally of low grade they are not insignificant comprising alopecia, muscle spasms, dysguesia, fatigue, weight loss and diarrhoea (Tawbi et al., 2011; Von Hoff et al., 2009). Furthermore from mouse models they appear to affect bone metabolism and their mode of action will clearly lead them to be significantly teratogenic. Therefore, in what population would SMO inhibitor therapy be best employed and how its additive effect would be measured are important translational questions.

The obvious patient population would be those who were resistant, refractory or sub-optimally responding to conventional TKI therapy. Their long term prognosis is substantially worse than optimal responders and Hh inhibitor therapy might offer an opportunity to intervene. Additionally it would be possible to measure augmented response over time through qRT-PCR as they, by definition, would have measurable residual disease. Another population might be those with evidence of progression that were not transplant candidates as their long term outlook is poor. As we have discussed, in advanced phase patients, there is a breakdown in the natural haemopoietic hierarchy with LSCs emerging from mature

populations that are dependent on developmental pathway signalling e.g. Wnt and from recently reported work with Hh inhibitors in BP CML, also Hh (Jamieson et al., 2004; Schairer et al., 2010). The abrogation of these signalling pathways might be effective in targeting these populations, however optimism in this regard must be tempered by the knowledge that by that stage multiple aberrations have accumulated in the malignant clone (Radich, 2007).

Conceptually there is an argument for the use of SMO inhibitor therapy in all CP CML patients. Early intervention designed to target the CML LSC prior to the development of significant secondary aberrations might be expected to be more effective in targeting the resistant LSC; improving speed and depth of response to therapy and possibly even eradicating the leukaemic clone. While potentially advantageous, this approach has various challenges: it would involve exposing patients to the risks of a second experimental therapy early in the disease course and it would be difficult to find appropriate and practical means to measure the efficacy of the combinatorial treatment over and above conventional therapy. Conceivably, one might measure the rate and depth of decline of the leukaemic clone by sensitive qRTPCR and ultimately genomic DNA quantification. Alternatively, quantification of residual leukaemic cells with LSC potential could be performed. Low levels of residual disease would make it impractical to perform xenotransplantation assays; however quantification of residual LSC could be performed by *in vitro* assays of stem cell function such as those that were performed here. While an extremely useful research tool these techniques are not available in most diagnostic laboratories and are difficult to standardise between centres. Clinical trials are currently underway testing SMO inhibition in all the contexts discussed above and detailed information about the clinical effectiveness of this approach should be available in the near future.

7.5 Concluding Remarks

The introduction of TKI therapy for CML is the culmination of decades of research into the pathophysiology of CML and stands as a paradigm for the application of rationally designed targeted therapy in cancer. The initial enthusiasm following the demonstration of the remarkable efficacy of TKI therapy in CML has been tempered by the realisation that it is, in all probability, not a curative intervention for the majority of patients. However investigation into the effect of these targeted therapies and the capacity of leukaemic cells to persist despite their action has yielded major insights into the structure, organisation and

process of leukaemogenesis that are potentially applicable in the wider oncological field. Not least is a firm validation of the concept of LSC in CML, an increasing understanding of their clinical relevance and a rapidly expanding repertoire of potential pharmacological targets, including Hh signalling, through which to effect their destruction. The challenge for subsequent years is to develop these insights into effective clinical interventions.

8 Appendices

8.1 Appendix 1: Samples, Cell Number and Total RNA for Microarray and Validation Experiments

Sample		Vol /μL	total RNA/ μg	cell number	Sample		Vol /μL	total RNA/ μg	cell number		
pool	normal	HSC	25	737	3.12E+05	264	CP CML	HSC	25	701	1.93E+05
pool	normal	MPP	25	800	4.00E+05	264	CP CML	CMP	55	14198	5.89E+06
pool	normal	CMP	55	3745	1.28E+06	264	CP CML	GMP	25	856	3.35E+05
pool	normal	GMP	55	5748	1.18E+06	264	CP CML	MEP	55	24872	1.73E+06
pool	normal	MEP	55	2308	6.16E+05	101	AP CML	HSC	24	371	1.45E+05
15	normal	HSC	25	611	3.92E+05	101	AP CML	CMP	55	16333	5.96E+06
15	normal	MPP	25	249	1.22E+05	101	AP CML	GMP	24	435	1.28E+05
15	normal	CMP	55	4635	2.14E+06	101	AP CML	MEP	55	1412	2.63E+05
15	normal	GMP	55	7587	1.44E+06	239	AP CML	HSC	25	694	2.00E+05
15	normal	MEP	55	10924	1.91E+06	239	AP CML	CMP	55	4462	1.00E+06
12	normal	HSC	25	424	2.37E+05	239	AP CML	GMP	25	1627	2.43E+05
12	normal	MPP	55	2636	8.64E+05	239	AP CML	MEP	55	16816	3.74E+06
12	normal	CMP	55	7207	1.21E+06	0104	AP CML	HSC	25	1282	6.49E+05
12	normal	GMP	55	8567	1.67E+06	0104	AP CML	MPP	25	880	5.99E+05
12	normal	MEP	55	10824	1.23E+06	0104	AP CML	CMP	25	13816	5.04E+06
107	CP CML	HSC	23	500	1.30E+05	0104	AP CML	GMP	55	8822	1.50E+06
107	CP CML	CMP	55	11414	2.11E+06	0104	AP CML	MEP	55	8541	2.76E+06
107	CP CML	GMP	17	213	4.00E+04	185	AP CML	HSC	25	910	4.18E+05
107	CP CML	MEP	55	11497	3.41E+06	185	AP CML	MPP	25	777	2.86E+05
111	CP CML	HSC	24	513	2.24E+05	185	AP CML	CMP	55	14025	3.90E+06
111	CP CML	CMP	55	9423	3.89E+06	185	AP CML	GMP	25	1280	4.37E+05
111	CP CML	GMP	55	4908	8.16E+05	185	AP CML	MEP	55	11456	3.58E+06
111	CP CML	MEP	55	22382	5.57E+06	AMCA	BC CML	HSC	25	505	2.86E+05
273	CP CML	HSC	23	710	5.55E+04	AMCA	BC CML	MPP	55	2321	7.76E+05
273	CP CML	CMP	55	12626	4.34E+06	AMCA	BC CML	CMP	55	5401	1.97E+06
273	CP CML	GMP	23	882	1.76E+05	AMCA	BC CML	GMP	55	8118	2.40E+06
273	CP CML	MEP	55	34544	8.64E+06	AMCA	BC CML	MEP	55	3586	9.35E+05
194	CP CML	HSC	24	338	1.95E+05	HP	BC CML	HSC	25	250	1.08E+05
194	CP CML	CMP	55	4092	1.20E+06	HP	BC CML	MPP	55	11895	2.91E+06
194	CP CML	GMP	25	1695	4.05E+05	HP	BC CML	CMP	55	5035	1.67E+07
194	CP CML	MEP	55	10989	2.00E+06	HP	BC CML	GMP	55	16808	3.32E+06
110	CP CML	HSC	25	430	1.93E+05	HP	BC CML	MEP	55	4212	1.07E+06
110	CP CML	CMP	55	21985	1.20E+06						
110	CP CML	GMP	25	1704	4.05E+05						
110	CP CML	MEP	55	7209	2.00E+06						

8.2 Appendix 2: Collaborative *in vivo* Murine Modelling Studies

8.2.1 Introduction

Immunodeficient murine models (e.g. NOD/SCID mice) can be used as hosts for transplantation of human primary CML cells although the engraftment rate and duration are much lower than with normal haemopoietic cells (Dazzi et al., 1998; Lewis et al., 1998; Sirard et al., 1996; Verstegen et al., 1999; Wang et al., 1998). One reason for this is the residual immune system in the transplant recipient mice and correspondingly, the development of more intensively immunocompromised recipient mice (e.g. the NOD/SCID/IL2 γ mouse) has led to improvements in engraftment rate and durability with human haemopoietic cells (McDermott et al., 2010; Shultz et al., 2005). Thus, primary CML tissue can be cultured *in vitro* in the presence of test compounds prior to transplantation into NSG hosts and the resultant engraftment of malignant cells quantified at a suitably distant time point (e.g. 10 weeks) from transplantation to ensure that they derived from the LSC population within the original graft. Variations in engraftment between treatment arms in an experiment would suggest differences in LSC numbers consequent on treatment effect.

An alternate approach is the *in vivo* treatment of a murine model of CML, followed by secondary transplantation to assess LSC function. This approach provides insight into the effect that the agent has over a prolonged period of treatment in a more physiological context, where the constituents of the murine haemopoietic system and microenvironment are complete. Two fundamental limitations to this approach are the degree to which CML is accurately reflected in the model system and that murine haemopoiesis differs in significant ways from human haemopoiesis (e.g. the role of the spleen and our understanding of the haemopoietic hierarchy) and in order to be clinically relevant, the resulting findings have to be extrapolated from one system to another.

A CML-like disease can be efficiently produced by retroviral transduction of human BCR-ABL into murine HSCs using a murine stem cell retroviral vector (Cuiffo and Ren, 2006; Pear et al., 1998). Following transplantation of these infected cells into lethally irradiated syngeneic mice, a myeloproliferative condition akin to CML is reliably produced and is transplantable to secondary hosts where a similar phenotype is produced. The main limitation of this model is that the

disease thus produced is rather unpredictable and often exhibits a very aggressive phenotype, more akin to advanced CML that results in early death (in about 3 weeks) of the murine host (Cuiffo and Ren, 2006). Thus, this model is not ideal for studying self-renewal and stem cell function in CP CML.

An alternate model is available, an inducible transgenic mouse where BCR-ABL is expressed under control of a tetracycline regulated 3' enhancer of the murine *Scf* gene (Koschmieder et al., 2005; Zhang et al., 2012a). This allows *BCR-ABL* expression to be targeted to the murine HSC population and to be switched on as desired by withdrawal of tetracycline. When *BCR-ABL* expression is induced, a chronic myeloproliferative condition akin to CP CML is produced. This condition is transplantable to secondary hosts and reversible with reintroduction of tetracycline. Thus in many respects this model is more attractive for the *in vivo* study of CP CML LSC function.

8.2.2 Inhibition of Hh signalling in primary CML samples and murine models of CML

Previous studies evaluating inhibition of Hh signalling in CML have utilised variations of the retroviral transduction model described above. Zhao *et al* used the *Vav-Cre-Lox* system to create *Smo*-deficient mice from which to isolate *Smo*^{-/-} HSCs, whereas Dierks *et al* used foetal liver cells from *Smo*-deficient mouse embryos (Dierks et al., 2008; Zhao et al., 2009). Both groups expressed human BCR-ABL in these cells before transplantation. Despite differing approaches, broadly similar results were obtained. *Smo* deletion reduced LSC numbers and reduced the incidence of leukemia with prolonged latency in primary transplantation and greatly reduced capacity to recrudescence disease in secondary hosts.

Dierks *et al* and Zhao *et al* also performed complementary pharmacologic inhibitor studies both *in vivo* and *in vitro*. *In vivo*, they demonstrated prolonged survival in diseased mice, reduced LSC population, and lower functional activity of the residual LSC. *In vitro*, both groups reported reduced primary colony formation derived from primary patient samples in CFC assays. Zhao *et al* found that *Smo*^{-/-} cells had higher levels of the cell determinant Numb and that expression of Numb correlated with reduced replating capacity. Dierks *et al* found that genetic deletion of *Smo* or *Ptch1* resulted in reduced secondary replating capacity and that

cyclopamine treatment of CD34⁺ BC CML cells resulted in reduced LTC-IC recovery suggesting a potential stem cell-specific effect in this context.

It is notable that in the paper by Dierks *et al*, the dose of cyclopamine used was generally in excess of the dose that Zhao *et al* reported as having off target toxicities (3 μ M) (Dierks et al., 2008; Zhao et al., 2009). While these seminal studies have established a role for the Hh pathway in LSC maintenance and propagation in CML, they have not provided a clinically applicable approach. Both studies largely utilised BC CML cells and a murine model of CML which has significant drawbacks for studying CP CML.

8.3 Collaborative Results

As discussed in the introduction to this chapter, the gold standard proof of HSC/LSC function extends from the reconstitution of a haemopoietic system in xenotransplantation experiments. Additionally, while the effect of treatment with LDE225 \pm nilotinib on LSC in a supportive stromal microenvironment had been addressed *in vitro* it was considered important to assess the effect of LDE225 and nilotinib treatment *in vivo* in a complete haemopoietic system.

In order to pursue these goals collaboration with Professor Ravi Bhatia's team at City of Hope, Duarte, California was instigated. Professor Bhatia and his team have considerable expertise in murine modelling of CML and have access to the severely immunocompromised NSG mice and the transgenic inducible *Scf-tTa-BCR-ABL-GFP* murine model of CML. The work discussed hereafter is a result of this collaboration and was performed in San Diego by Professor Bhatia and Dr Bin Zhang.

The effect of LDE225 \pm nilotinib on the capacity of normal CB CD34⁺ cells or CP CML CD34⁺ to engraft immunocompromised mice was assessed. Primary CP CML CD34⁺ (2×10^6) cells or CB cells (1×10^5) were transplanted via tail vein injection into lethally irradiated 8 week old NSG mice after 72h *in vitro* exposure to LDE225 (10nM) +/- nilotinib (5 μ M) or control (no treatment). Engraftment was assessed by flow cytometry for human CD45⁺ and CD45⁺/34⁺ cells in the BM after 6 weeks. The results indicated that engraftment of human CD45⁺/34⁺ cells was significantly reduced following combination treatment compared with the other experimental arms. As discussed in chapter 6, FISH analysis for BCR-ABL indicated that there was a preferential reduction in engraftment of BCR-ABL⁺ cells in mice transplanted with combination treated leukaemic cells compared to either

single arm treatment or no treatment. Engraftment of normal CB was not affected by pre-treatment. Additionally colony assays were performed with recovered murine BM cells, indicating a reduction in CFC formed in the nilotinib-containing arms compared to the untreated and LDE225 treated arms, see Figure 8-1. These results were consistent with the *in vitro* data described in chapter 6 and suggest that combination of LDE225 and nilotinib may selectively target primitive CML cells that are capable of repopulating the haemopoietic system in NSG mice.

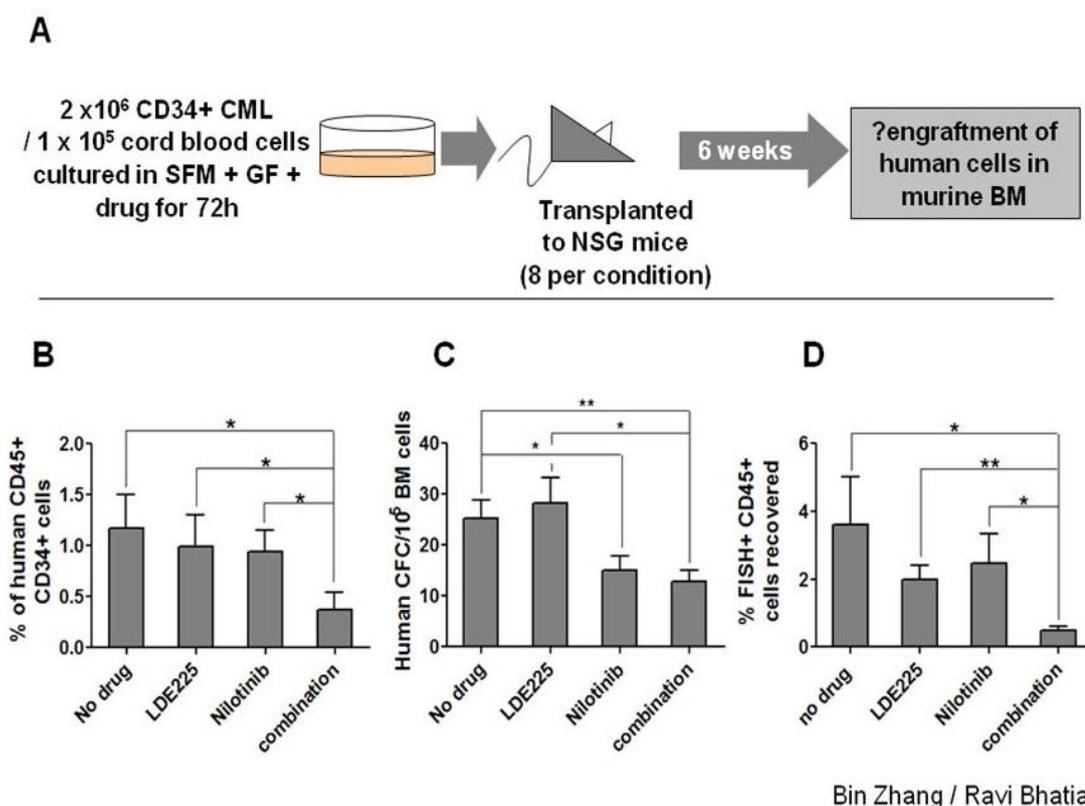


Figure 8-1: NSG murine model engraftment with normal and CP CML CD34⁺ cells after pre treatment with LDE225 +/- nilotinib.

The experimental schema is shown in (A) 2x10⁶ CD34⁺ CP CML cells or 1x10⁵ cells were cultured in SFM+LGF for 72h in the presence of the test compounds. The cells were then harvested, washed and injected into lethally irradiated 8 week old NSG mice. After 6 weeks the murine BM was harvested and assessed for human CD34⁺/45⁺ cell engraftment. (B) Indicates the mean percentage of human CD34⁺/45⁺ cells in the murine BM as indicated by flow cytometric analysis. (C) Indicates the relative number of CFC derived from BM cells obtained from NSG mice engrafted by cells from the different treatment arms per 100,000 BM cells. (D) FISH analysis for BCR-ABL positive cells was performed on recovered human CD45⁺ cells from the BM of NSG mice transplanted with cells treated in the different experimental arms. Error bars indicate SEM. ** indicates significance of p<0.01 and * significance of p<0.05. FISH analysis was performed by David Irvine

While the above experiments demonstrate the relative clonogenic potency of CD34⁺ cells pre-exposed to LDE225 ± nilotinib. In order to demonstrate the

effect of these agents alone and in combination on BCR-ABL⁺ cells where there was a fully intact haemopoietic microenvironment and immune system, the *Scf-tTa-BCR-ABL-GFP⁺* inducible transgenic mouse model of CML was utilised. Following withdrawal of tetracycline BCR-ABL expression is induced under control of the *Scf*/enhancer and so preferentially expressed in the primitive haemopoietic compartment. After 4 weeks, a pure GFP⁺ haemopoietic population was isolated from these mice and transplanted into irradiated Friend Virus B NIH (FVB/N) recipient mice. These mice developed a CML-like condition within 4 weeks and were treated with nilotinib (50mg/kg), LDE225 (80mg/kg), the combination of both or simply vehicle alone by oral gavage over the course of the following three weeks.

Interestingly, harvested LSC and progenitor cells from the CML mouse model demonstrated up regulation of several mediators and targets of Hh signalling. As briefly discussed in chapter 4, BM MPP exhibited increased expression of Hh target genes *Gli1* compared to non-leukaemic MPP. Likewise the LT-HSC portion demonstrated increased expression of *Gli1*, *Ptch1* and *Ptch2*. Following treatment with LDE225 there was significant reduction in expression of Hh targets, with reduction in *Gli1* expression noted particularly in murine LT-HSC (Figure 8-2).

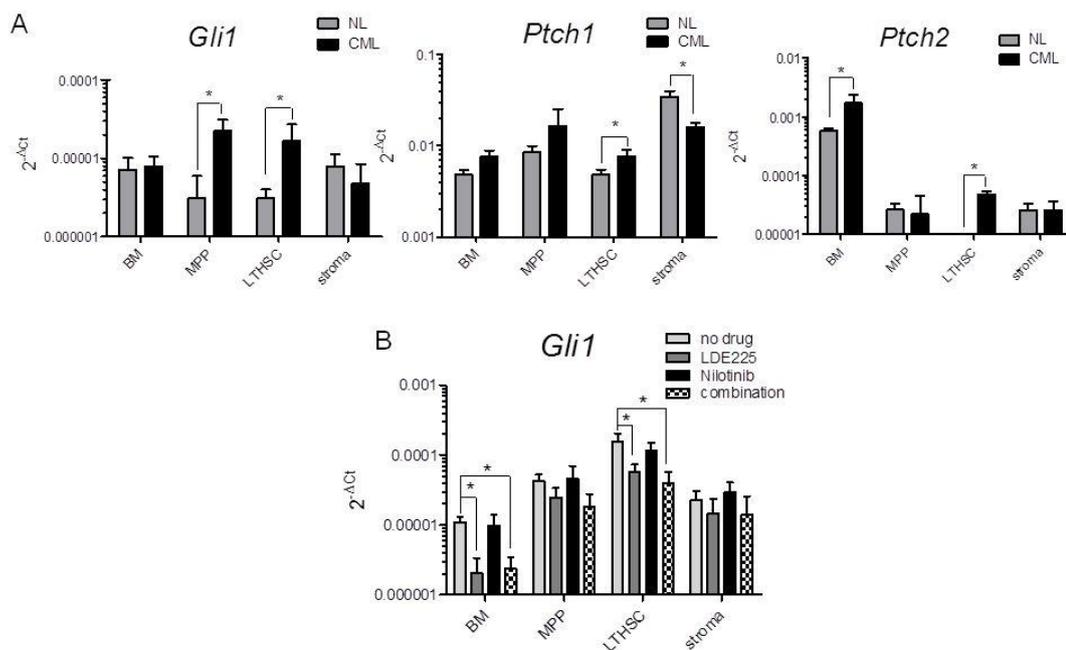


Figure 8-2: Expression of Hh mediators and targets in the *Scl-tTa-BCR-ABL-GFP*⁺ inducible transgenic mouse model of CML at baseline and following treatment.

(A) The expression levels of *Gli1*, *Ptch1* and *Ptch2* in whole BM, haemopoietic sub-populations and BM stroma recovered from the *Scl-tTa-BCR-ABL-GFP*⁺ inducible murine model of CML (leukaemic compared with non-leukaemic controls). (B) The expression levels of *Gli1* in whole BM, haemopoietic sub-populations and BM stroma recovered from the *Scl-tTa-BCR-ABL-GFP*⁺ inducible murine model of CML following treatment. Data and figures are courtesy of Professor Bhatia and Dr Zhang.

An initial analysis concluded that the recipient mice receiving nilotinib had reduced myeloid progenitors (CMP & GMP) in the spleen, but no reduction in splenic LT-HSC. However, the LDE225 treated mice demonstrated a reduction in LT-HSC in the spleen but not in splenic progenitors. The combination of both resulted in reduction of LT-HSC, CMP and GMP in the spleens of treated mice. Interestingly these results were not found in the BM of treated mice, where treatment was not shown to have any quantitative effect on stem or progenitor cell number (Figure 8-3).

A proportion of the treated mice were followed for 4 months following the 3 week treatment period. All the control mice died within 16d of treatment discontinuation. Improved survival compared with the control group was seen with nilotinib alone and a further improvement in survival seen in the combination treated arm compared to the control group (Figure 8-3). No increase in survival was seen in the LDE225 single arm treated mice alone (Figure 8-3).

Lastly BM and splenic cells were harvested from treated mice and pooled prior to transplantation into secondary recipient mice (5×10^6 cells per mouse).

Secondary recipient mice transplanted with BM or splenic cells from the control arm developed leukaemia, however secondary recipients receiving BM or splenic cells from mice treated in the combination arm demonstrated reduced levels of leukaemia compared with both the control treated and the single arm LDE225 or nilotinib treated mice (Figure 8-3).

It follows from this that the combination of nilotinib and LDE225 is affecting the capacity of LSC to recrudescence the disease in secondary hosts.

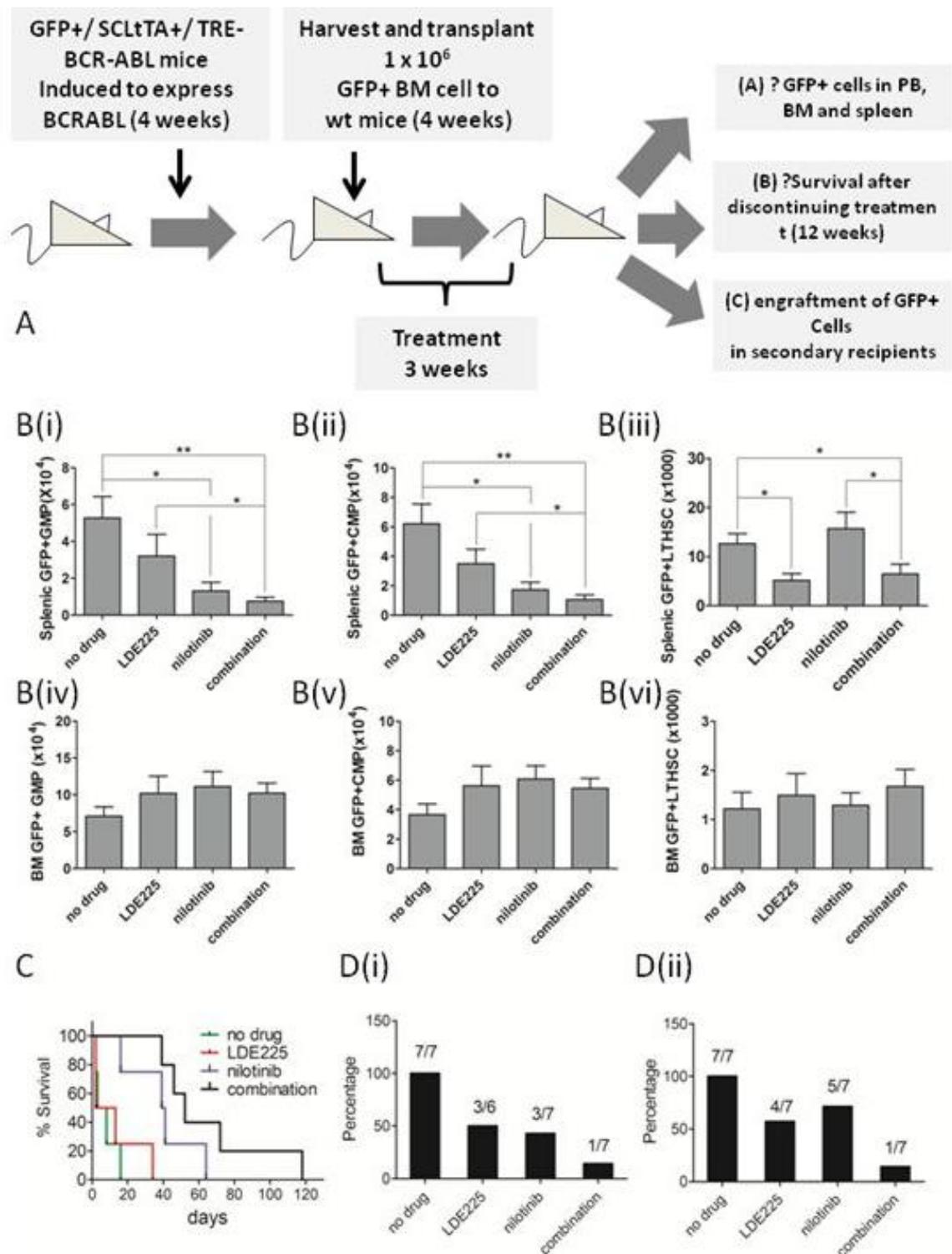


Figure 8-3: Studies in the *Scf-tTa-BCR-ABL-GFP*⁺ inducible transgenic mouse model of CML.

The experimental schema is summarised in (A). (Bi-vi) After 4 weeks these mice began daily treatment with vehicle, LDE225 ± nilotinib. After treatment had ceased, BM (Biv-vi) and splenic (Bi-iii) tissue was assessed by flow cytometry to quantify the number of LSC and progenitor cells present. These graphs indicate the mean absolute number of GMP (Bi,Biv), CMP (Bii/Bv) and LT-HSC (Biii/vi) assessed by surface phenotype in 8 mice per treatment arm. Error bars represent the SEM. Significance of $p < 0.05$ is represented by * and $p < 0.01$ represented by **. (C) Mice were followed for 4 months to ascertain survival following treatment. Survival in the different groups was assessed by creating Kaplan-Meier curves and the different survival rates between treatment arms were assessed by the log-rank test. Combination treatment was significantly different from single agent nilotinib ($p < 0.01$). (D) Pooled splenic or BM cells recovered from treated mice were transplanted into secondary hosts (8 per arm). Graph Di represents the proportion of recipients of BM cells developing leukaemia after 16 weeks, whereas graph Dii represents the proportion of recipients of splenic cells developing leukaemia after 16 weeks. Data and figures are courtesy of Professor Bhatia and Dr Zhang.

Thus, LDE225 in combination with nilotinib is confirmed to reduce engraftment of primary CD34⁺ CP CML cells in immunodeficient mice and to both enhance survival and reduce the capacity of BCR-ABL⁺ cells to recapitulate CML in secondary transplanted mice in the *Scf-tTa-BCR-ABL* mouse model of CML. These results are consistent with previous studies reported by Dierks *et al*/who demonstrated that BCR-ABL positive *Smo*^{-/-} foetal liver cells had reduced capacity to cause leukaemia in transplanted mice as compared to similarly transplanted WT, *Smo*^{+/-} or *Ptch*^{+/-} foetal liver cells, that *Smo*^{-/-} cells were completely unable to transmit leukaemia in second transplantation and *in vivo* treatment with the combination of the non-clinical grade SMO inhibitor cyclopamine in combination with nilotinib led to a significant prolongation of survival (mean 16d) in the combination treatment arm compared to single agent nilotinib (Dierks et al., 2008). Likewise Zhao *et al*/demonstrated prolonged survival in competitive transplant experiments with retrovirally transduced BCR-ABL⁺ *Smo*^{-/-} LSCs compared with similarly transduced *Smo*^{+/+} mice. They also demonstrated markedly reduced survival of secondary transplanted hosts receiving BCR-ABL⁺ cells also transduced to express constitutively active *Smo* (Zhao et al., 2009). The results presented in this study extend and expand the results reported by both authors into the CP of the disease and provide strong corroboration for the results from the *in vitro* assays described in chapter 6 of the main body of this work.

One interesting finding in the murine studies discussed above is the difference between the spleen and the BM in terms of LT-HSC and progenitor abundance. Whether this is due to differences in LT-HSC distribution due to changes wrought on their interaction and adhesion to the BM and splenic microenvironment, or whether Hh signalling is more important in directing LSC fate is not answered in this work. It is intriguing to speculate that primitive splenic haemopoietic cells may be more dependent on Hh signalling due to different microenvironmental conditions. All three Hh ligands are expressed in the spleen and recent reports have attributed functional roles in the regulation of splenic haemopoiesis in conjunction with other signalling pathways e.g. BMP4 particularly under conditions of haemopoietic stress (Lau et al., 2012; Perry et al., 2009).

9 Bibliography

- Abdi, H. (2007). The Bonferonni and Šidák Corrections for Multiple Comparisons. In *Encyclopedia of Measurement and Statistics* (Thousand Oaks), pp. 1-9.
- Abe, Y., Oda-Sato, E., Tobiume, K., Kawauchi, K., Taya, Y., Okamoto, K., Oren, M., and Tanaka, N. (2008). Hedgehog signaling overrides p53-mediated tumor suppression by activating Mdm2. *Proc Natl Acad Sci U S A* 105, 4838-4843.
- Abkowitz, J.L., Catlin, S.N., McCallie, M.T., and Gutter, P. (2002). Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. *Blood* 100, 2665-2667.
- Abkowitz, J.L., Golinelli, D., Harrison, D.E., and Gutter, P. (2000). In vivo kinetics of murine hemopoietic stem cells. *Blood* 96, 3399-3405.
- Abrahamsson, A.E., Geron, I., Gotlib, J., Dao, K.H., Barroga, C.F., Newton, I.G., Giles, F.J., Durocher, J., Creusot, R.S., Karimi, M., et al. (2009). Glycogen synthase kinase 3beta missplicing contributes to leukemia stem cell generation. *Proc Natl Acad Sci U S A* 106, 3925-3929.
- Abramovich, C., and Humphries, R.K. (2005). Hox regulation of normal and leukemic hematopoietic stem cells. *Curr Opin Hematol* 12, 210-216.
- Abramovich, C., Pineault, N., Ohta, H., and Humphries, R.K. (2005). Hox genes: from leukemia to hematopoietic stem cell expansion. *Ann N Y Acad Sci* 1044, 109-116.
- Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* 439, 599-603.
- Adolfsson J., Mansson R., Buza-Vidas N., Hultquist A., Luiba K., Jensen C.T., Bryder D., Yang L., Borge O.J., Thoren L.A., Sitnicka E., Sasaki Y., Sigvardsson M., Jacobsen S.E. (2005) Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121, 295-306.
- Affer, M., Dao, S., Liu, C., Olshen, A.B., Mo, Q., Viale, A., Lambek, C.L., Marr, T.G., and Clarkson, B.D. (2011). Gene Expression Differences between Enriched Normal and Chronic Myelogenous Leukemia Quiescent Stem/Progenitor Cells and Correlations with Biological Abnormalities. *Journal of Oncology* 2011, doi:10.1155/2011/798592.
- Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193-197.
- Alenzi, F.Q., Alenazi, B.Q., Ahmad, S., Salem, M.L., Al-Jabri, A.A., and Wysee, R.K.H. (2009). The Haemopoietic Stem Cell: Between Apoptosis and Self Renewal. *Yale J Biol Med* 82, 7-18.
- Alison, M.R., Lin, W.R., Lim, S.M., and Nicholson, L.J. (2012). Cancer stem cells: in the line of fire. *Cancer Treat Rev* 38, 589-598.
- Andaloussi, A.E., Graves, S., Meng, F., Mandal, M., Mashayekhi, M., and Aifantis, I. (2006). Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. *Nat Immunol* 7, 418-426.

- Anderson, K., Lutz, C., van Delft, F.W., Bateman, C.M., Guo, Y., Colman, S.M., Kempinski, H., Moorman, A.V., Titley, I., Swansbury, J., et al. (2011). Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature* 469, 356-361.
- Andreu, E.J., Lledo, E., Poch, E., Ivorra, C., Albero, M.P., Martinez-Climent, J.A., Montiel-Duarte, C., Rifon, J., Perez-Calvo, J., Arbona, C., et al. (2005). BCR-ABL induces the expression of Skp2 through the PI3K pathway to promote p27Kip1 degradation and proliferation of chronic myelogenous leukemia cells. *Cancer Res* 65, 3264-3272.
- Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2001). HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. *Exp Hematol* 29, 1125-1134.
- Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109, 39-45.
- Apperley, J.F. (2007). Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol* 8, 1018-1029.
- Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 118, 149-161.
- Argiropoulos, B., and Humphries, R.K. (2007). Hox genes in hematopoiesis and leukemogenesis. *Oncogene* 26, 6766-6776.
- Auer, H., Newsom, D., and Kornacker, K. (2009). Expression Profiling Using Affymetrix GeneChip Microarrays. In *Microchip Methods in Diagnostics*, U. Bilitewski, ed. (Humana Press), pp. 35-46.
- Ayers, K.L., and Therond, P.P. (2010). Evaluating Smoothed as a G-protein-coupled receptor for Hedgehog signalling. *Trends in Cell Biol* 20, 287-298.
- Ayroles, J.F., and Gibson, G. (2006). Analysis of variance of microarray data. *Methods Enzymol* 411, 214-233.
- Baccarani, M., Cortes, J., Pane, F., Niederwieser, D., Saglio, G., Apperley, J., Cervantes, F., Deininger, M., Gratwohl, A., Guilhot, F., et al. (2009). Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J Clin Oncol* 27, 6041-6051.
- Bansal, D., Scholl, C., Frohling, S., McDowell, E., Lee, B.H., Dohner, K., Ernst, P., Davidson, A.J., Daley, G.Q., Zon, L.I., et al. (2006). Cdx4 dysregulates Hox gene expression and generates acute myeloid leukemia alone and in cooperation with Meis1a in a murine model. *Proc Natl Acad Sci U S A* 103, 16924-16929.
- Barnes, E.A., Kong, M., Ollendorff, V., and Donoghue, D.J. (2001). Patched1 interacts with cyclin B1 to regulate cell cycle progression. *EMBO J* 20, 2214-2223.
- Baron, M. (2003). An overview of the Notch signalling pathway. *Semin Cell Dev Biol* 14, 113-119.
- Bartley, P.A., Ross, D.M., Latham, S., Martin-Harris, M.H., Budgen, B., Wilczek, V., Branford, S., Hughes, T.P., and Morley, A.A. (2010). Sensitive detection and quantification of minimal residual disease in chronic myeloid leukaemia using nested quantitative PCR for BCR-ABL DNA. *Int J Lab Hematol* 32, e222-228.

- Bartram, C.R., de Klein, A., Hagemeijer, A., van Agthoven, T., Geurts van Kessel, A., Bootsma, D., Grosveld, G., Ferguson-Smith, M.A., Davies, T., Stone, M., et al. (1983). Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 306, 277-280.
- Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci USA* 89, 2804-2808.
- Beachy, P.A., Cooper, M.K., Young, K.E., von Kessler, D.P., Park, W.J., Hall, T.M., Leahy, D.J., and Porter, J.A. (1997). Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. *Cold Spring Harb Symp Quant Biol* 62, 191-204.
- Beachy, P.A., Karhadkar, S.S., and Berman, D.M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature* 432, 324-331.
- Becker, A.J., McCulloch, E., and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* 197, 452-454.
- Beckmann, J., Scheitza, S., Wernet, P., Fischer, J.C., and Giebel, B. (2007). Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. *Blood* 109, 5494-5501.
- Bedi, A., Zehnauer, B.A., Collector, M.I., Barber, J.P., Zicha, M.S., Sharkis, S.J., and Jones, R.J. (1993). BCR-ABL gene rearrangement and expression of primitive hematopoietic progenitors in chronic myeloid leukemia. *Blood* 81, 2898-2902.
- Bellodi, C., Lidonnici, M.R., Hamilton, A., Helgason, G.V., Soliera, A.R., Ronchetti, M., Galavotti, S., Young, K.W., Selmi, T., Yacobi, R., et al. (2009). Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J Clin Invest* 119, 1109-1123.
- Bemmo, A., Benovoy, D., Kwan, T., Gaffney, D., Jensen, R., and Majewski, J. (2008). Gene Expression and Isoform Variation Analysis using Affymetrix Exon Arrays. *BMC Genomics* 9, 529.
- Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Statist Soc B* 57, 289-300.
- Benveniste, P., Cantin, C., Hyam, D., and Iscove, N.N. (2003). Hematopoietic stem cells engraft in mice with absolute efficiency. *Nat Immunol* 4, 708-713.
- Berman, D.M., Karhadkar, S.S., Hallahan, A.R., Pritchard, J.I., Eberhart, C.G., Watkins, D.N., Chen, J.K., Cooper, M.K., Taipale, J., Olson, J.M., et al. (2002). Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science* 297, 1559-1561.
- Berman, D.M., Karhadkar, S.S., Maitra, A., Montes De Oca, R., Gerstenblith, M.R., Briggs, K., Parker, A.R., Shimada, Y., Eshleman, J.R., Watkins, D.N., et al. (2003). Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 425, 846-851.
- Bernhardt, M., Galach, M., Novak, D., and Utikal, J. (2012). Mediators of induced pluripotency and their role in cancer cells - current scientific knowledge and future perspectives. *Biotechnol J* 7, 810-821.
- Bernt, K.M., and Armstrong, S.A. (2011). Targeting epigenetic programs in MLL-rearranged leukemias. *Hematology Am Soc Hematol Educ Program* 2011, 354-360.

- Bhardwaj, G., Murdoch, B., Wu, D., Baker, D.P., Williams, K.P., Chadwick, K., Ling, L.E., Karanu, F.N., and Bhatia, M. (2001). Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol* 2, 172-180.
- Bhaskaran, N., and Souchelnytskyi, S. (2008). Systemic analysis of TGFbeta proteomics revealed involvement of Plag1/CNK1/RASSF1A/Src network in TGFbeta1-dependent activation of Erk1/2 and cell proliferation. *Proteomics* 8, 4507-4520.
- Bhatia, M., Bonnet, D., Wu, D., Murdoch, B., Wrana, J., Gallacher, L., and Dick, J.E. (1999). Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J Exp Med* 189, 1139-1148.
- Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* 94, 5320-5325.
- Bhatia, R., Holtz, M., Niu, N., Gray, R., Snyder, D.S., and Sawyers, C.L. (2003). Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. *Blood* 101, 4701-4707.
- Bhattacharyya, J., Mihara, K., Yasunaga, S., Tanaka, H., Hoshi, M., Takihara, Y., and Kimura, A. (2009). BMI-1 expression is enhanced through transcriptional and posttranscriptional regulation during the progression of chronic myeloid leukemia. *Ann Hematol* 88, 333-340.
- Biernaux, C., Loos, M., Sels, A., Huez, G., and Stryckmans, P. (1995). Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood* 86, 3118-3122.
- Bigelow, R.L., Chari, N.S., Unden, A.B., Spurgers, K.B., Lee, S., Roop, D.R., Toftgard, R., and McDonnell, T.J. (2004). Transcriptional regulation of bcl-2 mediated by the sonic hedgehog signaling pathway through gli-1. *J Biol Chem* 279, 1197-1205.
- Bijl, J., Thompson, A., Ramirez-Solis, R., Kros, J., Grier, D.G., Lawrence, H.J., and Sauvageau, G. (2006). Analysis of HSC activity and compensatory Hox gene expression profile in Hoxb cluster mutant fetal liver cells. *Blood* 108, 116-122.
- Bijlsma, M.F., Spek, C.A., Zivkovic, D., van de Water, S., Rezaee, F., and Peppelenbosch, M.P. (2006). Repression of smoothelin by patched-dependent (pro-)vitamin D3 secretion. *Plos Biol* 4, e232.
- Bitgood, M.J., Shen, L., and McMahon, A.P. (1996). Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr Biol* 6, 298-304.
- Bjorkholm, M., Ohm, L., Eloranta, S., Derolf, A., Hultcrantz, M., Sjoberg, J., Andersson, T., Hoglund, M., Richter, J., Landgren, O., et al. (2011). Success story of targeted therapy in chronic myeloid leukemia: a population-based study of patients diagnosed in Sweden from 1973 to 2008. *J Clin Oncol* 29, 2514-2520.
- Blank, U., Karlsson, G., and Karlsson, S. (2008). Signaling pathways governing stem-cell fate. *Blood* 111, 492-503.
- Blotta, S., Jakubikova, J., Calimeri, T., Roccaro, A.M., Amodio, N., Azab, A.K., Foresta, U., Mitsiades, C.S., Rossi, M., Todoerti, K., et al. (2012). Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma. *Blood* 120, 5002-5013.

- Bockamp, E.O., McLaughlin, F., Gottgens, B., Murrell, A.M., Elefanty, A.G., and Green, A.R. (1997). Distinct mechanisms direct SCL/tal-1 expression in erythroid cells and CD34 positive primitive myeloid cells. *J Biol Chem* 272, 8781-8790.
- Bonifazi, F., de Vivo, A., Rosti, G., Guilhot, F., Guilhot, J., Trabacchi, E., Hehlmann, R., Hochhaus, A., Shepherd, P.C., Steegmann, J.L., et al. (2001). Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood* 98, 3074-3081.
- Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 3, 730-737.
- Bose, S., Deininger, M., Gora-Tybor, J., Goldman, J.M., and Melo, J.V. (1998). The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. *Blood* 92, 3362-3367.
- Bowie, M.B., Kent, D.G., Dykstra, B., McKnight, K.D., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. *Proc Natl Acad Sci U S A* 104, 5878-5882.
- Bowie, M.B., McKnight, K.D., Kent, D.G., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. *J Clin Invest* 116, 2808-2816.
- Branford, S., Rudzki, Z., Walsh, S., Parkinson, I., Grigg, A., and Szer, J. (2003). Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 102, 276-283.
- Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 7, 678-689.
- Brendel, C., Mohr, M., Schimmelpfennig, C., Müller, J., Bornhäuser, B., Schmidt, M., Ritter, M., Ehninger, G., and Neubauer, A. (1999). Detection of cytogenetic aberrations both in CD90 (Thy-1)-positive and (Thy-1)-negative stem cell (CD34) subfractions of patients with acute and chronic myeloid leukemias. *Leukemia* 13, 1770-1775.
- Brennan, S.K., Meade, B., Wang, Q., Merchant, A.A., Kowalski, J., and Matsui, W. (2010). Mantle cell lymphoma activation enhances bortezomib sensitivity. *Blood* 116, 4185-4191.
- Bresnick, E.H., Katsumura, K.R., Lee, H.Y., Johnson, K.D., and Perkins, A.S. (2012). Master regulatory GATA transcription factors: mechanistic principles and emerging links to hematologic malignancies. *Nucleic Acids Res* 40, 5819-5831.
- Brown, K., Xie, S., Qiu, X., Mohrin, M., Shin, J., Liu, Y., Zhang, D., Scadden, D.T., and Chen, D. (2013). SIRT3 Reverses Aging-Associated Degeneration. *Cell Rep* 3, 319-327.
- Brummendorf, T.H., Dragowska, W., Zijlmans, J., Thornbury, G., and Lansdorp, P.M. (1998). Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. *J Exp Med* 188, 1117-1124.
- Brun, A.C., Bjornsson, J.M., Magnusson, M., Larsson, N., Leveen, P., Ehinger, M., Nilsson, E., and Karlsson, S. (2004). Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. *Blood* 103, 4126-4133.

- Bruns, I., Czibere, A., Fischer, J.C., Roels, F., Cadeddu, R.P., Buest, S., Bruennert, D., Huenerlituerkoglu, A.N., Stoecklein, N.H., Singh, R., et al. (2009). The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence. *Leukemia* 23, 892-899.
- Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringham, F.R., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841-846.
- Canales, R.D., Luo, Y., Willey, J.C., Austermler, B., Barbacioru, C.C., Boysen, C., Hunkapiller, K., Jensen, R.V., Knight, C.R., Lee, K.Y., et al. (2006). Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol* 24, 1115-1122.
- Carow, C.E., Hangoc, G., Cooper, S.H., Williams, D.E., and Broxmeyer, H.E. (1991). Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential. *Blood* 78, 2216-2221.
- Carow, C.E., Hangoc, G., and H.E., B. (1993). Human multipotential progenitor cells (CFU-GEMM) have extensive replating capacity for secondary CFU-GEMM: an effect enhanced by cord blood plasma. *Blood* 81, 942-949.
- Cashman, J., Eaves, A.C., and Eaves, C.J. (1985). Regulated proliferation of primitive hematopoietic progenitor cells in long-term human marrow cultures. *Blood* 66, 1002-1005.
- Cashman, J.D., Lapidot, T., Wang, J.C., Doedens, M., Shultz, L.D., Lansdorp, P., Dick, J.E., and Eaves, C.J. (1997). Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* 89, 4307-4316.
- Catlin, S.N., Busque, L., Gale, R.E., Guttorp, P., and Abkowitz, J.L. (2011). The replication rate of human hematopoietic stem cells in vivo. *Blood* 117, 4460-4466.
- Cayuso, J., Ulloa, F., Cox, B., Briscoe, J., and Marti, E. (2006). The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. *Development* 133, 517-528.
- Cedar, H., and Bergman, Y. (2011). Epigenetics of haematopoietic cell development. *Nat Rev Immunol* 11, 478-488.
- Challen, G.A., Boles, N.C., Chambers, S.M., and Goodell, M.A. (2010). Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF- β 1. *Cell Stem Cell* 6, 265-278.
- Chambers, S.M., Boles, N.C., Lin, K.-Y.K., Tierney, M.P., Bowman, T.V., Bradfute, S.B., Chen, A.J., Merchant, A.A., Sirin, O., Weksberg, D.C., et al. (2007). Hematopoietic Fingerprints: An Expression Database of Stem Cells and Their Progeny. *Cell Stem Cell* 1, 578-591.
- Chen, J.K., Taipale, J., Cooper, M.K., and Beachy, P.A. (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* 16, 2743-2748.
- Chen, M., Gallipoli, P., Degeer, D., Sloma, I., Forrest, D.L., Chan, M., Lai, D., Jorgensen, H., Ringrose, A., Wang, H.M., et al. (2013). Targeting Primitive Chronic Myeloid Leukemia Cells by Effective Inhibition of a New AHI-1-BCR-ABL-JAK2 Complex. *J Natl Cancer Inst* 105, 405-423.

- Chen, Y., Wang, H., Kantarjian, H., and Cortes, J. (2012). Trends in chronic myeloid leukemia incidence and survival in the United States from 1975 to 2009. *Leukemia & lymphoma*, 1-7.
- Cheng, T., Rodrigues, N., Dombkowski, D., Stier, S., and Scadden, D.T. (2000a). Stem cell repopulation efficiency but not pool size is governed by p27kip1. *Nat Med* 6, 1235-1240.
- Cheng, T., Rodrigues, N., Shen, H., Yang, Y.G., Dombkowski, D., and Sykes, M. (2000b). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 287, 1804-1808.
- Cheshier, S.H., Morrison, S.J., Liao, X., and Weissman, I.L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A* 96, 3120-3125.
- Cheshier, S.H., Prohaska, S.S., and Weissman, I.L. (2007). The effect of bleeding on hematopoietic stem cell cycling and self-renewal. *Stem Cells Dev* 16, 707-717.
- Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383, 407-413.
- Chomel, J.-C., and Turhan, A.G. (2011). Chronic myeloid leukemia stem cells in the era of targeted therapies: resistance, persistence and long-term dormancy. *Oncotarget* 2.
- Chu, S., McDonald, T., Lin, A., Chakraborty, S., Huang, Q., Snyder, D.S., and Bhatia, R. (2011). Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. *Blood* 118, 5565-5572.
- Churchill, G. (2004). Using ANOVA to analyze microarray data. *Biotechniques* 37, 173-175.
- Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006). Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 66, 9339-9344.
- Clement, V., Sanchez, P., de Tribolet, N., Radovanovic, I., and Ruiz i Altaba, A. (2007). HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr Biol* 17, 165-172.
- Clevers, H. (2006). Wnt/b-Catenin Signaling in Development and Disease. *Cell* 127, 469-480.
- Cohen, M.M., Jr. (2009). Perspectives on RUNX genes: an update. *American journal of medical genetics Part A* 149A, 2629-2646.
- Collado, M., Blasco, M.A., and Serrano, M. (2007). Cellular Senescence in Cancer and Aging. *Cell* 130, 223-233.
- Collins, A.T., Berry, P.A., Hyde, C., Stower, M.J., and Maitland, N.J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 65, 10946-10951.
- Conneally, E., Cashman, J., Petzer, A., and Eaves, C. (1997). Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc Natl Acad Sci USA* 94, 9836-9841.

- Copland, M., Hamilton, A., Elrick, L.J., Baird, J.W., Allan, E.K., Jordanides, N., Barow, M., Mountford, J.C., and Holyoake, T.L. (2006). Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. *Blood* 107, 4532-4539.
- Copland, M., Pellicano, F., Richmond, L., Allan, E.K., Hamilton, A., Lee, F.Y., Weinmann, R., and Holyoake, T.L. (2008). BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors. *Blood* 111, 2843-2853.
- Corbin, A.S., Agarwal, A., Loriaux, M., Cortes, J., Deininger, M.W., and Druker, B.J. (2011). Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J Clin Invest* 121, 396-409.
- Corbit, K.C. (2005). Vertebrate Smoothed functions at the primary cilium. *Nature* 437, 1018-1021.
- Corcoran, R.B., and Scott, M.P. (2006). Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc Natl Acad Sci USA* 103, 8408-8413.
- Cortes, J.E., Kantarjian, H., Shah, N.P., Bixby, D., Mauro, M.J., Flinn, I., O'Hare, T., Hu, S., Narasimhan, N.I., Rivera, V.M., et al. (2012a). Ponatinib in refractory Philadelphia chromosome-positive leukemias. *N Engl J Med* 367, 2075-2088.
- Cortes, J.E., Kantarjian, H.M., Brummendorf, T.H., Kim, D.W., Turkina, A.G., Shen, Z.X., Pasquini, R., Khoury, H.J., Arkin, S., Volkert, A., et al. (2011a). Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. *Blood* 118, 4567-4576.
- Cortes, J.E., Kim, D.-W., Pinilla-Ibarz, J., le Coutre, P., Paquette, R., Chuah, C., Nicolini, F.E., Apperley, J., Khoury, H.J., Talpaz, M., et al. (2012b). A Pivotal Phase 2 Trial of Ponatinib in Patients with Chronic Myeloid Leukemia (CML) and Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia (Ph+ALL) Resistant or Intolerant to Dasatinib or Nilotinib, or with the T315I BCR-ABL Mutation: 12-Month Follow-up of the PACE Trial. *Blood (ASH Annual Meeting Abstracts)* 120, 163.
- Cortes, J.E., Kim, D.W., Kantarjian, H.M., Brummendorf, T.H., Dyagil, I., Griskevicius, L., Malhotra, H., Powell, C., Gogat, K., Countouriotis, A.M., et al. (2012c). Bosutinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: results from the BELA trial. *J Clin Oncol* 30, 3486-3492.
- Cortes, J.E., Maru, A., Souza, C.A.A.D., Guilhot, F., Duvillie, L., Powell, C., Countouriotis, A., and Gambacorti-Passerini, C. (2011b). Bosutinib Versus Imatinib in Newly Diagnosed Chronic Phase Chronic Myeloid Leukemia - BELA Trial: 24-Month Follow-up. *Blood (ASH Annual Meeting Abstracts)* 118, 455-.
- Coulombel, L., Eaves, C.J., Dube, I.D., Kalousek, D.K., and Eaves, A.C. (1984). Variable persistence of leukemic progenitor cells in long-term CML and AML marrow cultures. *Kroc Foundation series* 18, 243-254.
- Coulombel, L., Kalousek, D.K., Eaves, C.J., Gupta, C.M., and Eaves, A.C. (1983). Long-term marrow culture reveals chromosomally normal hematopoietic progenitor cells in patients with Philadelphia chromosome-positive chronic myelogenous leukemia. *N Engl J Med* 308, 1493-1498.

- Craig, W., Kay, R., Cutler, R.L., and Lansdorp, P.M. (1993). Expression of Thy-1 on human hematopoietic progenitor cells. *J Exp Med* 177, 1331-1342.
- Cridland, S.O., Keys, J.R., Papathanasiou, P., and Perkins, A.C. (2009). Indian hedgehog supports definitive erythropoiesis. *Blood Cells Mol Dis* 43, 149-155.
- Cristobal, I., Blanco, F.J., Garcia-Orti, L., Marcotegui, N., Vicente, C., Rifon, J., Novo, F.J., Bandres, E., Calasanz, M.J., Bernabeu, C., et al. (2010). SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* 115, 615-625.
- Crossman, L.C., Druker, B.J., Deininger, M.W., Pirmohamed, M., Wang, L., and Clark, R.E. (2005). hOCT 1 and resistance to imatinib. *Blood* 106, 1133-1134.
- Cuiffo, B., and Ren, R. (2006). Models of hematopoietic malignancies: chronic myeloid leukemia. *Drug Discovery Today: Disease Models* 3, 183-189.
- Cumano, A., and Godin, I. (2007). Ontogeny of the hematopoietic system. *Annu Rev Immunol* 25, 745-785.
- Curry, J.L., Trentin, J.J., and Wolf, N. (1967). Hemopoietic spleen colony studies. II. Erythropoiesis. *J Exp Med* 125, 703-720.
- Dai, P. (1999). Sonic Hedgehog-induced activation of the Gli1 promoter is mediated by GLI3. *J Biol Chem* 274, 8143-8152.
- Dai, Y., Rahmani, M., Corey, S.J., Dent, P., and Grant, S. (2004). A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem* 279, 34227-34239.
- Daley, G.Q., and Baltimore, D. (1988). Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. *Proc Natl Acad Sci U S A* 85, 9312-9316.
- Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1990a). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 247, 824-830.
- Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1990b). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 247, 824-830.
- Dalma Weiszhausz, D.D., Warrington, J., Tanimoto, E.Y., and Miyada, C.G. (2006). The Affymetrix GeneChip® Platform: An Overview. In *Methods in enzymology*, K. Alan, and O. Brian, eds. (Academic Press), pp. 3-28.
- Damm, F., Itzykson, R., Kosmider, O., Droin, N., Renneville, A., Chesnais, V., Gelsi-Boyer, V., de Botton, S., Vey, N., Preudhomme, C., et al. (2013). SETBP1 mutations in 658 patients with myelodysplastic syndromes, chronic myelomonocytic leukemia and secondary acute myeloid leukemias. *Leukemia*.
- Dayyani, F., Wang, J., Yeh, J.R., Ahn, E.Y., Tobey, E., Zhang, D.E., Bernstein, I.D., Peterson, R.T., and Sweetser, D.A. (2008). Loss of TLE1 and TLE4 from the del(9q) commonly deleted region in AML cooperates with AML1-ETO to affect myeloid cell proliferation and survival. *Blood* 111, 4338-4347.

- Dazzi, F., Capelli, D., Hasserjian, R., Cotter, F., Corbo, M., Poletti, A., Chinswangwatanakul, W., Goldman, J.M., and Gordon, M.Y. (1998). The Kinetics and Extent of Engraftment of Chronic Myelogenous Leukemia Cells in Non-Obese Diabetic/Severe Combined Immunodeficiency Mice Reflect the Phase of the Donor's Disease: An In Vivo Model of Chronic Myelogenous Leukemia Biology. *Blood* 92, 1390-1396.
- de Klein, A., van Kessel, A., Grosveld, G., Bartram, C., Hagemeijer, A., Bootsma, D., Spurr, N., Heisterkamp, N., Groffen, J., and Stephenson, J. (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* 300, 765-767.
- de Lavallade, H., Apperley, J.F., Khorashad, J.S., Milojkovic, D., Reid, A.G., Bua, M., Szydlo, R., Olavarria, E., Kaeda, J., Goldman, J.M., et al. (2008). Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol* 26, 3358-3363.
- Dean, M., Fojo, T., and Bates, S. (2005). Tumour stem cells and drug resistance. *Nat Rev Cancer* 5, 275-284.
- Deininger, M.W., Goldman, J.M., Lydon, N., and Melo, J.V. (1997). The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. *Blood* 90, 3691-3698.
- Deininger, M.W., Goldman, J.M., and Melo, J.V. (2000). The molecular biology of chronic myeloid leukemia. *Blood* 96, 3343-3356.
- Delaney, C., Heimfeld, S., Brashem-Stein, C., Voorhies, H., Manger, R.L., and Bernstein, I.D. (2010). Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat Med* 16, 232-236.
- Delpuech, O., Griffiths, B., East, P., Essafi, A., Lam, E.W., Burgering, B., Downward, J., and Schulze, A. (2007). Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. *Mol Cell Biol* 27, 4917-4930.
- Denef, N., Neubuser, D., Perez, L., and Cohen, S.M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothed. *Cell* 102, 521-531.
- Denell, R.E., and Frederick, R.D. (1983). Homoeosis in *Drosophila*: a description of the Polycomb lethal syndrome. *Dev Biol* 97, 34-47.
- Dexter, T.M., Allen, T.D., and Lajtha, L.G. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol* 91, 335-344.
- Diaz-Blanco, E., Bruns, I., Neumann, F., Fischer, J.C., Graef, T., Roskopf, M., Brors, B., Pechtel, S., Bork, S., Koch, A., et al. (2007). Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase. *Leukemia* 21, 494-504.
- Dick, J.E., Bhatia, M., Gan, O., Kapp, U., and Wang, J.C. (1997). Assay of human stem cells by repopulation of NOD/SCID mice. *Stem Cells* 15 Suppl 1, 199-203; discussion 204-197.
- Dick, J.E., Guenechea, G., Gan, O.I., and Dorrell, C. (2001). In vivo dynamics of human stem cell repopulation in NOD/SCID mice. *Annals of the New York Academy of Sciences* 938, 184-190.

- Dierks, C., Beigi, R., Guo, G.R., Zirlik, K., Stegert, M.R., and Manley, P. (2008). Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell* 14, 238-249.
- Dierks, C., Grbic, J., Zirlik, K., Beigi, R., Englund, N.P., Guo, G.R., Veelken, H., Engelhardt, M., Mertelsmann, R., Kelleher, J.F., et al. (2007). Essential role of stromally induced hedgehog signaling in B-cell malignancies. *Nat Med* 13, 944-951.
- Ding, L., Ley, T.J., Larson, D.E., Miller, C.A., Koboldt, D.C., Welch, J.S., Ritchey, J.K., Young, M.A., Lamprecht, T., McLellan, M.D., et al. (2012). Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* 481, 506-510.
- Domen, J., Cheshier, S.H., and Weissman, I.L. (2000). The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *J Exp Med* 191, 253-264.
- Domen, J., and Weissman, I.L. (2000). Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med* 192, 1707-1718.
- Donato, N.J., Wu, J.Y., Stapley, J., Gallick, G., Lin, H., Arlinghaus, R., and Talpaz, M. (2003). BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood* 101, 690-698.
- Doulatov, S., Notta, F., Eppert, K., Nguyen, L.T., Ohashi, P.S., and Dick, J.E. (2010). Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol* 11, 585-593.
- Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. *Cell Stem Cell* 10, 120-136.
- Drabkin, H.A., Parsy, C., Ferguson, K., Guilhot, F., Lacotte, L., Roy, L., Zeng, C., Baron, A., Hunger, S.P., Varella-Garcia, M., et al. (2002). Quantitative HOX expression in chromosomally defined subsets of acute myelogenous leukemia. *Leukemia* 16, 186-195.
- Drexler, H.G. (1994). Leukemia cell lines: in vitro models for the study of chronic myeloid leukemia. *Leukemia research* 18, 919-927.
- Druker, B.J., Guilhot, F., O'Brien, S.G., Gathmann, I., Kantarjian, H., Gattermann, N., Deininger, M.W., Silver, R.T., Goldman, J.M., Stone, R.M., et al. (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 355, 2408-2417.
- Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J., and Lydon, N.B. (1996). Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 2, 561-566.
- Du, J., Chen, Y., Li, Q., Han, X., Cheng, C., Wang, Z., Danielpour, D., Dunwoodie, S.L., Bunting, K.D., and Yang, Y.C. (2012). HIF-1alpha deletion partially rescues defects of hematopoietic stem cell quiescence caused by Cited2 deficiency. *Blood* 119, 2789-2798.
- Du, Y., Jenkins, N.A., and Copeland, N.G. (2005). Insertional mutagenesis identifies genes that promote the immortalization of primary bone marrow progenitor cells. *Blood* 106, 3932-3939.

- Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature* 417, 299-304.
- Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N., et al. (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* 6, 314-322.
- Dyer, M.A., Farrington, S.M., Mohn, D., Munday, J.R., and Baron, M.H. (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* 128, 1717-1730.
- Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S.J., Brinkman, R., and Eaves, C. (2007). Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* 1, 218-229.
- Eaves, A.C., Barnett, M.J., Ponchio, L., Cashman, J.D., Petzer, A.L., and Eaves, C.J. (1998a). Differences between normal and CML stem cells: potential targets for clinical exploitation. *Stem Cells* 16 Suppl 1, 77-83; discussion 89.
- Eaves, A.C., and Eaves, C.J. (1988). Maintenance and proliferation control of primitive hemopoietic progenitors in long-term cultures of human marrow cells. *Blood cells* 14, 355-368.
- Eaves, C., Cashman, J., and Eaves, A. (1998b). Defective regulation of leukemic hematopoiesis in chronic myeloid leukemia. *Leukemia research* 22, 1085-1096.
- Eaves, C., Coulombel, L., Dube, I., Kalousek, D., Cashman, J., and Eaves, A. (1985a). Behavior of human leukemic progenitor populations in long-term marrow culture. *Haematology and blood transfusion* 29, 163-167.
- Eaves, C., Coulombel, L., Dube, I., Kalousek, D., Cashman, J., and Eaves, A. (1985b). Maintenance of normal and abnormal hemopoietic cell populations in long-term cultures of CML and AML marrow cells. *Progress in clinical and biological research* 184, 403-413.
- Eaves, C.J., Cashman, J.D., Zoumbos, N.C., Barnett, M.J., and Eaves, A.C. (1993). Biological strategies for the selective manipulation of normal and leukemic stem cells. *Stem Cells* 11 Suppl 3, 109-121.
- Eaves, C.J., and Humphries, R.K. (2010). Acute myeloid leukemia and the Wnt pathway. *N Engl J Med* 362, 2326-2327.
- Eaves, C.J., Sutherland, H.J., Cashman, J.D., Otsuka, T., Lansdorp, P.M., Humphries, R.K., Eaves, A.C., and Hogge, D.E. (1991). Regulation of primitive human hematopoietic cells in long-term marrow culture. *Seminars in hematology* 28, 126-131.
- Ehtesham, M., Sarangi, A., Valadez, J.G., Chanthaphaychith, S., Becher, M.W., Abel, T.W., Thompson, R.C., and Cooper, M.K. (2007). Ligand-dependent activation of the hedgehog pathway in glioma progenitor cells. *Oncogene* 26, 5752-5761.
- El Andaloussi, A., Graves, S., Meng, F., Mandal, M., Mashayekhi, M., and Aifantis, I. (2006). Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus. *Nat Immunol* 7, 418-426.
- Elefanty, A.G., Hariharan, I.K., and Cory, S. (1990). bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice. *EMBO J* 9, 1069-1078.

- Ema, H., Takano, H., Sudo, K., and Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. *J Exp Med* 192, 1281-1288.
- Eppert, K., Takenaka, K., Lechman, E.R., Waldron, L., Nilsson, B., van Galen, P., Metzeler, K.H., Poepl, A., Ling, V., Beyene, J., et al. (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* 17, 1086-1093.
- Ernst, P., Fisher, J.K., Avery, W., Wade, S., Foy, D., and Korsmeyer, S.J. (2004). Definitive hematopoiesis requires the mixed-lineage leukemia gene. *Developmental Cell* 6, 437-443.
- Espinosa, L., Ingles-Esteve, J., Aguilera, C., and Bigas, A. (2003). Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem* 278, 32227-32235.
- Fang, B., Zheng, C., Liao, L., Han, Q., Sun, Z., Jiang, X., and Zhao, R.C. (2005). Identification of human chronic myelogenous leukemia progenitor cells with hemangioblastic characteristics. *Blood* 105, 2733-2740.
- Fialkow, P.J., Gartler, S.M., and Yoshida, A. (1967). Clonal origin of chronic myelocytic leukemia in man. *Proc Natl Acad Sci U S A* 58, 1468-1471.
- Fialkow, P.J., Jacobson, R.J., and Papayannopoulou, T. (1977). Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am J Med* 63, 125-130.
- Ficara, F., Murphy, M.J., Lin, M., and Cleary, M.L. (2008). Pbx1 Regulates Self-Renewal of Long-Term Hematopoietic Stem Cells by Maintaining Their Quiescence. *Cell Stem Cell* 2, 484-496.
- Fine, B.M., Stanulla, M., Schrappe, M., Ho, M., Viehmann, S., Harbott, J., and Boxer, L.M. (2004). Gene expression patterns associated with recurrent chromosomal translocations in acute lymphoblastic leukemia. *Blood* 103, 1043-1049.
- Finetti, F., Paccani, S.R., Riparbelli, M.G., Giacomello, E., Perinetti, G., Pazour, G.J., Rosenbaum, J.L., and Baldari, C.T. (2009). Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse. *Nat Cell Biol* 11, 1332-1339.
- Forozan, F., Karhu, R., Kononen, J., Kallioniemi, A., and Kallioniemi, O.P. (1997). Genome screening by comparative genomic hybridization. *Trends Genet* 13, 405-409.
- Forsberg, E.C., Bhattacharya, D., and Weissman, I.L. (2006). Hematopoietic stem cells: expression profiling and beyond. *Stem Cell Rev* 2, 23-30.
- Fortunel, N.O., Otu, H.H., Ng, H.H., Chen, J., Mu, X., Chevassut, T., Li, X., Joseph, M., Bailey, C., Hatzfeld, J.A., et al. (2003). Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". *Science* 302, 393; author reply 393.
- Forzati, F., Federico, A., Pallante, P., Abbate, A., Esposito, F., Malapelle, U., Sepe, R., Palma, G., Troncone, G., Scarfo, M., et al. (2012). CBX7 is a tumor suppressor in mice and humans. *J Clin Invest* 122, 612-623.
- Frank-Kamenetsky, M., Zhang, X.M., Bottega, S., Guicherit, O., Wichterle, H., Dudek, H., Bumcrot, D., Wang, F.Y., Jones, S., Shulok, J., et al. (2002). Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothed agonists and antagonists. *Journal of biology* 1, 10.

- Fuller, J.F., McAdara, J., Yaron, Y., Sakaguchi, M., Fraser, J.K., and Gasson, J.C. (1999). Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. *Blood* 93, 3391-3400.
- Fuller, M.T., and Spradling, A.C. (2007). Male and female *Drosophila* germline stem cells: two versions of immortality. *Science* 316, 402-404.
- Galy, A., Travis, M., Cen, D., and Chen, B. (1995). Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* 3, 459-473.
- Gao, J., Graves, S., Koch, U., Liu, S., Jankovic, V., Buonamici, S., El Andaloussi, A., Nimer, S.D., Kee, B.L., Taichman, R., et al. (2009). Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell* 4, 548-558.
- Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. *Mol Cell* 45, 344-356.
- Geay, J.F., Buet, D., Zhang, Y., Foudi, A., Jarrier, P., Berthebaud, M., Turhan, A.G., Vainchenker, W., and Louache, F. (2005). p210BCR-ABL inhibits SDF-1 chemotactic response via alteration of CXCR4 signaling and down-regulation of CXCR4 expression. *Cancer Res* 65, 2676-2683.
- Gering, M., and Patient, R. (2005). Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell* 8, 389-400.
- Giebel, B., Zhang, T., Beckmann, J., Spanholtz, J., Wernet, P., Ho, A.D., and Punzel, M. (2006). Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. *Blood* 107, 2146-2152.
- Giles, F.J., Kantarjian, H.M., le Coutre, P.D., Baccarani, M., Mahon, F.X., Blakesley, R.E., Gallagher, N.J., Gillis, K., Goldberg, S.L., Larson, R.A., et al. (2012). Nilotinib is effective in imatinib-resistant or -intolerant patients with chronic myeloid leukemia in blastic phase. *Leukemia* 26, 959-962.
- Goardon, N., Marchi, E., Atzberger, A., Quek, L., Schuh, A., Soneji, S., Woll, P., Mead, A., Alford, K.A., Rout, R., et al. (2011). Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell* 19, 138-152.
- Goldman, D.C., Bailey, A.S., Pfaffle, D.L., Al Masri, A., Christian, J.L., and Fleming, W.H. (2009). BMP4 regulates the hematopoietic stem cell niche. *Blood* 114, 4393-4401.
- Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., et al. (1999). Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 286, 531-537.
- Goodell M.A., Brose K., Paradis G., Conner A.S., Mulligan R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183, 1797-1806.
- Gordon, M.Y., Lewis, J.L., and Marley, S.B. (2002). Of mice and men...and elephants. *Blood* 100, 4679-4680.
- Gordon, M.Y., Marley, S.B., Apperley, J.F., Marin, D., Kaeda, J., Szydlo, R., and Goldman, J.M. (2003). Clinical heterogeneity in chronic myeloid leukaemia reflecting biological diversity in normal persons. *British journal of haematology* 122, 424-429.

- Gordon, M.Y., Marley, S.B., Lewis, J.L., Davidson, R.J., Nguyen, D.X., Grand, F.H., Amos, T.A., and Goldman, J.M. (1998). Treatment with interferon-alpha preferentially reduces the capacity for amplification of granulocyte-macrophage progenitors (CFU-GM) from patients with chronic myeloid leukemia but spares normal CFU-GM. *J Clin Invest* 102, 710-715.
- Gorlin, R.J. (2004). Nevoid basal cell carcinoma (Gorlin) syndrome. *Genetics in medicine : official journal of the American College of Medical Genetics* 6, 530-539.
- Gottesman, M.M., Fojo, T., and Bates, S.E. (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2, 48-58.
- Gough, S.M., Slape, C.I., and Aplan, P.D. (2011). NUP98 gene fusions and hematopoietic malignancies: common themes and new biologic insights. *Blood* 118, 6247-6257.
- Goyama, S., and Kurokawa, M. (2010). Evi-1 as a critical regulator of leukemic cells. *Int J Hematol* 91, 753-757.
- Grachtchouk, M., Mo, R., Yu, S., Zhang, X., Sasaki, H., Hui, C.C., and Dlugosz, A.A. (2000). Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nat Genet* 24, 216-217.
- Graham, S.M., Jorgensen, H.G., Allan, E., Pearson, C., Alcorn, M.J., Richmond, L., and Holyoake, T.L. (2002). Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 99, 319-325.
- Graham, S.M., Vass, J.K., Holyoake, T.L., and Graham, G.J. (2007). Transcriptional analysis of quiescent and proliferating CD34+ human hemopoietic cells from normal and chronic myeloid leukemia sources. *Stem Cells* 25, 3111-3120.
- Grant, H., Jiang, X., Stebbing, J., Foroni, L., Craddock, C., Griffiths, M., Clark, R.E., O'Brien, S., Khorashad, J.S., Gerrard, G., et al. (2010). Analysis of BCR-ABL1 tyrosine kinase domain mutational spectra in primitive chronic myeloid leukemia cells suggests a unique mutator phenotype. *Leukemia* 24, 1817-1821.
- Gratwohl, A., Brand, R., Apperley, J., Crawley, C., Ruutu, T., Corradini, P., Carreras, E., Devergie, A., Guglielmi, C., Kolb, H.J., et al. (2006). Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT). *Haematologica* 91, 513-521.
- Greaves, M., and Maley, C.C. (2012). Clonal evolution in cancer. *Nature* 481, 306-313.
- Groffen, J., Stephenson, J.R., Heisterkamp, N., Bartram, C., de Klein, A., and Grosveld, G. (1984a). The human c-abl oncogene in the Philadelphia translocation. *Journal of cellular physiology Supplement* 3, 179-191.
- Groffen, J., Stephenson, J.R., Heisterkamp, N., de Klein, A., Bartram, C.R., and Grosveld, G. (1984b). Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 36, 93-99.
- Groschel, S., Lugthart, S., Schlenk, R.F., Valk, P.J., Eiwien, K., Goudswaard, C., van Putten, W.J., Kayser, S., Verdonck, L.F., Lubbert, M., et al. (2010). High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. *J Clin Oncol* 28, 2101-2107.

- Guan, Y., Gerhard, B., and Hogge, D.E. (2003). Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). *Blood* 101, 3142-3149.
- Gunsilius, E., Duba, H.C., Petzer, A.L., Kahler, C.M., Grunewald, K., Stockhammer, G., Gabl, C., Dirnhofer, S., Clausen, J., and Gastl, G. (2000). Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* 355, 1688-1691.
- Guo, C.-Y., Liu, J., Xu, L., Yang, W.-J., Wang, X.-P., Xu, X.-F., Yu, Y.-C., and Xia, Y.-J. (2009). Gli1 maintains cell survival by up-regulating IGFBP6 and Bcl-2 through promoter regions in parallel manner in pancreatic cancer cells, Vol 8.
- Guzman, M.L., Neering, S.J., Upchurch, D., Grimes, B., Howard, D.S., Rizzieri, D.A., Luger, S.M., and Jordan, C.T. (2001). Nuclear factor- κ B is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* 98, 2301-2307.
- Ha, K., Coulombe-Huntington, J., and Majewski, J. (2009). Comparison of Affymetrix Gene Array with the Exon Array shows potential application for detection of transcript isoform variation. *BMC Genomics* 10, 519.
- Haferlach, T., Winkemann, M., Nickenig, C., Meeder, M., Ramm-Petersen, L., Schoch, R., Nickelsen, M., Weber-Matthiesen, K., Schlegelberger, B., Schoch, C., et al. (1997). Which compartments are involved in Philadelphia-chromosome positive chronic myeloid leukaemia? An answer at the single cell level by combining May-Grunwald-Giemsa staining and fluorescence in situ hybridization techniques. *British journal of haematology* 97, 99-106.
- Hamilton, A., Helgason, G.V., Schemionek, M., Zhang, B., Myssina, S., Allan, E.K., Nicolini, F.E., Muller-Tidow, C., Bhatia, R., Brunton, V.G., et al. (2012). Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival. *Blood* 119, 1501-1510.
- Hanfstein, B., Muller, M.C., Hehlmann, R., Erben, P., Lauseker, M., Fabarius, A., Schnittger, S., Haferlach, C., Gohring, G., Proetel, U., et al. (2012). Early molecular and cytogenetic response is predictive for long-term progression-free and overall survival in chronic myeloid leukemia (CML). *Leukemia* 26, 2096-2102.
- Hanson, A.J., Wallace, H.A., Freeman, T.J., Beauchamp, R.D., Lee, L.A., and Lee, E. (2012). XIAP monoubiquitylates Groucho/TLE to promote canonical Wnt signaling. *Mol Cell* 45, 619-628.
- Hantschel, O., Nagar, B., Guettler, S., Kretzschmar, J., Dorey, K., Kuriyan, J., and Superti-Furga, G. (2003). A myristoyl/phosphotyrosine switch regulates c-Abl. *Cell* 112, 845-857.
- Hao, L., Johnsen, R., Lauter, G., Baillie, D., and Burglin, T.R. (2006). Comprehensive analysis of gene expression patterns of hedgehog-related genes. *BMC Genomics* 7, 280.
- Hao, Q.-L., Thiemann, F.T., Petersen, D., Smogorzewska, E.M., and Crooks, G.M. (1996). Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* 88, 3306-3313.
- Harris, L.G., Samant, R.S., and Shevde, L.A. (2011). Hedgehog signaling: networking to nurture a promalignant tumor microenvironment. *Mol Cancer Res* 9, 1165-1174.

- Harrison, D., and Astle, C. (1982). Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure. *Journal of Experimental Medicine* 156, 1767-1779.
- Heaney, N.B., Pellicano, F., Zhang, B., Crawford, L., Chu, S., Kazmi, S.M., Allan, E.K., Jorgensen, H.G., Irvine, A.E., Bhatia, R., et al. (2010). Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. *Blood* 115, 2241-2250.
- Hegedus, C., Ozvegy-Laczka, C., Apati, A., Magocsi, M., Nemet, K., Orfi, L., Keri, G., Katona, M., Takats, Z., Varadi, A., et al. (2009). Interaction of nilotinib, dasatinib and bosutinib with ABCB1 and ABCG2: implications for altered anti-cancer effects and pharmacological properties. *Br J Pharmacol* 158, 1153-1164.
- Heidel, F.H., Bullinger, L., Feng, Z., Wang, Z., Neff, T.A., Stein, L., Kalaitzidis, D., Lane, S.W., and Armstrong, S.A. (2012). Genetic and pharmacologic inhibition of beta-catenin targets imatinib-resistant leukemia stem cells in CML. *Cell Stem Cell* 10, 412-424.
- Helgason, G.V., Karvela, M., and Holyoake, T.L. (2011). Kill one bird with two stones: potential efficacy of BCR-ABL and autophagy inhibition in CML. *Blood* 118, 2035-2043.
- Heretsch, P., Tzagkaroulaki, L., and Giannis, A. (2010). Modulators of the hedgehog signaling pathway. *Bioorg Med Chem* 18, 6613-6624.
- Hesson, L.B., Dunwell, T.L., Cooper, W.N., Catchpoole, D., Brini, A.T., Chiaramonte, R., Griffiths, M., Chalmers, A.D., Maher, E.R., and Latif, F. (2009). The novel RASSF6 and RASSF10 candidate tumour suppressor genes are frequently epigenetically inactivated in childhood leukaemias. *Molecular cancer* 8, 42.
- Heuser, M., Berg, T., Kuchenbauer, F., Lai, C.K., Park, G., Fung, S., Lin, G., Leung, M., Krauter, J., Ganser, A., et al. (2012). Functional role of BAALC in leukemogenesis. *Leukemia* 26, 532-536.
- Hidalgo, I., Herrera-Merchan, A., Ligos, J.M., Carramolino, L., Nunez, J., Martinez, F., Dominguez, O., Torres, M., and Gonzalez, S. (2012). Ezh1 Is Required for Hematopoietic Stem Cell Maintenance and Prevents Senescence-like Cell Cycle Arrest. *Cell Stem Cell* 11, 649-662.
- Hiwase, D.K., Saunders, V., Hewett, D., Frede, A., Zrim, S., Dang, P., Eadie, L., To, L.B., Melo, J., Kumar, S., et al. (2008). Dasatinib cellular uptake and efflux in chronic myeloid leukemia cells: therapeutic implications. *Clin Cancer Res* 14, 3881-3888.
- Hochhaus, A. (2011). Educational session: managing chronic myeloid leukemia as a chronic disease. *Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program 2011*, 128-135.
- Hochhaus, A., Baccarani, M., Deininger, M., Apperley, J.F., Lipton, J.H., Goldberg, S.L., Corm, S., Shah, N.P., Cervantes, F., Silver, R.T., et al. (2008a). Dasatinib induces durable cytogenetic responses in patients with chronic myelogenous leukemia in chronic phase with resistance or intolerance to imatinib. *Leukemia* 22, 1200-1206.
- Hochhaus, A., Baccarani, M., Deininger, M., Apperley, J.F., Lipton, J.H., Goldberg, S.L., Corm, S., Shah, N.P., Cervantes, F., Silver, R.T., et al. (2008b). Dasatinib induces durable cytogenetic responses in patients with chronic myelogenous leukemia in chronic phase with resistance or intolerance to imatinib. *Leukemia* 22, 1200-1206.

- Hochhaus, A., Kreil, S., Corbin, A.S., La Rosee, P., Muller, M.C., Lahaye, T., Hanfstein, B., Schoch, C., Cross, N.C., Berger, U., et al. (2002). Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 16, 2190-2196.
- Hochhaus, A., O'Brien, S.G., Guilhot, F., Druker, B.J., Branford, S., Foroni, L., Goldman, J.M., Muller, M.C., Radich, J.P., Rudoltz, M., et al. (2009). Six-year follow-up of patients receiving imatinib for the first-line treatment of chronic myeloid leukemia. *Leukemia* 23, 1054-1061.
- Hochman, E., Kinston, S., Harmelin, A., Gottgens, B., and Izraeli, S. (2006). The SCL 3' enhancer responds to Hedgehog signaling during hemangioblast specification. *Exp Hematol* 34, 1643-1650.
- Hofmann, I., Stover, E.H., Cullen, D.E., Mao, J., Morgan, K.J., Lee, B.H., Kharas, M.G., Miller, P.G., Cornejo, M.G., Okabe, R., et al. (2009). Hedgehog Signaling Is Dispensable for Adult Murine Hematopoietic Stem Cell Function and Hematopoiesis. *Cell Stem Cell* 4, 559-567.
- Hogan, C.J., Shpall, E.J., and Keller, G. (2002). Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. *Proc Natl Acad Sci U S A* 99, 413-418.
- Hogge, D.E., Lansdorp, P.M., Reid, D., Gerhard, B., and Eaves, C.J. (1996). Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor. *Blood* 88, 3765-3773.
- Holtz, M.S., Slovak, M.L., Zhang, F., Sawyers, C.L., Forman, S.J., and Bhatia, R. (2002). Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. *Blood* 99, 3792-3800.
- Holyoake, T., Jiang, X., Eaves, C., and Eaves, A. (1999). Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. *Blood* 94, 2056-2064.
- Holyoake, T.L., Horrocks, C., Thomas, T., Eaves, C.J., and Eaves, A.C. (2000). Cell separation improves the sensitivity of detecting rare human normal and leukemic hematopoietic cells in vivo in NOD/SCID mice. *Cytotherapy* 2, 411-421.
- Holyoake, T.L., Jiang, X., Jorgensen, H.G., Graham, S., Alcorn, M.J., Laird, C., Eaves, A.C., and Eaves, C.J. (2001). Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth in vitro in association with up-regulation of expression of interleukin-3. *Blood* 97, 720-728.
- Hu, Y., Chen, Y., Douglas, L., and Li, S. (2008). [beta]-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia. *Leukemia* 23, 109-116.
- Huang, S., and Terstappen, L.W. (1994). Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. *Blood* 83, 1515-1526.
- Huang, X., Cortes, J., and Kantarjian, H. (2012). Estimations of the increasing prevalence and plateau prevalence of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. *Cancer* 118, 3123-3127.

- Huber, T.L., Kouskoff, V., Joerg Fehling, H., Palis, J., and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 432, 625-630.
- Hughes, T.P., Hochhaus, A., Branford, S., Muller, M.C., Kaeda, J.S., Foroni, L., Druker, B.J., Guilhot, F., Larson, R.A., O'Brien, S.G., et al. (2010). Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). *Blood* 116, 3758-3765.
- Hughes, T.P., Kaeda, J., Branford, S., Rudzki, Z., Hochhaus, A., Hensley, M.L., Gathmann, I., Bolton, A.E., van Hoomissen, I.C., Goldman, J.M., et al. (2003). Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med* 349, 1423-1432.
- Hui, C.C., and Angers, S. (2011). Gli proteins in development and disease. *Annual review of cell and developmental biology* 27, 513-537.
- Humke, E.W., Dorn, K.V., Milenkovic, L., Scott, M.P., and Rohatgi, R. (2010). The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. *Genes Dev* 24, 670-682.
- Humphries, R.K., Eaves, A.C., and Eaves, C.J. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. *Proc Natl Acad Sci U S A* 78, 3629-3633.
- Huntly, B.J.P., Shigematsu, H., Deguchi, K., Lee, B.H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I.R., et al. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer Cell* 6, 587-596.
- Iacobucci, I., Rosti, G., Amabile, M., Poerio, A., Soverini, S., Cilloni, D., Testoni, N., Abruzzese, E., Montefusco, E., Ottaviani, E., et al. (2006). Comparison between patients with Philadelphia-positive chronic phase chronic myeloid leukemia who obtained a complete cytogenetic response within 1 year of imatinib therapy and those who achieved such a response after 12 months of treatment. *J Clin Oncol* 24, 454-459.
- Imamura, T., Morimoto, A., Takanashi, M., Hibi, S., Sugimoto, T., Ishii, E., and Imashuku, S. (2002). Frequent co-expression of HoxA9 and Meis1 genes in infant acute lymphoblastic leukaemia with MLL rearrangement. *British journal of haematology* 119, 119-121.
- Incardona, J.P., Gaffield, W., Kapur, R.P., and Roelink, H. (1998). The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. *Development* 125, 3553-3562.
- Incardona, J.P., Gruenberg, J., and Roelink, H. (2002). Sonic hedgehog induces the segregation of patched and smoothed in endosomes. *Curr Biol* 12, 983-995.
- Ingham, P.W., and Placzek, M. (2006). Orchestrating ontogenesis: variations on a theme by sonic hedgehog. *Nat Rev Genet* 7, 841-850.
- Ioannou, Y.A. (2000). The structure and function of the Niemann-Pick C1 protein. *Mol Genet Metab* 71, 175-181.
- Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4, 249-264.

- Irvine, D.A., and Copland, M. (2012). Targeting hedgehog in hematologic malignancy. *Blood* 119, 2196-2204.
- Iscove, N.N., and Nawa, K. (1997). Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr Biol* 7, 805-808.
- Ishikawa F., Yoshida S., Saito Y., Hijikata A., Kitamura H., Tanaka S., Nakamura R., Tanaka T., Tomiyama H., Saito N., Fukata M., Miyamoto T., Lyons B., Ohshima K., Uchida N., Taniguchi S., Ohara O., Akashi K., Harada M., Shultz L.D. (2007). Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* 25 1315-21
- Ishizawa, J., Kuninaka, S., Sugihara, E., Naoe, H., Kobayashi, Y., Chiyoda, T., Ueki, A., Araki, K., Yamamura, K., Matsuzaki, Y., et al. (2011). The cell cycle regulator Cdh1 controls the pool sizes of hematopoietic stem cells and mature lineage progenitors by protecting from genotoxic stress. *Cancer Sci* 102, 967-974.
- Isohata, N., Aoyagi, K., Mabuchi, T., Daiko, H., Fukaya, M., Ohta, H., Ogawa, K., Yoshida, T., and Sasaki, H. (2009). Hedgehog and epithelial-mesenchymal transition signaling in normal and malignant epithelial cells of the esophagus. *Int J Cancer* 125, 1212-1221.
- Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., et al. (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* 431, 997-1002.
- Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., et al. (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* 12, 446-451.
- Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. *Science* 298, 601-604.
- Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Katoh-Fukui, Y., Koseki, H., et al. (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* 21, 843-851.
- Jabbour, E., and Kantarjian, H. (2012). Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *American journal of hematology* 87, 1037-1045.
- Jabbour, E., Kantarjian, H., Jones, D., Talpaz, M., Bekele, N., O'Brien, S., Zhou, X., Luthra, R., Garcia-Manero, G., Giles, F., et al. (2006). Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia* 20, 1767-1773.
- Jabbour, E., Kantarjian, H., O'Brien, S., Shan, J., Quintas-Cardama, A., Faderl, S., Garcia-Manero, G., Ravandi, F., Rios, M.B., and Cortes, J. (2011). The achievement of an early complete cytogenetic response is a major determinant for outcome in patients with early chronic phase chronic myeloid leukemia treated with tyrosine kinase inhibitors. *Blood* 118, 4541-4546; quiz 4759.
- Jabbour, E.J., Kantarjian, H., Eliasson, L., Cornelison, A.M., and Marin, D. (2012). Patient adherence to tyrosine kinase inhibitor therapy in chronic myeloid leukemia. *American journal of hematology* 87, 687-691.
- Jamieson, C.H., Ailles, L.E., Dylla, S.J., Muijtjens, M., Jones, C., and Zehnder, J.L. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* 351, 657-667.

- Janssen, J.J.W.M., Deenik, W., Smolders, K.G.M., van Kuijk, B.J., Pouwels, W., Kelder, A., Cornelissen, J.J., Schuurhuis, G.J., and Ossenkoppele, G.J. (2012). Residual normal stem cells can be detected in newly diagnosed chronic myeloid leukemia patients by a new flow cytometric approach and predict for optimal response to imatinib. *Leukemia* 26, 977-984.
- Jaras, M., Johnels, P., Hansen, N., Agerstam, H., Tzapogas, P., Rissler, M., Lassen, C., Olofsson, T., Bjerrum, O.W., Richter, J., et al. (2010). Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. *Proc Natl Acad Sci U S A* 107, 16280-16285.
- Jiang, X., Ng, E., Yip, C., Eisterer, W., Chalandon, Y., Stuible, M., Eaves, A., and Eaves, C.J. (2002). Primitive interleukin 3 null hematopoietic cells transduced with BCR-ABL show accelerated loss after culture of factor-independence in vitro and leukemogenic activity in vivo. *Blood* 100, 3731-3740.
- Jiang, X., Smith, C., Eaves, A., and Eaves, C. (2007a). The challenges of targeting chronic myeloid leukemia stem cells. *Clinical lymphoma & myeloma* 7 Suppl 2, S71-80.
- Jiang, X., Zhao, Y., Smith, C., Gasparetto, M., Turhan, A., Eaves, A., and Eaves, C. (2007b). Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. *Leukemia* 21, 926-935.
- Jin, L., Tabe, Y., Konoplev, S., Xu, Y., Leysath, C.E., Lu, H., Kimura, S., Ohsaka, A., Rios, M.B., Calvert, L., et al. (2008). CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. *Mol Cancer Ther* 7, 48-58.
- Jing, D., Wobus, M., Poitz, D.M., Bornhauser, M., Ehninger, G., and Ordemann, R. (2012). Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. *Haematologica* 97, 331-339.
- Jonas, D., Lubbert, M., Kawasaki, E., Henke, M., Bross, K., Mertelsmann, R., and Herrmann, F. (1992). Clonal analysis of bcr-abl rearrangement in T lymphocytes from patients with chronic myelogenous leukemia. *Blood* 79, 1017-1023.
- Jones, R.J., Gocke, C.D., Kasamon, Y.L., Miller, C.B., Perkins, B., Barber, J.P., Vala, M.S., Gerber, J.M., Gellert, L.L., Siedner, M., et al. (2009). Circulating clonotypic B cells in classic Hodgkin lymphoma. *Blood* 113, 5920-5926.
- Jordan, C.T., Guzman, M.L., and Noble, M. (2006). Cancer stem cells. *N Engl J Med* 355, 1253-1261.
- Jordan, C.T., Yamasaki, G., and Minamoto, D. (1996). High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. *Exp Hematol* 24, 1347-1355.
- Jordanides, N.E., Jorgensen, H.G., Holyoake, T.L., and Mountford, J.C. (2006). Functional ABCG2 is overexpressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. *Blood* 108, 1370-1373.
- Jorgensen, H.G., Allan, E.K., Jordanides, N.E., Mountford, J.C., and Holyoake, T.L. (2007). Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. *Blood* 109, 4016-4019.
- Jude, C.D., Climer, L., Xu, D., Artinger, E., Fisher, J.K., and Ernst, P. (2007). Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. *Cell Stem Cell* 1, 324-337.

- Kaelin, W.G., Jr. (2005). The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* 5, 689-698.
- Kajiume, T., Ninomiya, Y., Ishihara, H., Kanno, R., and Kanno, M. (2004). Polycomb group gene *mel-18* modulates the self-renewal activity and cell cycle status of hematopoietic stem cells. *Exp Hematol* 32, 571-578.
- Kamminga, L.M., Bystrykh, L.V., Boer, A.C., Houwer, S., Douma, J., Weersing, E., Dontje, B., and de Haan, G. (2006a). The polycomb group gene *Ezh2* prevents hematopoietic stem cell exhaustion. *Blood* 107, 2170-2179.
- Kamminga, L.M., Bystrykh, L.V., de Boer, A., Houwer, S., Douma, J., Weersing, E., Dontje, B., and de Haan, G. (2006b). The Polycomb group gene *Ezh2* prevents hematopoietic stem cell exhaustion. *Blood* 107, 2170-2179.
- Kantarjian, H., Cortes, J., Kim, D.W., Dorlhiac-Llacer, P., Pasquini, R., DiPersio, J., Muller, M.C., Radich, J.P., Khoury, H.J., Khoroshko, N., et al. (2009). Phase 3 study of dasatinib 140 mg once daily versus 70 mg twice daily in patients with chronic myeloid leukemia in accelerated phase resistant or intolerant to imatinib: 15-month median follow-up. *Blood* 113, 6322-6329.
- Kantarjian, H., O'Brien, S., Shan, J., Huang, X., Garcia-Manero, G., Faderl, S., Ravandi-Kashani, F., Verstovsek, S., Beth Rios, M., and Cortes, J. (2008). Cytogenetic and molecular responses and outcome in chronic myelogenous leukemia: need for new response definitions? *Cancer* 112, 837-845.
- Kantarjian, H.M., Giles, F., Gattermann, N., Bhalla, K., Alimena, G., Palandri, F., Ossenkoppele, G.J., Nicolini, F.E., O'Brien, S.G., Litzow, M., et al. (2007). Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood* 110, 3540-3546.
- Kantarjian, H.M., Hochhaus, A., Saglio, G., De Souza, C., Flinn, I.W., Stenke, L., Goh, Y.T., Rosti, G., Nakamae, H., Gallagher, N.J., et al. (2011). Nilotinib versus imatinib for the treatment of patients with newly diagnosed chronic phase, Philadelphia chromosome-positive, chronic myeloid leukaemia: 24-month minimum follow-up of the phase 3 randomised ENESTnd trial. *Lancet Oncol* 12, 841-851.
- Kantarjian, H.M., Shah, N.P., Cortes, J.E., Baccarani, M., Agarwal, M.B., Undurraga, M.S., Wang, J., Ipina, J.J., Kim, D.W., Ogura, M., et al. (2012). Dasatinib or imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: 2-year follow-up from a randomized phase 3 trial (DASISION). *Blood* 119, 1123-1129.
- Karanu, F.N., Murdoch, B., Gallacher, L., Wu, D.M., Koremoto, M., Sakano, S., and Bhatia, M. (2000). The notch ligand *jagged-1* represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* 192, 1365-1372.
- Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* 124, 407-421.
- Katoh, M. (2007). Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. *Stem Cell Rev* 3, 30-38.
- Katoh, Y., and Katoh, M. (2009). Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr Mol Med* 9, 873-886.

- Kawamoto H., Ikawa T., Masuda K., Wada H., Katsura Y. (2010). A map for lineage restriction of progenitors during hematopoiesis: the essence of the myeloid-based model. *Immunol. Rev.* 238, 23-38.
- Kazuhito, N., Teruyuki, M., Takayuki, H., and Atsushi, H. (2008). Regulation of Reactive Oxygen Species and Genomic Stability in Hematopoietic Stem Cells Antioxid Redox Signal 10, 1183-1894.
- Keating, A., Wang, X.H., and Laraya, P. (1994). Variable transcription of BCR-ABL by Ph+ cells arising from hematopoietic progenitors in chronic myeloid leukemia. *Blood* 83, 1744-1749.
- Kelly, P.N., Dakic, A., Adams, J.M., Nutt, S.L., and Strasser, A. (2007). Tumor growth need not be driven by rare cancer stem cells. *Science* 317, 337.
- Kenney, A.M., Cole, M.D., and Rowitch, D.H. (2003). Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development* 130, 15-28.
- Kenney, A.M., and Rowitch, D.H. (2000). Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol Cell Biol* 20, 9055-9067.
- Kharas, M.G., Yusuf, I., Scarfone, V.M., Yang, V.W., Segre, J.A., Huettnner, C.S., and Fruman, D.A. (2007). KLF4 suppresses transformation of pre-B cells by ABL oncogenes. *Blood* 109, 747-755.
- Khorashad, J.S., de Lavallade, H., Apperley, J.F., Milojkovic, D., Reid, A.G., Bua, M., Szydlo, R., Olavarria, E., Kaeda, J., Goldman, J.M., et al. (2008). Finding of kinase domain mutations in patients with chronic phase chronic myeloid leukemia responding to imatinib may identify those at high risk of disease progression. *J Clin Oncol* 26, 4806-4813.
- Kiel, M.J., He, S., Ashkenazi, R., Gentry, S.N., Teta, M., Kushner, J.A., Jackson, T.L., and Morrison, S.J. (2007). Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature* 449, 238-242.
- Kiel, M.J., and Morrison, S.J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol* 8, 290-301.
- Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109-1121.
- Kikushige, Y., Yoshimoto, G., Miyamoto, T., Iino, T., Mori, Y., Iwasaki, H., Niino, H., Takenaka, K., Nagafuji, K., Harada, M., et al. (2008). Human Flt3 Is Expressed at the Hematopoietic Stem Cell and the Granulocyte/Macrophage Progenitor Stages to Maintain Cell Survival. *J Immunol* 180, 7358-7367.
- Kim, J., Kato, M., and Beachy, P.A. (2009). Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus. *Proc Natl Acad Sci U S A* 106, 21666-21671.
- Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol* 7, 1048-1056.

- Kise, Y., Morinaka, A., Teglund, S., and Miki, H. (2009). Sufu recruits GSK3beta for efficient processing of Gli3. *Biochem Biophys Res Commun* 387, 569-574.
- Knoblich, J.A. (2008). Mechanisms of asymmetric stem cell division. *Cell* 132, 583-597.
- Kobune, M. (2004). Indian hedgehog gene transfer augments hematopoietic support of human stromal cells including NOD/SCID-[beta]2m-/- repopulating cells. *Blood* 104, 1002-1009.
- Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H.R., and Radtke, F. (2008). Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood* 111, 160-164.
- Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661-672.
- Konig, H., Copland, M., Chu, S., Jove, R., Holyoake, T.L., and Bhatia, R. (2008a). Effects of dasatinib on SRC kinase activity and downstream intracellular signaling in primitive chronic myelogenous leukemia hematopoietic cells. *Cancer Res* 68, 9624-9633.
- Konig, H., Holtz, M., Modi, H., Manley, P., Holyoake, T.L., Forman, S.J., and Bhatia, R. (2008b). Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. *Leukemia* 22, 748-755.
- Koo, S., Huntly, B.J., Wang, Y., Chen, J., Brumme, K., Ball, B., McKinney-Freeman, S.L., Yabuuchi, A., Scholl, C., Bansal, D., et al. (2010). Cdx4 is dispensable for murine adult hematopoietic stem cells but promotes MLL-AF9-mediated leukemogenesis. *Haematologica* 95, 1642-1650.
- Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., and van Oers, M.H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84, 1415-1420.
- Koptyra, M., Falinski, R., Nowicki, M.O., Stoklosa, T., Majsterek, I., Nieborowska-Skorska, M., Blasiak, J., and Skorski, T. (2006). BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. *Blood* 108, 319-327.
- Koschmieder, S., Gottgens, B., Zhang, P., Iwasaki-Arai, J., Akashi, K., Kutok, J.L., Dayaram, T., Geary, K., Green, A.R., Tenen, D.G., et al. (2005). Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. *Blood* 105, 324-334.
- Koschmieder, S., and Schemionek, M. (2011). Mouse models as tools to understand and study BCR-ABL1 diseases. *Am J of Blood Res* 1, 65-75.
- Krause, D., Fackler, M., Civin, C., and May, W. (1996). CD34: structure, biology, and clinical utility [see comments]. *Blood* 87, 1-13.
- Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* 442, 818-822.
- Kronenwett, R., Butterweck, U., Steidl, U., Kliszewski, S., Neumann, F., Bork, S., Blanco, E.D., Roes, N., Graf, T., Brors, B., et al. (2005). Distinct molecular phenotype of malignant CD34(+) hematopoietic stem and progenitor cells in chronic myelogenous leukemia. *Oncogene* 24, 5313-5324.

- Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T., et al. (2003). Notch1 but Not Notch2 Is Essential for Generating Hematopoietic Stem Cells from Endothelial Cells. *Immunity* 18, 699-711.
- Kumar, A.R., Hudson, W.A., Chen, W., Nishiuchi, R., Yao, Q., and Kersey, J.H. (2004). Hoxa9 influences the phenotype but not the incidence of MLL-AF9 fusion gene leukemia. *Blood* 103, 1823-1828.
- Kumari, A., Brendel, C., Hochhaus, A., Neubauer, A., and Burchert, A. (2012). Low BCR-ABL expression levels in hematopoietic precursor cells enable persistence of chronic myeloid leukemia under imatinib. *Blood* 119, 530-539.
- Landrette, S.F., Kuo, Y.-H., Hensen, K., van Waalwijk van Doorn-Khosrovani, S.B., Perrat, P.N., Van de Ven, W.J.M., Delwel, R., and Castilla, L.H. (2005). Plag1 and Plagl2 are oncogenes that induce acute myeloid leukemia in cooperation with Cbfb-MYH11. *Blood* 105, 2900-2907.
- Lapidot, T., Pflumio, F., Doedens, M., Murdoch, B., Williams, D.E., and Dick, J.E. (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* 255, 1137-1141.
- Lapidot T., Sirard C., Vormoor J., Murdoch B., Hoang T., Caceres-Cortes J., Minden M., Paterson B., Caligiuri M.A., Dick J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367, 645-8
- Lau, C.I., Outram, S.V., Saldana, J.I., Furmanski, A.L., Dessens, J.T., and Crompton, T. (2012). Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. *Blood* 119, 4741-4751.
- Lauth, M., and Toftgard, R. (2007). Non-canonical activation of GLI transcription factors: implications for targeted anti-cancer therapy. *Cell Cycle* 6, 2458-2463.
- Lawrence, H.J., Christensen, J., Fong, S., Hu, Y.L., Weissman, I., Sauvageau, G., Humphries, R.K., and Largman, C. (2005). Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. *Blood* 106, 3988-3994.
- Lawrence, H.J., Sauvageau, G., Ahmadi, N., Lopez, A.R., LeBeau, M.M., Link, M., Humphries, K., and Largman, C. (1995). Stage- and lineage-specific expression of the HOXA10 homeobox gene in normal and leukemic hematopoietic cells. *Exp Hematol* 23, 1160-1166.
- le Coutre, P., Ottmann, O.G., Giles, F., Kim, D.W., Cortes, J., Gattermann, N., Apperley, J.F., Larson, R.A., Abruzzese, E., O'Brien, S.G., et al. (2008). Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is active in patients with imatinib-resistant or -intolerant accelerated-phase chronic myelogenous leukemia. *Blood* 111, 1834-1839.
- le Viseur, C., Hotfilder, M., Bomken, S., Wilson, K., Röttgers, S., Schrauder, A., Rosemann, A., Irving, J., Stam, R.W., Shultz, L.D., et al. (2008). In Childhood Acute Lymphoblastic Leukemia, Blasts at Different Stages of Immunophenotypic Maturation Have Stem Cell Properties. *Cancer Cell* 14, 47-58.
- Leary, A., Strauss, L., Civin, C., and Ogawa, M. (1985). Disparate differentiation in hemopoietic colonies derived from human paired progenitors. *Blood* 66, 327-332.

- Leary, A.G., and Ogawa, M. (1987). Blast cell colony assay for umbilical cord blood and adult bone marrow progenitors. *Blood* 69, 953-956.
- Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., and Beachy, P.A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* 266, 1528-1537.
- Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125, 301-313.
- Lefebvre, V. (2010). The SoxD transcription factors--Sox5, Sox6, and Sox13--are key cell fate modulators. *Int J Biochem Cell Biol* 42, 429-432.
- Lengerke, C., McKinney-Freeman, S., Naveiras, O., Yates, F., Wang, Y., Bansal, D., and Daley, G.Q. (2007). The cdx-hox pathway in hematopoietic stem cell formation from embryonic stem cells. *Annals of the New York Academy of Sciences* 1106, 197-208.
- Lengerke, C., Schmitt, S., Bowman, T.V., Jang, I.H., Maouche-Chretien, L., McKinney-Freeman, S., Davidson, A.J., Hammerschmidt, M., Rentzsch, F., Green, J.B., et al. (2008). BMP and Wnt specify hematopoietic fate by activation of the Cdx-Hox pathway. *Cell Stem Cell* 2, 72-82.
- Lessard, J., Baban, S., and Sauvageau, G. (1998). Stage-specific expression of polycomb group genes in human bone marrow cells. *Blood* 91, 1216-1224.
- Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukemic stem cells. *Nature* 423, 455-460.
- Lewis, E.B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* 276, 565-570.
- Lewis, I.D., McDiarmid, L.A., Samels, L.M., To, L.B., and Hughes, T.P. (1998). Establishment of a reproducible model of chronic-phase chronic myeloid leukemia in NOD/SCID mice using blood-derived mononuclear or CD34+ cells. *Blood* 91, 630-640.
- Li, X., Deng, W., Lobo-Ruppert, S.M., and Ruppert, J.M. (2007a). Gli1 acts through Snail and E-cadherin to promote nuclear signaling by [beta]-catenin. *Oncogene* 26, 4489-4498.
- Li, X., Ma, Q., Xu, Q., Liu, H., Lei, J., Duan, W., Bhat, K., Wang, F., Wu, E., and Wang, Z. (2012a). SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial-mesenchymal transition in vitro through non-canonical activation of Hedgehog pathway. *Cancer Lett* 322, 169-176.
- Li, Y., Clough, N., Sun, X., Yu, W., Abbott, B.L., Hogan, C.J., and Dai, Z. (2007b). Bcr-Abl induces abnormal cytoskeleton remodeling, beta1 integrin clustering and increased cell adhesion to fibronectin through the Abl interactor 1 pathway. *J Cell Sci* 120, 1436-1446.
- Li, Y., Hibbs, M.A., Gard, A.L., Shylo, N.A., and Yun, K. (2012b). Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. *Stem Cells* 30, 741-752.
- Lichtman, M.A., Beutler, E., Kipps, T.j., Seligsohn, U., Kaushansky, K., and Prchal, J. (2006). *Williams Hematology* (7th Edition), 7 edn.
- Lin, H., and Schagat, T. (1997). Neuroblasts: a model for the asymmetric division of stem cells. *Trends Genet* 13, 33-39.

- Lipshutz, R.J., Fodor, S.P., Gingeras, T.R., and Lockhart, D.J. (1999). High density synthetic oligonucleotide arrays. *Nat Genet* 21, 20-24.
- Litovchick, L., Sadasivam, S., Florens, L., Zhu, X., Swanson, S.K., Velmurugan, S., Chen, R., Washburn, M.P., Liu, X.S., and DeCaprio, J.A. (2007). Evolutionarily Conserved Multisubunit RBL2/p130 and E2F4 Protein Complex Represses Human Cell Cycle-Dependent Genes in Quiescence. *Molecular cell* 26, 539-551.
- Liu, S., Dontu, G., Mantle, I.D., Patel, S., Ahn, N.S., Jackson, K.W., Suri, P., and Wicha, M.S. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* 66, 6063-6071.
- Liu, X.L., Yuan, J.Y., Zhang, J.W., Zhang, X.H., and Wang, R.X. (2007). Differential gene expression in human hematopoietic stem cells specified toward erythroid, megakaryocytic, and granulocytic lineage. *Journal of leukocyte biology* 82, 986-1002.
- Long, B., Zhu, H., Zhu, C., Liu, T., and Meng, W. (2011). Activation of the Hedgehog pathway in chronic myelogenous leukemia patients. *Journal of experimental & clinical cancer research : CR* 30, 8.
- Lorenz, E., Uphoff, D., Reid, T.R., and Shelton, E. (1951). Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* 12, 197-201.
- LoRusso, P.M., Rudin, C.M., Reddy, J.C., Tibes, R., Weiss, G.J., Borad, M.J., Hann, C.L., Brahmer, J.R., Chang, I., Darbonne, W.C., et al. (2011). Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. *Clin Cancer Res* 17, 2502-2511.
- Lozzio, B.B., and Lozzio, C.B. (1977). Properties of the K562 cell line derived from a patient with chronic myeloid leukemia. *Int J Cancer* 19, 136.
- Lozzio, C.B., and Lozzio, B.B. (1975). Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 45, 321-334.
- Lu, L., Xiao, M., Clapp, D.W., Li, Z.H., and Broxmeyer, H.E. (1993a). High efficiency retroviral mediated gene transduction into single isolated immature and replatable CD34(3+) hematopoietic stem/progenitor cells from human umbilical cord blood. *J Exp Med* 178, 2089-2096.
- Lu, L., Xiao, M., Shen, R.N., Grigsby, S., and Broxmeyer, H.E. (1993b). Enrichment, characterization, and responsiveness of single primitive CD34 human umbilical cord blood hematopoietic progenitors with high proliferative and replating potential. *Blood* 81, 41-48.
- Lucas, C.M., Wang, L., Austin, G.M., Knight, K., Watmough, S.J., Shwe, K.H., Dasgupta, R., Butt, N.M., Galvani, D., Hoyle, C.F., et al. (2008). A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia* 22, 1963-1966.
- Lugo, T.G., Pendergast, A.M., Muller, A.J., and Witte, O.N. (1990). Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 247, 1079-1082.
- Luis, T.C., Ichii, M., Brugman, M.H., Kincade, P., and Staal, F.J.T. (2012). Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia* 26, 414-421.

- Luis, T.C., Weerkamp, F., Naber, B.A., Baert, M.R., de Haas, E.F., Nikolic, T., Heuvelmans, S., De Krijger, R.R., van Dongen, J.J., and Staal, F.J. (2009). Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood* 113, 546-554.
- Lund, A.H., and van Lohuizen, M. (2004). Polycomb complexes and silencing mechanisms. *Curr Opin Cell Biol* 16, 239-246.
- Lustig, B., and Behrens, J. (2003). The Wnt signaling pathway and its role in tumor development. *J Cancer Res Clin Oncol* 129, 199-221.
- Lyons, A.B., and Parish, C.R. (1994). Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 171, 131-137.
- Macdonald, B.T., Semenov, M.V., and He, X. (2007). SnapShot: Wnt/beta-catenin signaling. *Cell* 131, 1204.
- Maeda, O., Kondo, M., Fujita, T., Usami, N., Fukui, T., Shimokata, K., Ando, T., Goto, H., and Sekido, Y. (2006). Enhancement of GLI1-transcriptional activity by beta-catenin in human cancer cells. *Oncol Rep* 16, 91-96.
- Magee, J.A., Piskounova, E., and Morrison, S.J. (2012). Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* 21, 283-296.
- Mahon, F.-X., Rea, D., Guilhot, J., Guilhot, F., Huguet, F., Nicolini, F.E., Legros, L., Charbonnier, A., Guerci, A., Varet, B.R., et al. (2011). Discontinuation of Imatinib in Patients with Chronic Myeloid Leukemia Who Have Maintained Complete Molecular Response: Update Results of the STIM Study. *Blood (ASH Annual Meeting Abstracts)* 118, 603-.
- Mahon, F.X., Belloc, F., Lagarde, V., Chollet, C., Moreau-Gaudry, F., Reiffers, J., Goldman, J.M., and Melo, J.V. (2003). MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. *Blood* 101, 2368-2373.
- Mahon, F.X., Hayette, S., Lagarde, V., Belloc, F., Turcq, B., Nicolini, F., Belanger, C., Manley, P.W., Leroy, C., Etienne, G., et al. (2008). Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression. *Cancer Res* 68, 9809-9816.
- Mahon, F.X., Rea, D., Guilhot, J., Guilhot, F., Huguet, F., and Nicolini, F. (2010). Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* 11, 1029-1035.
- Majeti, R., Park, C.Y., and Weissman, I.L. (2007). Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* 1, 635-645.
- Majewski, I.J., Blewitt, M.E., de Graaf, C.A., McManus, E.J., Bahlo, M., Hilton, A.A., Hyland, C.D., Smyth, G.K., Corbin, J.E., Metcalf, D., et al. (2008). Polycomb repressive complex 2 (PRC2) restricts hematopoietic stem cell activity. *Plos Biol* 6, e93.
- Majewski, I.J., Ritchie, M.E., Phipson, B., Corbin, J., Pakusch, M., Ebert, A., Buslinger, M., Koseki, H., Hu, Y., Smyth, G.K., et al. (2010). Opposing roles of polycomb repressive complexes in hematopoietic stem and progenitor cells. *Blood* 116, 731-739.
- Malhotra, S., and Kincade, P.W. (2009). Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. *Cell Stem Cell* 4, 27-36.

- Mancini, S.J., Mantei, N., Dumortier, A., Suter, U., MacDonald, H.R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* 105, 2340-2342.
- Manley, P.W., Druce, P., Fendrich, G., Furet, P., Liebetanz, J., Martiny-Baron, G., Mestan, J., Trappe, J., Wartmann, M., and Fabbro, D. (2010). Extended kinase profile and properties of the protein kinase inhibitor nilotinib. *Biochim Biophys Acta* 1804, 445-453.
- Manz, M.G., Miyamoto, T., Akashi, K., and Weissman, I.L. (2002). Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci U S A* 99, 11872-11877.
- Marigo, V., and Tabin, C.J. (1996). Regulation of patched by sonic hedgehog in the developing neural tube. *Proc Natl Acad Sci U S A* 93, 9346-9351.
- Marin, D., Bazeos, A., Mahon, F.-X., Eliasson, L., Milojkovic, D., Bua, M., Apperley, J.F., Szydlo, R., Desai, R., Kozlowski, K., et al. (2010). Adherence Is the Critical Factor for Achieving Molecular Responses in Patients With Chronic Myeloid Leukemia Who Achieve Complete Cytogenetic Responses on Imatinib. *Journal of Clinical Oncology* 28, 2381-2388.
- Marin, D., Ibrahim, A.R., Lucas, C., Gerrard, G., Wang, L., Szydlo, R.M., Clark, R.E., Apperley, J.F., Milojkovic, D., Bua, M., et al. (2012). Assessment of BCR-ABL1 transcript levels at 3 months is the only requirement for predicting outcome for patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors. *J Clin Oncol* 30, 232-238.
- Marley, S.B., and Gordon, M.Y. (2005). Chronic myeloid leukaemia: stem cell derived but progenitor cell driven. *Clin Sci (Lond)* 109, 13-25.
- Marley, S.B., Lewis, J.L., and Gordon, M.Y. (2003). Progenitor cells divide symmetrically to generate new colony-forming cells and clonal heterogeneity. *British journal of haematology* 121, 643-648.
- Marley, S.B., Lewis, J.L., Zheng, B., Davidson, R.J., Davis, J.G., McDonald, C., Alenzi, F.Q., Goldman, J.M., and Gordon, M.Y. (2001). Peripheral blood progenitor cell mobilisation alters myeloid, but not erythroid, progenitor cell self-renewal kinetics. *Bone Marrow Transplant* 27, 241-248.
- Marschalek, R. (2011). Mechanisms of leukemogenesis by MLL fusion proteins. *British journal of haematology* 152, 141-154.
- Martin-Perez, D., Piris, M.A., and Sanchez-Beato, M. (2010). Polycomb proteins in hematologic malignancies. *Blood* 116, 5465-5475.
- Martin, P.J., Najfeld, V., Hansen, J.A., Penfold, G.K., Jacobson, R.J., and Fialkow, P.J. (1980). Involvement of the B-lymphoid system in chronic myelogenous leukaemia. *Nature* 287, 49-50.
- Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182, 1545-1556.
- Maru, Y. (2012). Molecular biology of chronic myeloid leukemia. *Cancer Sci* 103, 1601-1610.

- Maru, Y., and Witte, O.N. (1991). The BCR gene encodes a novel serine/threonine kinase activity within a single exon. *Cell* 67, 459-468.
- Massague, J. (2004). G1 cell-cycle control and cancer. *Nature* 432, 298-306.
- Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K., and Nakayama, Keiichi I. (2011). p57 Is Required for Quiescence and Maintenance of Adult Hematopoietic Stem Cells. *Cell Stem Cell* 9, 262-271.
- Matsuzaki, Y., Kinjo, K., Mulligan, R.C., and Okano, H. (2004). Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* 20, 87-93.
- Maximow, A. (1909). The Lymphocyte as a stem cell common to different blood elements in embryonic development and during the post-fetal life of mammals. *Folia Haematologica* 8, 125-134.
- Mazurier, F., Gan, O., McKenzie, J., Doedens, M., and Dick, J. (2004). Lentivector-mediated clonal tracking reveals intrinsic heterogeneity in the human hematopoietic stem cell compartment and culture-induced stem cell impairment. *Blood* 103, 545-552.
- McCune, J., Namikawa, R., Kaneshima, H., Shultz, L., Lieberman, M., and Weissman, I. (1988). The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science* 241, 1632-1639.
- McDermott, S.P., Eppert, K., Lechman, E.R., Doedens, M., and Dick, J.E. (2010). Comparison of human cord blood engraftment between immunocompromised mouse strains. *Blood* 116, 193-200.
- McKenzie, J.L., Gan, O.I., Doedens, M., Wang, J.C.Y., and Dick, J.E. (2006). Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. *Nat Immunol* 7, 1225-1233.
- McLaughlin, J., Chianese, E., and Witte, O.N. (1987). In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. *Proc Natl Acad Sci U S A* 84, 6558-6562.
- McMahon, K.A., Hiew, S.Y., Hadjur, S., Veiga-Fernandes, H., Menzel, U., Price, A.J., Kioussis, D., Williams, O., and Brady, H.J. (2007). Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. *Cell Stem Cell* 1, 338-345.
- Medvinsky, A., and Dzierzak, E. (1996). Definitive Hematopoiesis Is Autonomously Initiated by the AGM Region. *Cell* 86, 897-906.
- Medyouf, H., Gusscott, S., Wang, H., Tseng, J.-C., Wai, C., Nemirovsky, O., Trumpp, A., Pflumio, F., Carboni, J., Gottardis, M., et al. (2011). High-level IGF1R expression is required for leukemia-initiating cell activity in T-ALL and is supported by Notch signaling. *The Journal of Experimental Medicine* 208, 1809-1822.
- Melo, J. (1996). The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype [editorial; comment]. *Blood* 88, 2375-2384.
- Mendez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 452, 442-447.
- Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829-834.

- Merchant, A., Joseph, G., Wang, Q., Brennan, S., and Matsui, W. (2010). Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors. *Blood* 115, 2391-2396.
- Merkerova, M., Bruchova, H., Kracmarova, A., Klamova, H., and Brdicka, R. (2007). Bmi-1 over-expression plays a secondary role in chronic myeloid leukemia transformation. *Leukemia & lymphoma* 48, 793-801.
- Michael, L.E., Westerman, B.A., Ermilov, A.N., Wang, A., Ferris, J., Liu, J., Blom, M., Ellison, D.W., Lohuizen, M.v., and Dlugosz, A. (2008). Bmi1 is required for Hedgehog-pathway driven medulloblastoma expansion. *Neoplasia* 10, 1343-1349.
- Michallet, M., Philip, T., Philip, I., Godinot, H., Sebban, C., Salles, G., Thiebaut, A., Biron, P., Lopez, F., Mazars, P., et al. (2000). Transplantation with selected autologous peripheral blood CD34+Thy1+ hematopoietic stem cells (HSCs) in multiple myeloma: impact of HSC dose on engraftment, safety, and immune reconstitution. *Exp Hematol* 28, 858-870.
- Miller-Moslin, K., Peukert, S., Jain, R.K., McEwan, M.A., Karki, R., Llamas, L., Yusuff, N., He, F., Li, Y., Sun, Y., et al. (2009). 1-amino-4-benzylphthalazines as orally bioavailable smoothed antagonists with antitumor activity. *J Med Chem* 52, 3954-3968.
- Millour, J., de Olano, N., Horimoto, Y., Monteiro, L.J., Langer, J.K., Aligue, R., Hajji, N., and Lam, E.W. (2011). ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. *Mol Cancer Ther* 10, 1046-1058.
- Milner, L.A., and Bigas, A. (1999). Notch as a Mediator of Cell Fate Determination in Hematopoiesis: Evidence and Speculation. *Blood* 93, 2431-2448.
- Minakuchi, M., Kakazu, N., Gorrin-Rivas, M.J., Abe, T., Copeland, T.D., Ueda, K., and Adachi, Y. (2001). Identification and characterization of SEB, a novel protein that binds to the acute undifferentiated leukemia-associated protein SET. *Euro J Biochem* 268, 1340-1351.
- Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., et al. (2007). Foxo3a Is Essential for Maintenance of the Hematopoietic Stem Cell Pool. *Cell Stem Cell* 1, 101-112.
- Miyamoto, T., Weissman, I.L., and Akashi, K. (2000). AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci U S A* 97, 7521-7526.
- Miyazono, K. (2012). Tumour promoting functions of TGF-beta in CML-initiating cells. *J Biochem* 152, 383-385.
- Mochizuki-Kashio, M., Mishima, Y., Miyagi, S., Negishi, M., Saraya, A., Konuma, T., Shinga, J., Koseki, H., and Iwama, A. (2011). Dependency on the polycomb gene Ezh2 distinguishes fetal from adult hematopoietic stem cells. *Blood* 118, 6553-6561.
- Modi, H., McDonald, T., Chu, S., Yee, J.K., Forman, S.J., and Bhatia, R. (2007). Role of BCR/ABL gene-expression levels in determining the phenotype and imatinib sensitivity of transformed human hematopoietic cells. *Blood* 109, 5411-5421.
- Mohr, O.L. (1919). Character Changes Caused by Mutation of an Entire Region of a Chromosome in *Drosophila*. *Genetics* 4, 275-282.

- Mohty, M., Yong, A.S., Szydlo, R.M., Apperley, J.F., and Melo, J.V. (2007). The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. *Blood* 110, 380-383.
- Morey, L., and Helin, K. (2010). Polycomb group protein-mediated repression of transcription. *Trends Biochem Sci* 35, 323-332.
- Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of b-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 275, 1787-1790.
- Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997a). Identification of a lineage of multipotent hematopoietic progenitors. *Development* 124, 1929-1939.
- Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1, 661-673.
- Morrison, S.J., Wright, D.E., and Weissman, I.L. (1997b). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci U S A* 94, 1908-1913.
- Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature methods* 5, 621-628.
- Mullor, J.L., Dahmane, N., Sun, T., and Ruiz i Altaba, A. (2001). Wnt signals are targets and mediators of Gli function. *Curr Biol* 11, 769-773.
- Murray, L., Chen, B., Galy, A., Chen, S., Tushinski, R., Uchida, N., Negrin, R., Tricot, G., Jagannath, S., and Vesole, D. (1995). Enrichment of human hematopoietic stem cell activity in the CD34+Thy- 1+Lin- subpopulation from mobilized peripheral blood. *Blood* 85, 368-378.
- Nagao, R., Ashihara, E., Kimura, S., Strovel, J.W., Yao, H., Takeuchi, M., Tanaka, R., Hayashi, Y., Hirai, H., Padia, J., et al. (2011). Growth inhibition of imatinib-resistant CML cells with the T315I mutation and hypoxia-adaptation by AV65--a novel Wnt/beta-catenin signaling inhibitor. *Cancer Lett* 312, 91-100.
- Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* 112, 859-871.
- Naka, K., Hoshii, T., and Hirao, A. (2010a). Novel therapeutic approach to eradicate tyrosine kinase inhibitor resistant chronic myeloid leukemia stem cells. *Cancer Sci* 101, 1577-1581.
- Naka, K., Hoshii, T., Muraguchi, T., Tadokoro, Y., Ooshio, T., Kondo, Y., Nakao, S., Motoyama, N., and Hirao, A. (2010b). TGF-[bgr]-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 463, 676-680.
- Nemeth, M.J., and Bodine, D.M. (2007). Regulation of hematopoiesis and the hematopoietic stem cell niche by Wnt signaling pathways. *Cell Res* 17, 746-758.
- Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B.W., Liu, S., Mao, H., Chang, J.S., Galiotta, A., Uttam, A., et al. (2005). The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell* 8, 355-368.

- Nilsson, L., Åstrand-Grundström, I., Arvidsson, I., Jacobsson, B., Hellström-Lindberg, E., Hast, R., and Jacobsen, S.E.W. (2000). Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. *Blood* 96, 2012-2021.
- Nilsson, S.K., Johnston, H.M., and Coverdale, J.A. (2001). Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* 97, 2293-2299.
- Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertonecello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* 106, 1232-1239.
- Nitta, M., Kato, Y., Strife, A., Wachter, M., Fried, J., Perez, A., Jhanwar, S., Duigou-Osterndorf, R., Chaganti, R., and Clarkson, B. (1985). Incidence of involvement of the B and T lymphocyte lineages in chronic myelogenous leukemia. *Blood* 66, 1053-1061.
- Nolan-Stevaux, O., Lau, J., Truitt, M.L., Chu, G.C., Hebrok, M., Fernandez-Zapico, M.E., and Hanahan, D. (2009). GLI1 is regulated through Smoothed-independent mechanisms in neoplastic pancreatic ducts and mediates PDAC cell survival and transformation. *Genes Dev* 23, 24-36.
- Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J.E. (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333, 218-221.
- Novershtern, N., Subramanian, A., Lawton, L.N., Mak, R.H., Haining, W.N., McConkey, M.E., Habib, N., Yosef, N., Chang, C.Y., Shay, T., et al. (2011). Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell* 144, 296-309.
- Nowell, P.C., and Hungerford, D.A. (1960). A minute chromosome in chronic granulocytic leukemia. *Science* 132, 1497.
- Nowicki, M.O., Falinski, R., Koptyra, M., Slupianek, A., Stoklosa, T., and Gloc, E. (2004). BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. *Blood* 104, 3746-3753.
- Nowicki, M.O., Pawlowski, P., Fischer, T., Hess, G., Pawlowski, T., and Skorski, T. (2003). Chronic myelogenous leukemia molecular signature. *Oncogene* 22, 3952-3963.
- Nunoda, K., Tauchi, T., Takaku, T., Okabe, S., Akahane, D., Sashida, G., Ohyashiki, J.H., and Ohyashiki, K. (2007). Identification and functional signature of genes regulated by structurally different ABL kinase inhibitors. *Oncogene* 26, 4179-4188.
- Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287, 795-801.
- O'Brien, C.A., Kreso, A., and Jamieson, C.H. (2010). Cancer stem cells and self-renewal. *Clin Cancer Res* 16, 3113-3120.
- O'Brien, S.G., Guilhot, F., Larson, R.A., Gathmann, I., Baccarani, M., Cervantes, F., Cornelissen, J.J., Fischer, T., Hochhaus, A., Hughes, T., et al. (2003). Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 348, 994-1004.

- O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene *Ezh2* is required for early mouse development. *Mol Cell Biol* 21, 4330-4336.
- O'Hare, T., Eide, C.A., and Deininger, M.W. (2007). Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood* 110, 2242-2249.
- O'Hare, T., Shakespeare, W.C., Zhu, X., Eide, C.A., Rivera, V.M., Wang, F., Adrian, L.T., Zhou, T., Huang, W.S., Xu, Q., et al. (2009). AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. *Cancer Cell* 16, 401-412.
- O'Hare, T., Walters, D.K., Stoffregen, E.P., Jia, T., Manley, P.W., Mestan, J., Cowan-Jacob, S.W., Lee, F.Y., Heinrich, M.C., Deininger, M.W., et al. (2005). In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* 65, 4500-4505.
- O'Hare, T., Zabriskie, M.S., Eiring, A.M., and Deininger, M.W. (2012). Pushing the limits of targeted therapy in chronic myeloid leukaemia. *Nat Rev Cancer* 12, 513-526.
- Oakley, K., Han, Y., Vishwakarma, B.A., Chu, S., Bhatia, R., Gudmundsson, K.O., Keller, J., Chen, X., Vasko, V., Jenkins, N.A., et al. (2012). *Setbp1* promotes the self-renewal of murine myeloid progenitors via activation of *Hoxa9* and *Hoxa10*. *Blood* 119, 6099-6108.
- Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* 81, 2844-2853.
- Oguro, H., and Iwama, A. (2007). Life and death in hematopoietic stem cells. *Curr Opin Immunol* 19, 503-509.
- Oh, I.H. (2010). Microenvironmental targeting of Wnt/beta-catenin signals for hematopoietic stem cell regulation. *Expert Opin Biol Ther* 10, 1315-1329.
- Okabe, S., Tauchi, T., Tanaka, Y., Katagiri, S., and Ohyashiki, K. (2012). Effects of the Hedgehog Inhibitor GDC-0449, Alone or in Combination with Dasatinib, on BCR-ABL-Positive Leukemia Cells. *Stem Cells and Development* 21, 2939-2948.
- Okada, S., Fukuda, T., Inada, K., and Tokuhisa, T. (1999). Prolonged expression of c-fos suppresses cell cycle entry of dormant hematopoietic stem cells. *Blood* 93, 816-825.
- Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood* 80, 3044-3050.
- Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. *Immunity* 33, 387-399.
- Opferman, J.T., Iwasaki, H., Ong, C.C., Suh, H., Mizuno, S., Akashi, K., and Korsmeyer, S.J. (2005). Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 307, 1101-1104.
- Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996a). Long-term lymphohematopoietic reconstitution by a single CD34- low/negative hematopoietic stem cell. *Science* 273, 242-245.
- Osawa, M., Nakamura, K., Nishi, N., Takahashi, N., Tokuomoto, Y., Inoue, H., and Nakauchi, H. (1996b). In vivo self-renewal of c-Kit+ Sca-1+ Lin(low/-) hemopoietic stem cells. *J Immunol* 156, 3207-3214.

- Outram, S.V., Hager-Theodorides, A.L., Shah, D.K., Rowbotham, N.J., Drakopoulou, E., Ross, S.E., Lanske, B., Dessens, J.T., and Crompton, T. (2009). Indian hedgehog (Ihh) both promotes and restricts thymocyte differentiation. *Blood* 113, 2217-2228.
- Outram, S.V., Varas, A., Pepicelli, C.V., and Crompton, T. (2000). Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity* 13, 187-197.
- Packer, L.M., Rana, S., Hayward, R., O'Hare, T., Eide, C.A., Rebocho, A., Heidorn, S., Zabriskie, M.S., Niculescu-Duvaz, I., Druker, B.J., et al. (2011). Nilotinib and MEK inhibitors induce synthetic lethality through paradoxical activation of RAF in drug-resistant chronic myeloid leukemia. *Cancer Cell* 20, 715-727.
- Pajcini, K.V., Speck, N.A., and Pear, W.S. (2011). Notch signaling in mammalian hematopoietic stem cells. *Leukemia* 25, 1525-1532.
- Palandri, F., Iacobucci, I., Soverini, S., Castagnetti, F., Poerio, A., Testoni, N., Alimena, G., Breccia, M., Rege-Cambrin, G., Tiribelli, M., et al. (2009). Treatment of Philadelphia-positive chronic myeloid leukemia with imatinib: importance of a stable molecular response. *Clin Cancer Res* 15, 1059-1063.
- Pan, S., Wu, X., Jiang, J., Gao, W., Wan, Y., and Cheng, D. (2010). Discovery of NVP-LDE225, a potent and selective smoothed antagonist. *ACS Med Chem Lett* 1, 130-134.
- Pan, Y., Wang, C., and Wang, B. (2009). Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. *Dev Biol* 326, 177-189.
- Parcells, B.W., Ikeda, A.K., Simms-Waldrip, T., Moore, T.B., and Sakamoto, K.M. (2006). FMS-like tyrosine kinase 3 in normal hematopoiesis and acute myeloid leukemia. *Stem Cells* 24, 1174-1184.
- Park, I.-k., Qian, D., Kiel, M., Becker, M.W., Pihalja, M., Weissman, I.L., Morrison, S.J., and Clarke, M.F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423, 302-305.
- Passegue, E., Wagers, A.J., Giuriato, S., Anderson, W.C., and Weissman, I.L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med* 202, 1599-1611.
- Pathi, S., Pagan-Westphal, S., Baker, D.P., Garber, E.A., Rayhorn, P., Bumcrot, D., Tabin, C.J., Blake Pepinsky, R., and Williams, K.P. (2001). Comparative biological responses to human Sonic, Indian, and Desert hedgehog. *Mech Dev* 106, 107-117.
- Peacock, C.D., Wang, Q., Gesell, G.S., Corcoran-Schwartz, I.M., Jones, E., Kim, J., Devereux, W.L., Rhodes, J.T., Huff, C.A., Beachy, P.A., et al. (2007). Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proc Natl Acad Sci U S A* 104, 4048-4053.
- Pear, W.S., Miller, J.P., Xu, L., Pui, J.C., Soffer, B., Quackenbush, R.C., Pendergast, A.M., Bronson, R., Aster, J.C., Scott, M.L., et al. (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* 92, 3780-3792.
- Pearse, R.V., 2nd, Vogan, K.J., and Tabin, C.J. (2001). Ptc1 and Ptc2 transcripts provide distinct readouts of Hedgehog signaling activity during chick embryogenesis. *Dev Biol* 239, 15-29.

- Peeters, M., Ottersbach, K., Bollerot, K., Orelia, C., de Bruijn, M., and Wijgerde, M. (2009). Ventral embryonic tissues and Hedgehog proteins induce early AGM hematopoietic stem cell development. *Development* 136, 2613-2621.
- Pei, D. (2009). Regulation of pluripotency and reprogramming by transcription factors. *J Biol Chem* 284, 3365-3369.
- Pellicano, F., Simara, P., Sinclair, A., Helgason, G.V., Copland, M., Grant, S., and Holyoake, T.L. (2011). The MEK inhibitor PD184352 enhances BMS-214662-induced apoptosis in CD34+ CML stem/progenitor cells. *Leukemia* 25, 1159-1167.
- Peng, C., Chen, Y., Yang, Z., Zhang, H., Osterby, L., Rosmarin, A.G., and Li, S. (2010). PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice. *Blood* 115, 626-635.
- Perez-Caro, M., Cobaleda, C., Gonzalez-Herrero, I., Vicente-Duenas, C., Bermejo-Rodriguez, C., Sanchez-Beato, M., Orfao, A., Pintado, B., Flores, T., Sanchez-Martin, M., et al. (2009). Cancer induction by restriction of oncogene expression to the stem cell compartment. *EMBO J* 28, 8-20.
- Perry, J.M., Harandi, O.F., Porayette, P., Hegde, S., Kannan, A.K., and Paulson, R.F. (2009). Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood* 113, 911-918.
- Peterson, L.E. (2003). Partitioning large-sample microarray-based gene expression profiles using principal components analysis. *Comput Methods Programs Biomed* 70, 107-119.
- Pettengell, R., Luft, T., Henschler, R., Hows, J.M., Dexter, T.M., Ryder, D., and Testa, N.G. (1994). Direct comparison by limiting dilution analysis of long-term culture-initiating cells in human bone marrow, umbilical cord blood, and blood stem cells. *Blood* 84, 3653-3659.
- Petzer, A., Eaves, C., Lansdorp, P., Ponchio, L., Barnett, M., and Eaves, A. (1996). Characterization of primitive subpopulations of normal and leukemic cells present in the blood of patients with newly diagnosed as well as established chronic myeloid leukemia. *Blood* 88, 2162-2171.
- Petzer, A.L., Eaves, C.J., Barnett, M.J., and Eaves, A.C. (1997). Selective expansion of primitive normal hematopoietic cells in cytokine-supplemented cultures of purified cells from patients with chronic myeloid leukemia. *Blood* 90, 64-69.
- Piazza, R., Valletta, S., Winkelmann, N., Redaelli, S., Spinelli, R., Pirola, A., Antolini, L., Mologni, L., Donadoni, C., Papaemmanuil, E., et al. (2013). Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. *Nat Genet* 45, 18-24.
- Pierce, G.B., Dixon, F.J., and Verney, E. (1959). Testicular teratomas. I. Demonstration of teratogenesis by metamorphosis of multipotential Cells. *Cancer* 12, 573-583.
- Pineault, N., Abramovich, C., and Humphries, R.K. (2005). Transplantable cell lines generated with NUP98-Hox fusion genes undergo leukemic progression by Meis1 independent of its binding to DNA. *Leukemia* 19, 636-643.
- Pineault, N., Helgason, C.D., Lawrence, H.J., and Humphries, R.K. (2002). Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. *Exp Hematol* 30, 49-57.

- Po, A., Ferretti, E., Miele, E., De Smaele, E., Paganelli, A., Canettieri, G., Coni, S., Di Marcotullio, L., Biffoni, M., Massimi, L., et al. (2010). Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *EMBO J* 29, 2646-2658.
- Polakis, P. (2012). Wnt signaling in cancer. *Cold Spring Harb Perspect Biol* 4.
- Pronk, C.J., Rossi, D.J., Mansson, R., Attema, J.L., Norddahl, G.L., Chan, C.K., Sigvardsson, M., Weissman, I.L., and Bryder, D. (2007). Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell* 1, 428-442.
- Przygodzka, P., Ramstedt, B., Tengel, T., Larsson, G., and Wilczynska, M. (2010). Bomapin is a redox-sensitive nuclear serpin that affects responsiveness of myeloid progenitor cells to growth environment. *BMC Cell Biol* 11, 30.
- Ptasznik, A., Urbanowska, E., Chinta, S., Costa, M.A., Katz, B.A., Stanislaus, M.A., Demir, G., Linnekin, D., Pan, Z.K., and Gewirtz, A.M. (2002). Crosstalk Between BCR/ABL Oncoprotein and CXCR4 Signaling through a Src Family Kinase in Human Leukemia Cells. *J Exp Med* 196, 667-678.
- Punzel, M., Zhang, T., Liu, D., Eckstein, V., and Ho, A.D. (2002). Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34(+)CD38(-) cells. *Exp Hematol* 30, 464-472.
- Puttini, M., Coluccia, A.M., Boschelli, F., Cleris, L., Marchesi, E., Donella-Deana, A., Ahmed, S., Redaelli, S., Piazza, R., Magistrini, V., et al. (2006). In vitro and in vivo activity of SKI-606, a novel Src-Abl inhibitor, against imatinib-resistant Bcr-Abl+ neoplastic cells. *Cancer Res* 66, 11314-11322.
- Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Mansson, R., Thoren, L.A., Ekblom, M., Alexander, W.S., and Jacobsen, S.E. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell Stem Cell* 1, 671-684.
- Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., and Morrison, S.J. (2008). Efficient tumour formation by single human melanoma cells. *Nature* 456, 593-598.
- Quintas-Cardama, A., and Cortes, J. (2009). Molecular biology of bcr-abl1-positive chronic myeloid leukemia. *Blood* 113, 1619-1630.
- Quintas-Cardama, A., Cortes, J.E., and Kantarjian, H.M. (2011). Early cytogenetic and molecular response during first-line treatment of chronic myeloid leukemia in chronic phase: long-term implications. *Cancer* 117, 5261-5270.
- Quintas-Cardama, A., Kantarjian, H.M., and Cortes, J.E. (2009). Mechanisms of primary and secondary resistance to imatinib in chronic myeloid leukemia. *Cancer Control* 16, 122-131.
- Raaijmakers, M.H., Mukherjee, S., Guo, S., Zhang, S., Kobayashi, T., Schoonmaker, J.A., Ebert, B.L., Al-Shahrour, F., Hasserjian, R.P., Scadden, E.O., et al. (2010). Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 464, 852-857.
- Raaphorst, F.M., van Kemenade, F.J., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2000). Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. *Am J Pathol* 157, 709-715.

- Radich, J.P. (2007). The Biology of CML blast crisis. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program 2007, 384-391.
- Radich, J.P., Dai, H., Mao, M., Oehler, V., Schelter, J., Druker, B., Sawyers, C., Shah, N., Stock, W., Willman, C.L., et al. (2006). Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci U S A* 103, 2794-2799.
- Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10, 547-558.
- Radulovic, V., de Haan, G., and Klauke, K. (2013). Polycomb-group proteins in hematopoietic stem cell regulation and hematopoietic neoplasms. *Leukemia* 27, 523-533.
- Rajasekhar, V.K., and Begemann, M. (2007). Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. *Stem Cells* 25, 2498-2510.
- Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C., and Melton, D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* 298, 597-600.
- Raval, P. (1994). Qualitative and quantitative determination of mRNA. *J Pharmacol Toxicol Methods* 32, 125-127.
- Regl, G., Kasper, M., and Schnidar, H. (2004). Activation of the BCL2 promoter in response to Hedgehog/GLI signal transduction is predominantly mediated by GLI2. *Cancer Res* 64, 7724-7731.
- Reifenberger, J., Wolter, M., Knobbe, C.B., Kohler, B., Schonicke, A., Scharwachter, C., Kumar, K., Blaschke, B., Ruzicka, T., and Reifenberger, G. (2005). Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. *Br J Dermatol* 152, 43-51.
- Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* 434, 843-850.
- Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423, 409-414.
- Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* 414, 105-111.
- Richter, A.M., Pfeifer, G.P., and Dammann, R.H. (2009). The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta* 1796, 114-128.
- Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The *Drosophila* homology of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*. *Cell* 50, 649-657.

- Rix, U., Hantschel, O., Durnberger, G., Remsing Rix, L.L., Planyavsky, M., Fernbach, N.V., Kaupe, I., Bennett, K.L., Valent, P., Colinge, J., et al. (2007). Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets. *Blood* 110, 4055-4063.
- Rizo, A., Horton, S.J., Olthof, S., Dontje, B., Ausema, A., van Os, R., van den Boom, V., Vellenga, E., de Haan, G., and Schuringa, J.J. (2010). BMI1 collaborates with BCR-ABL in leukemic transformation of human CD34+ cells. *Blood* 116, 4621-4630.
- Rizo, A., Olthof, S., Han, L., Vellenga, E., de Haan, G., and Schuringa, J.J. (2009). Repression of BMI1 in normal and leukemic human CD34(+) cells impairs self-renewal and induces apoptosis. *Blood* 114, 1498-1505.
- Robbins, D.J., Fei, D.L., and Riobo, N.A. (2012). The Hedgehog signal transduction network. *Sci Signal* 5, re6.
- Robbins, D.J., and Hebrok, M. (2007). Hedgehogs: la dolce vita. Workshop on Hedgehog-Gli Signaling in Cancer and Stem Cells. *EMBO Rep* 8, 451-455.
- Robert-Moreno, A., Guiu, J., Ruiz-Herguido, C., Lopez, M.E., Ingles-Esteve, J., Riera, L., Tipping, A., Enver, T., Dzierzak, E., Gridley, T., et al. (2008). Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. *EMBO J* 27, 1886-1895.
- Robinson, M.D., and Speed, T.P. (2007). A comparison of Affymetrix gene expression arrays. *BMC Bioinformatics* 8, 449.
- Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.C., and Muenke, M. (1996). Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nat Genet* 14, 357-360.
- Rohatgi, R., Milenkovic, L., and Scott, M.P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372-376.
- Rosenwald, A., Wright, G., Chan, W.C., Connors, J.M., Campo, E., Fisher, R.I., Gascoyne, R.D., Muller-Hermelink, H.K., Smeland, E.B., Giltneane, J.M., et al. (2002). The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med* 346, 1937-1947.
- Ross, D.M., Branford, S., Seymour, J.F., Schwarzer, A.P., Arthur, C., Bartley, P.A., Slader, C., Field, C., Dang, P., Filshie, R.J., et al. (2010). Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR. *Leukemia* 24, 1719-1724.
- Roy, S., and Ingham, P.W. (2002). Hedgehogs tryst with the cell cycle. *J Cell Sci* 115, 4393-4397.
- Ruiz i Altaba, A. (2008). Therapeutic inhibition of Hedgehog-Gli signaling in cancer: epithelial, stromal, or stem cell targets? *Cancer Cell* 14, 281-283.
- Ruiz i Altaba, A., Mas, C., and Stecca, B. (2007). The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol* 17, 438-447.
- Sadlon, T.J., Lewis, I.D., and D'Andrea, R.J. (2004). BMP4: its role in development of the hematopoietic system and potential as a hematopoietic growth factor. *Stem Cells* 22, 457-474.

- Saglio, G., Hochhaus, A., Goh, Y.T., Masszi, T., Pasquini, R., Maloisel, F., Erben, P., Cortes, J., Paquette, R., Bradley-Garelik, M.B., et al. (2010). Dasatinib in imatinib-resistant or imatinib-intolerant chronic myeloid leukemia in blast phase after 2 years of follow-up in a phase 3 study: efficacy and tolerability of 140 milligrams once daily and 70 milligrams twice daily. *Cancer* 116, 3852-3861.
- Sallmyr, A., Fan, J., and Rassool, F.V. (2008). Genomic instability in myeloid malignancies: increased reactive oxygen species (ROS), DNA double strand breaks (DSBs) and error-prone repair. *Cancer Lett* 270, 1-9.
- Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999). Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. *Development* 126, 3915-3924.
- Sauvageau, G., Iscove, N.N., and Humphries, R.K. (2004). In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene* 23, 7223-7232.
- Sauvageau, G., Lansdorp, P.M., Eaves, C.J., Hogge, D.E., Dragowska, W.H., Reid, D.S., Largman, C., Lawrence, H.J., and Humphries, R.K. (1994). Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proc Natl Acad Sci U S A* 91, 12223-12227.
- Sauvageau, G., Thorsteinsdottir, U., Eaves, C.J., Lawrence, H.J., Largman, C., Lansdorp, P.M., and Humphries, R.K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev* 9, 1753-1765.
- Sauvageau, M., and Sauvageau, G. (2010). Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. *Cell Stem Cell* 7, 299-313.
- Savage, D.G., and Antman, K.H. (2002). Imatinib mesylate--a new oral targeted therapy. *N Engl J Med* 346, 683-693.
- Sawyers, C.L., Hochhaus, A., Feldman, E., Goldman, J.M., Miller, C.B., Ottmann, O.G., Schiffer, C.A., Talpaz, M., Guilhot, F., Deininger, M.W., et al. (2002). Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 99, 3530-3539.
- Schairer, A., Shih, A., Geron, I., Reya, T., Levin, W.J., Van Arsdale, T., and Jamieson, C. (2010). Human Blast Crisis Leukemia Stem Cell Inhibition with a Novel Smoothed Antagonist. *Blood (ASH Annual Meeting Abstracts)* 116, 1223-.
- Scharenberg, C.W., Harkey, M.A., and Torok-Storb, B. (2002). The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99, 507-512.
- Schemionek, M., Elling, C., Steidl, U., Baumer, N., Hamilton, A., Spieker, T., Gothert, J.R., Stehling, M., Wagers, A., Huettner, C.S., et al. (2010). BCR-ABL enhances differentiation of long-term repopulating hematopoietic stem cells. *Blood* 115, 3185-3195.
- Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467-470.
- Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nat Protocols* 3, 1101-1108.

- Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells* 4, 7-25.
- Scholl, C., Bansal, D., Dohner, K., Eiwien, K., Huntly, B.J., Lee, B.H., Rucker, F.G., Schlenk, R.F., Bullinger, L., Dohner, H., et al. (2007). The homeobox gene CDX2 is aberrantly expressed in most cases of acute myeloid leukemia and promotes leukemogenesis. *J Clin Invest* 117, 1037-1048.
- Schuettengruber, B., Chourrout, D., Vervoort, M., Leblanc, B., and Cavalli, G. (2007). Genome regulation by polycomb and trithorax proteins. *Cell* 128, 735-745.
- Seke Etet, P.F., Vecchio, L., and Nwabo Kamdje, A.H. (2012). Signaling pathways in chronic myeloid leukemia and leukemic stem cell maintenance: key role of stromal microenvironment. *Cell Signal* 24, 1883-1888.
- Semerad, C.L., Christopher, M.J., Liu, F., Short, B., Simmons, P.J., Winkler, I., Levesque, J.P., Chappel, J., Ross, F.P., and Link, D.C. (2005). G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. *Blood* 106, 3020-3027.
- Sengupta, A., Banerjee, D., Chandra, S., Banerji, S.K., Ghosh, R., Roy, R., and Banerjee, S. (2007). Deregulation and cross talk among Sonic hedgehog, Wnt, Hox and Notch signaling in chronic myeloid leukemia progression. *Leukemia* 21, 949-955.
- Sengupta, A., Ficker, A.M., Dunn, S.K., Madhu, M., and Cancelas, J.A. (2012a). Bmi1 reprograms CML B-lymphoid progenitors to become B-ALL-initiating cells. *Blood* 119, 494-502.
- Sengupta, R., Dubuc, A., Ward, S., Yang, L., Northcott, P., Woerner, B.M., Kroll, K., Luo, J., Taylor, M.D., Wechsler-Reya, R.J., et al. (2012b). CXCR4 activation defines a new subgroup of Sonic hedgehog-driven medulloblastoma. *Cancer Res* 72, 122-132.
- Shah, D.K., Hager-Theodorides, A.L., Outram, S.V., Ross, S.E., Varas, A., and Crompton, T. (2004). Reduced thymocyte development in sonic hedgehog knockout embryos. *J Immunol* 172, 2296-2306.
- Shah, N.P., Kantarjian, H.M., Kim, D.W., Rea, D., Dorlhiac-Llacer, P.E., Milone, J.H., Vela-Ojeda, J., Silver, R.T., Houry, H.J., Charbonnier, A., et al. (2008). Intermittent target inhibition with dasatinib 100 mg once daily preserves efficacy and improves tolerability in imatinib-resistant and -intolerant chronic-phase chronic myeloid leukemia. *J Clin Oncol* 26, 3204-3212.
- Shah, N.P., Nicoll, J.M., Nagar, B., Gorre, M.E., Paquette, R.L., Kuriyan, J., and Sawyers, C.L. (2002). Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2, 117-125.
- Shahi, M.H., Afzal, M., Sinha, S., Eberhart, C.G., Rey, J.A., Fan, X., and Castresana, J.S. (2010). Regulation of sonic hedgehog-GLI1 downstream target genes PTCH1, Cyclin D2, Plakoglobin, PAX6 and NKX2.2 and their epigenetic status in medulloblastoma and astrocytoma. *BMC Cancer* 10, 614.
- Shanmugam, K., Green, N.C., Rambaldi, I., Saragovi, H.U., and Featherstone, M.S. (1999). PBX and MEIS as Non-DNA-Binding Partners in Trimeric Complexes with HOX Proteins. *Mol Cell Biol* 19, 7577-7588.
- Shao, H., Huang, Q., and Liu, Z.-J. (2012). Chapter Seven - Targeting Notch Signaling for Cancer Therapeutic Intervention. In *Adv Pharm*, S.M.S. Keiran, ed. (Academic Press), pp. 191-234.

- Shepherd, B.E., Gutterop, P., Lansdorp, P.M., and Abkowitz, J.L. (2004). Estimating human hematopoietic stem cell kinetics using granulocyte telomere lengths. *Exp Hematol* 32, 1040-1050.
- Shimon, I., and Shpilberg, O. (1995). The insulin-like growth factor system in regulation of normal and malignant hematopoiesis. *Leukemia research* 19, 233-240.
- Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., et al. (2005). Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R γ null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. *J Immunol* 174, 6477-6489.
- Siggins, S.L., Nguyen, N.Y., McCormack, M.P., Vasudevan, S., Villani, R., Jane, S.M., Wainwright, B.J., and Curtis, D.J. (2009). The Hedgehog receptor Patched1 regulates myeloid and lymphoid progenitors by distinct cell-extrinsic mechanisms. *Blood* 114, 995-1004.
- Singh, A.P., Arora, S., Bhardwaj, A., Srivastava, S.K., Kadakia, M.P., Wang, B., Grizzle, W.E., Owen, L.B., and Singh, S. (2012). CXCL12/CXCR4 protein signaling axis induces sonic hedgehog expression in pancreatic cancer cells via extracellular regulated kinase- and Akt kinase-mediated activation of nuclear factor kappaB: implications for bidirectional tumor-stromal interactions. *J Biol Chem* 287, 39115-39124.
- Singh, R.R., Kim, J.E., Davuluri, Y., Drakos, E., Cho-Vega, J.H., and Amin, H.M. (2010). Hedgehog signaling pathway is activated in diffuse large B-cell lymphoma and contributes to tumor cell survival and proliferation. *Leukemia* 24, 1025-1036.
- Sirard, C., Lapidot, T., Vormoor, J., Cashman, J.D., Doedens, M., Murdoch, B., Jamal, N., Messner, H., Addey, L., Minden, M., et al. (1996). Normal and leukemic SCID-repopulating cells (SRC) coexist in the bone marrow and peripheral blood from CML patients in chronic phase, whereas leukemic SRC are detected in blast crisis. *Blood* 87, 1539-1548.
- Sloma, I., Imren, S., Beer, P.A., Zhao, Y., Lecault, V., Leung, D., Raghuram, K., Brimacombe, C., Lambie, K., Piret, J., et al. (2013). Ex vivo expansion of normal and chronic myeloid leukemic stem cells without functional alteration using a NUP98HOXA10homeodomain fusion gene. *Leukemia* 27, 159-169.
- Sloma, I., Jiang, X., Eaves, A.C., and Eaves, C.J. (2010). Insights into the stem cells of chronic myeloid leukemia. *Leukemia* 24, 1823-1833.
- Slupianek, A., Schmutte, C., Tomblin, G., Nieborowska-Skorska, M., Hoser, G., Nowicki, M.O., Pierce, A.J., Fishel, R., and Skorski, T. (2001). BCR/ABL regulates mammalian RecA homologs, resulting in drug resistance. *Mol Cell* 8, 795-806.
- Sobrinho-Simoes, M., Wilczek, V., Score, J., Cross, N.C., Apperley, J.F., and Melo, J.V. (2010). In search of the original leukemic clone in chronic myeloid leukemia patients in complete molecular remission after stem cell transplantation or imatinib. *Blood* 116, 1329-1335.
- Somervaille, T.C., and Cleary, M.L. (2006). Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell* 10, 257-268.
- Somervaille, T.C., Matheny, C.J., Spencer, G.J., Iwasaki, M., Rinn, J.L., Witten, D.M., Chang, H.Y., Shurtleff, S.A., Downing, J.R., and Cleary, M.L. (2009). Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. *Cell Stem Cell* 4, 129-140.

- South, A.P., Cho, R.J., and Aster, J.C. (2012). The double-edged sword of Notch signaling in cancer. *Semin Cell Dev Biol* 23, 458-464.
- Soverini, S., Hochhaus, A., Nicolini, F.E., Gruber, F., Lange, T., Saglio, G., Pane, F., Muller, M.C., Ernst, T., Rosti, G., et al. (2011). BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. *Blood* 118, 1208-1215.
- Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58-62.
- Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 6, 846-856.
- Spitz, F., and Duboule, D. (2008). Chapter 6 Global Control Regions and Regulatory Landscapes in Vertebrate Development and Evolution. In *Adv Genetics*, H. Veronica van, and E.H. Robert, eds. (Academic Press), pp. 175-205.
- St-Jacques, B., Hammerschmidt, M., and McMahon, A.P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 13, 2072-2086.
- Staal, F.J.T., and Luis, T.C. (2010). Wnt signaling in hematopoiesis: Crucial factors for self-renewal, proliferation, and cell fate decisions. *J Cell Biochem* 109, 844-849.
- Staal, F.J.T., Luis, T.C., and Tiemessen, M.M. (2008). WNT signalling in the immune system: WNT is spreading its wings. *Nat Rev Immunol* 8, 581-593.
- Stecca, B., Mas, C., Clement, V., Zbinden, M., Correa, R., Piguet, V., Beermann, F., and Ruiz, I.A.A. (2007). Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. *Proc Natl Acad Sci U S A* 104, 5895-5900.
- Stegmann, J.L., Cervantes, F., le Coutre, P., Porkka, K., and Saglio, G. (2012). Off-target effects of BCR-ABL1 inhibitors and their potential long-term implications in patients with chronic myeloid leukemia. *Leukemia & lymphoma* 53, 2351-2361.
- Steelman, L.S., Pohnert, S.C., Shelton, J.G., Franklin, R.A., Bertrand, F.E., and McCubrey, J.A. (2004). JAK//STAT, Raf//MEK//ERK, PI3K//Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* 18, 189-218.
- Steffen, B., Knop, M., Bergholz, U., Vakhrusheva, O., Rode, M., Kohler, G., Henrichs, M.P., Bulk, E., Hehn, S., Stehling, M., et al. (2011). AML1/ETO induces self-renewal in hematopoietic progenitor cells via the Groucho-related amino-terminal AES protein. *Blood* 117, 4328-4337.
- Steinleitner, K., Rampetsreiter, P., Koffel, R., Ramanathan, G., Mannhalter, C., and Weiser, R. (2012). EVI1 and MDS1/EVI1 Expression During Primary Human Hematopoietic Progenitor Cell Differentiation into Various Myeloid Lineages. *Anticancer Res* 32, 4883-4889.
- Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D.T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* 99, 2369-2378.

- Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., et al. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* 384, 129-134.
- Storey, J.D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A* 100, 9440-9445.
- Storms, R.W., Trujillo, A.P., Springer, J.B., Shah, L., Colvin, O.M., Ludeman, S.M., and Smith, C. (1999). Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci U S A* 96, 9118-9123.
- Strathdee, G., Holyoake, T.L., Sim, A., Parker, A., Oscier, D.G., Melo, J.V., Meyer, S., Eden, T., Dickinson, A.M., Mountford, J.C., et al. (2007). Inactivation of HOXA genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis. *Clin Cancer Res* 13, 5048-5055.
- Su, W., Meng, F., Huang, L., Zheng, M., Liu, W., and Sun, H. (2012). Sonic hedgehog maintains survival and growth of chronic myeloid leukemia progenitor cells through β -catenin signaling. *Exp Hematol* 40, 418-427.
- Suda, J., Suda, T., and Ogawa, M. (1984). Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. *Blood* 64, 393-399.
- Suda, T., Takubo, K., and Semenza, G.L. (2011). Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell* 9, 298-310.
- Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977-988.
- Sutherland, H.J., Eaves, C.J., Eaves, A.C., Dragowska, W., and Lansdorp, P.M. (1989). Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74, 1563-1570.
- Sutherland, H.J., Eaves, C.J., Lansdorp, P.M., Phillips, G.L., and Hogge, D.E. (1994). Kinetics of committed and primitive blood progenitor mobilization after chemotherapy and growth factor treatment and their use in autotransplants. *Blood* 83, 3808-3814.
- Sutherland, H.J., Eaves, C.J., Lansdorp, P.M., Thacker, J.D., and Hogge, D.E. (1991). Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. *Blood* 78, 666-672.
- Sutherland, H.J., Hogge, D.E., Cook, D., and Eaves, C.J. (1993a). Alternative mechanisms with and without steel factor support primitive human hematopoiesis. *Blood* 81, 1465-1470.
- Sutherland, H.J., Hogge, D.E., and Eaves, C.J. (1993b). Growth factor regulation of the maintenance and differentiation of human long-term culture-initiating cells (LTC-IC). *Leukemia* 7 Suppl 2, S122-125.
- Sutherland, H.J., Hogge, D.E., Lansdorp, P.M., Phillips, G.L., Eaves, A.C., and Eaves, C.J. (1995). Quantitation, mobilization, and clinical use of long-term culture-initiating cells in blood cell autografts. *Journal of hematotherapy* 4, 3-10.
- Sutherland, H.J., Lansdorp, P.M., Henkelman, D.H., Eaves, A.C., and Eaves, C.J. (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci U S A* 87, 3584-3588.

- Suzuki, Y., Montagne, K., Nishihara, A., Watabe, T., and Miyazono, K. (2008). BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and angiopoietin-1/Tie2 signalling. *J Biochem* 143, 199-206.
- Szanişzlo, P., Wang, N., Sinha, M., Reece, L.M., Van Hook, J.W., Luxon, B.A., and Leary, J.F. (2004). Getting the right cells to the array: Gene expression microarray analysis of cell mixtures and sorted cells. *Cytometry Part A : the journal of the International Society for Analytical Cytology* 59, 191-202.
- Taichman, R.S., and Emerson, S.G. (1994). Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *J Exp Med* 179, 1677-1682.
- Taipale, J. (2000). Effects of oncogenic mutations in Smoothed and Patched can be reversed by cyclopamine. *Nature* 406, 1005-1009.
- Takahashi, N., Miura, I., Saitoh, K., and Miura, A.B. (1998). Lineage Involvement of Stem Cells Bearing the Philadelphia Chromosome in Chronic Myeloid Leukemia in the Chronic Phase as Shown by a Combination of Fluorescence-Activated Cell Sorting and Fluorescence In Situ Hybridization. *Blood* 92, 4758-4763.
- Takanaga, H., Tsuchida-Straeten, N., Nishide, K., Watanabe, A., Aburatani, H., and Kondo, T. (2009). Gli2 is a novel regulator of sox2 expression in telencephalic neuroepithelial cells. *Stem Cells* 27, 165-174.
- Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. *J Exp Med* 199, 295-302.
- Talpaz, M., Silver, R.T., Druker, B.J., Goldman, J.M., Gambacorti-Passerini, C., Guilhot, F., Schiffer, C.A., Fischer, T., Deininger, M.W., Lennard, A.L., et al. (2002). Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* 99, 1928-1937.
- Taussig D.C., Miraki-Moud F., Anjos-Afonso F., Pearce D.J., Allen K., Ridler C., Lillington D., Oakerverve H., Cavenagh J., Agrawal S.G., Lister T.A., Gribben J.G., Bonnet D. (2008). *Blood*. 112, 568-75
- Tawbi, H.A., Ahnert, J.R., Dummer, R., Thomas, A.L., Granvil, C., Shou, Y., Dey, J., Mita, M.M., Amakye, D.D., and Mita, A.C. (2011). Phase I study of LDE225 in advanced solid tumors: Updated analysis of safety, preliminary efficacy, and pharmacokinetic-pharmacodynamic correlation. *J Clin Oncol* 29, Abs 3062.
- Taylor, M.D., Liu, L., Raffel, C., Hui, C.C., Mainprize, T.G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., et al. (2002). Mutations in SUFU predispose to medulloblastoma. *Nat Genet* 31, 306-310.
- Tedeschi, F.A., Cardozo, M.A., Valentini, R., and Zalazar, F.E. (2010). Co-expression of HoxA9 and bcr-abl genes in chronic myeloid leukemia. *Leukemia & lymphoma* 51, 892-896.
- Teglund, S., and Toftgard, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta* 1805, 181-208.
- Teh, M.-T., Wong, S.-T., Neill, G.W., Ghali, L.R., Philpott, M.P., and Quinn, A.G. (2002). FOXM1 Is a Downstream Target of Gli1 in Basal Cell Carcinomas. *Cancer Res* 62, 4773-4780.

- Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M.F., and Concordet, J.P. (2006). Multisite protein kinase A and glycogen synthase kinase 3 β phosphorylation leads to Gli3 ubiquitination by SCF β TrCP. *Mol Cell Biol* 26, 4316-4326.
- Terpstra, W., Ploemacher, R.E., Prins, A., van Lom, K., Pouwels, K., Wognum, A.W., Wagemaker, G., Lowenberg, B., and Wielenga, J.J. (1996). Fluorouracil selectively spares acute myeloid leukemia cells with long-term growth abilities in immunodeficient mice and in culture. *Blood* 88, 1944-1950.
- Terskikh, A.V., Miyamoto, T., Chang, C., Diatchenko, L., and Weissman, I.L. (2003). Gene expression analysis of purified hematopoietic stem cells and committed progenitors. *Blood* 102, 94-101.
- Thomas, E.D., and Epstein, R.B. (1965). Bone marrow transplantation in acute leukemia. *Cancer Res* 25, 1521-1524.
- Thomas, E.D., Lochte, H.L., Jr., Lu, W.C., and Ferrebee, J.W. (1957). Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med* 257, 491-496.
- Thomas, J., Wang, L., Clark, R.E., and Pirmohamed, M. (2004). Active transport of imatinib into and out of cells: implications for drug resistance. *Blood* 104, 3739-3745.
- Thomas, P.D., Mi, H., and Lewis, S. (2007). Ontology annotation: mapping genomic regions to biological function. *Curr Opin Chem Biol* 11, 4-11.
- Thorsteinsdottir, U., Mamo, A., Kroon, E., Jerome, L., Bijl, J., Lawrence, H.J., Humphries, K., and Sauvageau, G. (2002). Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood* 99, 121-129.
- Tian, H., Callahan, C.A., DuPree, K.J., Darbonne, W.C., Ahn, C.P., Scales, S.J., and de Sauvage, F.J. (2009). Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proc Natl Acad Sci U S A* 106, 4254-4259.
- Tiecke, E., Turner, R., Sanz-Ezquerro, J.J., Warner, A., and Tickle, C. (2007). Manipulations of PKA in chick limb development reveal roles in digit patterning including a positive role in Sonic Hedgehog signaling. *Dev Biol* 305, 312-324.
- Till, J., and McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14, 213.
- Till, J., and McCulloch, E. (1963). A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proc Natl Acad Sci USA* 51, 29-36.
- Ting, S.B., Deneault, E., Hope, K., Cellot, S., Chagraoui, J., Mayotte, N., Dorn, J.F., Laverdure, J.P., Harvey, M., Hawkins, E.D., et al. (2012). Asymmetric segregation and self-renewal of hematopoietic stem and progenitor cells with endocytic Ap2a2. *Blood* 119, 2510-2522.
- Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. (2007). FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. *Cell* 128, 325-339.
- Trentin, J.J. (1971). Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM). *Am J Pathol* 65, 621-628.

- Trowbridge, J.J., Scott, M.P., and Bhatia, M. (2006). Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proc Natl Acad Sci U S A* 103, 14134-14139.
- Udomsakdi, C., Eaves, C.J., Lansdorp, P.M., and Eaves, A.C. (1992a). Phenotypic heterogeneity of primitive leukemic hematopoietic cells in patients with chronic myeloid leukemia. *Blood* 80, 2522-2530.
- Udomsakdi, C., Eaves, C.J., Swolin, B., Reid, D.S., Barnett, M.J., and Eaves, A.C. (1992b). Rapid decline of chronic myeloid leukemic cells in long-term culture due to a defect at the leukemic stem cell level. *Proc Natl Acad Sci U S A* 89, 6192-6196.
- Udomsakdi, C., Lansdorp, P.M., Hogge, D.E., Reid, D.S., Eaves, A.C., and Eaves, C.J. (1992c). Characterization of primitive hematopoietic cells in normal human peripheral blood. *Blood* 80, 2513-2521.
- Uhmann, A., Dittmann, K., Nitzki, F., Dressel, R., Koleva, M., Frommhold, A., Zibat, A., Binder, C., Adham, I., Nitsche, M., et al. (2007). The Hedgehog receptor Patched controls lymphoid lineage commitment. *Blood* 110, 1814-1823.
- Uhmann, A., van den Brandt, J., Dittmann, K., Hess, I., Dressel, R., Binder, C., Luhder, F., Christiansen, H., Fassnacht, M., Bhandoola, A., et al. (2011). T cell development critically depends on prethymic stromal patched expression. *J Immunol* 186, 3383-3391.
- Unnisa, Z., Clark, J.P., Roychoudhury, J., Thomas, E., Tessarollo, L., Copeland, N.G., Jenkins, N.A., Grimes, H.L., and Kumar, A.R. (2012). Meis1 preserves hematopoietic stem cells in mice by limiting oxidative stress. *Blood* 120, 4973-4981.
- Valk, P.J., Verhaak, R.G., Beijen, M.A., Erpelinck, C.A., Barjesteh van Waalwijk van Doorn-Khosrovani, S., Boer, J.M., Beverloo, H.B., Moorhouse, M.J., van der Spek, P.J., Lowenberg, B., et al. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 350, 1617-1628.
- Van Etten, R.A. (1999). Cycling, stressed-out and nervous: cellular functions of c-Abl. *Trends Cell Biol* 9, 179-186.
- van Kemenade, F.J., Raaphorst, F.M., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P.E., Otte, A.P., and Meijer, C.J.L.M. (2001). Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. *Blood* 97, 3896-3901.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3, research0034.0031 - research0034.0011.
- Varnat, F., Duquet, A., Malerba, M., Zbinden, M., Mas, C., Gervaz, P., and Ruiz i Altaba, A. (2009). Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Mol Med* 1, 338-351.
- Vazquez, A., Bond, E.E., Levine, A.J., and Bond, G.L. (2008). The genetics of the p53 pathway, apoptosis and cancer therapy. *Nat Rev Drug Discov* 7, 979-987.

- Verstegen, M.M., Cornelissen, J.J., Terpstra, W., Wagemaker, G., and Wognum, A.W. (1999). Multilineage outgrowth of both malignant and normal hemopoietic progenitor cells from individual chronic myeloid leukemia patients in immunodeficient mice. *Leukemia* 13, 618-628.
- Viatour, P., Somerville, T.C., Venkatasubrahmanyam, S., Kogan, S., McLaughlin, M.E., Weissman, I.L., Butte, A.J., Passegué, E., and Sage, J. (2008). Hematopoietic Stem Cell Quiescence Is Maintained by Compound Contributions of the Retinoblastoma Gene Family. *Cell Stem Cell* 3, 416-428.
- Villuendas, R., Steegmann, J.L., Pollan, M., Tracey, L., Granda, A., Fernandez-Ruiz, E., Casado, L.F., Martinez, J., Martinez, P., Lombardia, L., et al. (2006). Identification of genes involved in imatinib resistance in CML: a gene-expression profiling approach. *Leukemia* 20, 1047-1054.
- Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* 103, 3258-3264.
- Vokes, S.A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W.J., Davidson, E.H., Wong, W.H., and McMahon, A.P. (2007). Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. *Development* 134, 1977-1989.
- Vokes, S.A., Ji, H., Wong, W.H., and McMahon, A.P. (2008). A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev* 22, 2651-2663.
- Von Hoff, D.D., LoRusso, P.M., Rudin, C.M., Reddy, J.C., Yauch, R.L., and Tibes, R. (2009). Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med* 361, 1164-1172.
- Wagers, A.J., Christensen, J.L., and Weissman, I.L. (2002). Cell fate determination from stem cells. *Gene Ther* 9, 606-612.
- Wang, G.G., Pasillas, M.P., and Kamps, M.P. (2006). Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes. *Mol Cell Biol* 26, 3902-3916.
- Wang, J.C., Lapidot, T., Cashman, J.D., Doedens, M., Addy, L., Sutherland, D.R., Nayar, R., Laraya, P., Minden, M., Keating, A., et al. (1998). High level engraftment of NOD/SCID mice by primitive normal and leukemic hematopoietic cells from patients with chronic myeloid leukemia in chronic phase. *Blood* 91, 2406-2414.
- Wang, J.C.Y., Doedens, M., and Dick, J.E. (1997). Primitive Human Hematopoietic Cells Are Enriched in Cord Blood Compared With Adult Bone Marrow or Mobilized Peripheral Blood as Measured by the Quantitative In Vivo SCID-Repopulating Cell Assay. *Blood* 89, 3919-3924.
- Wang, L., Pearson, K., Ferguson, J.E., and Clark, R.E. (2003). The early molecular response to imatinib predicts cytogenetic and clinical outcome in chronic myeloid leukaemia. *British journal of haematology* 120, 990-999.
- Wang, Y., Krivtsov, A.V., Sinha, A.U., North, T.E., Goessling, W., Feng, Z., Zon, L.I., and Armstrong, S.A. (2010). The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. *Science* 327, 1650-1653.

- Warr, M.R., Pietras, E.M., and Passegue, E. (2011). Mechanisms controlling hematopoietic stem cell functions during normal hematopoiesis and hematological malignancies. *Wiley interdisciplinary reviews Systems biology and medicine* 3, 681-701.
- Watkins, D.N., Berman, D.M., Burkholder, S.G., Wang, B., Beachy, P.A., and Baylin, S.B. (2003). Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 422, 313-317.
- Weisberg, E., Manley, P.W., Breitenstein, W., Bruggen, J., Cowan-Jacob, S.W., Ray, A., Huntly, B., Fabbro, D., Fendrich, G., Hall-Meyers, E., et al. (2005). Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* 7, 129-141.
- Weissman, I.L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 287, 1442-1446.
- Wetzler, M., Talpaz, M., Van Etten, R.A., Hirsh-Ginsberg, C., Beran, M., and Kurzrock, R. (1993). Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. *J Clin Invest* 92, 1925-1939.
- White, D.L., Saunders, V.A., Dang, P., Engler, J., Venables, A., Zrim, S., Zannettino, A., Lynch, K., Manley, P.W., and Hughes, T. (2007). Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity. *Blood* 110, 4064-4072.
- White, D.L., Saunders, V.A., Dang, P., Engler, J., Zannettino, A.C., Cambareri, A.C., Quinn, S.R., Manley, P.W., and Hughes, T.P. (2006). OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood* 108, 697-704.
- Wikenheiser-Brokamp, K.A. (2006). Retinoblastoma family proteins: insights gained through genetic manipulation of mice. *Cell Mol Life Sci* 63, 767-780.
- Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448-452.
- Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al. (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 135, 1118-1129.
- Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* 6, 93-106.
- Wisniewski, D., Affer, M., Willshire, J., and Clarkson, B. (2011). Further phenotypic characterization of the primitive lineage- CD34+CD38-CD90+CD45RA- hematopoietic stem cell/progenitor cell sub-population isolated from cord blood, mobilized peripheral blood and patients with chronic myelogenous leukemia. *Blood Cancer J* 1, e36, doi: 10.1038/bcj.2011.1035.
- Wolter, M., Reifenberger, J., Sommer, C., Ruzicka, T., and Reifenberger, G. (1997). Mutations in the human homologue of the *Drosophila* segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res* 57, 2581-2585.

- Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L., and Weissman, I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. *Science* 294, 1933-1936.
- Wu, A.M., Till, J.E., Siminovitch, L., and McCulloch, E.A. (1967). A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol* 69, 177-184.
- Wu, H.K., and Minden, M.D. (1997). Transcriptional activation of human LIM-HOX gene hLH-2 in chronic myelogenous leukemia is due to a cis-acting effect of Bcr-Abl. *Biochem Biophys Res Commun* 234, 742-747.
- Wu, J., Huang, L., Huang, M., Liu, W., Zheng, M., Cao, Y., Liu, Y., Zhang, Y., Lu, Y., Xu, G., et al. (2009). Dominant contribution of malignant endothelial cells to endotheliopoiesis in chronic myeloid leukemia. *Exp Hematol* 37, 87-91.
- Wu, M., Kwon, H.Y., Rattis, F., Blum, J., Zhao, C., Ashkenazi, R., Jackson, T.L., Gaiano, N., Oliver, T., and Reya, T. (2007). Imaging hematopoietic precursor division in real time. *Cell Stem Cell* 1, 541-554.
- Xie, T., and Spradling, A.C. (2000). A niche maintaining germ line stem cells in the *Drosophila* ovary. *Science* 290, 328-330.
- Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., and Nakauchi, H. (2011). Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* 147, 1146-1158.
- Yang, L., Bian, Y., Huang, S., Ma, X., Zhang, C., Su, X., Chen, Z.J., Xie, J., and Zhang, H. (2011). Identification of signature genes for detecting hedgehog pathway activation in esophageal cancer. *Pathol Oncol Res* 17, 387-391.
- Yang, L., Xie, G., Fan, Q., and Xie, J. (2010). Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. *Oncogene* 29, 469-481.
- Yao, J., Liu, Y., Husain, J., Lo, R., Palaparti, A., Henderson, J., and Stifani, S. (1998). Combinatorial expression patterns of individual TLE proteins during cell determination and differentiation suggest non-redundant functions for mammalian homologs of *Drosophila* Groucho. *Dev Growth Differ* 40, 133-146.
- Yauch, R.L., Gould, S.E., Scales, S.J., Tang, T., Tian, H., Ahn, C.P., Marshall, D., Fu, L., Januario, T., Kallop, D., et al. (2008). A paracrine requirement for hedgehog signalling in cancer. *Nature* 455, 406-410.
- Yilmaz, O.H., Kiel, M.J., and Morrison, S.J. (2006). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood* 107, 924-930.
- Yoffe, G., Chinault, A., Talpaz, M., Blick, M., Kantarjian, H., Taylor, K., and Spitzer, G. (1987). Clonal nature of Philadelphia chromosome-positive and -negative chronic myelogenous leukemia by DNA hybridization analyses. *Exp Hematol* 15, 725-728.
- Yoon, J.W., Gilbertson, R., Iannaccone, S., Iannaccone, P., and Walterhouse, D. (2009). Defining a role for Sonic hedgehog pathway activation in desmoplastic medulloblastoma by identifying GLI1 target genes. *Int J Cancer* 124, 109-119.

- Yoon, J.W., Kita, Y., Frank, D.J., Majewski, R.R., Konicek, B.A., Nobrega, M.A., Jacob, H., Walterhouse, D., and Iannaccone, P. (2002). Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *J Biol Chem* 277, 5548-5555.
- Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., Miyamoto, K., et al. (2007). Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* 1, 685-697.
- Zeisig, B.B., Milne, T., Garcia-Cuellar, M.P., Schreiner, S., Martin, M.E., Fuchs, U., Borkhardt, A., Chanda, S.K., Walker, J., Soden, R., et al. (2004). Hoxa9 and Meis1 are key targets for MLL-ENL-mediated cellular immortalization. *Mol Cell Biol* 24, 617-628.
- Zhang, B., Ho, Y.W., Huang, Q., Maeda, T., Lin, A., Lee, S.U., Hair, A., Holyoake, T.L., Huettner, C., and Bhatia, R. (2012a). Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. *Cancer Cell* 21, 577-592.
- Zhang, B., Li, M., McDonald, T., Holyoake, T.L., Moon, R.T., Campana, D., Shultz, L., and Bhatia, R. (2013). Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-beta-catenin signaling. *Blood* 121, 1824-1838.
- Zhang, H., Li, H., Xi, H.S., and Li, S. (2012b). HIF1alpha is required for survival maintenance of chronic myeloid leukemia stem cells. *Blood* 119, 2595-2607.
- Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425, 836-841.
- Zhao, C., Blum, J., Chen, A., Kwon, H.Y., Jung, S.H., Cook, J.M., Lagoo, A., and Reya, T. (2007). Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell* 12, 528-541.
- Zhao, C., Chen, A., Jamieson, C.H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H.Y., Kim, J., Chute, J.P., Rizzieri, D., et al. (2009). Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 458, 776-779.
- Zhou, S., Schuetz, J.D., Bunting, K.D., Colapietro, A.-M., Sampath, J., Morris, J.J., Lagutina, I., Grosveld, G.C., Osawa, M., Nakauchi, H., et al. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7, 1028-1034.
- Zhu, H., and Lo, H.W. (2010). The Human Glioma-Associated Oncogene Homolog 1 (GLI1) Family of Transcription Factors in Gene Regulation and Diseases. *Curr Genomics* 11, 238-245.
- Zimmermann, S., and Martens, U.M. (2008). Telomeres, senescence, and hematopoietic stem cells. *Cell and tissue research* 331, 79-90.
- Zon, L.I. (2008). Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature* 453, 306-313.