Analysis of the transcriptome: Investigation of human embryonic stem cells during directed differentiation to cardiovascular lineages

Lynsey Howard

B.Sc (hons)

Submitted in the fulfilment of the requirements of the degree of
Doctor of Philosophy in the College of Medical, Veterinary and Life Sciences, University of Glasgow.

British Heart Foundation Glasgow Cardiovascular Research Centre, Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow.

July 2012

Author's Declaration

I declare that this thesis has been written entirely by myself and is a record of work performed by myself with the exception of cardiomyocyte differentiation optimisation (Dr A. Kaupisch), embryoid body formation, lentivirus optimisation in SA461 hESC, the LC Sciences microRNA microarray (Dr N. M. Kane), Northern blotting (R Lui) and haematopoetic and neuronal differentiation samples (Dr A McCahill). This thesis has not been submitted previously for a higher degree. The research was carried out in the Division of Cardiovascular and Medical Sciences, University of Glasgow, under the supervision of Prof. A.H. Baker and Prof. G. Milligan.

Lynsey Howard

July 2012

Acknowledgements

Firstly, I would like to thank my main PhD supervisor Prof. Andrew H. Baker for all of his advice, help, support, constructive criticism, guidance, career advice, and for affording me the opportunity to work in his laboratory. I would also like to thank Prof. Graeme Milligan for his guidance, discussion and support in his role as secondary PhD supervisor. Additionally, thanks to the Medical Research Council and British Heart Foundation for the support and funding of this work.

Special thanks to my informal supervisor, Dr Nicole M Kane, for her help, support, friendship and at times blunt honesty(!) during my PhD.

I would like to thank all of the fantastic people that I have been fortunate enough to work with at the BHF GCRC and in the Davidson-based laboratories. I would especially like to thank Dr Angela Bradshaw for patiently teaching me how to clone in the early days, Dr Carolyn Clarke for her qRT-PCR expertise, Dr Laura Denby for answering all of my "stupid questions", Dr Martin McBride and Dr John McClure for patiently teaching a newbie the world of microarray analysis, Dr Alexandra Kaupisch for teaching me to make beating cells, Dr Ruth MacKenzie for her endothelial expertise and support when the ChIP's were down(!), Dr Angela McCahill for kindly providing samples and laughs, Dr Robert McDonald for his vascular cell expertise, Dr Rachel Shirley for her training in *in vivo* techniques and Dr Lorraine Work for talking me into *in vivo* training in the first place. Special thanks to Nicola Britton for teaching me too many things to mention and putting up with my taste in music, Pete Burton for stem cell expertise, interesting tissue culture discussion and introducing me to more genres of music than I knew existed, Gregor Aitcheson for tidying up after me(!) and Ruifang Lui for RNA expertise.

Heartfelt thanks to my office buddies Wendy Crawford, Margaret Duffy, Aiste Monkeviciute, and Emily Ord for the laughs, hugs, lunches and all round friendship. You girls were always able to put a smile on my face, even on the most stressful days! In addition, thanks to the mojito crew, Ange, Carolyn, Lynda, Helen S, Katie, Rachel, Nic, Stacy, Monica, Oscar, and Ruth for teaching me the art of cocktail making and the beauty of a good science discussion while slightly under the influence! Thanks to Scott Cowan for the late night TC rants.

Also, thanks to Dr Helen Spencer and Dr Rowan Orme for their endless advice on science from afar.

I'd like to thank my wonderful family for their love and support throughout my studies, and for always believing in me.

So many thanks to my Dad for his unconditional love, support, advice and expertly timed glasses of wine.

Thank you to the late Christine Howard, my Mum, who taught me optimism, patience and the importance of friendship and laughter. Thank you to my Nana, the late Lily Howard, who taught me perseverance and that you can always find the music in life. Thank you to my late friend Gary Skirving, who encouraged me to question the answers. I would like to dedicate this thesis to their memory.

Thank you to my friends outside of University, Alison, Louise and Karin for reminding me there is still a world outside and that we need very little excuse for a bottle of bubbly.

Massive thanks to my best friend Cheryl and the Andrew family for their constant love, support, advice, and the provision of dinner or a "hoose party" as and when needed.

Last, but by no means least, a massive thank you to Scott. You are the other half of my triangle (it is the strongest shape, fact!). Thanks for the love, laughs, hugs, support, advice, dinners, clean clothes and putting up with an absent-minded scientist.

Table of Contents

List of Tables

List of Figures

List of Publications

HOWARD, L., KANE, N. M., MILLIGAN, G. & BAKER, A. H. 2011. MicroRNAs regulating cell pluripotency and vascular differentiation. *Vascular Pharmacology*, 55, 69-78.

KANE, N. M., **HOWARD, L.**, DESCAMPS, B., MELONI, M., MCCLURE, J., LU, R., MCCAHILL, A., BREEN, C., MACKENZIE, R. M., DELLES, C., MOUNTFORD, J. C., MILLIGAN, G., EMANUELI, C. & BAKER, A. H. 2012. Role of MicroRNAs 99b, 181a, and 181b in the Differentiation of Human Embryonic Stem Cells to Vascular Endothelial Cells. *Stem Cells*, 30, 643-654.

List of Abbreviations/Definitions

293T cells	HEK transformed cell line
3’	3 prime end
5’	5 prime end
α	alpha
β	beta
μ g	microgram
μ g/mL	microgram per millilitre
μ L	microlitre
μ M	micromolar (micromoles per litre)
Ψ	packaging signal
Ψ Δ	packaging signal deletion
$\times$ g	relative centrifugal force
ABI	ankle brachial index
AIDS	acquired immune deficiency syndrome
amp	ampicillin
ampR	ampicillin resistance
ANOVA	analysis of variance
BCA	bicinchoninic acid

bFGF	basic fibroblast growth factor
BMMNC	bone-marrow mononuclear cell
BMP4	bone morphogenetic protein 4
bp	base pairs
BSA	bovine serum albumin
C-terminal	carboxyl terminal domain
CABG	coronary artery bypass grafting
cDNA	complementary DNA
CHD	coronary heart disease
ChIP	Chromatin immunoprecipitation
CIS	common integration site
CLI	critical limb ischaemia
cm	centimetres
CPC	cardiac progenitor cell
cPPT	central polypurine tract
ct	cycle-threshold
CVD	cardiovascular disease
DAPI	4 , 6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium

DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
E2F	elongation 2 transcriptional factor
EB	embryoid body
EC	endothelial cell
ECL	enhanced chemiluminescent
EDTA	ethylenediamine tetra-acetic acid
eGFP	enhanced green fluorescent protein
EPC	endothelial progenitor cell
ESCC	embryonic stem cell cycle
FACS	fluorescence-activated cell sorting
FAM	fluorescein amidite
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
g/L	gram per litre
g/mole	gram per mole
g/molecule	gram per molecule
GMP	good manufacturing practice

h	hour
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K27me3	Histone 3 lysine 27 tri-methylation
HEK	human embryonic kidney
HeLa cells	human cervical carcinoma cell line
hESC	human embryonic stem cells
HGF	hepatocyte growth factor
HIV-1	human immunodeficiency virus 1
HRP	horse radish peroxidise
HSC	haematopoietic stem cells
HSVEC	human saphenous vein endothelial cell
HSVSMC	human saphenous vein smooth muscle cell
iPSC	induced pluripotent stem cell
ICm	inner cell mass
int-LV	integrating lentiviral vector
iu/mL	infectious unit of LV per ml
L	litre
LB	Luria-Bertani
LNT	lentiviral vector

LVEF	left ventricular ejection fraction
M	molar (moles per litre)
MACS	magnetic activated cell sorting
MCS	multiple cloning site
MEM	minimum essential media
mESC	mouse embryonic stem cells
mg/L	milligram per litre
MI	myocardial infarction
min	minute
miR	microRNA
mL	millilitre
mM	millimolar (millimoles per litre)
MOI	multiplicity of infection
mRNA	messenger RNA
MSC	mesenchymal stem cell
ng	nanogram
ng/ μ l	nanogram per microlitre
nm	nanometre
nM	nanomolar (nanomoles per litre)

NO	nitric oxide
OD	optical density
ORF	open reading frame
PAD	peripheral artery disease
PBS	phosphate buffered saline
PCI	percutaneous coronary interventions
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEI	polyethylenimine
PFA	paraformaldehyde
qRT-PCR	quantitative real time PCR
RCF	relative centrifugal force
RISC	RNA induced silencing complex
RLU	relative luminescence unit
RNA	ribonucleic acid
rpm	revolutions per minute
RRM	ready reaction mix
RT	room temperature
SDS	sodium dodecyl sulphate

s	seconds
S.E.M	standard error of the mean
SFFV	spleen focus forming virus
SIN	self-inactivating
siRNA	silencing RNA
SKM	skeletal myocyte
SOC	super optimal broth with catabolite repression
SRF	serum response factor
TAMRA	tetramethylrhodamine
TBE	tris/borate
$TcPO_2$	transcutaneous oxygen pressure
TEMED	tetramethylethylenediamine
TF	transcription factor
TGF	tumour growth factor
T_m	melting temperature
U	unit of restriction enzyme activity
UV	ultraviolet light
V	voltage
v/v	volume per volume

VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSV-g	vesicular stomatitis virus glycoprotein
w	week
w/v	weight per volume
WAS	Wiskott-Aldrich syndrome
WPRE	woodchuck post-transcriptional regulatory element

Summary

To date, the need for effective treatments to tackle ischaemic diseases such as CHD and PAD remains unmet. As such, there has been a great deal of interest in developing cell therapies in order to address these important pathologies. The main goals of a cell therapy strategy for ischaemic disease remain prompt restoration of blood supply to the affected areas in order to salvage tissue and/or regeneration of tissues previously lost to ischaemia.

Derived from the Inner Cell Mass (ICM) of an embryo at the blastocyst stage, hESC have been proposed as a potential source of functional, transplantable cells for a variety of cell therapy applications. Although successful differentiation of multiple cell types from hESC has been demonstrated, the molecular processes governing the cell commitment process remain poorly understood, and differentiation efficiency often fails to provide the number of cells required to see clinical benefit in the patient. As such, a more thorough transcriptional characterisation of cardiovascular cell types derived from hESC was the goal of this study.

MicroRNAs (miRNA; miR) are small (~22nt), non-coding RNAs which negatively regulate mRNA. MiR-1 and miR-133 were previously shown to play a role in regulating cardiac differentiation with miR-1 potentiating cardiac differentiation and miR-133 having an inhibitory effect. Optimisation of lentiviral vectors showed generation of single pre-miR overexpression lentiviruses for miR-1 and miR-133 in a construct using the SFFV promoter to be possible. Furthermore, it was realised SA461 hESC were unsuitable for cardiac differentiation, however, using a modified version of the LaFlamme protocol in a monolayer system resulted in beating cells with a cardiomyocyte phenotype in H1 hESC. Despite successful overexpression of miR-1 and miR-133, there was very little effect on cardiac differentiation over no virus control.

Previously published methods for the generation of vascular endothelial cells (EC) have reported varying efficiency and target cell population purity (~3 – 30%). This laboratory recently reported the successful generation of functional EC-like cells from hESC in a feeder-free manner. HESC-EC were analysed by LC Sciences miRNA microarray at early time points day 0, day 2, day 4 and day 10

after initiation of differentiation with time-matched pluripotent controls. An induction of miR-99b, -181a and -181b over time was observed, and validated in H1 hESC. In addition, miR-99b, -181a and -181b were also found to be expressed in other mesodermal cell types including adult human saphenous vein endothelial cells (HSVEC). No statistically significant expression of these miRNAs could be found in representative cell types of ectoderm and endoderm germ layers, therefore it was hypothesised that these miRNAs were largely mesoderm specific. Despite initial data showing a significant difference in expression between HSVEC from control patients and patients undergoing coronary artery bypass grafting (CABG), classical pathophysiological stimuli to cause endothelial cell stress did not change the expression of miR-99b, -181a and -181b *in vitro*.

In order to understand more about gene expression in early lineage commitment, hESC-EC were analysed by Illumina microarray at early timepoints day 0, day 2, day 4 and day 10 after initiation of differentiation with time-matched pluripotent controls. In parallel, primary human saphenous vein endothelial cells (HSVEC) were analysed. Illumina technology permitted whole-genome profiling in a high throughput chip format. Due to overall expression levels being lower intensity than expected, no cut-off of fold-change was applied to the dataset. Analysis of the dataset showed a large number of significantly differentially expressed probes at each time point: Day 2 of endothelial differentiation compared to Day 0 pluripotent control showed 1040 significant differentially expressed probe changes, Day 4 of endothelial differentiation compared to Day 0 pluripotent control showed 2400 significant differentially expressed probe changes and Day 10 of endothelial differentiation compared to Day 0 pluripotent control showed 2157 significant differentially expressed probe changes (all False Discovery Rate <0.05). Although significant downregulation of pluripotency markers were observed, few endothelial associated genes were present at hESC-EC day 10. Analysis of HSVEC compared to hESC-EC Day 10 reveals 6133 significantly differentially expressed probes (FDR <0.05). This suggests that although day 10 hESC-ECs have previously been shown satisfy criteria for endothelial cells in *vitro* and *in vivo*, their transcriptional profiling demonstrates that they remain different in comparison to adult ECs.

A transient induction of several transcription factors was observed at hESC-EC day 2, accounting for some 10% of gene changes at this time point. We

hypothesised that these transcription factors may play key roles in the early mesoderm/EC commitment process. Of these, FOXA2, a transcription factor not previously associated with mesoderm or EC commitment, was upregulated, and this was further validated by both qRT-PCR and ICC in SA461, H1 and RC10 hESC lines. In addition to gene expression data, an *in silico* prediction of gene epigenetic status was made using a previously published chromatin immunoprecipitation sequencing (ChIP-SEQ) dataset performed in H9 hESC. Approximately 3000 genes are bivalently marked, meaning they have both H3K4me3 active chromatin and H3K27me3 repressive chromatin at their transcriptional start sites (TSS). This conveys a poised state, with the potential for the gene to be rapidly activated and/or repressed. Of these bivalent genes it was noted that FOXA2 was marked as being potentially bivalent. Upon further investigation using ChIP it was revealed that FOXA2 carried both H3K4me3 and H3K27me3 chromatin modifications in the TSS region, both in H9 and SA461 pluripotent hESC. It was hypothesised that epigenetic modification was responsible for the dynamic expression of FOXA2, although this hypothesis remains to be investigated further. Lastly, several of the miRNA targets for miR-99b, miR-181a and miR-181b were downregulated by hESC-EC day 10 compared to hESC-EC day 0, although whether these targets play a role in refining differentiation to EC warrants further investigation.

In summary, a range of molecular biology techniques were employed to investigate the master control of hESC differentiation. These studies have contributed to existing knowledge on mesodermal and cardiovascular lineage specification. They provide evidence to support the continued in depth investigation of these processes in order to develop a clinically relevant cell therapy for ischaemic diseases.

Chapter 1: Introduction

1.1 Cardiovascular Disease

Worldwide, the leading cause of non-infectious morbidity is cardiovascular disease (CVD) (http://www.who.int, 2009). The term cardiovascular disease encompasses diseases of the heart and circulatory systems, including coronary heart disease (CHD (associated with MI)) and peripheral artery disease (PAD). Atherosclerosis, a disease process where cholesterol accumulates in atheroma plaques on blood vessel walls is the primary cause of CVD including CHD and PAD.

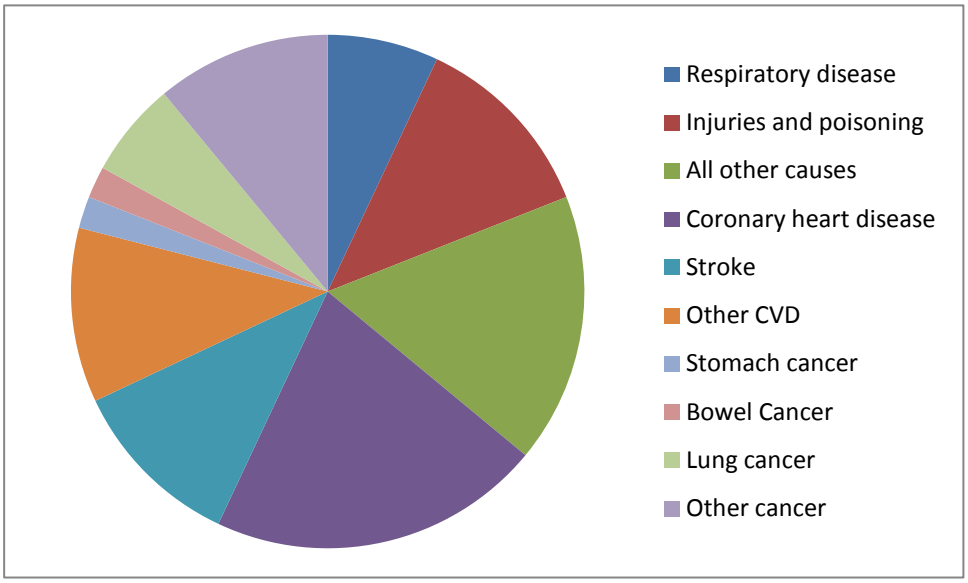
Coronary heart disease involves the coronary arteries becoming narrowed due to the presence of cholesterol rich atheroma plaques on the vessel walls. This leads to reduced blood flow to the heart and causes the symptoms of angina. CHD and stroke are responsible for the highest percentage of deaths worldwide (http://www.who.int, 2009). CHD has the highest mortality rate in the developed world compared to any other disease, including cancers (http://www.bhf.org.uk, 2008). In Europe 21% of all deaths in men (Figure 1.1A) and 22% of all deaths in women (Figure 1.1B) can be attributed to CHD. Approximately 2.5 million people are living with CHD in the UK, accounting for around 94,000 deaths per year (http://www.bhf.org.uk, 2008).

When an atherosclerotic plaque within a coronary artery is acutely disrupted it can completely occlude the vessel, causing ischaemia and often, myocardial infarction (MI). The resulting ischaemic damage to the heart vasculature and muscle causes survivors to suffer from negative cardiac remodelling, including tissue necrosis and formation of non-contractile scar tissue, resulting in debilitating cardiac insufficiency. The current best practice guidelines promote prompt revascularisation via administration of fibrinolytic drugs, percutaneous coronary intervention (PCI), or in more severe cases coronary artery bypass graft (CABG). The effectiveness of these treatments is largely based upon a rapid access to medical help. Optimal door to needle time for administration of fibrinolytic therapy is within 30 minutes and door-to-balloon time to be within 90 minutes (Members et al., 2004, Brodie et al., 2001). Achieving treatment within these timescales remains challenging. While procedural revascularisation reduces the risk of further infarction and ischaemia, it does nothing to heal injured tissues, reverse the ventricular dysfunction already established or

restore the substantial cardiomyocyte deficit. As many as 1 billion cardiomyocytes can be damaged as the result of a major MI (Murry et al., 2006). This leads to an inflammatory response in which macrophages remove the damaged cells, which are replaced with scar tissue. Pharmacological intervention does go some way to lessen symptom severity, however cannot stall the inevitable progression to congestive heart failure, for which the median survival without intervention is 1.7 years in men and 3.2 years in women (Lloyd-Jones, 2001).

In addition, peripheral arterial disease (PAD) is a marker of systemic atherosclerosis, in which build up of atheroma in arteries supplying the leg muscles can lead to critical limb ischaemia (CLI). PAD affects 4-12 % of people aged between 55 and 70 years of age, and 20% of those over 70 years of age worldwide (Robless et al., 2007). Patients with PAD suffer from a profound reduction in functional mobility and balance capacity, depression and overall poor quality of life (Ruo et al., 2007, McDermott et al., 2011). In addition, patients with PAD have the same risk as patients with CAD of death from cardiac or cerebrovascular causes (Hirsch et al., 2006). Currently, the management of PAD includes smoking cessation, strict control of diabetes (if present), control of hypertension (if present), control of cholesterol levels, exercise therapy, anti-platelet therapy and surgical or endovascular revascularisation using balloon angioplasty, stent placement or bypass surgery. Success of these treatments can be limited by restenosis after angioplasty or bypass surgery or by a lack of suitable vessels to perform the bypass. If these strategies fail then major amputation is often the only option left, which occurs in approximately 30% of all incidences of severe limb ischaemia (Winitsky et al., 2005, Bradbury et al., 2005). Therefore regenerative medicine therapies could allow new strategies for revascularisation of ischaemic limbs, with the goal being to stimulate new vessel formation.

Regenerative medicine can be defined as focus on the “repair, replacement or regeneration of cells, tissues or organs” (Mason and Dunnill, 2007) and has seen a surge in popularity in recent years. If such technology achieves its full potential, an alternative cell-based regenerative treatment for cardiovascular diseases would be extremely attractive.



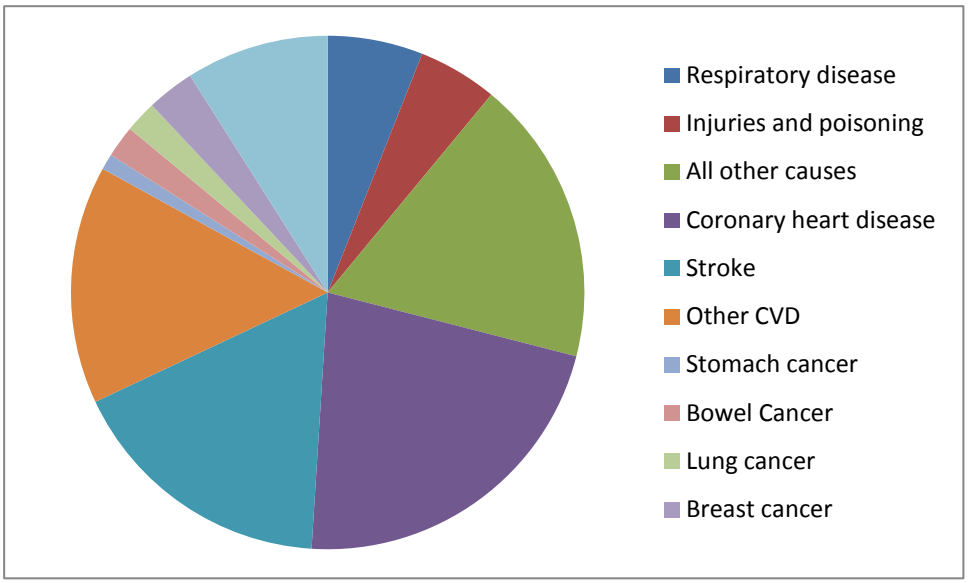


Figure 1.1: All cause European deaths in 2009.

All cause mortality in 2009 in Europe for men (A), women (B). Source: BHF.

1.2 Cardiovascular regenerative medicine

1.2.1 Intrisic regeneration

Unlike tissues such as skeletal muscle or liver, the postnatal human heart has very little (if any) capacity for regeneration after damage. A key goal of cardiovascular regenerative medicine is to repopulate the ischaemically damaged area with functional cardiomyocytes and vascular endothelial cells, in

order to effectively increase the functionality of the damaged heart as demonstrated in animal models (Segers and Lee, 2008).

The candidate cell population for these regenerative strategies would need to have several key properties; it must have myogenic and/or angiomyogenic potential to replace the elements of tissues that have been lost, be easily produced in large quantities, have high survival rates once transplanted and be non immunogenic, preferably useable without the immunosuppressive drug regimes in force for donor transplant.

The regeneration of the heart has remained a controversial yet intensely investigated area for over 150 years. Replacement of lost myocytes to improve cardiac function is a key aim for cell therapy. Similarly, improvement in blood flow in tissue ischaemia, both cardiac and peripheral, is an important endpoint for cell therapy strategies.

It seems possible that the mammalian heart has some endogenous regenerative potential, but that it remains sub-optimal for repair of the damage caused by cardiovascular complications (Winitsky et al., 2005, Jackson et al., 2001, Oh et al., 2004).

Although amphibious, zebrafish have been extensively used as a model organism for heart regeneration due to its amenability to genetic modification and capacity for cardiac regeneration (Poss et al., 2002). In landmark studies involving surgical amputation of the cardiac apex (which accounts for approximately 20% of the ventricular mass) zebrafish were able to completely regenerate the area, initially with a fibrin/collagen clot which was then replaced steadily with new, functional heart tissue within 30 days (Jopling et al., 2010). In transgenic zebrafish where all pre-existing cardiomyocytes were labelled GFP+ by tamoxifen treatment, it was shown that the new tissue was also GFP+ and was therefore derived from division of existing cardiomyocytes and not from a progenitor population as was previously hypothesised (Kikuchi et al., 2010, Jopling et al., 2010).

Limited cardiac regeneration has also been documented in rodent studies (Soonpaa and Field, 1998). In order to ascertain whether cardiomyocyte renewal

was due to existing cardiomyocyte division similar to zebrafish, or the result of migratory cardiac progenitor stem cells, studies using labelled cardiomyocytes in transgenic adult mice were performed. GFP was only expressed in cardiomyocytes with 83% successfully labelled (Hsieh et al., 2007). After 1 year of normal aging the percentage of GFP+ cardiomyocytes remained the same, however after MI the percentage dropped to 67.5% GFP+ cells in areas around the infarction (Hsieh et al., 2007). This would suggest the regeneration of these areas was not the result of existing cardiomyocytes dividing, but that a progenitor/stem cell population was responsible.

Recent studies performed in neonatal mice have highlighted the fundamental differences between cardiac regeneration in zebrafish and mammals. There are important differences between fish and mammalian cardiac structure with the 4 chambered double circulation heart of mammals working at high pressure compared to the 2 chambered single circulation fish heart, which more closely resembles the mammalian heart before septation in the embryo (Porrello et al., 2011). Researchers hypothesised that due to the similarities between the embryonic mammalian heart and the adult zebrafish heart that the mechanisms of regeneration might be conserved (Porrello et al., 2011). Resection of ~15% of the embryonic mouse heart of 1 day old neonatal mice resulted in a similar zebrafish-like process being observed; complete regeneration beginning with a clot which is gradually replaced with functional cardiac tissue derived from existing cardiomyocytes and full function appearing to be restored by 21 days (Porrello et al., 2011). However, performing this procedure in 7 day old neonates did not induce cardiac regeneration, but instead resulted in significant fibrosis and scar formation. Therefore, the ability of the neonatal mammalian heart to regenerate was lost within the first week of life (Porrello et al., 2011). Many questions remain, for example whether the loss of regeneration is due to adult cardiomyocytes intrinsic withdrawal from cell cycling or a loss of mitogenic cell signals in the adult heart.

Cardiac regeneration in the adult human remains difficult to study and a contentious subject area, with a variety of techniques attempted to overcome the many technical difficulties. Studies using direct measurement of DNA synthesis or expression of markers associated with proliferation, pulse-chase experiments and measurement of ^{14}C levels (caused by nuclear bomb tests in the

1950's incorporated into DNA) as an indication of cell turnover appear to suggest that human cardiomyocytes do divide, albeit at low levels and that this is increased post-MI (Bergmann et al., 2009). However, the rate at which this turn over occurs depends on the experimental technique used, ranging between complete replacement every 4.5 years to ~50% of cells being replaced over a lifetime (Steinhauser and Lee, 2011). Regardless of the most accurate estimates, the rate of innate regeneration is not sufficient to repair damage after MI. Clearly, novel interventions are therefore required.

The physiological response to PAD involves vascular remodelling of the collateral blood vessels. In a process termed arteriogenesis, the diameter of the pre-existing vessels in an effort to restore perfusion to the ischaemic area and is thought to be stimulated by shear stress (van Royen et al., 2001). Angiogenesis is the process of new capillary formation within a tissue and is thought to be stimulated by ischaemia (Hershey et al., 2001) This effect is blunted in those with diabetes as a co-morbidity, which accounts for a substantial proportion of those affected (Spinetti et al., 2008) and often is inadequate to rescue ischaemic tissue, hence the requirement for new therapeutic strategies.

1.2.2 Cardiovascular cell therapy

Use of cell therapy in cardiovascular regenerative medicine remains an attractive prospect and substantial work is ongoing worldwide. The main goals of a cell therapy strategy for heart disease and PAD remain prompt restoration of blood supply to the affected areas in order to salvage ischaemic tissue and/or regeneration of tissues previously lost to ischaemia.

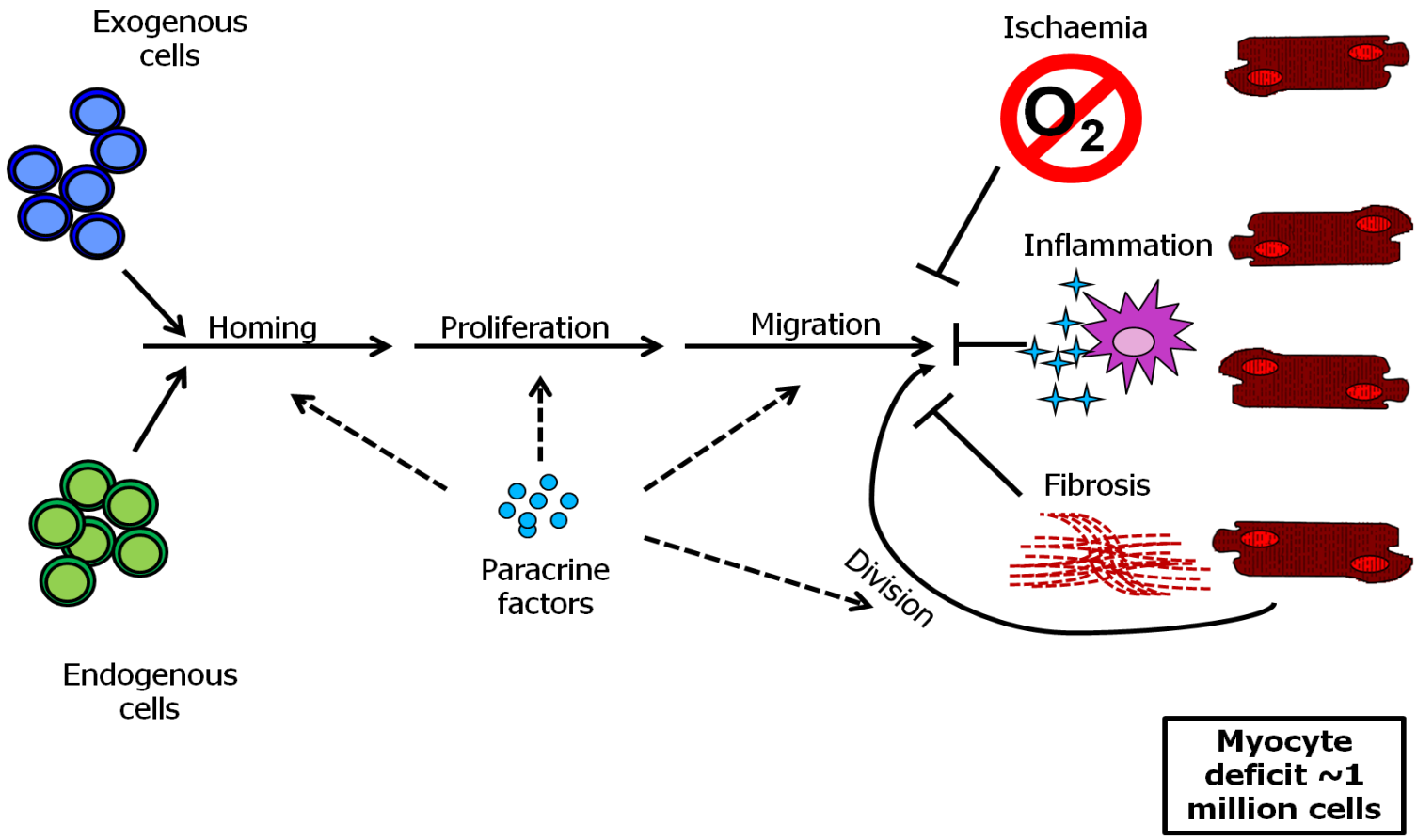


Figure 1.2: Schematic representation of cardiovascular cell therapy, and the major barriers to success.

The hostile environment that any cell therapy population will face upon introduction after MI or CLI, including ischaemia, immune system activation and inflammation and extensive scarring. It is not yet known whether the cells themselves will elicit a regenerative response or if paracrine factors produced by the cells will exert any benefit seen. Adapted from (Steinhauser and Lee, 2011).

1.2.2.1 Skeletal myoblasts

One of the first regenerative strategies to improve myocardial ischaemia involved direct injection of autologous skeletal myoblasts (SKM) (Menasche, 2007). Although not able to differentiate into cardiomyocytes (Laflamme and Murry, 2005), myoblasts are contractile, can be harvested for autologous transplantation, are resistant to ischaemia, able to differentiate to myotubes *in vivo* and can improve ventricular function in animal models (Menasche, 2007). However they do not couple electrically to the existing cardiomyocytes and therefore beat asynchronously (Menasche, 2007). Clinical trials have focused on chronic ischaemic cardiomyopathy rather than post MI due to the need for autologous SKM to undergo expansion *ex vivo* before being used therapeutically. However, although good safety data was observed there was an increase in arrhythmia events in the phase 1 clinical trial (Menasché et al., 2003). Upon further investigation little benefit was observed in the prospective randomised placebo-controlled phase 2 trial (MAGIC) in patients with chronic ischaemic cardiomyopathy, with the trial discontinued prematurely (Menasché et al., 2008). In mouse skeletal muscle a population of cells that can give rise to spontaneously beating cells resembling cardiomyocytes has been identified (Winitsky et al., 2005) however, no such population of cells have been described in the human. As such, it is unlikely that skeletal myoblasts are the most effective cell population to utilise in cell therapy for human CVD.

1.2.2.2 Bone-marrow-derived cells

Early studies reporting transdifferentiation of bone-marrow-derived haematopoietic stem cells to cardiomyocytes highlighted the regenerative potential of bone-marrow derived cells (Orlic et al., 2001). It has since been reported that upon transplantation, haematopoietic stem cells do not differentiate into cardiomyocytes but become mature blood cells (Balsam et al., 2004, Murry et al., 2004a) and elicit no regenerative effect on the myocardium (Figure 1.3). Despite these findings, a number of clinical trials involving bone marrow derived stem cells as therapy for MI are in progress. Results from the clinical trials suggest that delivery of bone marrow derived stem cells is safe, but the clinical benefits have been subtle with left ventricular ejection fraction

increased by no more than ~3%, below the minimum change of ~5% thought to be required to improve quality of life and survival for the patient (Abdel-Latif et al., 2007). Other measurements of infarct remodelling or exercise capacity seemed to be positively affected by bone marrow stem cell therapy, suggesting that bone marrow derived-stem cells are acting in a paracrine fashion to salvage ischaemic areas of the heart by secretion of angiogenic growth factors (Korf-Klingebiel et al., 2008). *In vitro* studies designed to interrogate this paracrine effect further showed that media pre-conditioned on hypoxic and genetically modified Akt+ mesenchymal stem cells inhibits apoptosis and can stimulate rat cardiomyocytes to contract (Gnecchi et al., 2006). When the conditioned media was injected into a rat model of MI, infarct size and LV function was measurably improved within 72 hours (Gnecchi et al., 2006). This was considered to be due to the presence of several protein including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) being upregulated in hypoxic Akt-MSCs, as these factors could mediate the beneficial effects shown (Gnecchi et al., 2006).

In 2002, the first small clinical trial using autologous bone-marrow mononuclear cells (BMMNC) in CLI demonstrated an improvement in ankle-brachial index (ABI), transcutaneous oxygen pressure ($TcPO_2$), rest pain and pain-free walking distance at both 4 and 24 weeks post treatment. In contrast, those injected with peripheral blood mononuclear cells showed no improvement (Tateishi-Yuyama et al., 2002). This promising result stimulated great interest in the therapeutic angiogenic capacity of a readily available autologous cell source. Recent clinical trials performed with bone marrow derived cells can be found in Table 1.1. Bone marrow progenitor cell therapy remains an interesting prospect, and further work to investigate the benefits of these treatments in the long term is essential.

Table 1.1: Studies performed on Bone marrow mononuclear cells for PAD.

Clinical trials performed using BMMNC in patients with PAD and/or CLI. Adapted from (Lawall et al., 2010). ABI = Ankle brachial index, $TcPO_2$ = trans cutaneous partial pressure of oxygen, NM = not mentioned, + = increase, - = decrease, +/- = no change.

Year	Reference	Study Type	Number of Subjects	ABI	$TcPO_2$	Pain	Amputation	+/- overall result
2002	(Tateishi-Yuyama et al., 2002)	Randomised, non-blinded controlled trial	45	+	+	-	-	+
2002	(Esato et al., 2002)	Patient series/uncontrolled trial	8	+	NM	-	-	+
2004	(Saigawa et al., 2004)	Patient series/uncontrolled trial	8	+	+	-	-	+
2004	(Higashi et al., 2004)	Patient series/uncontrolled trial	8	+	+	-	-	+
2004	(Miyamoto et al., 2004)	Patient series/uncontrolled trial	12	+	NM	-	NM	+
2005	(Nizankowski et al., 2005)	Patient series/uncontrolled trial	10	+	+	-	-	+
2006	(Durdu et al., 2006)	Randomised, non-blinded controlled trial	28	+	+	-	-	+
2006	(Bartsch et al., 2006)	Patient series/uncontrolled trial	10	+	+	NM	NM	+
2006	(Miyamoto et al., 2006)	Patient series/uncontrolled trial	8	NM	NM	-	NM	+
2007	(Kajiguchi et al., 2007)	Patient series/uncontrolled trial	7	+/-	+	-	?	+/-
2007	(Huang et al., 2007)	Controlled trial	74	+	+	-	NM	+
2007	(Hernández et al., 2007)	Patient series/uncontrolled trial	12	+	+	-	-	+
2008	(Gu et al., 2008)	Patient series/uncontrolled trial	16	+	+	-	-	+
2008	(Chochola et al., 2008)	Patient series/uncontrolled trial	28	+	+	-	-	+
2008	(Wester et al., 2008)	?	8	NM	NM	-	-	+
2008	(Van Tongeren et al., 2008)	Patient series/uncontrolled trial	27	+	+	-	?	+
2008	(Vriese et al., 2008)	Patient series/uncontrolled trial	16	+/-	+	-	?	+/-
2009	(Amann et al., 2009)	Patient series/uncontrolled trial	51	+	+	-	-	+
2009	(Prochazka et al., 2009)	Patient series/uncontrolled trial	37	+	+	-	NM	+

1.2.2.3 Endothelial progenitor cells

In a landmark study by Asahara *et al.* in 1997, it was reported that a sub-population of bone marrow derived cells have the ability to differentiate into endothelial cells and as such are termed endothelial progenitor cells (EPCs) (Young et al., 2007, Asahara et al., 1997). However, there is a lack of consensus as to the definition of an EPC, with more detailed characterisation required. CD34+ haematopoietic cells were shown to give rise to cells expressing endothelial markers both *in vitro* and *in vivo* (Asahara et al., 1997). As CD34 is also expressed on endothelial cells, other groups have used CD133 as a marker for EPCs (Gehling et al., 2000). However, further investigation called into question the suitability of these markers when another group were not able to derive endothelial-like cells from progenitors that were CD34+CD133+KDR+ (Case et al., 2007). In addition, when this cell population was subjected to chromatin immunoprecipitation (ChIP) analysis, it was found that the transcriptional start site of endothelial nitric oxide synthase (eNOS), a gene which appears to be important in EPC mediated functional vascular improvement, was populated by repressive histone modifications suggesting that this population of cells are not solely fated to give rise to endothelial progenitors (Ohtani et al., 2011). Alternatively, several groups have previously shown several different CD34+ or CD133+ populations to possess the ability to express endothelial genes and form vessel-like structures both *in vitro* or *in vivo* (Kawamoto et al., 2006, Kocher et al., 2001, Masuda et al., 2011). Furthermore, in the zebrafish model, human CD34+ cells contributed to the embryonic vasculature when injected pre-gastrulation, and when injected post-gastrulation were recruited into newly-forming vessels (Pozzoli et al., 2011). Further studies involving a subpopulation of CD34+ cells which co-express KDR, reported that these cells gave rise to a higher incidence of EPCs (Madeddu et al., 2004). In contrast CD133+CD34-KDR+ cells were found to be more potent in vascular repair, but appeared to be an immature cell type which could further differentiate into CD133+CD34+KDR+ cells (Friedrich et al., 2006). CD45 is expressed at a low level on 90% CD34+ progenitor cell types, and it was previously reported that it is those cells types which remain CD45- which give rise to EPCs, forming endothelial-like structures *in vitro* (Case et al., 2007). Whilst these cells have not been shown to differentiate into cardiomyocytes *in vivo*, it is suggested they play a role in promoting angiogenesis (Rubart and Field, 2006, Young et al., 2007) both by

directly contributing to the vasculature and by having a paracrine effect on cardiomyocyte survival (Narmoneva et al., 2004). However, EPCs isolated from patients with diabetes or hypertension display a reduction in their capacity for endothelialisation of denuded arteries and blood flow recovery after ischaemia when introduced into immunocompromised mice (Giannotti et al., 2010, Landmesser et al., 2004, Sorrentino et al., 2007) meaning that the potential of EPCs for cardiac regeneration or revascularisation in PAD may be hampered by existing co-morbidities in patients.

1.2.2.4 Cardiac progenitor cells

The discovery of cardiac progenitor cells (CPCs) have been reported by several different groups (Beltrami et al., 2003, Peng et al., 2011). CPC populations appear to express different markers suggesting multiple CPC types or CPCs at different stages of maturation being isolated and studied, that would perhaps converge given the correct stimuli (Pfister et al., 2008). C-KIT expressing CPCs have been studied in greater detail than others (Laflamme and Murry, 2011). C-KIT is expressed on mature cells such as haematopoietic stem cells and mast cells, and immature endothelial and cardiomyocytes (Tallini et al., 2009). CPCs expressing c-KIT were discovered in the perivascular compartment of the adult heart and following isolation from both rat and human hearts, have given rise to cardiomyocytes, smooth muscle cells and endothelial cells (Bearzi et al., 2007). Studies have also indicated that upon transplantation in the rat, c-KIT+ CPCs can induce regeneration in the infarcted heart with myogenesis and angiogenesis reportedly being stimulated in the damaged area (Bearzi et al., 2007). A phase I clinical trial to test the safety, feasibility and efficacy of this treatment in humans is currently underway (SCIPIO, ClinicalTrials.gov identifier NCT00474461) (Bolli et al., 2011). Researchers recruited patients with post-MI left ventricular dysfunction (LVEF) classified as ejection fraction of less than 40% before coronary artery bypass grafting and 1 million autologous CPCs were administered by intracoronary infusion at a mean of 113 days post surgery (Bolli et al., 2011). Control patients were given no treatment. Interim analyses performed at 4 months after 14 patients received CPC treatment showed an increase in LVEF from 30.3% to 38.5% ($p<0.001$) with no change in 7 control patients over the same time period (Bolli et al., 2011). Infarct size in 7 treated patients on whom cardiac MRI could be performed was reduced by 24% at 4 months and 30% at 1

year. Importantly, there were no CPC related adverse effects reported (Bolli et al., 2011).

Other CPC populations currently under investigation include cardiosphere-forming cells, so-called for their ability to migrate out of cardiac tissue in culture and form spheroids in suspension culture (Chimenti et al., 2010, D'Amario et al., 2011). These cells are a mixed population with some expressing c-KIT. This population has been reported to be able to differentiate to cardiomyocytes both *in vitro* and *in vivo* following transplantation (Chimenti et al., 2010). An improvement in cardiac function post MI was also observed (Chimenti et al., 2010). A phase I clinical trial using CPC cardiospheres forming cells after myocardial infarction enrolled patients 2-4 weeks post-MI, and infused autologous CPC's into the affected artery 1.5-3 months post-MI. They recently reported that CPC treatment was safe and resulted in reduction of scar mass, increase in viable heart mass and regional contractility however end-diastolic volume, end-systolic volume and EF did not differ by 6 months (Makkar et al., 2012).

Although the recent findings in the CPC field are encouraging, there is a lack of research on the endogenous role of CPCs in development, normal physiology and ageing, with most of the studies performed involving transplantation and alleviation of disease (Laflamme and Murry, 2011).

Table 1.2: Current Cell therapy clinical trials for cardiovascular disease.

Recent clinical trials using cell therapy for cardiovascular disease, including trial identifier, trial name, patient number, cell type used, primary endpoints of the study, route of cell delivery, outcome (if any available) and references.

ID	Trial Name	No. of Patients	Cell Type	Primary End Point	Route of Delivery	Outcome	Ref
NCT00711542	REPAIR-ACS	100	Bone marrow-derived progenitor cells	Coronary flow reserve in the infarct vessel	Intracoronary	Ongoing	
NCT00355186	SWISS-AMI	150	Bone marrow mononuclear cells	LVEF	Intracoronary	No data published	(Sürder et al., 2010)
NCT00984178	TECAM2	120	Bone marrow mononuclear cells	LVEF; Left ventricular end-systolic volume	Intracoronary	No data published	
NCT00350766	EMRTCC	300	Bone marrow mononuclear cells	LVEF	Intracoronary	No data published	(Dohmann et al., 2008)
NCT00684021	The TIME Study	120	Bone marrow mononuclear cells	LVEF	Intracoronary	No data published	(Traverse et al., 2009)
NCT00684060	The Late TIME Study	87	Bone marrow mononuclear cells	LVEF	Intracoronary	Safe but no improvement at 6 months	(Traverse Jh and et al., 2011)
NCT00691834	ReNeW	50	Bone marrow mononuclear cells	LVEF; Occurrence of arrhythmia, heart failure and death	Intracoronary	No data published	
NCT00874354	REVI-TALIZE	30	Bone marrow mononuclear cells	Safety and feasibility; LVEF	Intracoronary	No data published	
NCT00268307	-	60	Bone marrow mononuclear cells	Safety	Intracoronary	Safe and favourable remodelling	(Traverse et al., 2010)
NCT00939042	-	40	Bone marrow mononuclear cells	LVEF	Intracoronary	Ongoing	
NCT00765453	REGEN-AMI	102	Bone marrow-derived progenitor cells	LVEF	Intracoronary	Ongoing	
NCT00437710	CARDIAC	50	Bone marrow-derived stem cells	Mortality; Mortality and Morbidity; Left ventricular function	Intracoronary	No data published	
NCT00275977	-	10	Bone marrow-derived stem cells	LVEF	Intracoronary	No data published	(Wollert et al., 2004)
NCT00529932	SELECT-AMI	60	CD133+ enriched bone marrow cells	Safety; Myocardial thickening in non-viable akinetic/hypokinetic LV wall segments	Intracoronary	Ongoing	

NCT00725738	TRACIA STUDY	80	CD34+ cells	LVEF	Intracoronary	No data published	
NCT00936819	ENACT-AMI	100	Early endothelial progenitor cells	LVEF	Intracoronary	No data published	
NCT00501917	MAGIC	116	Peripheral blood stem cells	LVEF	Intracoronary	No data published	(Kang et al., 2011)
NCT00555828	-	25	Mesenchymal precursor cells	Feasibility and safety	Transendocardial	Ongoing	
NCT00877903	Prochymal®	220	Adult human mesenchymal stem cells	Left ventricular end systolic volume	Intravenous	Ongoing	
NCT00893360	CADUCEUS	30	Cardiosphere-derived stem cells	Safety	Intracoronary	Safe and favourable remodelling	(Makkar et al., 2012)
NCT00326989	Cellwave Study	100	Bone marrow progenitor cells	LVEF	Intracoronary	No data published	
NCT00824005	FOCUS	87	Bone marrow mononuclear cells	Maximal oxygen consumption, left ventricular end systolic volume, reversible defect size	Transendocardial	No data published	(Willerson et al., 2010)
NCT00810238	C-Cure	240	Bone marrow-derived cardiosphere cells	LVEF	Transendocardial	Significantly improved LVEF and 6 min walk test	Reported at BCS conference 2011.
NCT00690209	-	30	Bone marrow-derived stem cells	Left ventricular columns and contractility	Transepicardial during CABG	Ongoing	
NCT00418418	-	60	Bone marrow-derived stem cells	LVEF	Transepicardial during CABG	No data published	
NCT01049867	-	10	CD133+ Endothelial precursor cells	Regional and global myocardial contractility	Intracoronary	Ongoing	
NCT01033617	IMPACT-CABG	20	CD133+ bone marrow stem cells	Freedom from major adverse cardiac event; Freedom from major arrhythmia	Transepicardial during CABG	Ongoing	
NCT00950274	PERFECT	142	CD133+ bone marrow stem cells	LVEF	Transepicardial during CABG	Ongoing	
NCT00462774	Cardio133	60	CD133+ marrow cells	LVEF	Transepicardial during CABG	Significantly improved LVEF	(Stamm et al., 2007)
NCT00346177	-	30	CD34+ cells	Safety, LVEF, heart failure symptoms	Transendocardial	Withdrawn due to lack of funding	

NCT00620048	-	10	CD34+ cells	Safety, LVEF, heart failure symptoms	Transendocardial	Withdrawn due to lack of funding	
NCT00221182	-	10	CD34+ cells	Myocardial perfusion abnormality/Safety		Withdrawn due to lack of participants	
NCT00721045	-	60	Mesenchymal precursor cells	Safety and feasibility	Transendocardial	No data published	
NCT01076920	MESAMI	10	Mesenchymal stem cells	Safety and feasibility	Transendocardial	Ongoing	
NCT01087996	The POSEIDON-Pilot Study	30	Bone-marrow derived mesenchymal stem cells	TE-SAE	Transendocardial	No data published	
NCT00768066	TAC-HFT	60	Mesenchymal cells/Bone marrow cells	TE-SAE	Transendocardial	No data published	(Trachtenberg et al., 2011)
NCT00587990	PROMETHEUS	45	Mesenchymal stem cells	Serious adverse events	Transepicardial during CABG	No data published	
NCT00908622	PERCUTANEO	50	Skeletal myoblasts	LVEF; wall motion score index	Percutaneous Implantation	Ongoing	
NCT00526253	MARVEL	390	Skeletal myoblasts	6 minute walk test; Quality of Life Questionnaire	Transendocardial	Ongoing	
NCT00474461	SCIPIO	40	Cardiac stem cells	Safety/Efficacy Study	Intracoronary	Ongoing	(Bolli et al., 2011)
NCT00981006	ALCADIA	6	Cardiac-derived stem cells	Safety	Transepicardial during CABG	Ongoing	

1.2.2.5 Human embryonic stem cells

Derived from the Inner Cell Mass (ICM) of an embryo at the blastocyst stage (Thomson et al., 1998, Reubinoff et al., 2000, Shamblott et al., 1998), human embryonic stem cells (hESC) have been proposed as a potential source of functional, transplantable cells for a variety of cardiovascular-related applications including repair of infarcted myocardium, improvement in cardiac function and induction of angiogenesis in ischaemic tissues. This broad potential is due to the capacity of hESC for self-renewal, scale-up and ability to become any cell type in the human body (Evans and Kaufman, 1981, Reubinoff et al., 2000, Thomson et al., 1998). The ability of hESC to undergo indefinite self-renewing division, retain a normal diploid karyotype and remain pluripotent makes a powerful tool in regenerative medicine. Early studies demonstrated that directing differentiation to bias for the cell type of choice was possible, with early studies being published for neural generation (Carpenter et al., 2001, Reubinoff et al., 2001, Zhang et al., 2001), cardiomyocyte generation (Xu et al., 2002, Kehat et al., 2001).

Recently, hESC have been approved for use in phase I clinical trials, with Geron conducting the first ever trial in patients with severe spinal cord injury (2009). The trial was halted by the FDA when safety concerns were raised over benign tumours found in animal models following treatment, however extensive in vivo characterisation demonstrated the therapy was safe enough to continue, and patients began to be recruited in October 2010. Disappointingly, in November 2011, Geron discontinued the trial citing financial reasons (Scott and Huggett, 2012). The 4 patients already enrolled will be followed up as per the original study design, however as yet no data has been published and it is thought the numbers remain too low to be able to demonstrate efficacy.

In addition, phase I clinical trials using hESC therapy for the eye disorders Stargardt's macular dystrophy and dry age-related macular degeneration were initiated by ACT (NCT01345006 and NCT01344993) (Schwartz et al., 2012). Preliminary findings from the first 2 patients to receive the treatment indicate that hESC-derived retinal pigment epithelial cells (RPE) transplanted attached and persisted during the study, and no adverse effects such as abnormal cell growth or immune rejection were present in the first 4 months (Schwartz et al.,

2012). Furthermore, there was no detrimental effect on the patients existing vision, and slight improvement in vision was observed in both patients, although more so in the patient with Stargardt's macular dystrophy who went from being able to see hand motions, to being able to count fingers (Schwartz et al., 2012). These results appear promising, however clearly many more patients require to be tested and with longer follow-up times.

Alternate sources of "stem cells", produced via the ability to reprogram differentiated somatic cells back into a pluripotent stem cell-like state (induced Pluripotent Stem Cells, iPSC) by exogenous expression of transcription factors such as Kruppel-like factor 4 (Klf4), v-Myc myelocytomatosis viral oncogene homolog (avian) (C-Myc), POU class 5 homeobox 1 (POU5F1, Oct4), SRY (sex determining region Y)-box 2 (Sox2), Lin-28 homolog A (Lin28) and homeobox transcription factor Nanog (Nanog) has been extensively reported and reviewed (Lai et al., 2011, Takahashi and Yamanaka, 2006, Yu et al., 2007). Collectively, hESC and iPSC offer substantial potential for regenerative medicine.

Safety concerns with using hESC have largely limited the number of clinical trials and difficulties with licensing a therapy for use in humans. Steps should be taken to genetically profile the hESC line used to minimise risk of conferring genetic mutations (Hovatta, 2011). In addition, cell culture protocols should be good manufacturing process (GMP) compliant and xeno-free, however this often results in differentiation efficiency being reduced (Sidhu et al., 2008). Furthermore, adequate purification measures to ensure no contaminating pluripotent cells are left in the population are crucial to reduce the risk of teratoma formation, such as FACS or MACS (Tang et al., 2011). As with most transplanted tissues, there is a risk of immune rejection (Zhao et al., 2011). Steps can and have been taken to address these safety issues, however, these technologies should be developed towards the clinic with thorough attention to detail, and due caution.

1.2.2.5.1 Pluripotency

The self-renewing and pluripotent potential of these cell types represent complex biological processes, with stringent regulatory processes in place that govern cell phenotype. During development, pluripotency in embryonic cells is

transient, existing for a brief duration. Shortly after embryogenesis initiates, cells become “fated” to form the extra-embryonic tissues or pluripotent progenitors that give rise to the 3 germ layers, from which all foetal tissues are derived. It is these cells which, if isolated and cultured, remain indefinitely pluripotent in long term culture (Thomson et al., 1998). However, the phenotype of these cells can vary, with some differentiation bias seen. The shift from pluripotency to a cell differentiation program involves the inhibition of self-renewal in order for the cell to commit to a specific lineage. The mechanisms controlling this shift remain incompletely understood. HESC differentiation to specific lineages is controlled by complex transcriptional activation of gene networks. Concomitantly, regulation in the genome to suppress expression of genes driving towards a differentiated cell type is required to maintain pluripotency. This is governed by networks including transcriptional activation/suppression, epigenetic regulation at the genome level as well as post-transcriptional regulation via non-coding RNA molecules including miRNAs (Smale, 2010, Kashyap et al., 2009). To maintain pluripotency, a core set of pluripotency factors, Oct4 (encoded by *Pou5f1*), Sox2 and Nanog are essential. Their importance was made apparent from several studies; disruption of Oct4 through *Pou5f1* knockdown resulted in blastocyst stage embryos, however the ICM of the embryos were not pluripotent and could only give rise to extraembryonic trophoblast lineages (Nichols et al., 1998), knockdown of Nanog results in nonviable embryos unable to reach epiblast stage (Silva et al., 2009) and knockdown of Sox2 also resulted in embryonic lethality (Avilion et al., 2003). Although Nanog is not strictly required in order for ESC to proliferate, it promotes a stable pluripotent state and co-occupies most sites with Oct4 and Sox2 in the genome of ESC (Marson et al., 2008, Chambers et al., 2007). These core transcription factors not only occupy and activate essential pluripotency genes, they also collaborate to positively regulate their own promoters (Boyer et al., 2005) (Figure 1.3). This phenomenon of autoregulatory positive feedback explains why overexpression of TF’s can reprogram cells to iPSC, with the exogenously expressed factors initiating the endogenous TF transcription (Jaenisch and Young, 2008).

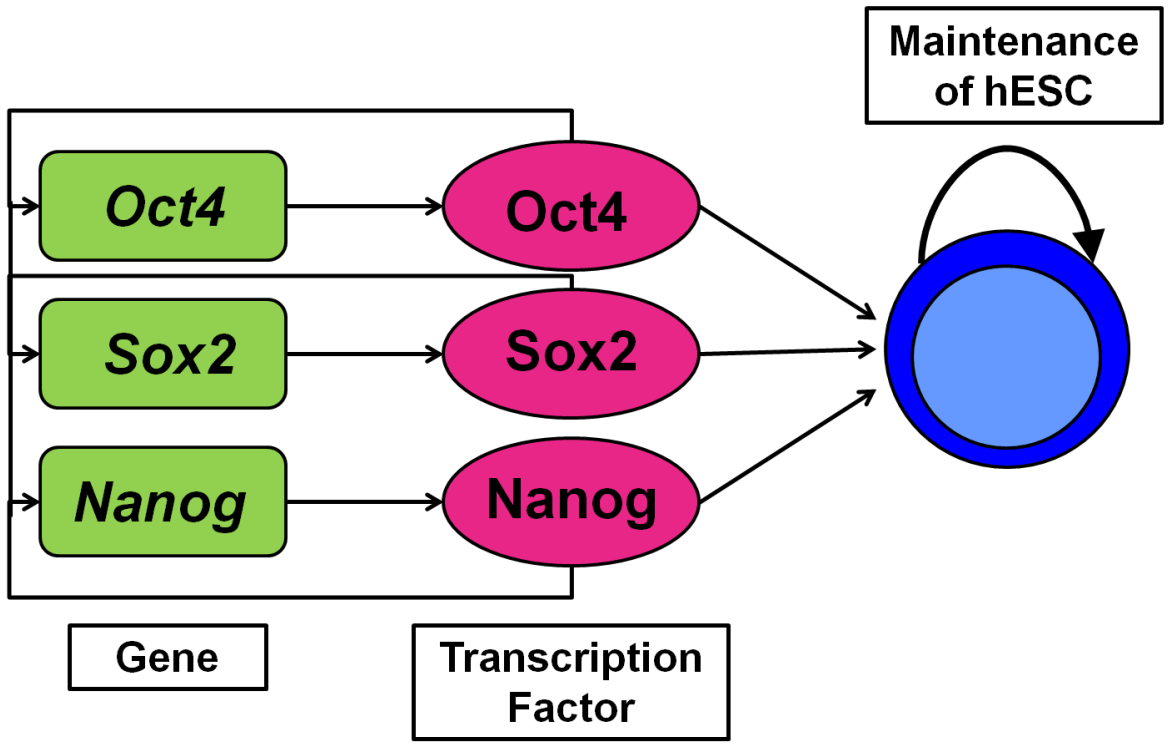


Figure 1.3: Regulation by core pluripotency transcription factors.

Oct4, Sox2 and Nanog transcription factors bind to and cause expression of *Oct4, Sox2* and *Nanog* genes, resulting in more transcription factor expression. These in turn bind to several hESC maintenance genes.

Genome-wide chromatin immunoprecipitation (ChIP) investigation in hESC has shown that Oct4, Sox2 and Nanog can bind or indeed co-bind to transcriptional start sites of several genes including miRNAs, genes involved in epigenetic regulation and genes encoding signalling proteins (Boyer et al., 2005). This suggests that Oct4, Sox2 and Nanog are some of the master regulators of pluripotency and additionally activate themselves and each other in a complex regulatory circuit (Rodda et al., 2005, Boyer et al., 2005).

1.2.2.5.2 HESC cardiomyocyte differentiation

Differentiation methods for hESC lineage commitment to cardiomyocytes shortly followed the initial discovery of hESC (Kehat et al., 2001, Mummery et al., 2003, Xu et al., 2002). Developmental biology studies using mouse and other organisms have allowed a better understanding of the molecular processes at work, with examples of published cardiac differentiation protocols listed in Table 1.2 and an overview of marker acquisition during cardiomyocyte differentiation from pluripotent hESC in Figure 1.4.

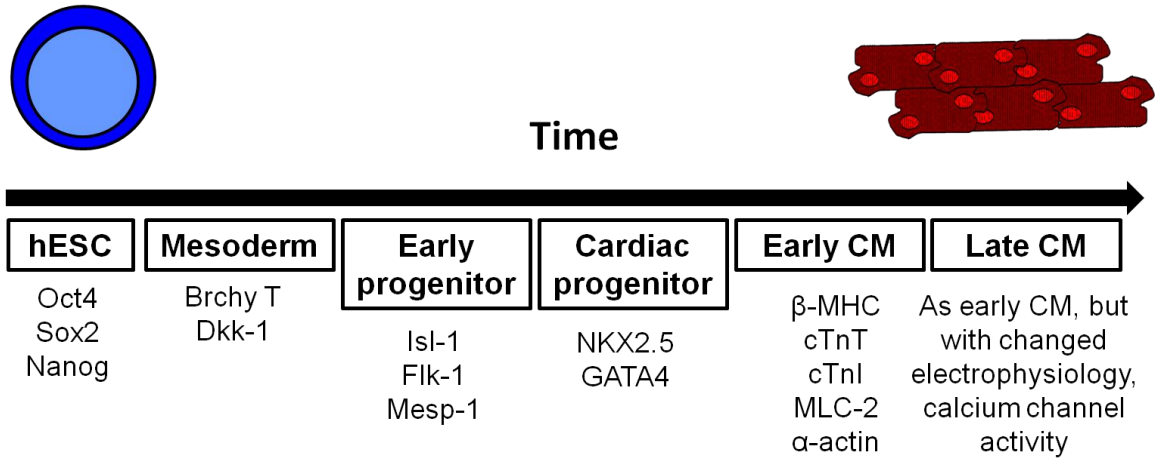


Figure 1.4: Overview of marker acquisition during hESC-CM commitment.

A schematic timeline of hESC-CM differentiation, showing the stages of differentiaton with the markers acquired. Adapted from (Adam Young et al., 2011).

Embryoid body (EB) formation involves allowing hESC in suspension to form cell aggregates, thereby recapitulating early embryogenesis and allowing differentiation to all 3 germ layers, including mesoderm and hence EC, blood and cardiomyocytes (Kehat et al., 2001, Mummery et al., 2003, Xu et al., 2002). EBs can then be partially dissociated and plated out, with some adhering areas spontaneously beating several days later (Laflamme et al., 2007). This typically results in less than 1% of cells becoming cardiomyocytes, and as such further improvements (such as removal of serum and forced aggregation) to this protocol as seen in table 1.2 can greatly increase differentiation efficiency.

Through detailed research of embryonic development in the mouse and other organisms, it was realised that endoderm can play a role in inducing cardiac differentiation (Mummery et al., 2003, Mummery et al., 1991). As such, co-culture of hESC with END2 cells, derived from mouse P19 embryonal carcinoma cells, which are a visceral endoderm-like cell line, has been found to induce cardiomyocyte differentiation, even in those lines that had been unable to differentiate through an EB protocol (Mummery et al., 2003). Indeed, media conditioned upon END2 cells was sufficient to induce differentiation in hESC at approximately a 10% efficiency (Graichen et al., 2008).

Directed differentiation with cells in a monolayer exposed to growth factors has been shown to be successful with just 2 factors; activin A and BMP4 (Laflamme et al., 2007, Takahashi et al., 2007, Xu et al., 2010). Activin A and BMP4 are both members of the TGFβ superfamily, and have established roles in mesodermal development and cardiogenesis in the embryo (Ladd et al., 1998, Schultheiss et al., 1997). Other growth factors shown to have an effect on mesoderm specification and cardiogenesis, include Wnt/β-catenin member Wnt3A and dickkopf homolog 1 (DKK1) (Tran et al., 2009). Wnt3A treatment of day 0-2 EBs increased mesoderm and cardiac differentiation (Tran et al., 2009), and also when Wnt3A was added along with activin A when plating EBs out the resulting differentiations contained as much as 50% cardiomyocytes (Paige et al., 2010). In contrast, addition of DKK1 (which is a canonical Wnt inhibitor) in the early stages of differentiation inhibited cardiogenesis (Tran et al., 2009) although addition at day 5-10 improved differentiation efficiency (Paige et al., 2010).

It is apparent that cardiac differentiation efficiency can be dramatically affected by seemingly small changes in concentration, combination or timing of growth factor treatment, and it is likely that protocols will require some tailoring to individual cell lines.

Table 1.3: Examples of published cardiac differentiation protocols.

Details of published hESC-CM differentiation protocols, listing differentiation style, conditions, growth factor or small molecules used and how many days for, whether serum is included, the approximate percentage of cardiomyocyte-like cells derived and references.

Differentiation	Conditions	Factor/molecule	Duration (days)	Serum	% Cardiomyocytes	References
Embryoid body	Spontaneous			Yes	<1%	Kehat *et al*. 2001., LaFlamme *et al*. 2007.
	Growth Factors	BMP4 (0.5 ng/mL)	0 to 1	No	~40% with low O2 before D12	Yang *et al*. 2008.
		Activin A (3 ng/mL)	1 to 4			
		BMP4 (10 ng/mL)	1 to 4			
		VEGF (10 ng/mL)	1 to 4			
		bFGF (5 ng/mL)	1 to 4			
		DKK1 (150 ng/mL)	4 to 8			
	Growth Factors	BMP4 (1ng/mL)	0 to 1	No	~60%	Kattman *et al*. 2011.
		bFGF (5 ng/mL)	1 to 3			
		Activin A (6 ng/mL)	1 to 3			
		BMP4 (10 ng/mL)	1 to 3			
		DKK1 (150 ng/mL)	3 to 5			
		VEGF (10 ng/mL)	3 to 5			
		SB431542 (5.4 μM)	3 to 5			
		Dorsomorphin (0.25 μM)	3 to 5			
		DKK1 (150 ng/mL)	5 to 7			
		VEGF (10 ng/mL)	5 to 7			
		VEGF (10 ng/mL)	7 onwards			
		bFGF (5 ng/mL)	7 onwards			

	Growth Factors	1-thioglycerol (400 μM)	0 to 2		~95%	Burridge *et al*. 2011
		PVA (4 mg/mL)	0 to 2			
		Hr-insulin (10 μg/mL)	0 to 2			
		BMP4 (25 ng/mL)	0 to 2			
		FGF2 (5 ng/mL)	0 to 2			
		Chemically defined lipids (1%)	0 to 2			
		Y-27632 (1 μM)	0 to 2			
		1-thioglycerol (400 μM)	2 to 4			
		Chemically defined lipids (1%)	2 to 4			
		HSA (5 mg/mL)	2 to 4			
		L-ascorbic acid (280 μM)	2 to 4			
		1-thioglycerol (400 μM)	4 onwards			
		Hr-insulin (10 μg/mL)	4 onwards			
		Chemically defined lipids (1%)	4 onwards			
END2	Co-culture			Yes or No	Higher yield if no serum	Mummery *et al. 2003.*, Passier *et al*. 2005.
	Conditioned medium	SB203580 (5 μM)	0 to 3	No	~20%	Graichen *et al*. 2008
	Defined medium	PG12 (2 μM)	0 to 12	No	~10%	Xu *et al*. 2008.
		SB203580 (5 μM)	0 to 12			
Adherant culture	Growth Factors	Activin A (100 ng/mL)	0 to 1	No	~30%	LaFlamme et al. 2007
		BMP4 (10 ng/mL)	1 to 5			
	Growth Factors	Activin A (100 ng/mL)	0 to 1	No	~50%	Paige *et al*. 2010.
		Wnt3A (100 ng/mL)	0 to 1			

		BMP4 (10 ng/mL)	1 to 5			
	Growth Factors	BMP4 (25 ng/mL)	0 to 1	No	~65%	Zhang *et al*. 2011.
		bFGF (6 ng/mL)	0 to 1			
		Activin A (100 ng/mL)	1 to 2			
		Noggin (250 ng/mL)	3 to 5			
		DKK1 (200 ng/mL)	5 to 8			
		RA (1 μM)	5 to 8			
		DKK1 (200 ng/mL)	8 onwards			
	Growth factors	Activin A (100 ng/mL)	0 to 1	No	~30-70%	Uosaki *et al*. 2011.
		BMP4 (10 ng/mL)	1 to 5			
		bFGF (10 ng/mL)	1 to 5			
		DKK1 (100 ng/mL)	5 to 7			
	Growth factors	BMP4 (20-40 ng/mL)	0 to 3	No	~24%	Elliot *et al*.2011
		Activin A (20 ng/mL)	0 to 3			
		VEGF (30 ng/mL)	0 to 3			
		SCF (40 ng/mL)	0 to 3			
		Wnt3A (50-80 ng/mL)	0 to 3			
		Hr-insulin (1 μg/mL)	0 to 3			
	Growth Factors	BMP4 (20 ng/mL)	0 to 3	No	~60%	Hudson *et al*. 2012
		Activin A (6 ng/mL)	0 to 3			
		IWP-4	4 to 15			

1.2.2.5.3 Endothelial differentiation

Differentiation of hESC to endothelial cells (EC) is a complex and poorly defined process and remains suboptimal for cell therapy in humans. Models of hindlimb ischaemia in the mouse have previously demonstrated that implantation of endothelial cells derived from hESC improves perfusion and limb viability (Sone et al., 2007, Yamahara et al., 2008, Cho et al., 2007, Kane et al., 2010a). Endothelial differentiation can be achieved by EB, co-culture with other cell types or by directed differentiation using growth factors, shear stress, or isolation of a progenitor cell phenotype. As with commitment to any lineage, the cells undergo dramatic changes in gene expression, miRNA signature, growth characteristics and morphology. No standard protocol exists for EC derivation from hESC.

EB differentiation, which gives rise to spontaneous differentiation of several cell types simultaneously, can generate ECs but at just 1-3% efficiency and requires the use of a problematic dissociation step to yield isolated populations (Levenberg et al., 2002, Wang et al., 2004, Cho et al., 2007, Lu et al., 2007). Co-culture of hESC with a murine fibroblast feeder layer generated functional endothelial cells capable of vessel formation *in vivo,* although differentiation efficiency after isolation of CD34+ cells remained low at ~10% (Wang et al., 2007b). Directed differentiation protocols offer a feeder-free method of inducing differentiation negating the potential for contaminating cell populations to be carried over during selection of the cell type of choice. With the serum-free culture method reported by Lagarkova *et al.*, fast differentiation can be achieved with ~50% cells expressing CD31 and VE-cadherin within 4 days (Lagarkova et al., 2008). However, there were no *in vivo* functionality assessments made and a significant population of pluripotent cells remained (Lagarkova et al., 2008). Recently, our laboratory described a feeder-free, serum-free method of generating functional endothelial-like cells by directed differentiation (Kane et al., 2010a). *In vitro* the hESC-EC produced nitric oxide, migrated across a wound, and formed vessel-like structures in matrigel (Kane et al., 2010a). *In vivo* hESC-EC subjected to differentiation conditions for 10 days before being transplanted into the ischaemic hindlimb of a mouse induced revascularisation of the limb, an increase in the number of vessels and were incorporated into the host vasculature (Kane et al., 2010a).

Whether the improvements in hindlimb ischaemia models are a result of the injection, the integration of cells into the host vasculature, of a paracrine action or a combination of some or all of these actions requires further investigation. Coaxing cells to an endothelial cell lineage remains a challenging prospect for vascular cell therapy, with several methods resulting in viable prospective cell populations. The question of which population of cells would be most clinically useful and at what stage to transplant, remains to be interrogated. Our laboratory previously reported that mature EC were unable to elicit an angiogenic response in animal models of ischaemia, therefore suggesting that an endothelial progenitor type cell may be more suited for cell therapy applications (Kane et al., 2010a).

1.2.2.5.4 Epigenetic Regulation of human embryonic stem cells

Control of gene expression can also come from epigenetic modification, where post-translational alteration of histone proteins leads to changes in chromatin structure (Jenuwein and Allis, 2001, Margueron et al., 2005) . Histone H3 lysine 4 tri-methylation (H3K4me3) and lysine 27 tri-methylation (H3K27me3) are particularly pertinent to hESC, as they are associated with heritable lineage-specific gene expression profiles as regulated by trithorax- and Polycomb-group proteins (Ringrose and Paro, 2004). H3K4me3 recruits nucleosome remodelling enzymes and histone deactylases (HDACs), resulting in positive regulation of transcription (Santos-Rosa et al., 2003, Pray-Grant et al., 2005, Sims et al., 2005, Wysocka et al., 2005). H3K27me3 negatively regulates transcription by promoting compact chromatin (Grau et al., 2011, Francis et al., 2004, Ringrose et al., 2004). During mammalian development, chromatin undergoes crucial changes (Park et al., 2012, Delaval and Feil, 2004). Large scale chIP studies performed in mESC and hESC report the presence of both H3K4me3 and H3K27me3 marks over ~3000 transcriptional start sites (TSS) of genes associated with development and lineage specification, thus the term “bivalent” in the context of epigenetic regulation was introduced (Bernstein et al., 2006, Ku et al., 2008, Azuara et al., 2006). It is thought that these bivalent genes are held in a poised state so that dynamic gene expression changes can be quickly and precisely effected upon differentiation of pluripotent cells to a defined lineage (Bernstein et al., 2006).

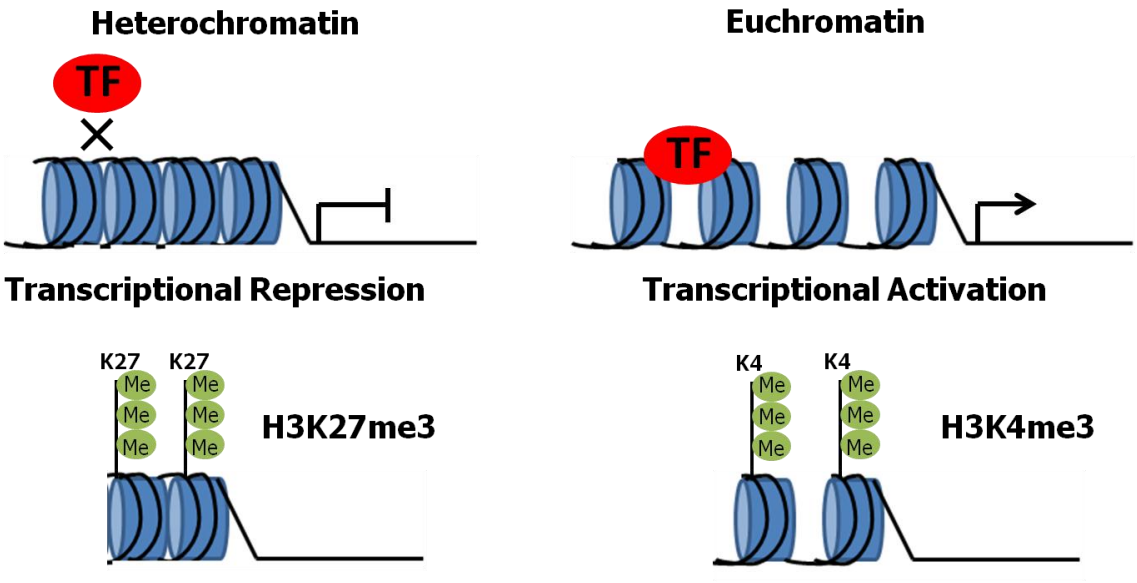


Figure 1.5: Description of how histone modifications can alter transcription.

When DNA is wrapped around tightly packed nucelosomes, Transcription Factors (TF) cannot gain access therefore the gene remains silenced, as in H3K27me3. In H3K4me3 nucleosomes are less tightly packed, allowing TF to transcribe gene.

1.2.2.5.5 MicroRNAs

First reported in *C. Elegans* in 1993, microRNAs (miRNA; miR) are small non-coding RNAs that control translation of mRNA by induction of mRNA degradation or blockade of translation (He and Hannon, 2004, Mendell, 2005, Lee et al., 1993). Lin-4 was the first of these powerful molecules to be discovered by the joint efforts of the Ambros and Ruvkin laboratories, when it was noted that lin-4 mutant C. Elegans were unable to lay eggs and displayed a failure in temporal development (Chalfie et al., 1981, Lee et al., 2004). It was then discovered that a null mutation in lin-14 gene had the opposite phenotype from lin-4 mutation (Ferguson et al., 1987, Lee et al., 2004). When the lin-4 gene was cloned it was found that the gene had no conventional start and stop codons, and any mutation in the putative open reading frame (ORF) did not result in a change in function of lin-4 (Lee et al., 1993, Lee et al., 2004). This led to the belief that lin-4 did not encode a protein, and upon further investigation it was found to encode for 2 transcripts of 61 bp and 22bp in length (Lee et al., 1993, Lee et al., 2004). Independantly, the Ruvkin laboratory noted that lin-14 was post-transcriptionally downregulated, and when the 2 groups shared unpublished data they realised that the lin-4 transcripts were complementary to a repeated sequence in the 3' UTR of the lin-14 gene (Lee et al., 2004). As a result both

groups published that lin-4 regulates lin-14 through its 3' UTR region, and the miRNA field was born (Lee et al., 1993, Wightman et al., 1993) Although small, each microRNA can target up to several hundred genes, providing a widespread method of regulation. MiRNAs are transcribed into long primary transcripts termed pri-microRNA (pri-miRNA) which are processed within the nucleus into ~70 nucleotide pre-miRNAs by Drosha, a RNase III type protein, and the double stranded RNA binding domain (dsRBD) protein DGCR8 (Lee et al., 2003). Exportin 5 then transports the pre-miRNAs to the cytoplasm (Lund et al., 2004) where they are processed by Dicer into a mature miRNA duplex ~22nt in length (Knight and Bass, 2001). One strand of the duplex is then preferentially loaded into the RNA-induced silencing complex (RISC) which, depending on the level of complementarity between the microRNA and its target, blocks translation or cleaves the mRNA (Figure 1.6) (Huntzinger and Izaurralde, 2011).

The focus of many recent studies has been to elucidate the role of miRNAs in embryonic stem cells, with research performed either on mouse embryonic stem cells (mESC) or hESC.

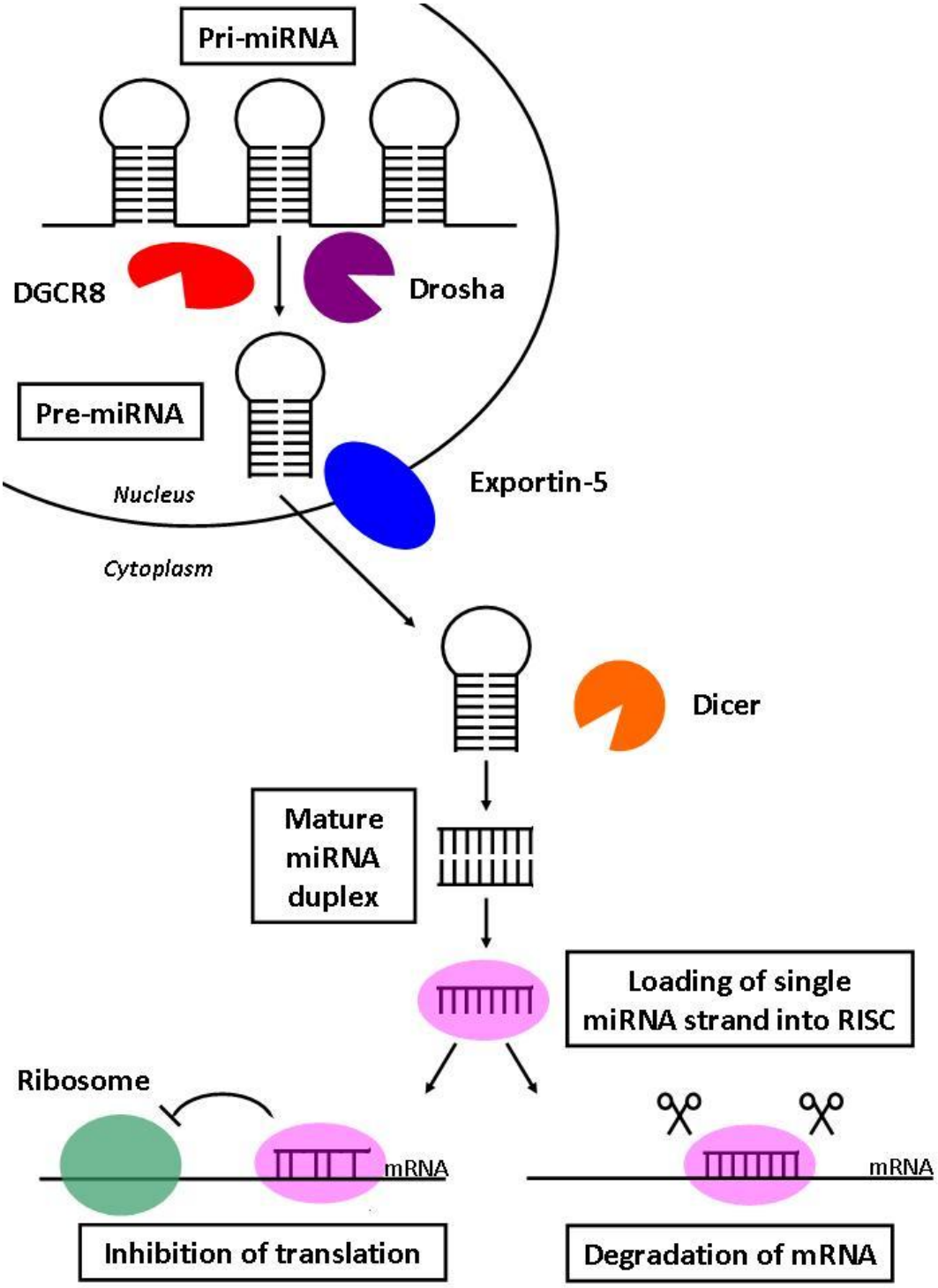


Figure 1.6: microRNA biogenesis.

In the nucleus, the pri-miRNA is transcribed from the miRNA gene, then processed by DGCR8 and Drosha to form the pre-miRNA. The pre-miRNA is then transported to the cytoplasm, where it undergoes further processing by Dicer to form a mature miRNA duplex. One strand of this duplex is preferentially loaded into the RNA induced silencing complex (RISC), where, dependant on the level of complementarity of the miRNA for its target sequence, translation is inhibited or the mRNA is degraded.

Early evidence for the contribution of miRNA in the regulation of ESC was shown by the use of DGCR8 and Dicer knockout ESC, which lack in all mature miRNAs

(Kanellopoulou et al., 2005, Wang et al., 2007a). Whilst DGCR8 is not required for siRNA processing, Dicer is essential for both miRNA and siRNA. (Denli et al., 2004, Gregory et al., 2004, Wang et al., 2007a). DGCR8 knockout cells are defective in differentiation, abnormally display markers of germ line differentiation and are unable to effectively downregulate pluripotency markers. (Wang et al., 2007a).

Dicer-knockout ESC exhibit a slower proliferation rate and an altered cell cycle phenotype with an increase in the population of cells at the non-dividing G1 and G0 stages of the cell cycle. When differentiated as EBs, Oct4 fails to silence completely, in conjunction with the lack of classical differentiation markers (Kanellopoulou et al., 2005).

Although miRNAs are expressed widely in a variety of cell types and tissues, there are lower numbers in pluripotent hESC (Gangaraju and Lin, 2009). Specific pluripotent miRNAs decrease when a cell differentiation program is initiated (Lakshmipathy et al., 2007). The role for these key miRNAs in pluripotent cells has been the subject of several studies. MiRNAs involved in self renewal appear to be distinct; the miR-302 cluster is expressed in both mESC and hESC, but miR-290 cluster is expressed only in mESC (Figure 1.6) (Kim, 2009). As mentioned previously, DGCR8 knockout mESC exhibit an abnormal cell cycle (Wang et al., 2007a). To interrogate this further, 266 mouse miRNAs were investigated against the DGCR8 knockout background in mESC and assayed for rescue of the cell cycle phenotype (Wang et al., 2008b). A set of miRNAs were identified; miR-291a-3p, miR-291b-3p, miR-294, miR-295, and miR-302 which were able to restore cell cycle, and denoted as ES cell-specific cell cycle-regulating (ESCC) miRNAs (Figure 1.6). They are reported to function by promoting the G1-S phase transition by targeting inhibitors of the cyclinE-Cdk2 complex, Cdkn1a (an experimentally validated target), and Rb1, Rbl1, Rbl2 and Lats2 (all predicted targets by Targetscan) (Wang et al., 2008b).

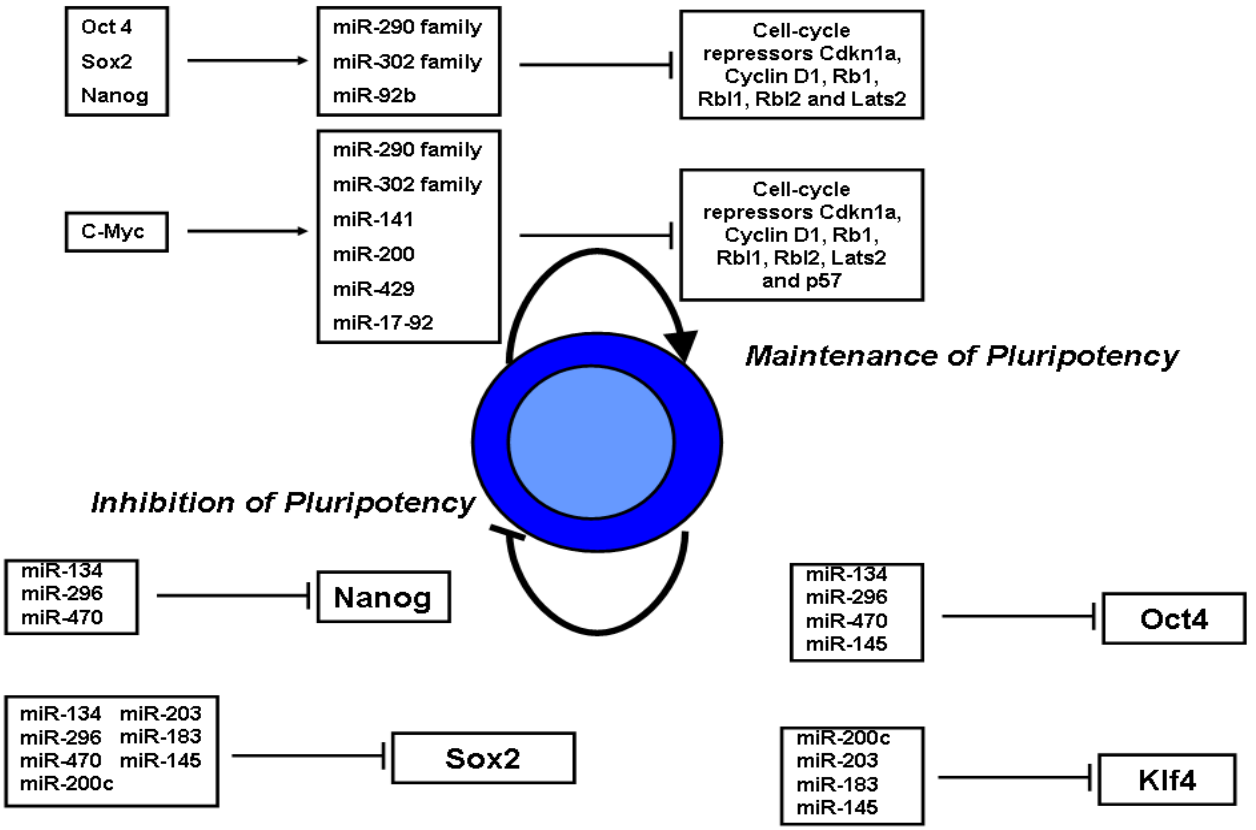


Figure 1.7: MiRNAs and targets involved in maintenance and inhibition of pluripotency.

In the maintenance of pluripotent ESC, transcription factors Oct4, Sox2 and Nanog upregulate miR-290 family, -302 family and -92b which in turn negatively regulate cell cycle repressors *Cdkn1a*, *Cyclin D1*, *Rb1*, *Rbl1*, *Rbl2* and *Lats2*. *C-Myc* upregulates miR-290 family, -302 family, -141, -200, -429, and -17-92 which target cell cycle repressors *Cdkn1a*, *Cyclin D1*, *Rb1*, *Rbl1*, *Rbl2*, *Lats2* and *p57*. Involved in the inhibition of pluripotency; miR-134, -296 and -470 negatively regulate *Nanog*, miR-134, -296, -470, -200c, -203, -183 and -145 repress *Sox2*, miR-134, -296, -470, and -145 target *Oct4* and miR-200c, -203, -183 and -145 decrease expression of *Klf4*.

The proliferation defect observed in Dicer1-knockout hESCs also highlights a potential role for miRNAs in cell cycle, with several of the predicted targets of the miR-302 family involved in cell cycle regulation (Greer Card et al., 2008). In mice, research to elucidate the pathways involved in the failure of Dicer knockout ESC to successfully differentiate reveals incomplete promoter methylation, leading to deficient silencing of Oct4 expression in these cells (Sinkkonen et al., 2008, Benetti et al., 2008).

Included in the ESCC miRNA group, the miR-302 family share a similar seed sequence to those other miRNAs that are prevalent in pluripotent hESC (Stadler et al., 2010). This suggests that there is some crossover between genes regulated by the ESCC miRNA and is evidence of the high level of redundancy often reported in miRNA regulated processes (Wang et al., 2008b). As previously mentioned, the transcription factors Sox2 and Oct 4 are essential for the maintenance of pluripotency in hESC. It has been shown that Sox2 and Oct 4 bind to the promoter region of the miR-302 family, and that expression is dependant on Sox2 and Oct4 (Figure 1.6). During embryogenesis, Oct4 and miR 302 family are co-expressed in a spatial and temporal manner during development (Greer Card et al., 2008). The miRNA-302 family are highly expressed in pluripotent hESC in comparison to other differentiated cell types (Suh et al., 2004). Expressed as a long transcript containing (in order 5' to 3') miR-302b, miR-302c, miR-302a, miR-302d and miR-367, this family shares an identical seed sequence and are 89% homologous in the mature miRNA form (Barroso-del Jesus et al., 2009). As with most miRNA, there is a high degree of conservation of the transcript miR-302 and targets between species.

In differentiated or lineage-committed cells, the cell cycle displays a longer G1 phase than the S phase. In pluripotent hESC it is the opposite; with the G1 phase shorter than the S phase (Wang et al., 2008b). This accounts for the fast self renewal which is one of the defining characteristics of the pluripotent hESC (Qi et al., 2009). These differences in cell cycle can be attributed to changes in expression of regulatory molecules, such as an increase in expression of cyclin D2/CDK4 before the transition of G1 to S phase (Becker et al., 2007). miR-302 members are predicted (by targetscan) to target several regulators of cell cycle. miR-302a translationally represses cyclin D1 in hESC, without degradation of mRNA levels. When miR-302 is inhibited in pluripotent hESC, an accumulation of

cells in G1 phase can be observed (Greer Card et al., 2008). The overexpression of miR-302 in differentiated cell types causes a decrease in G1 phase cells with an increase in S phase, associated with reprogramming towards iPS cells (Lin et al., 2011).

Several other miRNA polycistrons, including that of miR-302, were shown to be occupied at the predicted promoter region by Oct4, Sox2, Nanog and Tcf3, indicating a previously unknown regulatory mechanism (Figure 1.6) (Marson et al., 2008). It is thought that stem cell-related transcription factors can, and do, activate these miRNAs, which in turn regulate the levels of proteins involved in both pluripotency and differentiation. Examples of proteins regulated include Lefty1 ,which is involved in transcriptional activity in pluripotent ESC, and DNA methyltransferases 3a and 3b, which are essential for differentiation (Marson et al., 2008, Nakatake et al., 2006, Sinkkonen et al., 2008).

In addition, Sox2, Oct4 and Nanog associate with the predicted promoter regions of tissue-specific miRNAs, which remain silenced in ESC (Marson et al., 2008). The binding of these transcription factors to promoter regions suggests they are primed for upregulation when differentiation is begun, but silencing is achieved by co-habitation of the transcription factors with inhibitory polycomb protein Suz12, which remodels chromatin (Marson et al., 2008).

C-Myc, another essential gene involved in stem cell self renewal, also forms a regulatory negative feedback loop with the miRNA let-7, where mature let-7 biogenesis is inhibited by c-Myc activating Lin28 (Chang et al., 2009). Mature let-7 can also inhibit c-Myc expression (Melton and Blelloch, 2010). In a study using DGCR8 knockout mESC, it was shown that whilst ESCC miRNA rescued the cell cycling defect, the introduction of let-7 family miRNAs induced cellular differentiation (Melton and Blelloch, 2010). However, Introducing let-7 family miRNAs into wildtype mESC had no effect, suggesting ESCC miRNAs can redress the balance (Melton and Blelloch, 2010).

Further investigation of ESCC miRNAs produced data showing c-Myc is able to bind to the promoter of the miR-290 family in mESC but not the miR-302 family (Chen et al., 2008b), which is also expressed in hESC where miR-290 is not. It is thought c-Myc indirectly promotes expression of miR-302 however the

mechanism of action remains unknown (Lin et al., 2009). C-Myc has also been shown to occupy the promoter regions and promote expression of miR-141, miR-200 and miR-429. Upon overexpression in mESC these miRs prevented differentiation when cells were exposed to differentiation inducing conditions (Lin et al., 2009). The miR-17-92 family of miRNAs is expressed in pluripotent stem cells, as well as several cancers, and have seed sequences remarkably similar to those in the ESCC group (Laurent et al., 2008, Marson et al., 2008, Mendell, 2008, Wang et al., 2008b and Judson et al., 2009). Although the function in ESC has not yet been elucidated, c-Myc has been shown to stimulate expression of miR-17-92 cluster in tumour cells (O'Donnell et al., 2005). These c-Myc miRNAs also appear to play an important role in stem cell self-renewal (Smith et al., 2010). Also miR-92b has been shown to repress the Cdk inhibitor p57, again promoting the G1 to S phase transition (Sengupta et al., 2009). When Marson et al conducted their investigation of transcription factor binding sites in mESC, it was shown that Sox2 and Oct4 could occupy the promoter region of miR-92b, which suggests it is also important in pluripotency maintenance in mESC.

ESCC miRNAs and the let-7 family of miRNAs are thought to be important regulators of ESC function (Melton and Blelloch, 2010). The let-7 promoter region is occupied by Sox2, Oct4 and Nanog, with high levels of pri-let-7 transcripts regulated by the expression of Oct4, however, expression of mature let-7 miRNAs is not detected in pluripotent ESC (Melton and Blelloch, 2010). The conversion of pri-let-7 to its mature form is inhibited by RNA binding protein, and reprogramming factor, Lin28, preventing both Drosha (Viswanathan et al., 2008) and Dicer (Rybak et al., 2008) processing. Lin 28 and let-7 form a negative feedback loop where let-7 inhibits expression of Lin28 (Rybak et al., 2008) which is also necessary for the translation of Oct4 (Qiu et al., 2010).

As discussed previously, miRNA deficient ESC are unable to silence pluripotency markers during differentiation (Kanellopoulou et al., 2005, Wang et al., 2007a). For successful silencing of pluripotency markers DNA methylation must occur (Feldman 2006) but in Dicer deficient cells DNA methyltransferases, Dnmt3a and Dnmt3b, are lacking (Benetti et al., 2008, Sinkkonen et al., 2008). However, this could be restored via overexpression of the miR-209 family. Interestingly, when dicer knockout cells were further investigated, it was determined that the

transcriptional repressor Rbl2 was a target of the miR-290 family (Benetti et al. 2008; Sinkkonen et al. 2008). These results indicate that in the absence of miR-209 family, levels of Rbl2 are increased resulting in transcriptional repression of DNA methyltransferases leading to poor silencing of the pluripotency markers and inefficient differentiation observed in these Dicer null cells.

Other miRNAs have been shown to be required for differentiation of ESC; in differentiating mESC miR-134, miR-296 and miR-470 are expressed targeting Nanog, Oct4 and Sox2 (Tay et al., 2008) and targeting Sox2 and Klf4 are miR-200c, miR-203 and miR-183 (Wellner et al., 2009). In hESC undergoing differentiation miR-145 is expressed, targeting Oct4, Sox2 and Klf4 (Xu et al., 2009). Therefore, tight regulation of ESC pluripotency is achieved through a complex network of transcription factors and DNA methylation, with miRNAs adding a further layer of precision control.

Research performed on the development of knockout Dicer mice suggested miRNAs play a role in development of endothelium, when the embryos displayed reduced blood vessel formation, altered expression of VEGF, Flt1, Kdr and Tie1 and died between days 12.5 and 14.5 of gestation (Yang et al., 2005). To further assess the importance of miRNAs in endothelial cells, Suárez *et al*. Knocked down Dicer using a siRNA approach and examined the cell phenotype. They found differential expression of several endothelial associated genes including Tie-2, Kdr, and Tie-1, and increased activity of the endothelial nitric oxide synthase pathway. It was also noted that cells had reduced capability to form tubes in a matrigel assay and had reduced proliferation (Suarez et al., 2007).

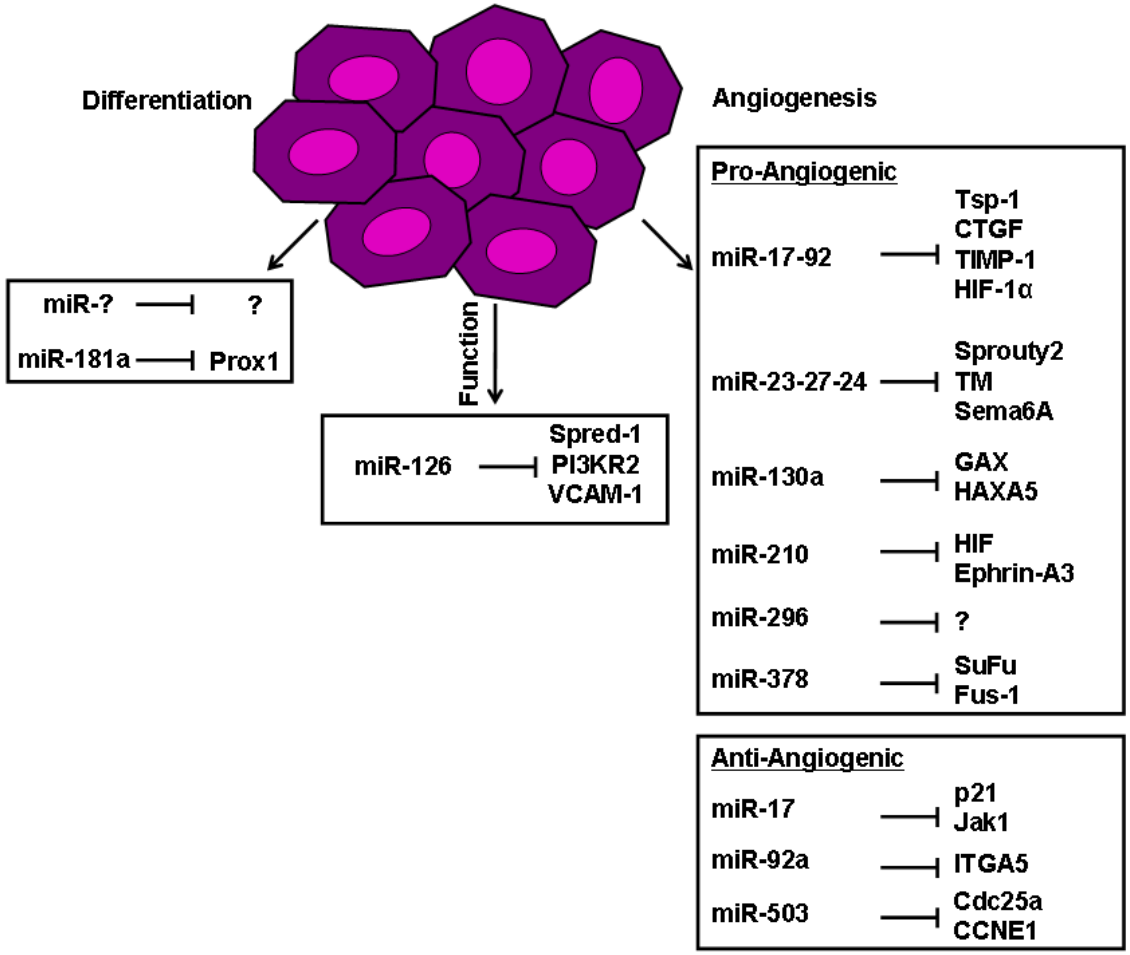


Figure 1.8: MiRNAs and targets involved in endothelial cell function and angiogenesis.

As yet, there are no named miRNA regulating endothelial differentiation. Involved in endothelial function, miR-126 inhibits expression of *Spred-1*, *PI3KR2* and *VCAM-1*. Implicated in angiogenesis; miR-17-92 targets *Tsp-1*, *CTGT*, *TIMP-1*, *HIF-1α*, miR-130a represses *GAX* and *homeobox A5*, miR-181a negatively regulates *Prox1*, miR-210 suppresses *HIF* and *Ephrin-A3*, direct targets of miR-296 are unknown and miR-378 downregulates *SuFu* and *Fus-1*.

In Dicer mutant zebrafish endothelial cells were still present but the circulatory system was disrupted (Giraldez et al., 2005). In mice, endothelial cell – specific knockdown of Dicer did not result in lethality (probably due to incomplete excision of the Dicer gene) but a decreased VEGF-induced angiogenesis and retarded recovery after ischaemic challenge were observed (Suárez et al., 2008).

The first miRNA reported to be expressed at high levels in mesodermal progenitors derived from ESC was miR-126 (Figure 1.7) (Ivey et al., 2008b), which in the human genome is situated within the epidermal growth factor like-7 gene. Found to be essential in maintaining the integrity of vessels in both zebrafish and mice (Fish et al., 2008, Wang et al., 2008a), morpholino antisense to miR-126 didn't affect endothelium morphology in zebrafish, but vessels were leakier and haemorrhages more frequent (Fish et al., 2008). In mice, the targeted knockdown of miR-126 resulted in a similar phenotype, along with attenuated cell migration, proliferation and angiogenesis (Wang et al., 2008a). That the deletion of miR-126 did not result in embryonic lethality suggests that miR-126 does not play a crucial role in differentiation of endothelial cells, and this is corroborated by findings showing miR-126 does not control differentiation of ESC to the endothelial cell lineage, instead playing a role in the integrity and function of the vessel (Fish et al., 2008). Sprouty-related proteins (Spreds) act as inhibitors of growth factor-induced ERK activation, as can be brought about by VEGF and bFGF (Wakioka et al., 2001). MiR-126 is predicted to target Spred-1 (Figure 1.7) (Fish et al., 2008). PI3 kinase regulatory subunit 2 (PI3KR2/p85) which in turn negatively regulates PI3 kinase, is also a target of miR-126 (Figure 1.7). In EC with suppressed miR-126 levels, a siRNA knockdown of PIK3R2 rescued the VEGF-dependent Akt/PKB phosphorylation (Fish et al., 2008). miR-126 has also been shown to negatively regulate VCAM-1 in a post-translational manner in endothelial cells (Figure 1.7) (Harris et al., 2008).

Fish *et al*. repeated that miR-146b (known to negatively regulate NF-kB activity in studies of cancer (Hurst et al., 2009)), miR-625 (no reported function) and miR-197 (reported to induce proliferation in follicular thyroid carcinoma (Weber et al., 2006)) were upregulated in their CD31+ cell population compared to CD31- cells, but as miR-126 was the most dramatic change, their function in endothelial cells was not studied further.

As well as having a potential role in pluripotent ESC, the miR-17-92 family are upregulated in tumour angiogenesis; promoting cell proliferation, inhibiting cell apoptosis and promoting angiogenesis, which is heightened in Myc-induced tumours (Figure 1.7) (Dews et al., 2006). MiR-17-92 members are predicted to target anti-angiogenic thrombospondin-1 (Tsp-1), connective tissue growth factor (CTGF), and tissue inhibitor of metalloproteinase 1 (TIMP-1) as well as HIF1-alpha (Dews et al., 2006, Ohtsuka and Dalton, 2008, Taguchi et al., 2008). In mouse models, which displayed impaired angiogenesis due to Dicer deficiency, partial restoration of vascularisation could be seen following an injection of miR-17-92 member, miR-17-5p (Ohtsuka and Dalton, 2008).

Also previously mentioned, the Let-7 family play a role in angiogenesis and endothelial cell biology, and are highly expressed in endothelial cells (Ohtsuka and Dalton, 2008). With a role in control of EC proliferation and motility by targeting TIMP-1 suggested for Let-7b (Ohtsuka and Dalton, 2008) and let-7f promoting sprout formation *in vitro,* the Let-7 family represent more functionally important miRNAs, but their role in differentiation of endothelial cells from ESC remains unknown.

Present in cycling endothelial cells (Chen and Gorski, 2008) and endothelial cells derived from hESC (Kane et al., 2010a), the role of miR-130a in differentiation of endothelial cells is unclear, but its role in angiogenesis has been extensively studied. With its 3' UTR containing 2 seed sequences for miR-130a, growth arrest specific homeobox (GAX) inhibits angiogenesis and regulates EC phenotype along with homeobox A5, and expression of both these genes appear to be modulated in response to expression of miR130a (Chen and Gorski, 2008).

Also expressed in hESC derived endothelial cells as well as adult endothelial cells, miR-210 is involved in migration and cell survival (Fasanaro et al., 2008) stimulating VEGF-driven cell migration and tubule formation on Matrigel when overexpressed; when absent these processes were inhibited. miR-210 is thought to achieve this by targeting HIF and ephrin-A3 which, when downregulated changes the angiogenic response to hypoxia, a key determinant of tissue pathology during tumour development (Fasanaro et al., 2008). Although never reported in endothelial cells derived from ESC, miR-378 has been implicated in angiogenesis, tumour cell survival and growth through repression of tumour

suppressors suppressor of fused (SuFu) and Fus-1 (Lee et al., 2007). miR-296 has also been suggested as an important part of the angiogenic process, although has not been characterised in ESC-EC (Würdinger et al., 2008).

A key gene involved in lymphatic endothelial cell identity, Prox1, has been shown to be bound by miR-181a, and as levels of miR-181a increased, so too did the number of cells which switched phenotype towards a blood vascular lineage *in vitro* (Figure 1.7) (Kazenwadel, Michael, and Harvey, 2010).

Although these miRs have been demonstrated to be expressed in EC, ESC or both, the role played in the differentiation of EC from ESC remains unknown and warrants further investigation.

Regulation of cardiomyocyte differentiation from ESC involves miR-1 and 133, which were first described in skeletal muscle (Chen et al., 2006) and then cardiac muscle (Figure 1.8) (Ivey et al., 2008b). Transcriptionally controlled by key regulators of muscle lineage commitment SRF, MyoD and Mef2; miR-1 and 133 are bicistronic (Kwon et al., 2005a, Zhao et al., 2007a, Rao et al., 2006). miR-1 supports differentiation of cardiac progenitor cells and exit from the cell cycle in both mammals and drosophila (Kwon et al., 2005a, Zhao et al., 2007a), whereas miR-133 has an inhibitory effect on skeletal myoblast differentiation whilst maintaining the cells' capacity for proliferation (Figure 1.8) (Chen et al., 2006).

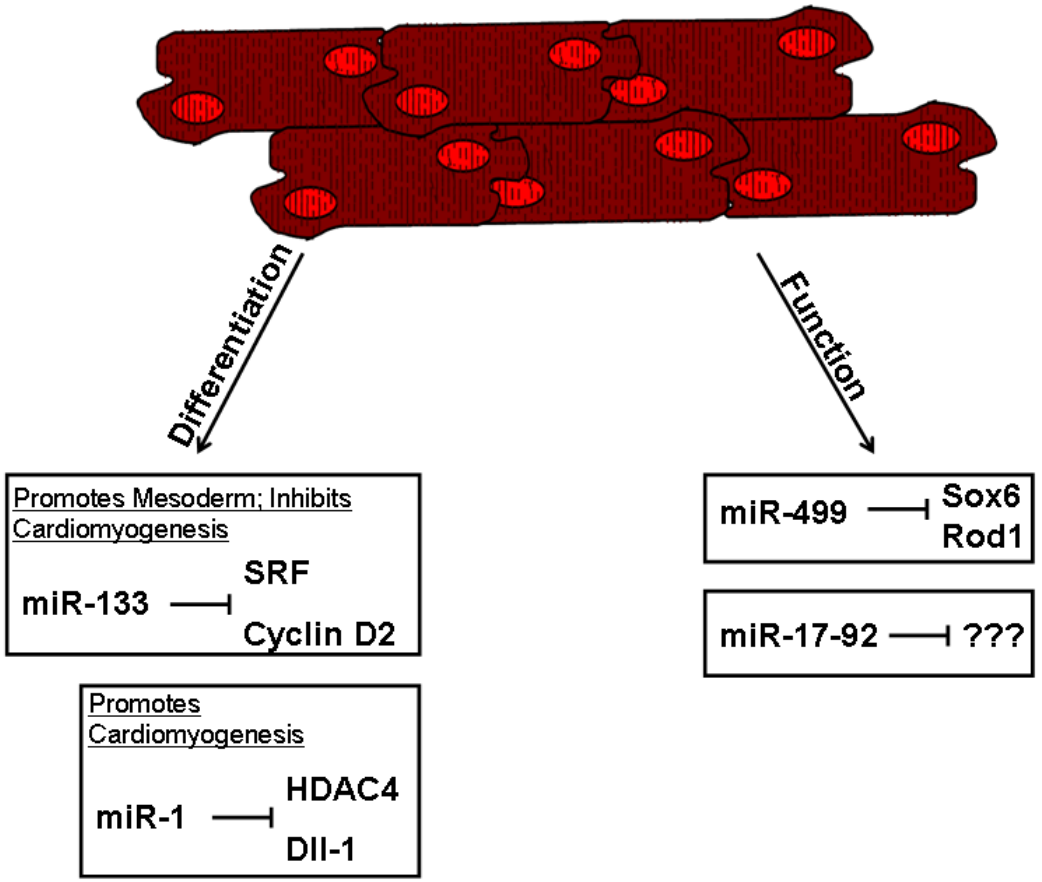


Figure 1.9: MiRNAs and targets involved in Cardiomyocyte differentiation and function.

Involved in the differentiation of cardiomyocytes, miR-1 targets *HDAC4* and *Dll-1* and miR-133 targets *SRF* and *CyclinD2*. MiRNAs with a role in the function of cardiomyocytes are miR-499 that represses *Sox6* and miR-17-92 for which the cardiomyocyte specific targets are unknown.

Although co-transcribed, there are differences in the modus operandi of miR-1 and 133. MiR-1 acts by suppressing non muscle genes and upregulating homeobox protein NK2 transcription factor related, locus 5 (Nkx2.5), an early mesoderm marker in both mESC and hESC (Ivey et al., 2008b). MiR-1 also increases the number of beating areas in adult cardiac progenitor cells, as well as upregulating several cardiac genes (Sluijter et al., 2010). In hESC embroid bodies, miR-1 has also been shown to upregulate myosin heavy chain (Wilson et al., 2010), although the timing of miR-1 upregulation is likely to be crucial (Takaya et al., 2009a). Direct targets are thought to include histone deacetylase 4 (HDAC4) and Notch ligand Delta-like 1 (Dll-1) (Figure 1.8) (Chen et al., 2006, Ivey et al., 2008b). Targeted deletion of one of the two allelles of miR-1-2 in mice resulted in defects in cardiac morphogenesis, electrical conduction and cell cycle (Zhao et al., 2007a).

miR-133 appears to act in a different way to its bicistronic partner. Overexpression in either hESC or mESC inhibits expression of cardiac genes and stimulated myoblast proliferation by repression of SRF and cyclin D2 (Ivey et al., 2008b, Takaya et al., 2009a, Chen et al., 2006). Deletion of either of the miR-133a genes resulted in phenotypically normal mice, but deletion of both caused abnormalities in cardiomyocyte proliferation and in the ventricular septum, leading to neonatal or late embryonic lethality (Liu et al., 2008).

The actions of miR-1 and miR-133 are not thought to be confined to cardiac lineage commitment, but are also involved in cardiac disease (Condorelli et al., 2010).

MiR-208 has an important role in the stress adaptation of the adult heart, and shares several common gene targets with miR-499 (van Rooij and Olson, 2007), which has recently been reported to be expressed in both adult cardiac progenitors and hESC (Sluijter et al., 2010, Wilson et al., 2010). Upon overexpression of miR-499 in mESC and hESC, expression of myosin heavy chain genes and cardiac transcription factors increase (Sluijter et al., 2010, Wilson et al., 2010) whilst downregulation of miR-499 inhibited cardiac differentiation (Sluijter et al., 2010).

Members of the miR-17-92 cluster may also play a role in cardiac differentiation, as knockout mice display retardation of cardiovascular development (Ventura et al., 2008), although the phenotype has not yet been fully characterised (Bonauer et al., 2009).

That miRs play an essential role in regulating ESC self renewal and commitment to lineages is highly likely, and indeed a hot topic for research. Several studies have mapped specific miR expression to a defined role in maintenance of pluripotency or differentiation of ESC, with miRs shown to regulate expression of transcription factors, cell cycle proteins, epigenetic modifiers and other proteins with regulatory roles (Figure 1.8).

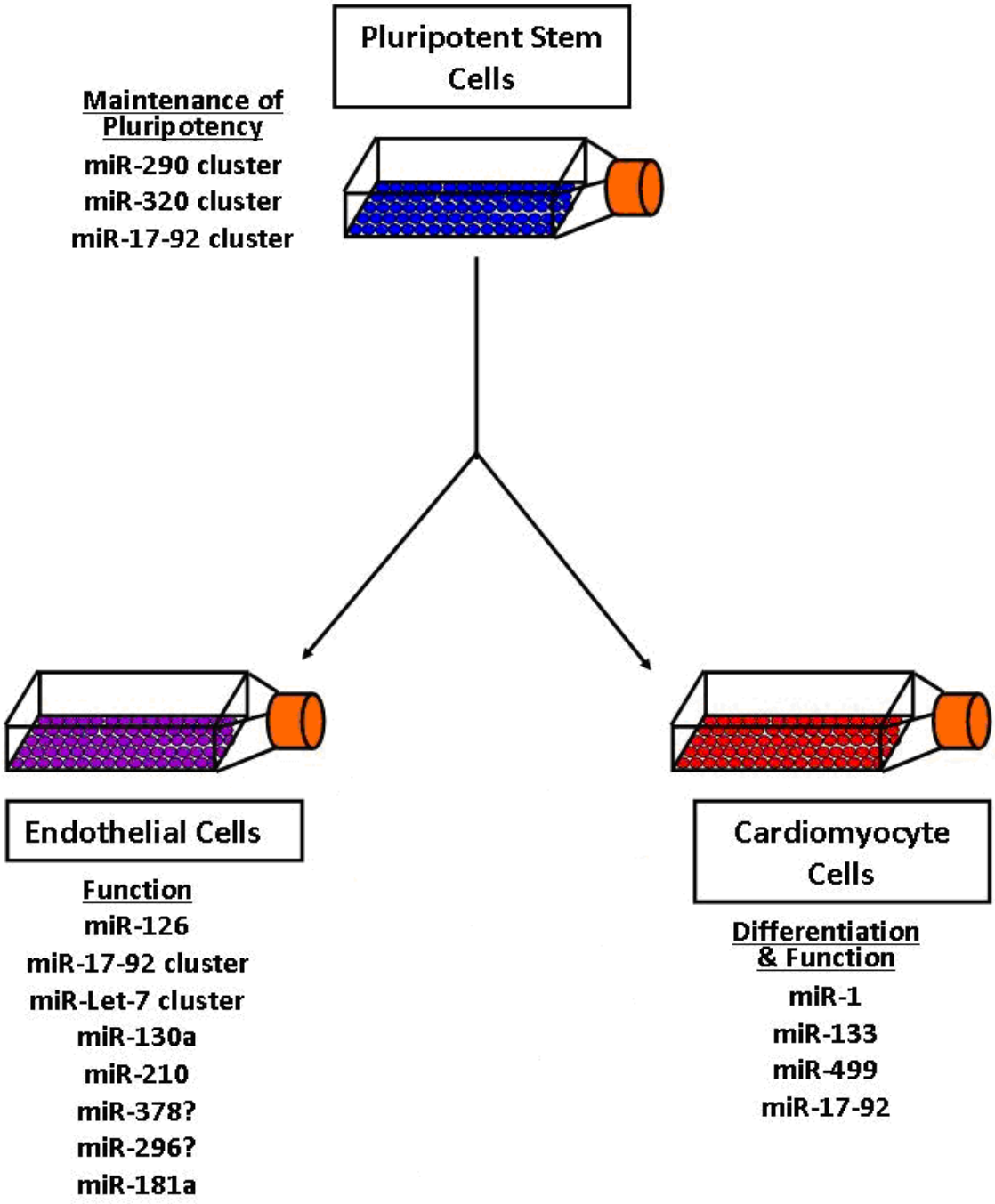


Figure 1.10: Summary of MiRNAs involved in Pluripotency and Cardiovascular Differentiation and Function.

MiRNAs found in pluripotent ESC include miR-290 cluster, -320 cluster,-17-92 cluster, -141, -200, -429 and -92b. Endothelial cell associated miRNAs include miR-126, -17-92, -Let-7, -130a, -210, -378, -296 and -181a. MiRNAs present in vascular smooth muscle cells are miR-1, -143/145, -25 and -221. MiR-1, -133, -499 and -17-92 cluster are present in cardiomyocytes cells.

The negative regulation of whole pathways of genes made possible by miRNAs make for a precision tool able to fine tune gene expression patterning. However, knockout of single miRNAs in mice rarely results in embryonic lethality, suggesting miRNAs are used to refine differentiation, rather than being the master control.

1.3 Aims of thesis

The aims of this thesis were to observe the effect of miRNA modulation during differentiation of hESC to cardiomyocytes and to gain insight into the role gene expression, microRNAs and epigenetic status played in both primary human endothelial cells and hESC derived endothelial cells. It was the overall aim of this study to uncover new information on the "master control" mechanisms that govern hESC differentiation to cardiovascular lineages.

These aims were addressed by the following studies:

- Optimisation and delivery of miRNA-overexpressing lentiviruses to hESC undergoing cardiac differentiation.

- MicroRNA, gene expression and epigenetic characterisation of primary endothelial cells and hESC derived endothelial cells.

Chapter 2: Materials and Methods

2.1 General Laboratory Practice

Laboratory reagents and equipment were of the highest commercially available grades. Unless otherwise stated, all chemicals were supplied by Sigma-Aldrich, Dorset, UK. Hazardous chemicals were handled as described in the Control Of Substances Hazardous to Health (COSHH) guidelines, with a laboratory coat and powder-free nitrile gloves worn throughout and using laboratory spectacles, facemask or fumehood if appropriate.

2.2 Cell biological methods

All tissue culture was performed in biological safety class II vertical laminar flow cabinets under sterile conditions. Cells were cultivated at 37°C in a humidified incubator atmosphere maintained at 5% CO_2.

2.2.1 Stem Cell culture

2.2.1.1 Maintenance

Human embryonic stem cells (hESC) were cultured as a monolayer using feeder- and serum free systems with media replaced every 24 or 48 h. Studies were performed on SA461 cells (Cellartis, Dundee, UK), RC10 (Roslin Cells Ltd., Roslin, UK) and H1 cells (WiCell, Madison, USA).

SA461 hESC were maintained pluripotent as described previously (Kane et al., 2010a) in maintenance medium composed of equal parts of a chemically defined media (Liu et al., 2006) comprising DMEM/F12, N-2 supplement (Invitrogen (Gibco), Paisley, UK) and B-27 supplement , 1 mM L-glutamine, penicillin (50 µg/mL), streptomycin (50 µg/mL)and 0.1 mM ß-mercaptoethanol (Invitrogen) with human Umbilical Wharton Inner Layer media (huWil) (Cellartis) and 10 ng/mL basic fibroblast growth factor (bFGF) (R&D systems, Abington, UK) and routinely passaged at approximately 90%. To passage SA461, cells were washed in PBS, incubated in 500 µL Tryple Select (Invitrogen) and observed under a light microscope until the cells showed defined borders. Tryple Select activity was neutralised by addition of 3 mL Pluripotency Media containing 10 µM ROCK Inhibitor Y-27632 (Calbiochem, Merck Chemicals Ltd., Nottingham, UK). Cells were detached from the growth surface using a cell scraper (Corning, Corning BV

Life Sciences, Amsterdam, Netherlands), resuspended homogenously and seeded out into T25 flasks for maintenance or plates pre-coated with fibronectin at 1 mg/mL (Merck Chemicals Ltd.) at a density of 50,000 cells/cm^2.

RC10 and H1 were later introduced to the laboratory and were routinely passaged in corning 6 well dishes pre-coated with Cell Start™ matrix (Invitrogen) at approximately 80% confluence to prevent cells from spontaneous differentiation through overgrowth. Due to discontinuation of huWil media and a move toward animal contaminant free culture systems both cell lines were maintained in StemPro™ media (Invitrogen) with the addition of 20 ng/mL bFGF. For routine passage of RC10 or H1, cells were washed in PBS, fresh maintenance media added and colonies created with the EZ-passage tool (Invitrogen). The floating colonies were cluster seeded into StemPro™ maintenance media.

2.2.1.2 Differentiation towards mesoderm/vascular endothelial cell lines (EC)

Experiments often required single cells in which case confluent hESC were treated with Tryple Select™ and cells were counted using a haemocytometer before plating to ensure accurate seeding density. Cells were differentiated in 12-well plates at 2 x 10^4 cells per well, 24-well plates at 1 x 10^4 cells per well, or in T25cm^3 flasks at a density of 1.25 x 10^7 cells per flask.

To direct cells towards endothelial differentiation, cells were incubated with Large Vessel Endothelial growth media (500 mL) with the addition of Large Vessel Endothelial cell growth supplement singles pack (final concentrations: hydrocortisone, 1 mg/mL; human epidermal growth factor, 10 ng/mL; basic fibroblast growth factor, 3 ng/mL; and heparin, 10 mg/mL) (TCS CellWorks, Buckingham, UK). This will be referred to as “Endothelial differentiation media”.

During endothelial differentiation media was replaced every 24 h for day 1-4, and every 48 h onwards.

2.2.1.3 Differentiation towards cardiomyocytes (CM)

Differentiation towards CM required single cells as described in section 2.1.1.2. H1 were seeded onto Matrigel™ (BD Biosciences, San Jose, CA)-coated 48-well plates at $3x10^4$ cells per well and infected with a lentivirus with Spleen Focus Forming Virus (SFFV) promoter driving either Green Fluorescent Protein (GFP) expression, pre-miR-1 or pre-miR-133 as described in 2.1.2.4. The CM differentiation protocol is an optimised version of the published by LaFlamme et al (Laflamme et al., 2007). After transduction with lentivirus the cells recovered for one day. The medium was then changed to RPMI 1640 and 1x of a 50x B27 stock (RPMI/B27) (Invitrogen) with the addition of 100 ng/mL Activin A (R&D systems, Abington, UK). After 24 h the media was changed to RPMI/B27 with 10 ng/mL BMP4 (R&D systems, Abington, UK) and left for 4 days without media change. On day 5 a new medium, advanced RPMI/B27 was used as it was found to be more beneficial for cell survival. Media change was then performed every 48 h until the cells were harvested for total RNA at day 14. At around 7-10 days after initiation of the protocol spontaneous beating was observed.

2.2.1.4 Differentiation towards neuronal lines

Differentiation towards neuronal cells was performed as reported by Gerrard *et al*. and required single cells as described in section 2.1.1.2 (Gerrard et al., 2005). H1 were seeded onto Matrigel™ (BD Biosciences) coated 12 well plates at $1x10^5$ cells per well in N2B27 media (1:1 mix of D-MEM/F12 supplemented with N2, and Neurobasal medium supplemented with B27 (all from Invitrogen) supplemented with 100 ng/mL mouse recombinant noggin (R&D Systems) (Gerrard et al., 2005). Media was replenished every 48 h with samples being harvested for total RNA at days 0, 4, 7, 10 and 14.

2.2.1.5 Differentiation towards haematopoetic lines

Haematopoetic samples were kindly provided by Dr Angela McCahill, Institute of Cardiovascular and Medical Sciences. Differentiation towards haematopoetic cells was performed as reported by Yung *et al*. and required single cells as described in section 2.1.1.2 (Yung et al., 2011).

2.2.2 Tissue culture

2.2.2.1 Human saphenous vein endothelial cells (HSVEC)

Saphenous veins were obtained from patients with Coronary Artery Disease (CAD) undergoing coronary bypass graft at the Golden Jubilee National Hospital, or control patients undergoing elective varicose vein stripping at the Glasgow Gartnaval General. These collections were covered by the Glasgow West Ethics Co mMittee VASCAB and all participants gave their written informed consent prior to participation.

HSVEC were isolated on the day of surgery by collagenase digestion. Briefly, the saphenous vein was removed from the sterile saline solution and gently flushed with wash medium (Dulbecco's Modified Eagle's Medium (DMEM), 100 IU/ mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Paisley, UK)). The vessel was clamped at one end using a small artery clip and filled with a solution of filter-sterilised collagenase (2 mg/mL in wash medium (Sigma-Aldrich)) clamped at the top and incubated in a sterile petri dish for 15 min at 37°C in 5% CO2. The vein was then flushed with wash medium and the flow-through containing endothelial cells collected. A second collagenase digestion was performed for 10 min, flow-through collected and cells sedimented by centrifugation for 5 min at 2,000 x g at RT. Cells were resuspended in endothelial differentiation media and placed into a T25cm^2 flask, incubated for 24 h at 5% CO_2 followed by a media change.

Primary human saphenous vein endothelial cells (HSVEC) cells were cultured in endothelial differentiation media (TCS CellWorks) with the addition of 20% heat inactivated foetal calf serum (FCS) (Invitrogen). Cells were passaged no more than 6 times before use to avoid loss of endothelial phenotype in culture (Scoumanne et al., 2002).

2.2.2.2 Human Embryonic Kidney 293T (HEK293T)

HEK293T cells (ATCC, Teddington, UK) were maintained in HEK293T standard medium comprised of Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% FCS, penicillin (100 µg/mL), streptomycin (100 µg/mL), L-Glutamate (2 mM) and sodium pyruvate (1 mM) (Invitrogen, Paisley, UK). When 60-70% confluent, HEK293T were passaged using 1x citric saline (10x: 100g KCl, 44g sodium citrate (both Sigma-Aldrich) in 1L distilled H_2O, autoclaved) up to 1:6 for maintenance or 1:2 for transfection of lentiviral producing plasmids the following day. The cells were used for Lentivirus production between passage 10-30.

2.3 Lentiviral vectors

Lentiviral vectors were second-generation, self-inactivating (SIN), HIV-1 based and produced as described previously (Demaison et al., 2002a, Buckley et al., 2008).

2.3.1 Production of lentivirus

Lentiviral vectors were produced by triple transient transfection of HEK293T cells with a packaging plasmid (pCMVΔ8.74), a plasmid encoding the envelope of vesicular stomatitis virus (VSVg) (pMDG) (Plasmid Factory, Bielefeld, Germany) and an expression plasmid (pHR'SIN-cPPT-SFFV-eGFP-WPRE) containing the desired gene under the control of the SFFV promoter (kind gift of Prof. Adrian Thrasher, Institute of Child Health, University College London, London, UK). The transfection reagent polyethylenimine (PEI; Sigma-Aldrich) was used as previously described (Demaison *et al.* 2002). Per T150 flask of 70% confluent HEK293T, 17.5µg envelope plasmid, 32.5 µg packaging plasmid and 50 µg of expression plasmid were added to 5 mL OptiMEM I reduced serum media with GlutaMax™ I (plus 2400 mg/l sodium bicarbonate, HEPES, sodium pyruvate, hypoxanthine, thymidine, trace elements, growth factors and 1.1 mg/l phenol red) (Invitrogen), filter-sterilised through a 0.22 µM filter and combined with (sterile-filtered) 5 mL OptiMEM containing 1 µl of 10 mM PEI. The mixture was left for 20 min in the flow cabinet with the light off at RT to allow formation of polyplexes, which give efficient DNA binding, have endosomolytic activity and are protected from lysosomal degradation. The culture MEM was removed from

HEK293T cells and they were washed with 10 mL of OptiMEM. 10 mL of DNA/PEI polyplexes were added to each flask and the cells placed back in the incubator at 37°C in 5% CO_2 for 4 h, after which the transfection mix was removed and replaced with 25 mL of fresh HEK293T standard medium (2.1.2.3). At 48 h post-transfection, the media containing lentiviral particles was taken off, sterile-filtered through a 0.22 µM filter unit and kept at 4°C with a further 25 mL HEK293T standard medium added (2.1.2.3). At 72 h post transfection the media containing lentiviral particles was taken off, sterile-filtered through a 0.22 µM filter unit and kept at 4°C for a maximum of 24 h prior to concentration.

2.3.2 Concentration of lentivirus

Lentiviruses were concentrated by ultracentrifugation before storage and use. Media containing lentiviral particles was added to sterile 14 mL ultra-clear centrifuge tubes (Beckman Coulter Ltd, Buckinghamshire, UK) which in turn were loaded into a SW-32.1 Ti rotor bucket (Beckman Coulter) and placed in the SW32 Ti rotor (Beckman Coulter) in an Optima L-80 XP Ultracentrifuge (Beckman Coulter). Lentiviral particles were subjected to ultracentrifugation at 92752 x g for 1 h and 7 min at 4°C. Immediately after centrifugation the supernatant was decanted carefully to avoid disruption to the lentivirus pellet, and the procedure repeated until no media remained. The tubes were drained carefully by placing them upside down on fresh tissue paper for 2 min, then turned right way up and 50 µL of OptiMEM added per tube. The tubes were then covered with bijou lids and placed on ice for 20 min. Each tube had the volume of OptiMEM pipetted up and down the walls of the tube to resuspend the lentivirus pellet thoroughly, before aliquots of 20 µL of concentrated lentivirus were stored at -80°C until required.

2.3.3 Determination of functional lentiviral titre

Lentiviral titres were determined by TaqMan® quantitative real-time PCR (qRT-PCR) detecting linear double-stranded DNA pre-integration complex as previously described (Butler et al., 2001) using the following primer and probe sequences: forward, 5'-TGTGTGCCCGTCTGTTGTGT-3'; reverse, 5'-

GAGTCCTGCGTCGAGAGAGC-3'; probe, 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3'. Briefly, HEK293T cells were seeded into a 12-well plate at a density of $5x10^4$ cells per well in standard media and left overnight at 37°C 5% CO_2 to attach. Freeze-thawed lentivirus was serially diluted and added as indicated in Figure 2.1. The transduction was left for 72 h, cells washed once with PBS, 200 µL of PBS was added to each well and the plate stored at -20°C for minimum 30 min. Plates could be stored for several weeks at this stage. The plate was then subjected to DNA extraction (see chapter 2.2.1). Serial dilutions of the expression plasmid were used to generate a standard curve ranging from 1×10^{12} to 1×10^0 plasmid copy number. The most concentrated standard was calculated from the following equations:

- To determine the molecular weight of the expression plasmid:

(bp) x (330 Daltons x 2nt/bp)= Daltons

Daltons = g/mole

- To determine the weight of one copy of plasmid:

(g per mole ÷ Avogadro's Constant) = g per molecule.

- To determine the copy number of plasmids per mL in the plasmid stock:

(concentration of plasmid in g per mL ÷ g per molecule) = copy number of plasmid per mL.

- To prepare the top standard:

(copy number of plasmid per mL ÷ top standard required) = initial dilution for top standard.

(1000 ÷ initial dilution factor for top standard) = µL of plasmid stock required to make 1 mL of top standard.

Titres ranged from 1×10^8 to 1×10^9 transducing functional U/ mL. Multiplicity of Infection (MOI) is the ratio of virus to cells, and ranged between 20 and 200. Viral dosing was calculated from the following equation:

((Cell number) x MOI x No. Of Replicates) ÷ Viral titre = Volume of virus to use (µL)

Virus suspension aliquots were stored at -80°C, thus avoiding repeated freeze thaw cycles. Thawed aliquots were briefly centrifuged and kept on ice.

2.3.3.1 Lentiviral infection protocol

hESC were seeded into 24 well plates coated with fibronectin (SA461) or Matrigel™ (H1) with the addition of ROCK Inhibitor (see section 2.1.1.1.) and allowed to attach overnight at 37°C, 5% CO_2 in a humidified incubator. Lentivirus was added to appropriate pluripotency maintenance media along with 4 µg/ mL polybrene (Sigma-Aldrich) and cells incubated overnight in the incubator. The media containing lentivirus was then removed and fresh pluripotency maintenance media added for 24 h to allow cells to recover. Cells could then either be maintained in pluripotency (section 2.1.1.1.) or directed to differentiate to the lineage of choice.

2.3.4 Cryo-preservation and recovery of cell lines

Cells were collected as detailed above and resuspended in 1 mL pluripotency maintenance media for SA461 or StemPro™ for RC10 and H1 with the addition of 10% dimethyl sulphoxide (DMSO) and 30% FCS per T-25 cells culture flask of cells. 1 mL of the cell suspension was aliquoted into cryo-preservation vials and subjected to cooling using isopropanol at a constant -1°C per minute to -80°C. Vials were stored under liquid nitrogen indefinitely. Recovery of cryo-preserved cells was performed by adding media pre-warmed to 37°C to the frozen cell

suspension, then adding this drop wise to a pre-coated flask (SA461) or plate (RC10 or H1). Media was replenished the following day.

2.4 Molecular biology

2.4.1 Extraction of DNA from cells

Total cellular DNA was extracted from cells using the QIAamp DNA Mini and Blood Mini kit (Qiagen, Crawley, UK) as per manufacturer's instructions. DNA binds to the silica of the QIAamp Mini Spin Column which was washed to remove contaminants. Cells were washed in PBS, 200 µL PBS added to each well of a cell culture plate and cells detached from the growth surface using a rubber policeman. The cell suspension was transferred to a clean 1.5 mL microcentrifuge tube and cells sedimented by centrifugation at 300 x g for 5 min. The supernatant was discarded, the cell pellet resuspended in 200 µL PBS, 20 µL QIAGEN proteinase K added, 200 µL buffer AL added and the sample pulse-vortexed for 15 sec. Buffer AL contains chaotropic salt guanidine hydrochloride, allowing the nucleic acids to adsorb to the silica membrane of the column while the polysaccharides and proteins do not bind and are washed away. The sample was incubated at 56°C in a waterbath for 10 min, 200 µL ethanol added and mixed by pulse-vortexing for 15 sec. The sample mixture was applied to a QIAamp Mini Spin Column and centrifuged for 1 minute at 6000 x g. The spin columns were washed with 500 µL of buffer AW1 (containing guanidine hydrochloride and ethanol) and then 500 µL of buffer AW2 (containing ethanol) was added and subjected to centrifugation for 3 min at 20, 000 x g followed by another centrifugation step for 1 minute at 20, 000 x g to eliminate likelihood of buffer AW2 carryover. The column was placed into a clean 1.5 mL microcentrifuge tube, 50 µL sterile distilled nuclease-free water added directly to the membrane, incubated for 1 minute at RT and then centrifuged at 6, 000 x g for 1 minute. This was repeated with the eluate to ensure highest possible yield. Extracted DNA was stored at -20°C.

2.4.2 Total RNA Extraction

Using the miRNAeasy Mini Kit (Qiagen, Crawley, UK), total cellular RNA was extracted with DNA contamination removed by treatment with TurboDNase™ (Ambion, Applied Biosystems, Warrington, UK) as per manufacturer protocols. Briefly, 700 µL QIAzol™ Lysis Reagent was added to the cell monolayer which was then scraped using a cell scraper, 200 µL pipette tip or a rubber policeman. Phenol and guanidine based, QIAzol™ allows efficient lysis of cells and chaotropic disruption allows the RNA to adsorb to the column membrane. The cell lysate was added into a nuclease-free 1.5 mL microcentrifuge tube and vortexed for 1 minute to ensure complete homogenisation. Allowing the sample to stand on the benchtop at RT for 5 min to promote dissociation of nucleoprotein complexes, 140 µL chloroform was then added and the tube shaken vigorously for 15 sec. The tube was placed on the benchtop at RT for 2-3 min and then centrifuged at 12,000 x g at 4°C for 15 min to allow the sample to separate into an upper, colourless phase containing total RNA, a white interphase containing DNA and a lower pink phase containing organic chemicals and proteins. The upper colourless phase was transferred to a fresh nuclease-free 1.5 mL microcentrifuge tube and 1.5 volumes 100% ethanol added and pipetted up and down 3 times to provide optimal conditions for RNA molecules greater than 18 nucleotides to bind to the silica-gel membrane of the column. Up to 700 µL of the sample was then loaded onto a RNeasy Mini spin column in a 2 mL collection tube and centrifuged at ≥8000 x g for 15 sec at RT with the flow-through discarded. This was repeated until the entire sample had passed through the spin column and then 700 µL of Buffer RWT (containing guanidine hydrochloride and ethanol) added and centrifuged at ≥8000 x g for 15 sec to wash. Following this, the spin column was washed twice with 500 µL Buffer RPE (a mild washing buffer containing ethanol), the first wash performed for 15 sec at ≥8000 x g and the second wash performed for 2 min at ≥8000 x g , both at RT. The spin column was then placed in a fresh 2 mL collection tube and centrifuged at full speed (13100 x g) for 1 minute at RT to eliminate any possible carryover of Buffer RPE. The spin column was transferred to a fresh 1.5 mL nuclease-free microcentrifuge tube, 30 µL nuclease free water added directly to the membrane and then centrifuged at ≥8000 x g for 1 minute with the eluate passed through the spin column again at ≥8000 x g for 1 minute to increase yield of RNA. The concentration of RNA was then determined by a NanoDrop™ (ND-1000

spectrophotometer, Labtech International, Ringmer, UK). At this point the RNA could be stored at -80 °C.

2.4.3 DNase Digestion

DNase digestion was performed using the TURBO DNA-free™ kit (Ambion). The reaction mixture for a 30 µL RNA sample comprised 3.5 µL 10x TURBO DNase buffer, 1 µL TURBO DNase (2 units/ µL) and 0.5 µL of nuclease-free water. This was placed at 37°C for 30 min, after which 5 µL DNase inactivation reagent was added and incubated at RT for 2 min. Following this, the sample was centrifuged at 6000 x g for 2 min at RT and the supernatant containing the DNase treated RNA carefully aspirated off and stored at -80 °C.

2.4.4 cDNA synthesis

To allow analysis of mRNA, cDNA was prepared from DNAse treated Total RNA using random primers and the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Warrington, UK). Up to 1 µg of RNA was diluted in nuclease-free water to a volume of 7.7 µL. The following reagents were added to create a reaction volume of 20 µL: 2 µL of 10x Buffer, 4.4 µL of 25 mM $MgCl_2$ (5 mM), 4 µL of dNTPs mix (500 µM each), 1 µL of Random Hexamers (2.5 µM), 0.4 µL of RNase Inhibitor and 0.5 µL of MultiScribe. Samples were placed in a MJ Research PTC Gradient Cycler set to 25°C for 10 min to allow pre-annealing, 48°C for 30 min for reverse transcription, 95°C for 5 min to inactivate reverse transcription and then held at 12°C. cDNA was stored at -20°C.

2.4.5 mRNA TaqMan® qRT-PCR analysis

TaqMan® qRT-PCR was used to quantify the relative gene expression levels of genes of interest (Table 1). Based on the detection of a fluorescent signal produced proportionally during amplification of a PCR product, the intensity of fluorescence released during the amplification cycle is directly proportional to the concentration of product produced in each cycle. Short primer sequences are designed against a target DNA sequence, between which a probe can anneal.

The probe is labelled at the 5' end with a reporter fluorophore and a quencher fluorophore at the 3' end. When intact, the quencher suppresses all fluorescence by the reporter. When the target sequence is present, the probe anneals and when the target sequence is amplified by the forward and reverse primers during the PCR, the probe is cleaved by AmpliTaq Gold. This means the quencher is separated from the reporter, resulting in a detectable increase in fluorescence. The fluorescence can be measured directly or relative to a control sample. Data is acquired whilst PCR is in the exponential phase and is measured when the reporter dye emission intensity rises above a background level, called the cycle threshold (C_t). A housekeeping gene which would remain stable and constant allows for corrections due to RNA concentration to be made. The ΔC_t is the difference between the gene C_t and the housekeeper C_t, allowing the signal to be corrected against the total RNA content. The mean ΔC_t between the triplicates on the 384 well PCR plate was calculated which was then used to calculate the $\Delta\Delta C_t$ which relates the amount of cDNA of the target gene to the ΔC_t value and the control sample within the experiment. The $2^{-\Delta\Delta Ct}$ shows the fold increase or decrease in gene expression of each sample in comparison to the appropriate biological control, which has the value 1 (Livak and Schmittgen, 2001).

QRT-PCR reactions were carried out in 384-well plates in technical triplicate, using TaqMan® probes (Table 2.1) and normalised to 18s or GAPDH housekeeping genes (Applied Biosystems). Briefly, the reaction mixture of a 10 µL reaction comprised 5 µL of 2x Taqman Mastermix, 0.5 µL of probe, 3 µL of water and 1.5 µL of cDNA. This was added per well of the 384-well plate and run on an ABI Prism® 7900Ht sequence Detection System (Applied Biosystems) and mean values used in subsequent analyses.

Table 2.1: List of TaqMan® qRT-PCR Gene Expression Assays.

List of gene expression assays used in this study, with RefSeq Gene Identifiers and Applied Biosystems Assay Identifiers.

Gene	RefSeq Gene ID	Assay ID
18S	N/A	Hs99999901_s1
ACTN2	NM_001103.2	Hs00153809_m1
ANKRA2	NM_023039.4	Hs00969413_g1
ASCL2	NM_005170.2	Hs00944285_s1
CXXC1	NM_001101654.1	Hs00969406_g1
DNMT3L	NM_175867.1	Hs01081364_m1
EHMT2	NM_006709.3	Hs00938384_g1
FOXA2	NM_021784.4	Hs00232764_m1
GABPB1	NM_002041.4	Hs01065385_m1
GAPDH	NM_002046.3	Hs02758991_g1
HDAC5	NM_001015053.1	Hs01108316_g1
L3MBTL2	NM_031488.4	Hs01002038_g1
MLL3	NM_170606.2	Hs01005539_m1
MNT	NM_020310.2	Hs00947071_g1
NKX2.5	NM_004387.3	Hs00231763_m1
RAX2	NM_032753.3	Hs00262109_m1
RBM4	NM_006328.3	Hs01056358_m1
RFX1	NM_002918.4	Hs00958823_m1
SNAPC4	NM_003086.2	Hs00967025_g1
SRA1	NM_001035235.2	Hs00398296_g1
T	NM_003181.2	Hs00610080_m1
TAF6L	NM_006473.2	Hs01008038_m1
TFAP4	NM_003223.2	Hs01558245_m1
TNNT2	NM_000364.2	Hs00165960_m1
TSC22D3	NM_001015881.1	Hs00608272_m1
UBTF	NM_001076683.1	Hs00610733_g1
USF2	NM_003367.2	Hs01100995_g1
ZNF35	NM_003420.3	Hs01071488_m1

2.4.6 MicroRNA (miRNA) TaqMan® qRT-PCR analysis

MiRNA expression was assayed by using specific miRNA primers included with the TaqMan® microRNA Expression Assays (Applied Biosystems) to reverse transcribe sample Total RNA using the miRNA Reverse Transcription Kit. Briefly, in a 7.5 µL reaction, 0.075 µL of dNTPS, 0.5 µL of multiscribe, 0.75 µL of 10x RT buffer, 0.095 µL of RNA inhibitor, 2.08 µL of water, 1.5 µL of 5x RT primer and 2.5 µL of Total RNA diluted to 2 ng/µL were added to 1 well of a 96-well plate. This was then run on a thermal cycler at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and held at 12°C before being stored at -20°C.

To perform the qRT-PCR portion of the experiment, 5 µL of 2x TaqMan Mastermix, 0.5 µL of specific miRNA probe, 3.835 µL of water and 0.67 µL of miRNA RT product were assembled in 1 well of a 384-well plate. Samples were normalised to RNU48 housekeeping assay, run in technical triplicate on an ABI Prism® 7900Ht sequence Detection System (Applied Biosystems) and mean values used in subsequent analyses. A list of assays used can be found in Table 2.2. Relative Quantification (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method previously described in Section 2.2.5 (Livak and Schmittgen, 2001), where each miRNA was compared to RNU48 and a control sample.

Table 2.2: TaqMan® MicroRNA expression assays.

List of qRT-PCR TaqMan® MicroRNA expression assays used in this study, including miRNA name, MIRBASE accession number and Applied Biosystems Identifier.

Gene	MIRBASE Accession	Assay ID
hsa-miR-1-1	MIMAT0000416	002222
hsa-miR-23a	MIMAT0000078	000399
hsa-miR-23b	MIMAT0000418	000400
hsa-miR-99b	MIMAT0000689	000436
hsa-miR-125a-5p	MIMAT0000443	002198
hsa-miR-133b	MIMAT0000770	002247
hsa-miR-181a	MIMAT0000256	000480
hsa-miR-181b	MIMAT0000270	001098
RNU48	N/A	001006

2.4.7 miRNA microarray

SA461 hESC were assayed for miRNA expression using a 2-channel human miRNA microarray (LC Sciences, Houston, TX, USA). Total RNA was extracted and DNase treated as detailed previously. The miRNA probes were designed around miRBase version 10.1 (miRBase, University of Manchester, UK). To assess significance in the resulting intergroup comparisons within the dataset Rank Products were utilised as previously described (Breitling et al., 2004) (Caruso et al., 2010) allowing statistical significance to be established at a False Discovery Rate (FDR) of <0.05.

2.4.8 Northern Blotting

miRNA expression was further quantified by Northern Blotting. Briefly, 10µg total RNA was run overnight at RT on a 15% denaturing polyacrylamide gel and then transferred for 90 min using a Biorad semidry transfer system onto a nylon membrane (Hybond, GE Healthcare Life Sciences, UK). Nylon membranes are most appropriate as they have a positive charge and the negatively charged nucleic acid backbone has a high affinity for them. Following 1-Ethyl-3-[3-dimethylaminopropyl] carbodimide hydrochloride (EDC) crosslinking at 60°C for 60 min, the membrane was subject to pre-hybridisation in hybridisation buffer

(50% formamide), for 60 min before hybridisation overnight with digoxigenin-labelled probe specific to each miRNA (Exiqon, Denmark). Formamide lowers the annealing temperature of the probe, preventing RNA degradation associated with higher temperatures. The membranes were washed with low and high stringency wash buffer (Ambion). A mouse anti-digoxigenin antibody (Sigma) was then used and bands visualised by development with CDP-Star®Chemiluminescent Substrate (Invitrogen).

2.4.9 Illumina whole genome expression microarray

Amplification of sample RNA for the Illumina array was achieved by using the Illumina® TotalPrep RNA Amplification Kit (AMIL1791) as per manufacturer's instructions. The kit generates biotinylated, amplified RNA for hybridization with Illumina arrays. The RNA amplification protocol is based on the method developed in the laboratory of James Eberwine (Van Gelder et al., 1990). The method included reverse transcription with an oligo(dT) primer bearing a T7 promoter using Array Script, a reverse transcriptase (RT) designed to yield higher concentrations of first strand cDNA than wild-type enzymes which resulted in the synthesis of virtually full-length cDNA. The cDNA was then subjected to second strand synthesis and cleanup, where it became a template for in vitro transcription (IVT) with T7 RNA polymerase. IVT along with biotin-UTP was used to generate hundreds to thousands of biotinylated copies of antisense RNA. This labelled antisense RNA (cRNA; aRNA) was then hybridised to Illumina Beadchips.

Starting RNA was subjected to QC checks by NanoDrop to ensure no contaminating proteins, DNA, phenol ethanol and salts remained after miRneasy RNA isolation. The Agilent® 2100 bioanalyser was employed to ensure RNA sample integrity. RNA Integrity Number (RIN) of 8.0+ was deemed acceptable for samples to go forward for microarray analysis. In a non-stick, sterile, RNase-free tube, 350 ng of Total RNA (50-500 ng is recommended) was brought to 11 µL with nuclease-free water. Separately, Reverse Transcription Master Mix was assembled at RT in a nuclease-fee tube. For a single 20 µL reaction, 1 µL of T7 Oligo(dT) Primer, 2 µL of 10X First Strand Buffer, 4 µL of dNTP Mix, 1 µL of RNase Inhibitor and 1 µL of ArrayScript were mixed gently, centrifuged briefly and placed on ice. 9 µL of Reverse Transcription Master Mix was transferred to each RNA sample and the sample mixed by pipetting up and down 2-3 times then

flicking the tube 3-4 times with brief centrifugation to bring the sample to the bottom of the centrifuge tube. The reactions were then placed in a thermal cycler set to 42°C for 2 h, after which the samples were centrifuged and placed on ice. On ice, a Second Strand Master Mix was prepared in a nuclease-free tube comprising, in order, 63 µL nuclease-free water, 10 µL 10X Second Strand Buffer, 4 µL dNTP Mix, 2 µL DNA Polymerase and 1 µL RNase H for a single 100 µL reaction. This was mixed gently and briefly centrifuged. 80 µL of Second Strand Master Mix was added to each sample, the sample mixed by pipetting up and down 3-4 times and centrifuged briefly, put in a thermal cycler set to 16°C for 2 h and placed on ice. 250 µL of cDNA Binding Buffer was added to each sample, mixed by pipetting up and down 2-3 times and flicking the tube 3-4 times with brief centrifugation and the sample loaded onto the cDNA Filter Cartridge. The cDNA cartridge was centrifuged at 10000 x g for 1 minute at RT and the flow-through discarded. 500 µL Wash Buffer was added to the cDNA Filter Cartridge and centrifuged at 10,000 x g for 1 minute with the flow-through discarded and the empty cDNA Filter Cartridge centrifuged for an additional minute to remove all traces of Wash Buffer. The cDNA Filter Cartridge was transferred to a cDNA Elution Tube. 20 µL of pre-heated nuclease-free water at 55°C was added to the centre of the filter in the cDNA Filter Cartridge and left at RT for 2 min. The cDNA Filter Cartridge was centrifuged for 1 minute at 10,000 x g, leaving the double stranded cDNA in the eluate. The cDNA was stored overnight at -20°C at this point, as directed by the protocol. Each cDNA sample was transferred to a 0.5 mL nuclease-free microcentrifuge tube and at RT an IVT Master Mix assembled, comprising 2.5 µL T7 10X Reaction Buffer, 2.5 µL T7 Enzyme Mix and 2.5 µL Biotin-NTP Mix per 25 µL reaction. This was mixed gently, centrifuged briefly and placed on ice. 7.5 µL of IVT Master Mix was added to each cDNA sample, mixed by pipetting up and down 2-3 times and flicking the tube 3-4 times with brief centrifugation, placed in a thermal cycler set to 37°C for 14 h. The reaction was stopped by the addition of 75 µL Nuclease-free water to each cRNA sample, bringing the final volume to 100ul. 350 µL of cRNA Binding Buffer was added to each cRNA sample, 250 µL of ACS reagent grade 100% ethanol added to sample and the mixture pipetted up and down 3 times. The mixture was immediately added to the centre of the filter of a cRNA Filter Cartridge, centrifuged at 10,000 x g for 1 minute and the flow-through discarded. 650 µL Wash Buffer was then added to the cRNA Filter Cartridge and centrifuged at 10,

000 x g for 1 minute, the flow-through discarded and the cRNA Filter Cartridge centrifuged for an additional minute to remove all trace of Wash Buffer. The cRNA Filter Cartridge was transferred to a fresh cRNA Collection Tube, 200 µL nuclease-free water heated to 55°C added to the centre of the filter in the cRNA Filter Cartridge and placed in a hybridisation oven at 55°C for 10 min. The cRNA Filter Cartridge was centrifuged at 10, 000 x g for 1.5 min, eluting the cRNA. At this stage samples were subjected to Nanodrop analysis to ascertain cRNA concentration was at least 150ng/µL, if not then samples were concentrated by vacuum centrifugation.

The resultant samples were then hybridised to the Illumina® HumanHT-12 v3 Expression BeadChip as per manufacturers protocol (cat number BD-901-1002). Briefly, the cRNA was pre-heated to 65°C for 5 min, vortexed, pulse centrifuged at 250 x g and allowed to cool to RT and 750ng pipetted into a microcentrifuge tube with the appropriate volume of nuclease-free water added to bring the sample to 5 µL. 10 µL of HYB was added to the sample and the Hyb Chamber assembled with 200 µL of HCB in each humidifying buffer reservoir. The sample was pipetted onto the centre of each inlet port on the beadchip, the beadchip placed into the Hyb chamber and the Hyb chamber placed into an illumina Hybridisation over at 58°C for 14 h. The beadchips were removed from the Hyb chambers, submerged in diluted wash E1BC buffer and the cover-seal removed. Following this the beadchips were placed in a slide rack submerged in a glass staining dish containing diluted wash E1BC then transferred to a Hybex Waterbath containing High-Temp Wash buffer at 55°C for 10 min. The slide rack was placed into a staining dish containing 250 mL fresh Wash E1BC buffer and plunged in and out of the solution 5-10 times before being put back into the staining dish and placed on an orbital shaker for 5 min at RT at medium speed. The beadchips were subsequently moved to a new staining dish containing 250 mL fresh 100% Ethanol, the sliderack plunged in and out of the solution 5-10 times and the staining dish placed on an orbital shaker for 10 min at RT. The beadchips were transferred back to the original staining dish containing 250 mL Wash E1BC buffer, plunged in and out of the solution 5-10 times and placed on an orbital shaker for 2 min at RT. The beadchips were subsequently put face up into a beadchip wash tray with 4 mL Block E1 buffer and placed on a rocker mixer at medium speed for 10 min at RT. The beadchips were transferred to

another wash tray containing 2 mL Block E1 buffer and 1:1,000 dilution of Cy3-Streptavidin and placed on a rocker mixer at medium speed for 10 min at RT. Subsequently, the beadchips were transferred to fresh Wash E1BC, plunged in and out of the solution 5 times before being placed submerged in Wash E1BC and placed on an orbital shaker at medium-low for 5 min at RT. The slide rack containing the beadchips was then transferred to a centrifuge and subjected to 1,400 x rpm for 4 min at RT after which they were imaged on an Illumina® BeadArray Reader.

Whole genome gene expression was determined for SA461 hESC differentiating to endothelial lineage as per design of the miRNA microarray, with data quantile normalised and exported from Genome Studio (Illumina, CA, USA) for uploading to Partek (Partek Inc., MO, USA) where the data was subjected to transformation by Principle Components Analysis (PCA). An analysis of variance (ANOVA) model was then created with step-up false discovery rate (FDR), taking into account chip effects and allowing pairwise comparisons. The data were then uploaded to Ingenuity Pathway Analysis (IPA, CA, USA, www.ingenuity.com) and pathways with the most significantly different genes interrogated, as well as dynamic expression of genes expressed at specific timepoints. In addition, a primary bivalency dataset from H9 hESC (Ku et al., 2008) was integrated with the data from the Illumina, allowing a prediction of bivalent status to be made for around ~2,500 genes.

2.4.10 Immunocytochemistry

Cells in culture plates were washed 3 times in PBS and fixed with 4% paraformaldehyde (PFA) for 15 min at RT. The PFA was removed and cells washed 3 times in PBS, and could be stored with a volume of PBS covering the cells at 4°C before continuing with the staining protocol. Cells were then exposed to permeabilising/blocking solution (P/B) (PBS, 10% donkey serum, 1% BSA and 0.1% Triton-X-100) for 30 min at RT. Primary antibodies were diluted in P/B solution as indicated in table X and incubated for 4 h at RT. Excess primary antibody was removed by 3 x 5 minute washes with PBS. Cells were subsequently incubated with the appropriate secondary antibody diluted in P/B solution as indicated in table x for 1 h. Plates were protected from light to prevent photo bleaching of sample. Cells were washed for 5 min with PBS on a rocker 3 times

to remove excess secondary antibody, a drop of ProLong Gold with DAPI (Invitrogen) added to the cell surface and a glass coverslip placed over the top. Cells were imaged using a Zeiss Axiovert 200M.

Table 2.3: Primary Antibodies.

Primary antibodies used for Immunocytochemistry studies, with information of the species raised in, dilution used, catalogue number and the supplier.

Protein	Species Raised In	Dilution Used	Catalogue Number	Company
Oct 4	Mouse	1 in 200	SC5279	Santa Cruz
FOXA2	Goat	1 in 50	AF2400	R&D Systems
Troponin T	Rabbit	1 in 200	AB45932	Abcam
NKX2.5	Rabbit	1 in 100	SC14033	Santa Cruz
Sarcomeric α-actinin	Mouse	1 in 400	A7811	Sigma

Table 2.4: Secondary Antibodies

Secondary antibodies used for immunocytochemistry studies, with information of the species raised in, dilution used, catalogue number and the supplier.

Fluorophore	Species Raised In	Dilution Used	Catalogue Number	Company
Alexafluor-555	Goat Anti-Mouse	1 in 400	A21424	Invitrogen
Alexafluor-488	Donkey Anti-Goat	1 in 400	A11055	Invitrogen
Alexafluor-488	Goat Anti-Rabbit	1 in 400	A11008	Invitrogen
Alexafluor-555	Goat Ant-Rabbit	1 in 400	A21428	Invitrogen

2.4.11 DNA Cloning

Eukaryotic expression plasmids were employed to create miRNA and transcription factor overexpression lentiviruses as well as reporter viruses driving GFP.

2.4.12 Small Scale Plasmid DNA Purification: QIAprep Mini Prep kit

Single bacterial colonies were picked from Luria Agar plates in the morning and used to inoculate starter cultures grown in 10 mL Luria Broth containing 100 µg/mL ampicillin at 37°C in a shaking incubator for 7 h. 2 mL of this was aliquotted and stored at 4°C for DNA extraction by QIAprep Mini Prep Kit, diagnostic restriction endonuclease digestion and sequencing to ensure cloning success.

Small scale plasmid purification was achieved by using the QIAprep Mini Prep kit (Qiagen, Crawley, UK). The kit works based on the alkaline lysis of bacterial cells and the binding of DNA to a silica membrane in the presence of high salt buffer followed by elution in low salt buffer. Bacteria were harvested by centrifugation at 6800 x g, for 3 min at RT. The bacterial pellet was then resuspended in 250 µL buffer P1 (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A), 250 µL of buffer P2 (200 mM NaOH, 1% SDS) was added to break down the bacterial cell wall and denature proteins, lysing the cells. The lysis was then neutralised by the addition of 350 µL buffer N3 (proprietary) and allowed high salt binding and precipitation of SDS, cellular debris and chromosomal DNA. Samples were then centrifuged at 17,900 x g for 10 min at RT and the supernatant applied to a

QIAprep spin column containing a silica membrane. The QIAprep spin column was then centrifuged at 17, 900 x g for 1 minute and the DNA bound to column washed with 500 µL buffer PB to remove endonucleases. A further wash with 750 µL of buffer PE removed salts and incubation of the column with 50 µL sterile nuclease-free water followed by centrifugation 18,000 x g for 1 minute eluted plasmid DNA. Plasmid DNA was routinely stored at -20°C.

2.4.13 Large Scale Plasmid DNA Purification: Qiagen Plasmid Maxi Kit

Large scale plasmid purification was achieved by using the Qiagen Plasmid Maxi Kit. The kit is also based on the alkaline lysis principle but DNA is bound to the column using an anion-charge membrane. In summary, a single colony of transformed bacteria was grown in 10 mL of Luria broth as previously detailed. After diagnostics performed to ensure correct clone selected, the starter culture was transferred to a conical flask containing 500 mL Luria broth and placed in an oscliating incubator overnight at 37°C. Bacteria were collected by centrifugation at 6, 000 x g for 15 min at 4°C (centrifuge model). The resulting pellet was then resuspended with 10 mL of P1 buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A) and 10 mL of buffer P2 (200 mM NaOH, 1% SDS) added and incubated for 5 min at RT. SDS disrupts the bacterial membrane and NaOH denatures the chromosomal DNA and proteins. 10 mL of chilled buffer P3 (3M potassium acetate at pH5.5) was then added to neutralise lysis of the bacterial cells, mixed vigorously and incubated on ice for 20 min. The precipitated lysate was then subjected to centrifugation at 4°C for 30 min at 20, 000 x g and the supernatant applied to a QIAGEN-tip 500 column pre-wetted with 10 mL buffer QBT (750 mM NaCl, 50 mM 3-morpholinopropanesulfonic acid pH7 (MOPS), 15% isopropanol, 0.15% Triton-X 100). The QIAGEN-tip 500 was allowed to empty by gravity flow, washed twice with 30 mL with buffer QC (1M NaCl, 50 mM MOPS pH7, 15% isopropanol) and DNA eluted with 15 mL of buffer QF (1.25M NaCl, 50 mM Tris pH8.5, 15% isopropanol) into a round bottom polypropylene centrifuge tube. Eluted DNA was desalted, precipitated by addition of 10.5 mL of isopropanol and subjected to centrifugation for 30 min at 15, 000 x g at 4°C. The resulting DNA pellet was washed with 5 mL of 70% ethanol and centrifuged for 10 min at 15, 000 x g at 4°C, the ethanol carefully removed and the DNA pellet allowed to air dry before resuspension in 300 µL sterile water.

2.4.14 Restriction Digestion

Digestion of double stranded plasmid DNA was achieved by use of Restriction Endonuclease Enzymes. This allowed insertion of specific DNA sequences (inserts) into plasmid backbones for expression. Both inserts and plasmid backbones were digested with appropriate restriction enzymes to generate compatible ends suitable for ligation. Briefly, plasmid DNA was digested with restriction endonucleases in either a small scale 20 µL reaction for diagnostic purposes (containing 100-200ng of DNA, 2 µL of restriction endonuclease, 2 µL of buffer, 1 µL of BSA and made to 20 µL with sterile water, incubated for 1-2 h at 37°C), or a larger scale 50 µL reaction for isolation and subsequent purification of insert or plasmid backbone (comprising 10-20 µg of DNA, 5 µL of restriction endonuclease, 5 µL of buffer, 5 µL of BSA and made up to 50 µL with sterile water, incubated overnight at 37°C). Reactions were then subjected to analysis by agarose gel electrophoresis.

2.4.15 Agarose gel electrophoresis

Gel electrophoresis separates nucleic acids or proteins by using a crosslinked polymer exposed to an electrical current. This works to separate nucleic acids or proteins as they have a negative charge and run towards a positive electrode or anode. As the samples migrate through the polymer, pores of the gel allow separation dependant on size. Restriction endonuclease digests were electrophoresed through an appropriate percentage (0.6-2% w/v) agarose gel (Invitrogen) in 1x Tris/Borate/EDTA (TBE) (10 mM Tris, 10 mM boric acid, 10 mM EDTA, pH 8.3) with ethidium bromide (Sigma-Aldrich) added at 10 ng/mL. Samples and DNA marker ladders (100 bp or 1 Kb) (Promega, Southampton, UK) were mixed with 6x blue/green loading dye (Invitrogen) before being loaded onto the gel and electrophoresed at a constant voltage of 30-100 V with 1x TBE used as running buffer. Bands on the gel were visualised using trans UV illumination on a ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.4.16 Gel Extraction of DNA

The Wizard® SV Gel and PCR Clean-Up System is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. To purify

restriction endonuclease digested DNA, samples were electrophoresed on an agarose gel and resultant bands localised using UV illumination as described in agarose gel elctrophoresis. The bands were excised using a clean scalpel blade and placed into a clean, pre-weighed 1.5 mL microcentrifuge tube. DNA was extracted from the agarose using the Wizard® SV Gel and PCR Clean-up System (Promega, Southampton, UK) following the manufacturers protocol. Briefly, 10 µL of Membrane Binding Solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate, pH 5.0) per 10 µg of gel was added and mixed thoroughly before being placed in a 65°C waterbath until the gel was dissolved fully. The melted gel mixture was then transferred to a silica column where it was incubated for 1 minute at RT and then subjected to centrifugation at 16, 000 x g for 1 minute at RT. The column was washed twice with membrane wash solution (10 mM potassium acetate pH 5, 80% ethanol, 16.7µM EDTA pH 8.0) with 700 µL and then 500 µL respectively and centrifuged at 16, 000 x g for 1 minute at RT. The column was then centrifuged for 16, 000 x g for 5 min to ensure no ethanol carry over. The purified DNA was eluted from the column by addition of 50 µL sterile nuclease-free water to the column membrane and centrifugation at 16, 000 x g for 1 minute and the eluate passed through the column a second time to ensure the highest possible yield. DNA was stored at -20°C.

2.4.17 Dephosphorylation of plasmid DNA

To prevent re-circularisation of plasmid backbone DNA after restriction endonuclease digestion, dephosphorylation was performed using the Antartic Alkaline Phosphatase (New England Biolabs UK Ltd (NEB), Hertfordshire, UK) according to manufacturer protocol. Briefly, 1-5µg of restriction endonuclease digested DNA was added to 1/10 volume of 10x Antartic Phosphatase Reaction Buffer, 1 µL Antartic Phosphatase and the reaction volume made up with sterile water. Following incubation at 37°C for 15 min the reaction was heated to 65°C for 5 min to inactivate the enzyme.

2.4.18 Ligation of insert into plasmid backbone

To ligate inserts into dephosphorylated plasmid backbone, T4 ligase (NEB) was used as per manufacturer instructions. In summary, ligation reactions were carried out at a range of molar ratios to increase the likelihood of the correct clone being present. Ratios of 5:1, 3:1 and 1:1 were routinely used. The formula for calculating molar ratios was:

$$\text{ng of Insert: } \frac{\text{(ng of vector) x (Kb size of insert)}}{\text{Kb size of vector}} \text{ x (molar ratio)}$$

DNA for ligation, 1 µL of T4 ligase, 1 µL of 10x T4 ligase buffer were added to a clean 0.5 mL microcentrifuge tube and reactions were made up to 10 µL using sterile water. Reactions were incubated for 1-4 h at RT or overnight at 4°C. Ligations could then be transformed.

2.4.19 Transformation of Chemically Competent Bacteria

Both JM109 and Top10 chemically competent *Escherichia coli* (*E. coli*) bacteria (Invitrogen, Paisley, UK) were used as hosts for plasmids detailed in this study. The plasmids used had ampicillan resistance to allow for selection of clones. To transform, 40 µL of competent cells per transformation were thawed on ice and 2ng of intact plasmid or 5 µL of ligated plasmid/insert mixes were added. After gentle mixing, cells were left on ice for 30 min before being heat-shocked at 42°C for 30 sec and returned to ice for 2 min. 250 µL of Super Optimal broth with Catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate and 20 mM glucose per 1L)(Invitrogen) was added and tubes placed in a shaking incubator (incubator model here) at 37°C for 1 h, 180 oscillations per minute. Transformation mixtures were plated out onto Luria Agar plates containing 100 µg/mL ampicillin (Sigma); for all transformations 20 µL and 200 µL were plated. Cultures were allowed to dry, plates inverted and placed into a 37°C incubator (Heraeus) overnight, and checked for resultant bacterial colonies.

2.4.20 DNA sequence analysis

DNA sequences were analysed by dideoxy sequencing. Based on the use of dideoxynucleotides labelled with a different fluorophore at the 3' end with missing hydroxyl groups so that another nucleotide cannot bind, this method generates a series of different size DNA fragments each with a fluorescently labelled dNTP as its terminal nucleotide. This can then be translated by excitation with a laser and recording of the wavelength each fluorophore emits, building up nucleotide by nucleotide the sequence information.

Sequencing of cloned plasmids was performed routinely to check for cloning efficiency. DNA was prepared as previously detailed and 300ng used as template for the sequencing reaction. Briefly, 2nM of forward or reverse primers were used (see table for primer sequences), 0.5 µL of Ready Reaction Mix (Applied Biosystems, UK), 3.5 µL Sequencing buffer were added to template DNA and sterile water to result in a 20 µL volume. This was subjected to 25 PCR cycles of 96°C for 50 sec, 50°C for 20 sec and 60°C for 3 min. The PCR products were then cleaned with CleanSEQ (Agencourt Bioscience Corporation, MA, USA) following manufacturers protocol. The resultant sample was then analysed using an ABI 3730 automated sequencer and SeqScape v2.0 software (Applied Biosystems).

2.4.21 Promoter activity assay

SA461 hESC were infected with GFP-expressing lentiviruses, either under control of a CytoMegaloVirus (CMV) or spleen focus-forming virus (SFFV) promoter with the control experiment of uninfected cells of the same passage number. The cells were either maintained in pluripotency (section 2.1.1.1), underwent directed differentiation to vascular endothelial cells (section 2.1.1.2) , were allowed to passively differentiate by addition of untreated MEM media (Invitrogen, Paisley, UK), or underwent directed to cardiomyocyte lineage (section 2.1.1.3). Visual analysis of promoter-driven GFP was assessed on a daily basis.

Cellular protein was harvested at 2 days and 7 days post infection by washing in PBS twice, 0.2% Triton-X-100 in PBS (v/v) added and cells scraped from the growth surface using a rubber policeman. Plates were placed at -20°C to ensure complete cell lysis and could be stored indefinitely before protein samples were subjected to a Green Fluorescent Protein (GFP) luminescence assay and protein quantification by BCA assay.

2.4.21.1 Green Fluorescent Protein (GFP) Luminescence assay

Cells were prepared as detailed in 2.2.21. Dilutions of recombinant eGFP (Clontech, Basingstoke, UK) in cell lysis buffer were used to create a standard curve as follows: 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL, 0.1 µg/mL, 0.05 µg/mL and 0.01 µg/mL. 20 µL of each standard was added in duplicate and of each sample in triplicate to a pre-chilled black 96-well plate with 80 µL of cell lysis buffer. The plate was then analysed immediately on a Wallac Victor^2 plate reader spectrophotometer (Wallac, Turku, Finland) at absorbance 507 nm. For both standards and samples average relative fluorescent units (RFU) were calculated and a linear standard curve generated. Expression of eGFP was normalised to total protein amount determined by BCA assay (2.2.21.2) and results shown as RFU/mg of protein (Nicklin et al., 2001).

2.4.21.2 Determination of protein concentration

Protein concentration in cellular samples was quantified using a colourimetric detection protocol, the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Illinois, USA) following manufacturer's instructions. In summary, a standard curve from the following Bovine Serum Albumen (BSA) dilutions was created: 2 mg/mL, 1.5 mg/mL, 1 mg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 25 µg/mL. Working reagent (WR) is created by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B with 200 µL of WR added to 25 µL of standard or sample in duplicate in a 96-well plate. The plate was incubated for 30 min at 37°C in the dark and then analysed at absorbance 570 nm using a Wallac Victor^2 plate reader spectrophotometer (Wallac). Results were extrapolated by plotting the unknown samples onto the standard curve created by the standard BSA dilutions.

2.4.22 Manipulation of miRNA expression – overexpression

A library of human pre-miRNA clones was obtained (under MTA from Reuven Agami, Netherlands Cancer Institute) that encode for over 350 human miRNAs. These are in retroviral and lentiviral clones under the control of the CMV promoter.

The miRNA's of interest were cloned in intermediate pri-miRNA form from the retrovirus plasmid to lentiviral expression vector under control of the SFFV promoter via the intermediate vector pCDNA3.1/Zeo(+) (Figure 5). The success of the cloning was verified by sequencing, diagnostic gels and TaqMan® miRNA expression assay.

The revised microRNA overexpression strategy involved designing inserts containing individual pre-miRNA stem loop sequences between appropriate restriction endonuclease sites, kozak sequence and stop codon (Figure ??). These were then chemically synthesised by GeneArt® (Life Technologies, Warrington, UK) and subjected to step-wise cloning by restriction endonuclease digestion to liberate from the GeneArt® plasmid, ligation into PCDNA3.1+/Zeo (Invitrogen) followed by ligation into a lentivirus expression plasmid, pSFFV-MCS. Success of cloning was verified by sequencing, diagnostic agarose gel electrophoresis and TaqMan® miRNA expression assay.

2.4.23 Chromatin ImmunoPrecipitation

In order to ascertain which methylation signature the transcriptional start site (TSS) of FOXA2 had, chromatin immunoprecipitation (ChIP) was performed.

Adherant hESC either maintained pluripotent or differentiated using endothelial differentiation media were first subjected to cross-linking by the addition of 1% w/v formaldehyde (Sigma-Aldrich) diluted in culture media with flasks placed on a rocker for 15 minutes at room temperature. To quench the cross-linking reaction, glycine was added to a final concentration of 125 mM with a further 5 minutes rocking at room temperature. The media was discarded and cells washed twice in standard PBS (Gibco). PBS with protease inhibitor cocktail (Roche, Hertfordshire, UK) was used to scrape cells from the growth surface, before samples were centrifuged at 500 x g for 5 minutes at 4°C. The

supernatant was carefully aspirated and cell pellets were snap frozen and stored at -80°C indefinitely until required.

To prepare chromatin, cell pellets were resuspended in Sarkosyl lysis buffer (SLB, 10mM TRIS pH 8.0, 0.5% w/v Sarkosyl, 5mM EDTA, 2.5mM EGTA) plus protease inhibitors and incubated on ice for 10 minutes. Cells were then collected by centrifugation at 200 x g, for 5 minutes at 4°C, the supernatant carefully aspirated and the pellet resuspended in SLB again. 5 µL of the cell suspension was placed onto a glass slide and checked by microscope to ensure cells were homogenously resuspended. The cells were then aliquotted into 2 mL microcentrifuge tubes and subjected to sonication in a Diagenode Bioruptor XL on H, 2 seconds on, 2 seconds off for 15 minutes at 4°C. After sonication, 5µL of the sample was placed on a glass slide and visualised by Olympus IX51 microscope to ensure sonication of cells a success. Samples were subjected to centrifugation at 13,100 x g for 5 minutes at 4°C twice with the supernatant moved to fresh tubes in between centrifugations. The supernatant was carefully collected and identical chromatin samples pooled into fresh tubes. The samples were measured by Qubit dsDNA kit (Invitrogen) and diluted with lysis buffer used to normalise the concentration of the samples. After that sonicated chromatin was diluted at 1:4 with Immunoprecipitation Dilution Buffer (IPDB; 20mM Tris pH8.0; 1mM EDTA pH8.0; 1mM EGTA pH8.0; 1% Triton X100; 0.01%SDS; 150mM NaCl) to prepare input.

For immunoprecipitation, 200µL of magnetic Dynabeads® M-280 Sheep anti-Rabbit IgG (Invitrogen) were simultaneously washed and blocked with 0.5% w/v BSA in PBS twice, then 2mL of 0.5% w/v BSA in PBS added to the beads to resuspend and 500µL of bead suspension aliquotted for each IP. 2.5µg of total H3 antibody (AB1791, Abcam), H3K27me3 antibody (C36B11, Cell Signalling, NEB) or H3K4me3 antibody (AB8580, Abcam) was added to beads and incubated for 1 hour at room temperature with agitation. The beads were washed in IPDB 3 times before 450µL of chromatin sample being added to each tube, and samples placed at 4°C for 2-3 hours with agitation. The beads were then washed twice with IPDB, once with high salt wash (20mM Tris pH8.0; 1mM EDTA pH8.0; 1% Triton X100; 0.01%SDS; 500mM NaCl), once with IPWB2 (10mM Tris pH8.0; 1mM EDTA pH8.0; 1% NP40; 1% Na-DOC; 250mM LiCl) and twice with TE buffer (10mM Tris pH8.0; 1mM EDTA). 50 µL of 10% Chelex-100 suspension (BioRAD) was then

added per IP, vortexed, and incubated for 15 min at 97C. The IPs were vortexed, briefly centrifuged and 450µL of nuclease-free water added before being vortexed and spun down again ready for PCR.

To perform qPCR using primer pairs directed at TSS of the gene of interest FOXA2 (Table 2.5), the DyNAmo ColorFlash kit (Finnzymes, Thermo Scientific, Fisher Scientific UK Ltd, Leicestershire, UK) was used. Briefly, 5µL of chromatin, 10µL of F416 Mastermix, 2µL of primer pair mix (2.5µM) and 3µL of water were added per well of a 96 well white PCR plate. Samples were run in quadruplicate along with a NTC and input control on a Bio-Rad Chromo 4 machine and qPCR data analysed using Opticon3 software. Data are shown normalised to 2% input sample, which represents the amount of chromatin used in the ChIP.

Table 2.5: Primers used for FOXA2 ChIP.
Sequences of forward and reverse primers used at the FOXA2 TSS.

Region	Forward Primer	Reverse Primer
2	5'-AAGGCCTTGCTCAAAGTTCC-3'	5'-TCACTTTTCCATCCCCTCTG-3'
7	5'-TTTCAGTCACAACCGAGGTG-3'	5'-TCAACTCCATATGCCCCATC-3'
10	5'-TGGAAAAGACGAGCGCTTAC-3'	5'-TTGCTGGTCGTTTGTTGTG-3'
17	5'-CCGAGTCTGTTTCATTCAGG-3'	5'-TCTAGAATCTGGGCGAGGTG-3'

2.4.24 Statistical Analyses

Where appropriate, values were presented as mean ± standard error of the mean (SEM). Analysis of experiments with multiple treatment groups was performed by repeated measures analysis of variance (ANOVA). Significant differences were determined by Tukey post-hoc testing and significance was cut off at $p<0.05$. Specific software suites were used in data analysis, including GraphPad Prism and Partek.

Chapter 3: Optimisation and delivery of miRNA overexpressing lentiviruses to hESC undergoing cardiac differentiation.

3.1 Introduction

After an MI, the death of cardiomyocytes impairs the pump capacity of the heart, which, if left untreated, can (and often does) lead to heart failure. Current treatments are limited, with transplantation being the only treatment which addresses the loss of cardiac tissue directly. The amount of transplants carried out is severely limited by lack of healthy donor tissue available, and an alternative source of tissue would go some way to address this issue. HESC have been proposed and investigated as such a potential therapy, with their properties of indefinite self renewal and capacity to differentiate to any cell type found in the human proving extremely attractive. Several laboratories have studied cardiac differentiation in hESC, although no standardised protocol to generate functional cardiomyocytes exists (Laflamme et al., 2007, Ivey et al., 2008a, Xu et al., 2002, Graichen et al., 2008, Yang et al., 2008).

MicroRNAs have been proposed to regulate several physiological and pathological processes, including cardiac differentiation and development (Ivey et al., 2008a, Elia et al., 2009), angiogenesis, arrhythmias, hypertrophy and remodelling (van Rooij et al., 2006, van Rooij et al., 2007, Zhao et al., 2007b, Care et al., 2007). As part of the same transcriptional unit, miR-1 and miR-133 have been shown to have specialised roles in cardiac development, disease and differentiation. MiR-1, which is widely conserved between species, is muscle specific and has been previously studied in cardiac development (Kwon et al., 2005b, Zhao et al., 2007b, Zhao et al., 2005). The relationship between miR-1 expression and cardiac differentiation has been further understood by studies which show its regulation of several cardiac markers such as Hand2, MyoD, Mef2, Nkx2.5, cyclin-dependant kinase-9, α-actin, myogenin and myosin heavy chain (Chen et al., 2006, Takaya et al., 2009a, Zhao et al., 2005).

MiR-133 has also been reported to be associated with muscle and cardiac function and disease. Double deletion of miR-133a-1 and miR-133a-2 in mice resulted in cardiac morphogenetic defects and a proliferative phenotype of cardiomyocytes, however single deletion resulted in a normal phenotype being observed, suggesting that these miRNAs function in concert and that if 25% of the normal levels of miR-133 are present, then the organism has no ill effects (Liu et al., 2008).

In a previous publication, Ivey *et al*. reported that miR-1 and miR-133 were enriched in hESC-derived cardiomyocytes when differentiated using an embryoid body formation protocol (Ivey et al., 2008a). Interestingly, over-expression of these miRNAs elicited opposing effects, with miR-1 over-expression resulting in promotion of cardiac lineage commitment, and miR-133 over-expression appearing to block specification to cardiac muscle, suggesting that miR-1 and miR-133 are critically important miRNA in mesoderm and cardiac differentiation (Ivey et al., 2008a).

HESC are refractory to several transfection methods, and due to their potent self-renewal capacity long lasting and stable expression would be useful qualities in any miRNA modulation approach. Useful due to their ability to integrate into the host genome providing long term gene expression (Naldini et al., 1996), lentiviruses provide opportunities for long lived and efficient microRNA manipulation. Lentiviruses are a member of the retrovirus family *retroviridae*, and include human immunodeficiency virus 1 (HIV-1), feline immunodeficiency virus (FIV) and simian immunodeficiency virus (SIV). Other advantages to using lentiviral technology include their ability to transduce both dividing and non dividing cells, the ease at which the transgene or promoter can be modified and their relatively simple production (Naldini et al., 1996). Lentiviral technologies used in this study were based on HIV-1 Self Inactivating vectors, that is, they were unable to replicate and form new infectious virus particles. Lentiviral vectors have been produced by deletion of viral genes gag, pol and env and replacement with a transgene of interest under the control of a promoter of choice. Most of these self inactivating vectors are produced by transient triple transfection of a packaging plasmid, an expression plasmid containing the promoter driving the transgene of interest and a vesicular stomatitis virus glycoprotein (VSV-g) envelope plasmid in the presence of polyethylenimine (PEI) (Sinn et al., 2005, Cockrell and Kafri, 2007). VSV-g remains a common choice for a lentiviral vector envelope protein as it has broad cell tropism (Seganti et al., 1986). The lentiviral vectors used in this study are known as “second generation” vectors; that is they had *vif, vpr, vpu* and *nef* deleted from the packaging plasmid thereby ensuring if mutation or recombination events had occurred, the viral particles would not have contained virulence factors. These modifications have also been shown to have no significant effect on efficiency or vector titres

(Zufferey et al., 1997). The expression plasmid had further modifications for improved biosafety profile, namely the deletion of key transcriptional regulatory sequences including the homogenous viral enhancer-promoter unit. This has been achieved by a deletion within the U3 region of the 3' Long Terminal Repeat (LTR) of the DNA transcript. This was reverse transcribed conferring the deletion to the 5' LTR of the proviral DNA. Transcriptional activity was therefore sufficiently hampered so that no full length viral RNA could be produced (Zufferey et al., 1998). Modifications created to increase the overall functionality of the lentiviral vectors included the introduction of the rev-responsive regulatory element (RRE), the central polypurine tract (cPPT) element and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). RRE is important as it allowed the Rev-mediated shuttle of mRNA from the nucleus to the cytoplasm for translation to occur and as such is also present on the packaging plasmid (Cochrane et al., 1990, Dull et al., 1998). The cPPT element enhanced the nuclear translocation, degree of vector genome integration into the host genome and had also been shown to increase the achievable titre (Demaison et al., 2002a, Logan et al., 2004, VandenDriessche et al., 2002). The WPRE improved viral genome transcript packaging, regulated the stability of transgene mRNA by polyadenylation, increased RNA export from the nucleus and enhanced translation of transgene mRNA (Zufferey et al., 1999, Demaison et al., 2002a).

It was previously assumed that integrating lentiviruses were not suitable for clinical therapies due to their propensity to integrate semi-randomly but with some common integration sites (CIS) within oncogenes. However, upon further study it was discovered that for the most part the CIS lie in benign genes (Modlich et al., 2009, Cavazzana-Calvo et al., 2010, Biffi et al., 2011) and there are current studies using lentivirus in patients with Wiscott-Aldrich syndrome (Scaramuzza et al., 2012). Lentiviral vectors remain very useful tools in preclinical studies, allowing interrogation of the miRNA involved in hESC lineage commitment.

The aim of this chapter was to design and produce suitable lentiviral vectors for miRNA overexpression, to allow interrogation of miR-1 and miR-133b in cardiac lineage commitment in a feeder-free monolayer hESc culture system (Figure 3.1).

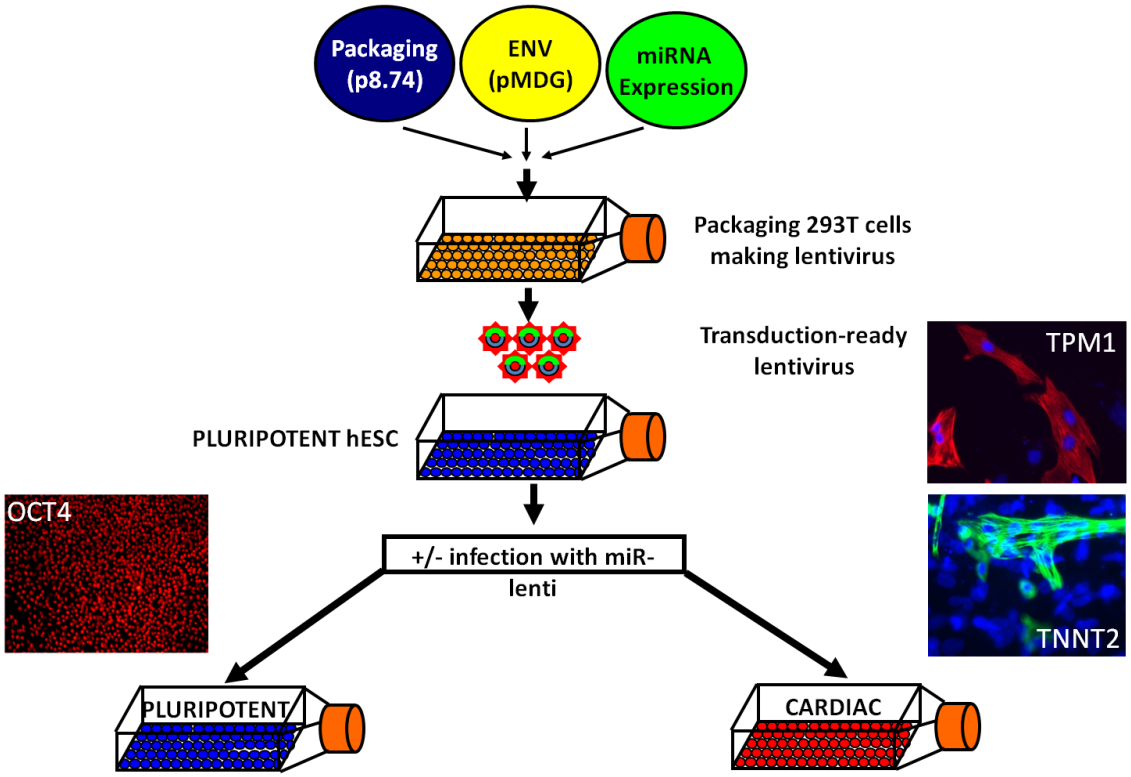


Figure 3.1: Schematic of cardiac differentiation and lentivirus infection of hESC.

Lentiviruses were generated by triple transfection of a packaging plasmid (p8.74) an envelope plasmid (pMGD) and a microRNA expression plasmid (LNT-SFFV-miR) in the presence of PEI in HEK293T cells. Lentiviruses were collected in the cell media at 48 and 72 hours before being sterile filtered, concentrated by ultracentrifugation and subjected to titration to determine functional virus titre. Lentiviral transduction in the presence of polybrene was then carried out in pluripotent hESC, after which cells were maintained pluripotent as shown by Oct 4 staining, or differentiated towards cardiac lineage as shown by positive staining of TNNT2 and TPM1.

3.2 Results

3.2.1 Lentiviral infection does not alter hESC pluripotency or differentiation

To understand what effect, if any, transduction with lentiviral vectors may have on hESC, SA461 cells were infected with a lentivirus expressing green fluorescent protein (GFP) under the spleen focus forming virus promoter at an MOI of 100 in the presence of 4 µg/mL of polybrene for 72 hours.

DAPI staining for intact nuclei in blue shows the location of cells (Figure 3.2, Panel I). Cells infected with a lentivirus expressing GFP under the SFFV promoter showed GFP positive cells indicating successful transduction (Figure 3.2, Panel II). Oct4 staining in red indicated the cells were indeed pluripotent (Figure 3.2, Panel III). The merged image of hESC infected with lentivirus expressing GFP and also expressing Oct 4 demonstrates lentivirally transduced cells remained pluripotent (Figure 3.2, Panel IV). Transduced hESC were still able to undergo embryoid body formation (Figure 3.2, Panel V) and passively differentiate to all 3 germ layers as confirmed by Taqman qRT-PCR for α-fetoprotein (AFP), ß-tubulin and α-actin cardiac muscle (Figure 3.2, Panel VI).

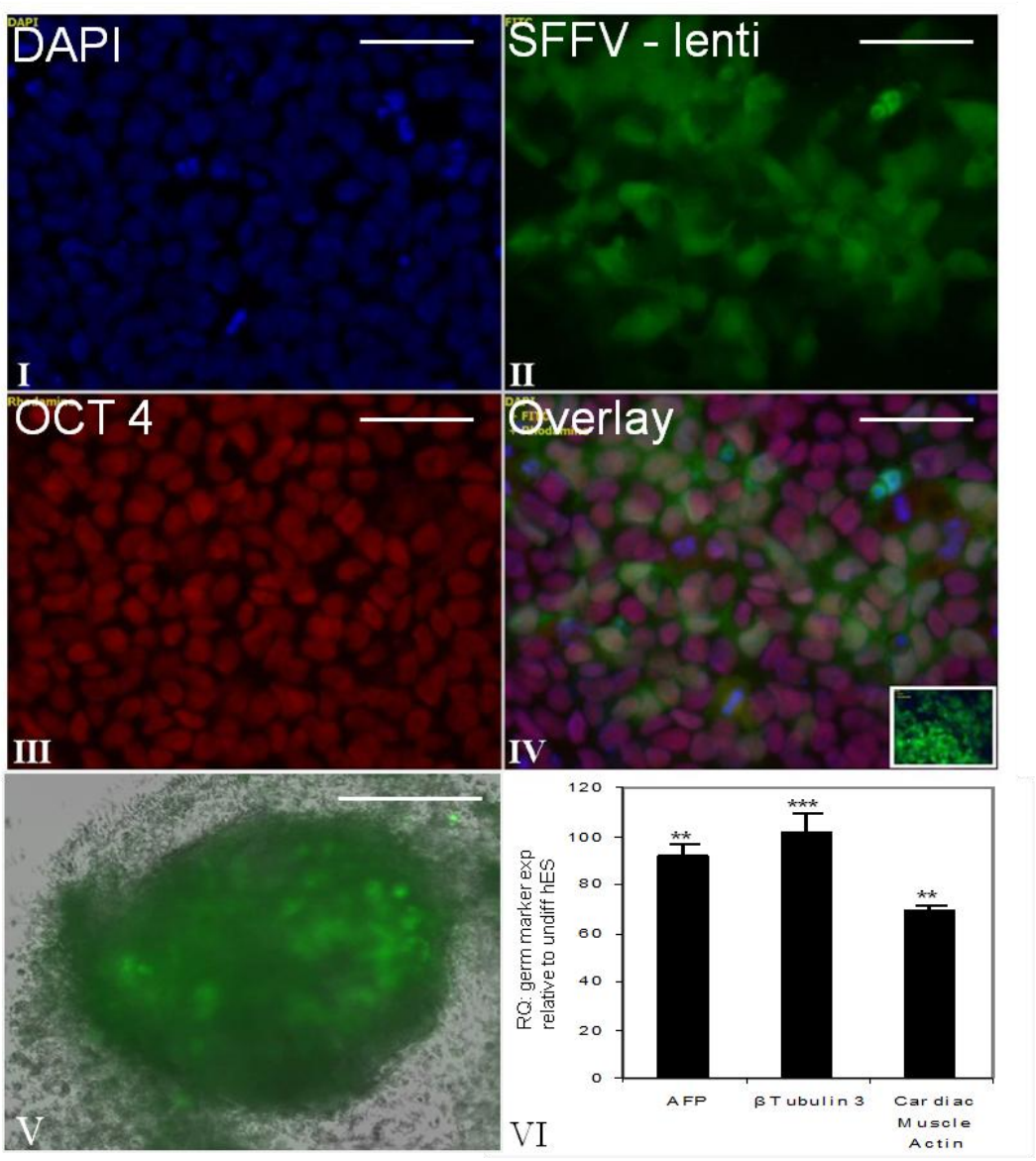


Figure 3.2: Lentiviral transduction of hESC does not affect pluripotency.

Panel I shows DAPI staining for intact nuclei in blue, in panel II cells infected with a lentivirus expressing GFP under SFFV promoter show GFP positive cells. Panel III shows Oct 4 expressed by transduced cells in red as detected by ICC. Panel IV shows a merged image of transduced hESC and positive expression of Oct 4. Panel V shows that the hESC were able to undergo embryoid body formation and passively differentiate to all 3 germ layers as confirmed by Taqman qRT-PCR for α-fetoprotein (AFP), β-tubulin and α-actin cardiac muscle shown in graph in panel VI. Scale bars panel I-IV = 50 µm. Scale bar panel V = 500 µm.

3.2.2 Spleen focus forming virus promoter achieves superior transgene expression in hESC

In order to choose the best promoter for high transgene expression from lentiviral vectors in hESC, two lentiviruses were compared; one which expressed GFP under the popular cytomegalovirus (CMV) promoter or one which expressed GFP under the SFFV promoter. Although it had previously been described that CMV was not an ideal choice for transgene overexpression in hESC (Liew et al., 2007), we received a large plasmid library of miRNAs under the CMV promoter that we wished to use in hESC. Therefore, cells were seeded in a 24 well plate at $1x10^4$ cells/well and either left uninfected (UI), infected with a lentivirus expressing GFP from a CMV promoter and compared to cells infected with a lentivirus expressing GFP from a SFFV promoter (LNT-GFP-SFFV), both at 150 MOI and all conditions had polybrene. Cells were then either maintained pluripotent, subjected to endothelial differentiation media, subjected to a cardiac differentiation protocol, or allowed to passively differentiate in 10% MEM. In cells harvested 2 days post infection, GFP expression relative to mg of protein was highest in those cells tranduced with the LNT-GFP-SFFV. The highest transgene expression relative to milligrams of protein was found in the endothelial and passive differentiation conditions. Additionally, expression was still relatively high under pluripotency and cardiac conditions (Figure 3.3). In cells harvested 7 days post infection again LNT-GFP-SFFV proved to be much more efficient at expressing GFP (Figure 3.3).

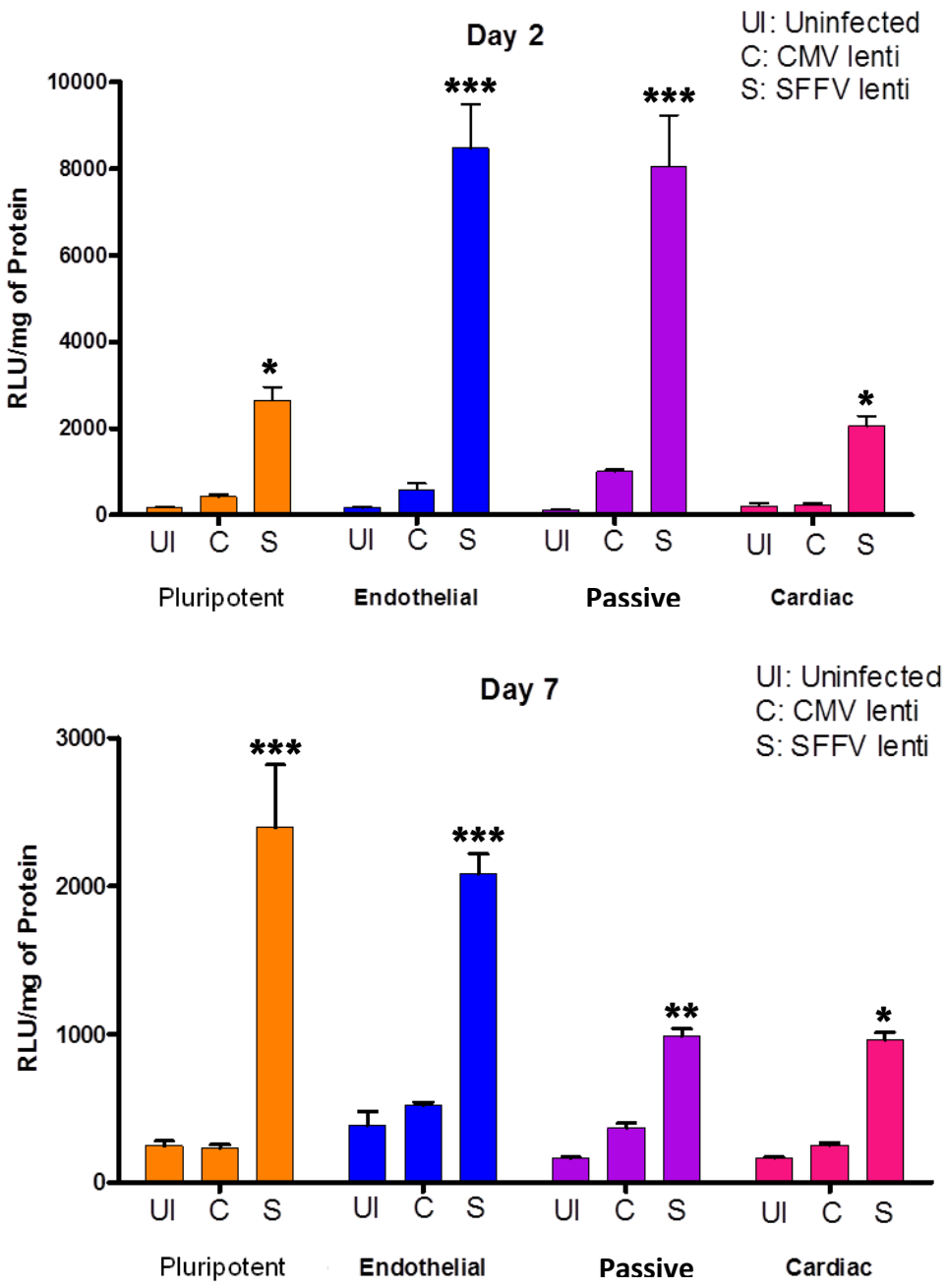
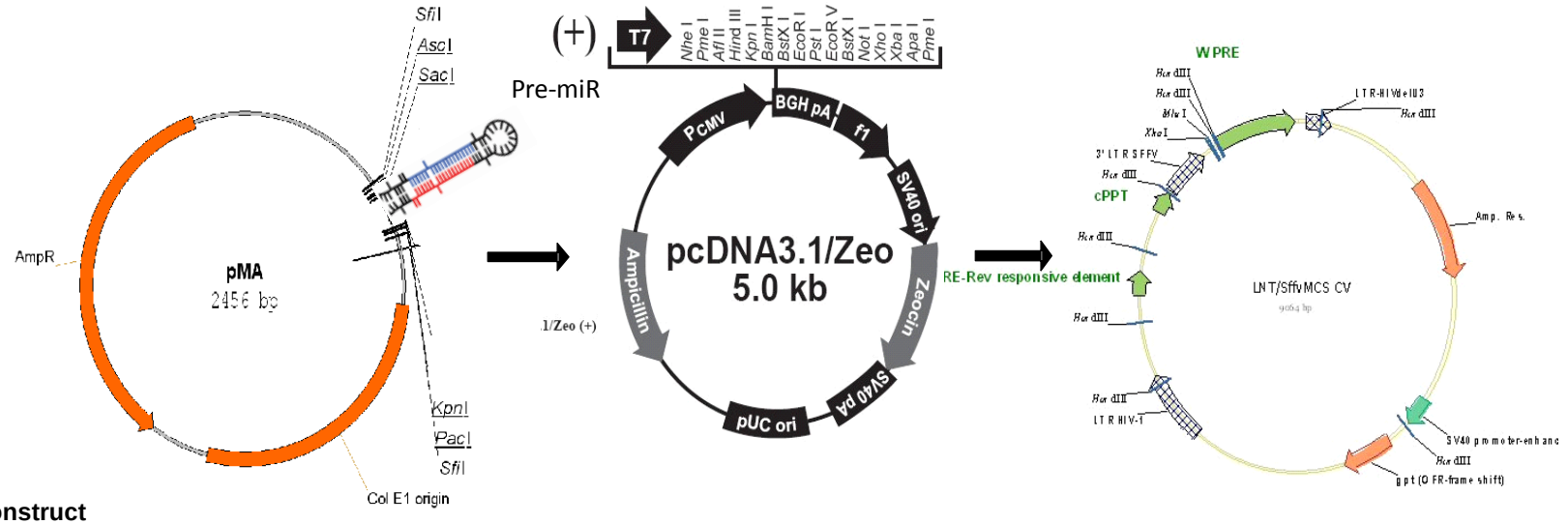


Figure 3.3: Analysis of CMV and SFFV promoters in hESC.

HESC were uninfected (UI), transduced with a lentivirus expressing GFP from a CMV promoter or transduced with a lentivirus expressing GFP from a SFFV promoter (LNT-GFP-SFFV), both at 150 MOI. Cells were then either maintained pluripotent, subjected to endothelial differentiation media, allowed to passively differentiate in 10% MEM or subjected to a cardiac differentiation protocol and analysed at day 2 and day 7 of differentiation. * = $p<0.05$ vs UI, **= $p<0.01$ vs UI, ***= $p<0.001$ vs UI.

3.2.3 Design optimisation of miRNA overexpressing lentiviral vectors

To create miRNA overexpression lentiviral vectors a sub-cloning strategy was designed and employed. A retroviral library of 350 pri-miRNA clones was kindly gifted from Reuven Agami at the Netherlands Cancer Institute. As the retroviral plasmid (pMSCV-Blast-miR) which contained each miRNA was driven by CMV, it was necessary to subclone the miRNA inserts into the LNT-MCS-SFFV plasmid, which has the same design as the LNT-SFFV-GFP plasmid but with no GFP tag. The restriction endonuclease sites used to excise the insert from the retroviral plasmid pMSCV-Blast-miR were BamHI and EcoRI. As the LNT-SFFV-GFP plasmid does not contain an EcoRI restriction endonuclease site, it was necessary to add the correct restriction endonuclease sites to the miRNA insert. In order to do this, the miRNA insert was subcloned into the PCDNA3.1(+)/Zeo multiple cloning site by digesting both plasmids with BamHI and EcoRI. PCDNA3.1(+)/Zeo was then subjected to dephosporylation before the miRNA insert of choice was ligated using T4 ligase into PCDNA3.1(+)/Zeo. At this stage diagnostic restriction endonuclease digests were run on an agarose gel and visualised and dideoxynucleotide sequencing was carried out showing that the miRNA insert had been successfully incorporated.



MiR-1 construct

5' AAGCTT GGATCC ACCATGG TGGGAAACATACTTCTTTATATGCCCATATGGACCTGCTAAGCTATGGAATGTAAAGAAGTATGTATCTCA **TAG TTTTTT CTCGAG GATATC 3'**
3' TTCGAA CCTAGG TGGTACC ACCCTTTGTATGAAGAAATATACGGGTATACCTGGACGATTCGATACCTTACATTTCTTCATACATAGAGT **ATC AAAAAA GAGCTC CTATAG 5'**

MiR-133 construct

5' AAGCTT GGATCC ACCATGG CCTCAGAAGAAAGATGCCCCCTGCTCTGGCTGGTCAAACGGAACCAAGTCCGTCTTCCTGAGAGGTTTGGTCCCCTTCAACCAGCTACAGCAGGGCTG
GCAATGCCCAGTCCTTGGAGA **TAG TTTTTT CTCGAG GATATC 3'**

3' TTCGAA CCTAGG TGGTACC GGAGTCTTCTTTCTACGGGGGACGAGACCGACCAGTTTGCCTTGGTTCAGGCAGAAGGACTCTCCAAACCAGGGGAAGTTGGTCGATGTCGTCCCGAC
CGTTACGGGTCAGGAACCTCT **ATC AAAAAA GAGCTC CTATAG 5'**

Figure 3.4: Cloning scheme of miR-1 and miR-133 overexpression lentiviruses.

Expression vector maps of GeneArt plasmid, PCDNA3.1/Zeo(+) and LNT-SFFV-MCS and sequences of miRNA pre-miR inserts as synthesised by GeneArt.

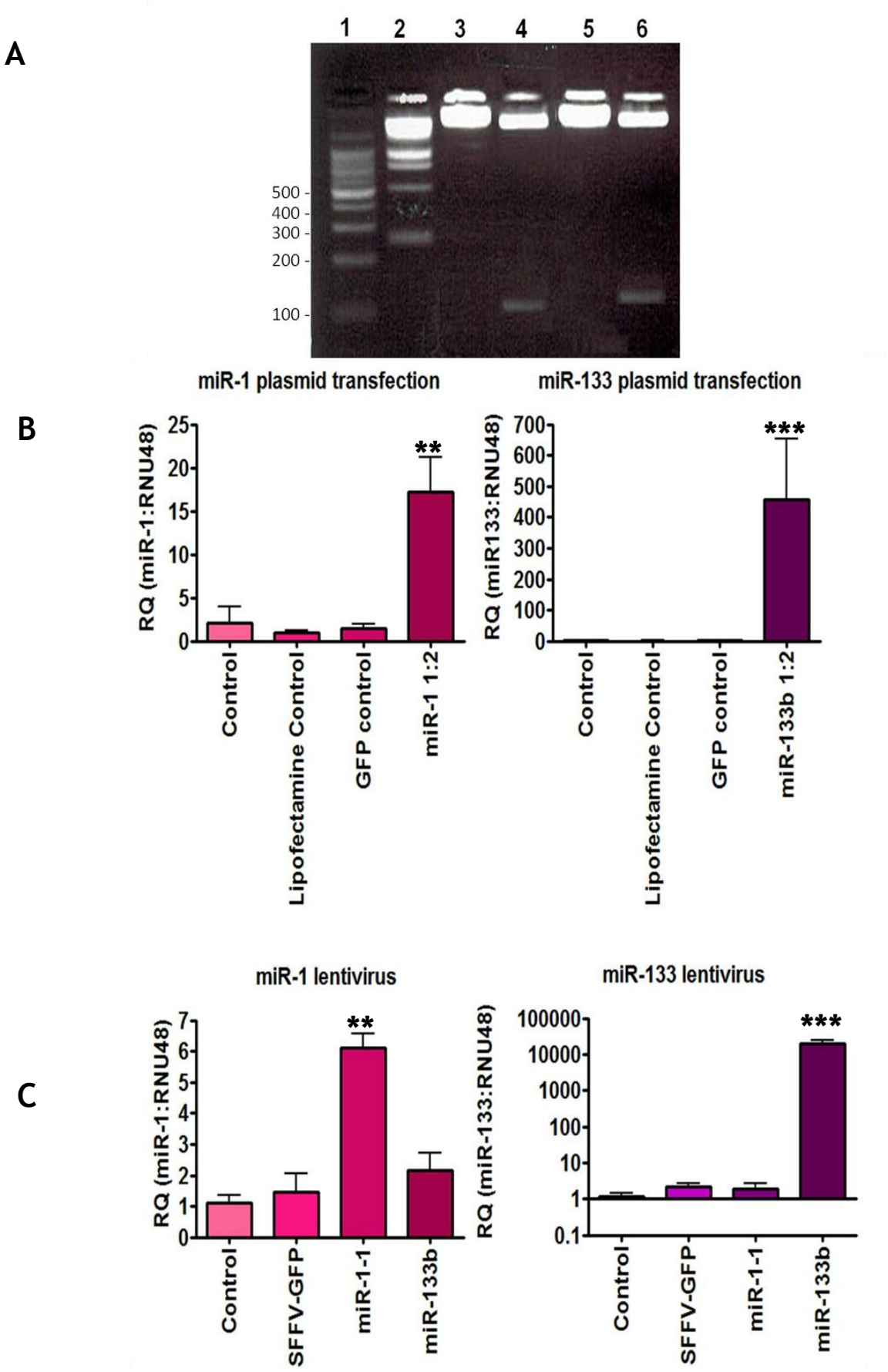


Figure 3.5: Cloning of miRNA overexpression lentiviruses.

(A) Diagnostic agarose gel electrophoresis of restriction endonuclease digests. Lane 1 is 100bp ladder, lane 2 is 1kb ladder, lane 3 is uncut LNT-SFFV-miR-1, lane 4 is LNT-SFFV-miR-1 cut with

BamHI and XhoI, lane 5 is uncut LNT-SFFV-miR-133 and lane 6 is LNT-SFFV-miR-133 cut with BamHI and XhoI. (B) Transfection by lipofectamine 2000 of PCDNA3.1 + miR-1 and PCDNA3.1 + miR-133 expression vectors into HeLa cells. (C) Transduction of HeLa cells with LNT-SFFV-GFP, LNT-SFFV-miR-1 and LNT-SFFV-miR-133 and qRT-PCR for miRNA expression. ** = $p<0.01$, *** = $p<0.001$.

As PCDNA3.1(+)/Zeo drives transgene expression under a CMV promoter and hESC cells are refractory to conventional transfection methods, correct expression of mature miRNA was assessed by transfection using Lipofectamine 2000 into HeLa cells, which were then miRNeasy extracted and subjected to miRNA TaqMan using appropriate primer and probe sets for each miRNA of interest. Expression levels compared to that of the original pMSCV-Blast-miR plasmids were similar for miR-133b, however miR-1-1 did not evoke as high levels of expression.

Upon further investigation, it was discovered that the large 500-700 bp pri-miR inserts containing pri-miRNA sequences were not only expressing the chosen miRNA, but contained fragments of other miRNAs too. This was most likely due to the nature of construction of the pri-miR library, where large sections of sequence surrounding the miR of interest were preserved in an effort to ensure correct biological processing upon expression. In order to simplify cloning by reducing the insert size and have a more targeted approach, a redesign of the strategy for specific overexpression of miRNAs of interest was carried out. The pre-miRNA stem loop sequences were obtained from miRbase (University of Manchester) and used to design new inserts for creation by GeneArt (Life Technologies) (Figure 3.4). In addition to the pre-miRNA stem loop, the inserts also contained multiple flanking restriction endonuclease sites so that the pre-miRNAs could be cloned into several different vectors and kozak and stop sequences to ensure correct processing (Figure 3.4). The pre-miRNA constructs were received in a standard plasmid with ampicillin resistance, grown up in *E.coli* under selection of ampicillin, restriction endonuclease digested and electrophoresed on a 2% agarose gel to isolate the inserts, gel extracted and ligated into PCDNA3.1. The plasmids were then subjected to diagnostic restriction endonuclease digests, dideoxynucleotide sequencing and transfection into Hela cells to quantify expression of mature miRNA as detailed previously (Figure 3.5). Once the pre-miRNA constructs passed these quality control checks, they were restriction endonuclease digested using BamHI and XhoI and ligated into the LNT-SFFV-MCS expression plasmid ready for lentiviral vector production.

Diagnostic endonuclease digestion (Figure 3.5) and dideoxynucleotide sequencing was then performed to ensure correct insertion of the pre-miRNA contructs, and lentivirus produced.

3.2.4 Cardiac differentiation optimisation

Discovering the most successful route of deriving cardiomyocytes from hESC involved optimisation of a previously published cardiac protocol (Laflamme et al., 2007) as detailed in section 2.1.1.3. SA461 cells were seeded on Matrigel™ and subjected to cardiac differentiation. Despite culture under these conditions for up to 28 days, no spontaneous beating could be observed. At day 7, moderate expression of tropomyosin, myosin heavy chain, α-actinin and troponin T could be observed however, upon further culture at day 14 this expression appeared to be lost (Figure 3.6). Therefore, SA461 did not exhibit enough cardiogenic potential to be used in cardiac differentiation studies.

We then went on to test H1 hESC for cardiogenic potential by the modified Laflamme cardiac differentiation protocol for 14 days (Laflamme et al., 2007). Cells were then fixed and ICC performed or harvested for total RNA extraction by miRneasy and subjected to qRT-PCR. H1 hESC were found to be an excellent choice for cardiac differentiation. Spontaneous beating occurred at approximately day 9 and by day 14, expression of of troponin T, tropomyosin, NKX2.5 and α-actinin cardiac markers and expression of for alpha actinin-2 (ACTN2), Brachyury (T), NK2 homeobox 5 (NKX2.5) and troponin T type 2 (cardiac) (TNNT2) could be observed (Figure 3.7)

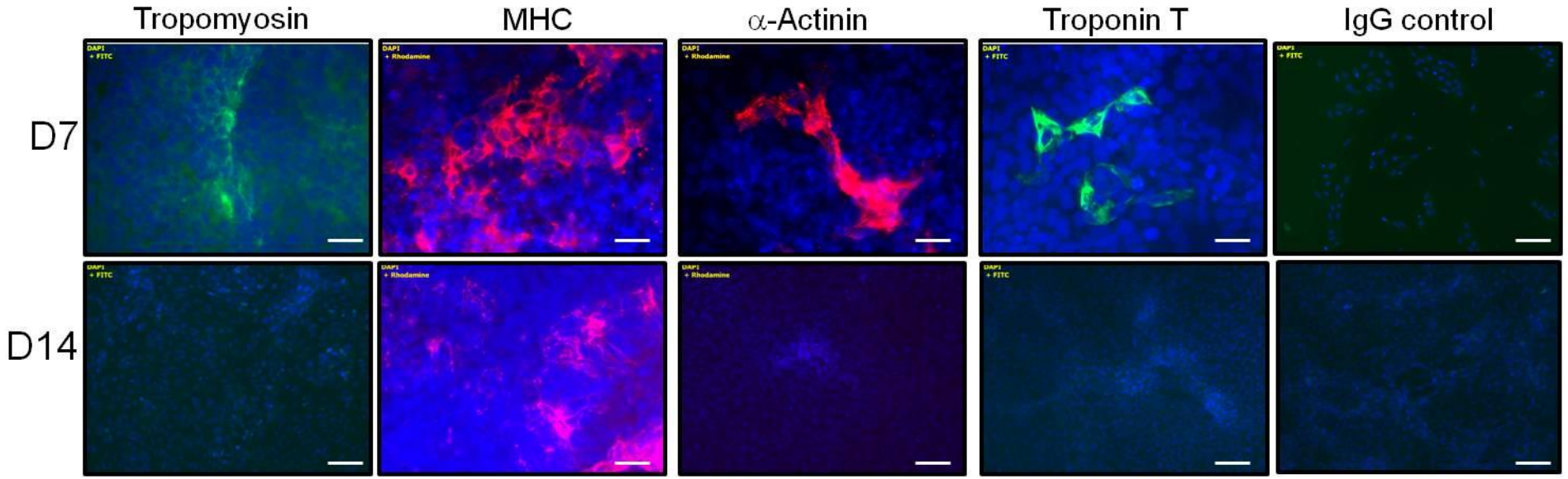


Figure 3.6: SA461 subjected to cardiac differentiation.

After 7 and 14 days of cardiac differentiation, ICC was performed for DAPI in blue, tropomyosin (green), myosin heavy chain (MHC) (red), α-Actinin (red), Troponin T (green) and control IgG. Scale bar 200 µm.

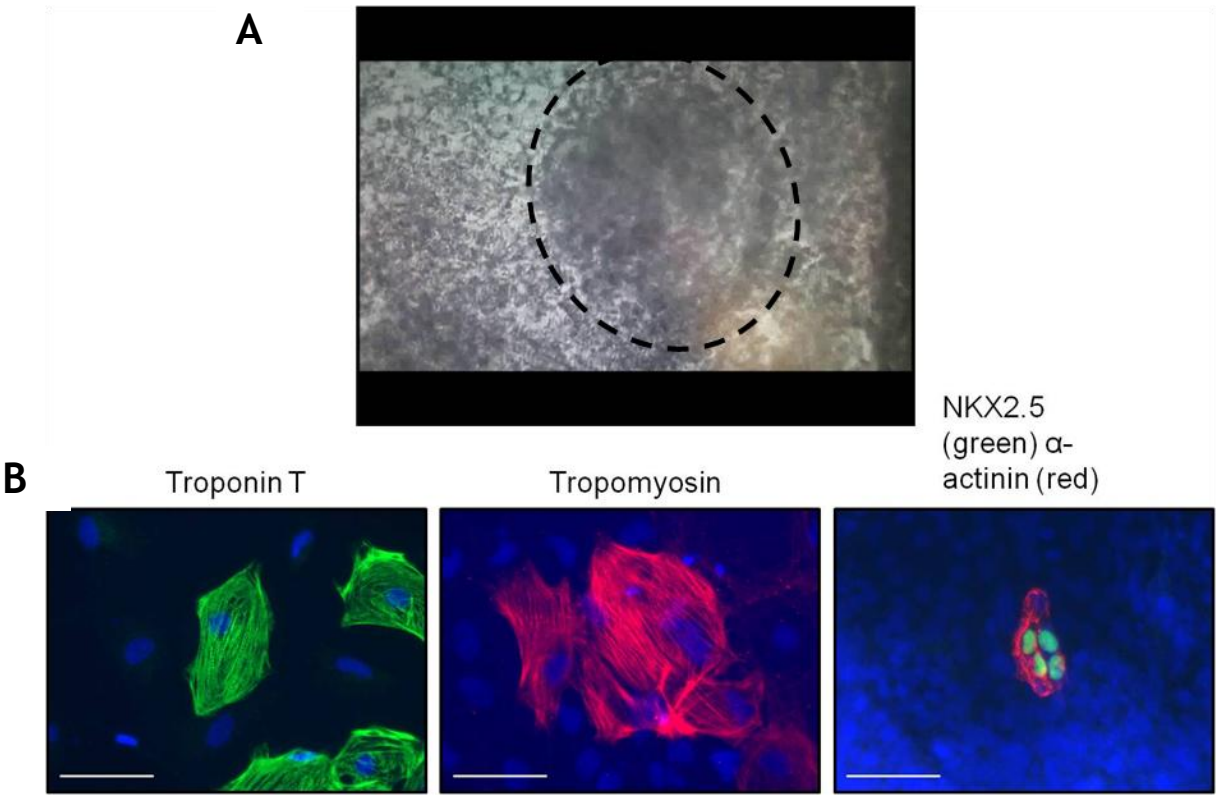


Figure 3.7: H1 cardiac differentiation.

(A) H1 hESC subjected to cardiac differentiation for 21 days. Dotted ellipse denotes beating area. (B) ICC performed for DAPI (blue), Troponin T (green), Tropomyosin (red), NKX2.5 (green) and α-actinin (red). Scale bars = 10 µm.

3.2.5 Overexpression of miR-1-1 and miR-133b in cardiac differentiation of hESC

These experiments were conducted to ascertain whether forced over-expression of miR-1-1 or miR-133b had an impact on monolayer cardiac differentiation in H1 hESC cells.

H1 hESC were seeded into 48-well plates pre-coated with Matrigel™ at 3x104 cells per well, and either maintained pluripotent, or differentiated towards cardiac lineage. In addition to differing media conditions, cells were either uninfected or transduced with LNT-SFFV-GFP, LNT-SFFV-pre-miR-1 or LNT-SFFV-pre-miR-133b, all at 25 MOI in the presence of 4 µg/mL of polybrene. The cells were allowed to differentiate for 14 days, with spontaneous beating observed from day 9. The samples were subjected to miRNA qRT-PCR to ensure the

overexpression of both miRNAs from the lentiviruses (Figure 3.8) and gene expression qRT-PCR for Oct 4 as a marker of pluripotency, and cardiac markers alpha actinin-2 (ACTN2), Brachyury (T), NK2 homeobox 5 (NKX2.5) and troponin T type 2 (cardiac) (TNNT2) (Figure 3.9).

Oct 4 expression was significantly downregulated between pluripotent samples and those subjected to cardiac conditions, with no statistically significant difference observed between samples transduced with GFP, miR-1 or miR-133b overexpression viruses (Fig9 A). ACTN2 was significantly upregulated in the sample group transduced with GFP-expressing lentivirus under cardiac conditions, and was significantly downregulated in those samples transduced with miR-133b lentivirus compared to the GFP virus control (Fig 9 B). Brachyury expression did not attain significance in any of the test groups, although it appeared to be upregulated in the GFP lentivirus transduced cardiac group and the miRNA-133b overexpression lentivirus transduced cardiac group (Fig 9 C). NKX2.5 was significantly upregulated in cardiac GFP lentivirus group as compared with pluripotent samples. NKX2.5 was also significantly downregulated in those cardiac samples transduced with miR-133b overexpressing lentivirus compared to the GFP virus control (Fig 9 D). TNNT2 was the most upregulated gene of those tested, with all cardiac samples showing significant increase over pluripotent samples, however miR-133b overexpressing lentivirus tranduced samples showed significant downregulation compared to GFP virus control (Fig 9 E).

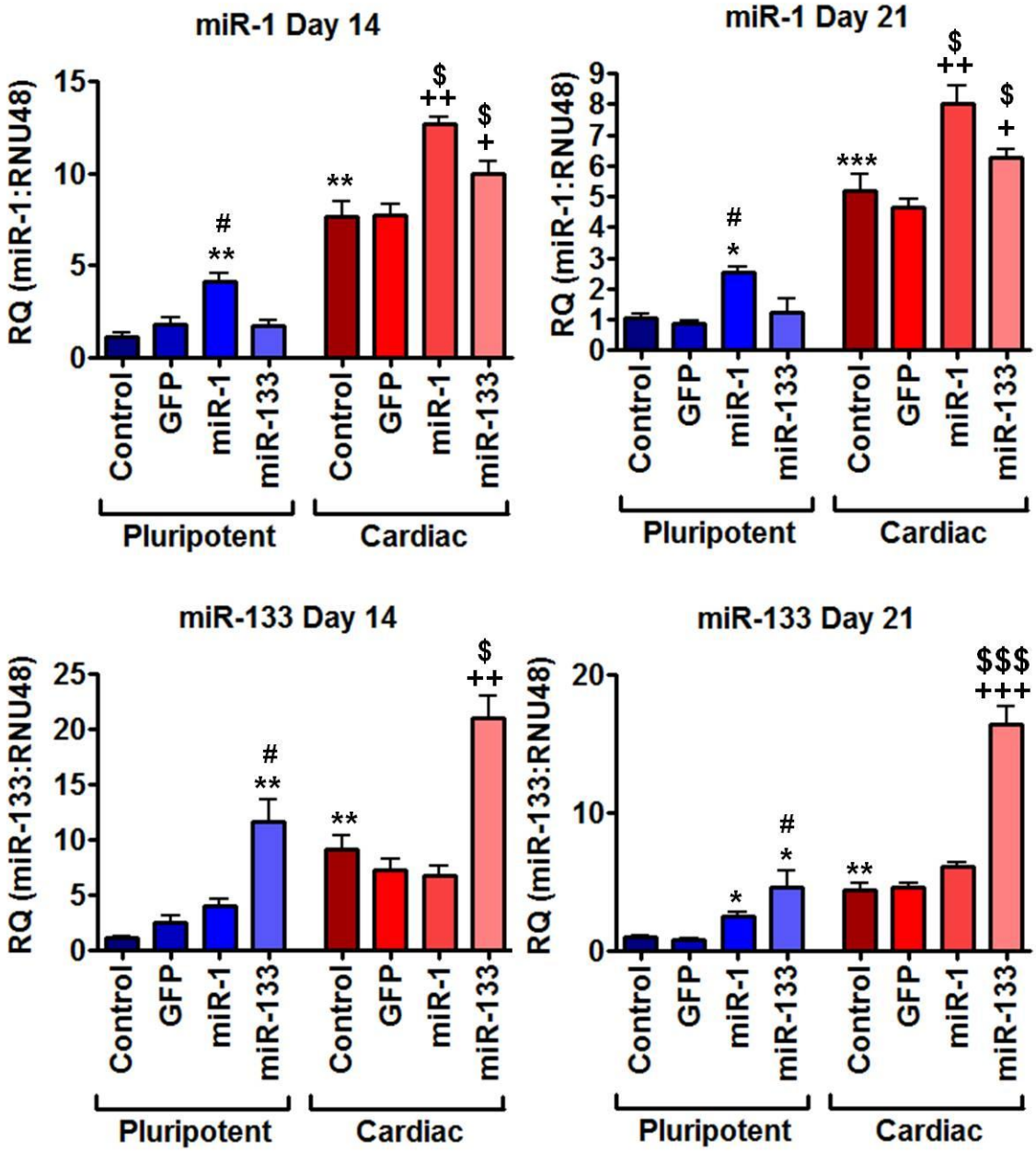


Figure 3.8: Expression of miR-1 and miR-133b from lentiviruses.

Samples maintained pluripotent or cardiac differentiated for 14 or 21 days, uninfected (control), LNT-SFFV-GFP control virus (GFP), LNT-SFFV-MiR-1 transduced (miR-1) or LNT-SFFV-MiR-133 transduced (miR-133). * = $p<0.05$ vs pluripotent control, ** = $p<0.01$ vs pluripotent control, *** = $p<0.001$ vs pluripotent control, # = $p<0.05$ vs pluripotent GFP, + = $p<0.05$ vs cardiac control, ++ = $p<0.01$ vs cardiac control, +++ = $p<0.001$ vs cardiac control, $ = $p<0.05$ vs cardiac GFP, $$ = $p<0.01$ vs cardiac GFP and $$$ = $p<0.001$ vs cardiac GFP.

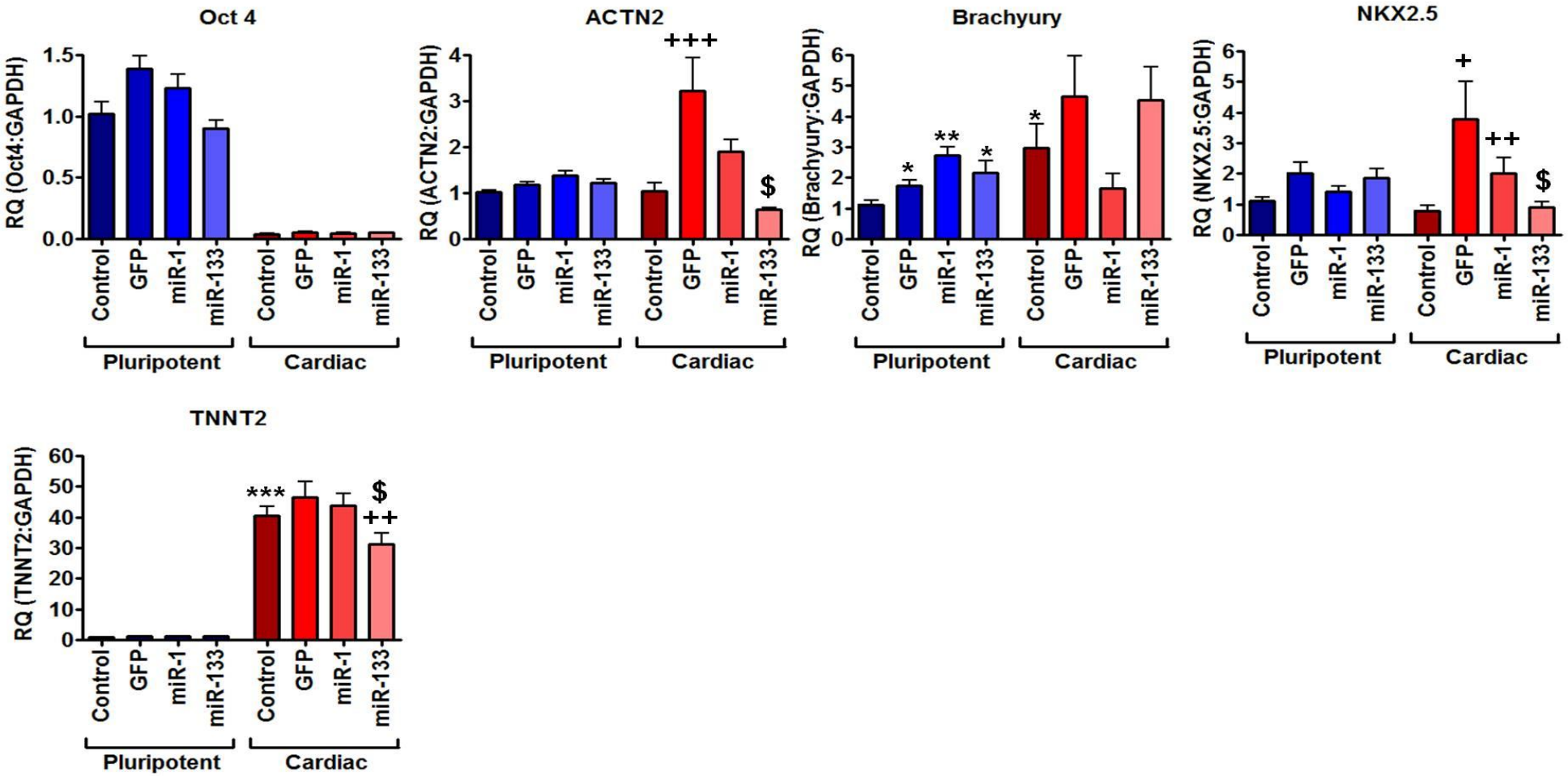


Figure 3.9: Overexpression of miR-1 and miR-133 in hESC during cardiac differentiation.

Samples were maintained pluripotent or subjected to cardiac differentiation and were either uninfected or transduced with LNT-SFFV-GFP, LNT-SFFV-pre-miR-1 or LNT-SFFV-pre-miR-133b. The samples were subjected to qRT-PCR after 14 days to ensure gene expression for Oct 4, alpha actinin-2 (ACTN2), brachyury (T), NK2 homeobox 5 (NKX2.5) and troponin T type 2 (cardiac) (TNNT2) (n=2, $p<0.05$). * = $p<0.05$ vs pluripotent control, ** = $p<0.01$ vs pluripotent control, *** = $p<0.001$ vs pluripotent control, # = $p<0.05$ vs pluripotent GFP, + = $p<0.05$ vs cardiac control, ++ = $p<0.01$ vs cardiac control, +++ = $p<0.001$ vs cardiac control, $ = $p<0.05$ vs cardiac GFP, $$ = $p<0.01$ vs cardiac GFP and $$$ = $p<0.001$ vs cardiac GFP.

3.3 Discussion

In this study, it was found that targeted overexpression of single pre-miRs was achievable in H1 hESC, that SA461 hESC were less cardiogenic than H1 hESC under cardiac differentiation conditions, and that the role of miR-1 and miR-133 overexpression in altering cardiac differentiation was unclear.

To interrogate of the role of miRNAs miR-1-1 and miR-133 in cardiac differentiation a lentiviral strategy was required. With hESC reportedly resistant to most transfection methods, lentiviral vectors offered an attractive alternative to allow overexpression of genes of interest with the VSVG envelope broad cell tropism coupled with the ability to infect non-dividing cells. Initially it was important to ascertain whether lentivirus infection in itself had an effect on hESC pluripotency or differentiation. As previously reported, lentiviruses integrate into the host genome in a semi-random fashion, often in proto-oncogenes (Modlich et al., 2009, Cavazzana-Calvo et al., 2010). So that this effect could be accounted for, a control lentivirus expressing GFP from a SFFV promoter was employed. It was observed that lentivirus infection has no effect on the hESC expression of pluripotency marker Oct 4, with cells expressing GFP also staining red for Oct4 expression. Importantly, upon embryoid body formation, the GFP expressing hESC could still differentiate to form representative cell types of all three germ layers, α-fetoprotein (AFP) for endoderm, ß-tubulin 3 for ectoderm and cardiac muscle actin for mesoderm. This confirms the pluripotent nature of hESC is not adversely affected by lentiviral infection.

As the existing library of pre-miRs was in a retrovirus format under the CMV promoter, the next experiments were designed to show whether CMV promoter could be active in hESC, as it had been previously reported that CMV promoter was not ideal for use in stem cells with inactivation of more than 95% of eGFP transgenes reported in H1 and H9 hESC (Liew et al., 2007, Xia et al., 2007). A lentivirus driving GFP under a CMV promoter and a lentivirus driving GFP under a SFFV promoter were employed, with SA461 hESC infected and either maintained as pluripotent or differentiated using an endothelial protocol, early cardiac protocol or placed in standard 10% DMEM to allow passive differentiation. There was also a no virus control. At both day 2 and day 7, it was observed that that

SFFV was a much more efficient promoter at driving GFP expression compared to the CMV promoter in all conditions tested although expression of the transgene was considerably lower at day 7 compared to day 2. This showed that to successfully overexpress miRNAs of interest in hESC, an SFFV promoter containing lentivirus expression plasmid would be the vector of choice. Low transgene expression in hESC can be attributed to poor transduction efficiency or gene inactivation (Xia et al., 2007). As successful transgene expression could be observed from the SFFV promoter, the poor transgene expression seen in those samples transduced with LNT-CMV-GFP can be attributed to promoter suppression as previously described (Xia et al., 2007). Rather than traditional silencing, where expression falls off over time, it has been reported that the mechanism to involve transgene blockage occurrs very soon after viral transduction and integration (Xia et al., 2007). Additionally, promoter suppression appeared to be associated with viral transduction, as transient transfection delievered transgenes were not observed to be suppressed (Xia et al., 2007). However, silencing of CMV-driven transgene expression after transient transfection was observed during further studies which reported the mechanism of suppression to be mediated through epigenetic modification (Meilinger et al., 2009). It was shown that CMV promoter suppression was dependant upon Np65 (also known as Uhrf1), a nuclear protein that recruits DNA methyltransferases Dnmt3a and Dmnt3b to hemi-methylated sites (Meilinger et al., 2009). ESC which were deficient in the 3 major DNA methyltransferases, or deficient in Np65 displayed no silencing of GFP or mRFP driven by CMV (Meilinger et al., 2009). We therefore used the LNT-SFFV-MCS lentivirus expression plasmid (kindly gifted by Prof. Thrasher, UCL, UK) for miRNA overexpression construct engineering.

Subsequently, a strategy was designed to subclone the pri-miRNA sequences to the LNT-SFFV-MCS expression plasmid. Further investigation of the initial cloning strategy sequencing data for the pri-miR-1-1 and pri-miR-133b showed that each insert not only contained the miRNA of choice, but other miRNA sequences. As such, a new miRNA overexpression strategy was required. On the basis of shRNA work, it was decided that the stem loop precursor miRNA would be sufficient to drive expression of each miRNA in a smaller, more precise construct (Fire et al., 1998, Paul et al., 2002). The sequence of each stem loop pre-miR was taken

from miRbase (http://www.mirbase.org/, (Griffiths-Jones, 2004)) and placed between a kozak and stop sequence along with appropriate restriction endonuclease digest sites to allow cloning of the pre-miRs into several different plasmid vectors. Both miR-1 and miR-133b LNT-SFFV vectors expressed mature miRNA as detected by TaqMan qRT-PCR, although miR-1 was expressed at lower levels then miR-133b. It is not known whether miR-1-1 undergoes more strict criteria for processing than miR-133b or that there is perhaps some sort of inhibition leading to a decreased level of expression. It has been reported that miR-1 is under the transcriptional control of muscle differentiation regulators including serum response factor (SRF) (Zhao et al., 2005) and therefore there is a possibility that this pathway is responsible for the lower levels of miR-1 observed. Interestingly, although the kozak sequence is thought to enhance protein expression (Kozak, 1987) and should therefore not be required for miRNA processing, constructs omitting the sequence did not produce detectable mature miRNA.

It has been proposed that different hESC lines display differing capacities for differentiation to each germ layer (Hong et al., 2011, Rooney et al., 2010, Wu et al., 2007, Pal et al., 2009) and reviewed in (Allegrucci and Young, 2007). Differences in cardiac potential between hESC lines were also reported previously (Burridge et al., 2007). SA461 hESC were subjected to a previously published protocol capable of generating beating clusters in other hESC lines (Laflamme et al., 2007). However, SA461 were unable to generate beating clusters regardless of length of time cells were subjected to culture conditions. H1 hESC had previously been reported to successfully differentiate to cardiac phenotype cells (Moore et al., 2008, Xu et al., 2002). When H1 hESC were tested in our laboratory, they consistently displayed ~30% beating clusters per well by day 14 of differentiation. The decision was made to switch to H1 hESC for cardiac differentiation experiments due to their ready ability to differentiate to beating cells which stained positive for tropomyosin, myosin heavy chain, α-actinin and troponin T.

To investigate whether the role of miR-1-1 and miR-133b was specific to embryoid body differentiation to cardiomyocytes, a monolayer cardiac differentiation protocol was employed along with miRNA overexpression lentiviruses. As expected, Oct 4 expression is present in those samples

maintained pluripotent, with no significant difference seen between different virus groups and the uninfected samples. This would suggest that, as previously shown in SA461 hESC, infection with a lentivirus has no effect on pluripotency genes, and that overexpression of miR-1 or miR-133b was not sufficient to initiate differentiation when cells remain under pluripotent media conditions. To date, no studies have shown miRNA overexpression under pluripotent conditions to be powerful enough to direct cells to a particular lineage, but rather it has been shown miRNAs can refine differentiation during embryoid body or directed differentiation (Ivey et al., 2008a, Kane et al., 2012, Tzur et al., 2008). It has recently been reported in mice, that overexpression of miR-1, -133, -208 and -499 in fibroblasts can induce differentiation of cardiac-like cells, both *in vitro* and *in vivo* (Jayawardena et al., 2012). ACTN2 appeared to be significantly upregulated in those samples transduced with LNT-SFFV-GFP control virus compared with uninfected cells. There has been no previously published information on lentiviral infection in itself having an impact on cardiac differentiation in hESC, or indeed any other system. As the hallmark of a retrovirus (and indeed a lentivirus) is the ability to integrate into the host cell genome in a semi-random fashion, it is not excluded that the virus could integrate into an area which causes disregulation of genes involved in cardiac or mesodermal differentiation. This possibility is of course increased with using a higher MOI, and previous studies in our laboratory have shown transduction with LNT-SFFV-GFP control virus to be powerful enough to create iPSC like cells from fibroblasts at a MOI of 200 (Kane et al., 2010b). However, in this case, the cells were transduced at an MOI of 20. ACTN2 is significantly downregulated in samples transduced with miR-133b overexpression lentivirus. As ACTN2 is not reported or predicted to be a direct target of miR-133b, it is likely that the downregulation is caused by a general inhibition of cardiac commitment as previously reported (Ivey et al., 2008a). Brachyury is encoded by the T gene in humans, is a member of the t box transcription factor family and an early marker of mesoderm (Wilkinson et al., 1990, Showell et al., 2004). Brachyury expression was significantly different with GFP, miR-1 and miR-133 overexpression in those samples maintained pluripotent however did not reach significant levels compared to either uninfected control or SFFV-GFP lentivirus control in the cardiac differentiated samples. These results suggested that lentiviral transduction had some effect on the expression of Brachyury in hESC

maintained pluripotent, but the levels did not reach that of the cardiac differentiated samples.

NKX2.5 is known as an early cardiac marker, involved in cardiac commitment, and is significantly upregulated in the SFFV-GFP control virus group compared to uninfected. NKX2.5 is also significantly downregulated in cardiac samples transduced with miR-133b overexpressing virus compared to LNT-SFFV-GFP control virus transduced samples, however there was no significant difference when compared to uninfected controls. This is consistent with findings previously reported, although in this setting it is not known whether this effect is due to a generally lower level expression of NKX2.5 per cell or a lower number of cells expressing NKX2.5 (Ivey et al., 2008a, Takaya et al., 2009b). TNNT2 expression achieved the highest levels of those genes tested, with all samples subjected to cardiac media conditions displaying significant increase over matched pluripotent samples. A significant decrease in TNNT2 levels was observed in miR-133 overexpression samples compared to SFFV-GFP control samples.

MiR-1 overexpression did not, as previously reported, appear to enhance cardiac differentiation of H1 hESC in a monolayer system. In all of the genes tested, those transduced with miR-1 overexpression virus showed no significant improvement over SFFV-GFP virus control or no virus control.

In previously published work, miR-133 overexpression appeared to inhibit cardiogenesis in an embryoid body cardiac protocol (Ivey et al., 2008a). Our results show a down regulation of cardiac genes in samples transduced with LNT-SFFV-miR-133 but only when compared to samples transduced with LNT-SFFV-GFP . In comparison to uninfected control samples no significant difference in cardiac potential could be ascertained by qRT-PCR. That miR-1 did not appear to potentiate cardiac differentiation could be due to the differences in culture technique (embryoid body vs monolayer), hESC line used or the fact our hESC were maintained under feeder-free conditions. Therefore the expected action of miR-1 and miR-133 in H1 hESC monolayer cardiac differentiation does not appear to be defined between protocols and laboratories.

In summary, we successfully showed generation of pre-miR overexpression lentiviruses for miR-1 and miR-133 in a construct using the SFFV promoter, that SA461 hESC were unsuitable for cardiac differentiation, and that using a modified version of the LaFlamme protocol in a monolayer system resulted in beating cells with a cardiomyocyte phenotype (Laflamme et al., 2007). However our investigation into miRNA refinement of cardiac differentiation from hESC was inconclusive. We were unable to confirm the findings published previously (Ivey et al., 2008a) and due to time constraints could not investigate the hypothesis further.

Chapter 4: MicroRNA, gene expression and epigenetic characterisation of primary endothelial cells and hESC derived endothelial cells.

4.1 Introduction

Endothelial cells provide an essential interface between the blood and the tissues. The revascularisation of the infarcted heart would play a major beneficial role in a regenerative medicine strategy to treat myocardial infarction. The differentiation of vascular endothelial cells in clinically relevant numbers for injection into the ischaemic area could stimulate angiogenesis, rescuing at risk myocytes, thus limiting the development of scar tissue and improving cardiac function. Similarly, for peripheral ischaemia, injection of cells into the ischaemic regions to create/stimulate new blood vessel growth could be beneficial.

Although successful generation of functional endothelial-like has been demonstrated (Wang et al., 2007b, Kane et al., 2010a, Strahl and Allis, 2000, Kane et al., 2012), very little is understood about the early commitment of pluripotent hESC to mesoderm and subsequently to the endothelial-like lineage. How mesodermal tissues are formed during development remains a difficult area to study due to lack of specific markers of early mesodermal commitment (Evseenko et al., 2010). Differentiation of hESC *in vitro* recapitulates early embryogenesis, and although hESC are increasingly studied, research is usually focussed on defined, intermediate to late differentiated cell products (Murry and Keller, 2008). As such, many questions remain about the initial processes involved as the hESC becomes more specified towards a chosen lineage.

At present, methods of generating stem cell-derived vascular endothelial cells differ greatly between laboratories and hESC lines used. Upon differentiation, cell morphology changes along with expression of various markers of differentiation which can be used to classify cell populations. Embryoid body (EB) differentiation, in which cells are encouraged to aggregate in culture, allows spontaneous differentiation to endothelial cells as well as potentially all cell-types from mesodermal, ectoderm and endoderm germ layers. The shortfalls of this technique include low efficiency of differentiation with just 1-3% endothelial cells reported (Levenberg et al., 2002, Li et al., 2009, Cho et al., 2007) and the technically difficult cell isolation procedures needed to obtain a homogenous cell population, such as fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS) (Levenberg et al., 2002, Li et al.,

2009). Recent modifications to EB differentiation include culture under hypoxic conditions (Prado-Lopez et al., 2010), the introduction of VEGF-A to the culture media (Nourse et al., 2010), and inhibition of the TGF-ß signalling pathway (James et al., 2010). These changes have improved the yield of an appropriate cell population, however issues such as animal contamination through use of serum and scale-up of production to produce a clinically relevant number of cells, still remain. Alternative endothelial cell differentiation strategies include a 2-D system involving co-culture with mouse embryonic fibroblast feeder layers followed by MACS sorting for CD34 positive EPC-like cells and further expansion of this population (Wang et al., 2007b). Further functional assays confirmed this cell population isolated was able to form structurally normal vessels *in vivo*, although the efficiency for this method remains at 10% (Wang et al., 2007b). Improvements to this method involving serum free media conditions was reported to yield ~50% CD31 and VE Cadherin positive cells within 6-7 days, although no *in vivo* functional characterisation was carried out (Lagarkova et al., 2008). Previous research from our group described a method to generate functional endothelial-like cells by directed differentiation without the need for serum or a feeder cell layer (Kane et al., 2010a). Cells derived using this protocol were ~80% positive for CD31 and VE Cadherin after 21 days and significantly improved neogenesis in a mouse model of hindlimb ischaemia following injection (Kane et al., 2010a).

MiRNAs have been implicated in primary endothelial cell function and involved in the regulation of formation of blood vessels in the developing embryo (Nicoli et al., 2010). Research performed on *Dicer* knockout (KO) mice suggested that miRNAs play a role in development of endothelium, with embryos displaying reduced blood vessel formation, altered expression of VEGF and endothelial-associated genes Flt1, Kdr and Tie1 and knockouts were embryonic lethal phenotype between days 12.5 and 14.5 of gestation (Yang et al., 2005). To further assess the importance of miRNAs in endothelial cells, Suárez *et al.* knocked down *Dicer* using a siRNA approach and examined the cell phenotype of human umbilical vascular endothelial cells (HUVECs) (Suarez et al., 2007). They reported differential expression of several endothelial-associated genes including Tie-2, Kdr, and Tie-1, and increased activity of the endothelial nitric oxide synthase pathway. It was also noted that Dicer KO cells had reduced

capability to form tubes in a tube forming assay and exhibited reduced proliferation (Suarez et al., 2007). Similar results were demonstrated in Dicer mutant zebrafish, where endothelial cells were present but the circulatory system was disrupted (Giraldez et al., 2005). Specific knockdown of Dicer in murine endothelial cells did not result in a lethal phenotype (likely owing to incomplete excision of the *Dicer* gene) however, a decreased VEGF-induced angiogenesis and retarded recovery after ischemic challenge were observed (Suárez et al., 2008).

Epigenetic control of the poised state classically associated with hESC is due to changes at the chromatin level, where post translational modification of histones can "prime" a gene for activation or repression dependant on which modification has occurred (Berger, 2007). ~3000 genes are thought to be primed for both repression and activation and have both tri-methylation of lysine 27 of histone H3 (H3K27Me3) for repression and tri-methylation of lysine 4 of histone H3 (H3K4Me3) for activation present (Bernstein et al., 2006)(Figure 1.4). Owing to both types of chromatin being present, the gene is termed bivalent, meaning they can be rapidly activated upon differentiation or remain silenced if committing to a lineage which does not require expression of that particular gene (Bernstein et al., 2006). Unsurprisingly, genes with these bivalent marks have been proposed as highly important cell fate deciders, and the role of these genes in cardiovascular differentiation remains to be investigated.

With the control of hESC differentiation so complex, a strategic dissection of regulation is required. The studies discussed in this chapter were designed to create a microRNA and gene expression profile, in addition to epigenetic status of hESC-EC and primary HSVEC.

4.2 Results

4.2.1 microRNA profile of SA461 hESC during endothelial-like differentiation

To allow characterisation of miRNA expression in SA461 hESC undergoing a mesodermal endothelial differentiation (Kane et al., 2010a), a LC Sciences 2 channel microarray was initially employed. Samples were collected from cells undergoing differentiation for 2, 4 and 10 days with pluripotent control samples collected at day 0 (initiation of differentiation) as well as at each of the timepoints (Figure 4.1). Of the 374 mature miRNAs tested, hsa-miR-99b, hsa-miR-181a and hsa-miR-181b showed a reproducible and statistically significant increase in expression over time in the endothelial differentiated samples (Figure 4.2A)($n=3$, $p<0.05$). These findings were subsequently validated by miRNA TaqMan qRT-PCR (Figure 4.2B) and Northern blotting (Figure 4.2C), confirming the expression of hsa-miR-99b, hsa-miR-181a and hsa-miR-181b human embryonic stem cell-derived endothelial-like cells (hESC-EC)($n=3$, $p<0.05$). These miRNAs were subsequently termed “novel endothelial-associated miRNA”.

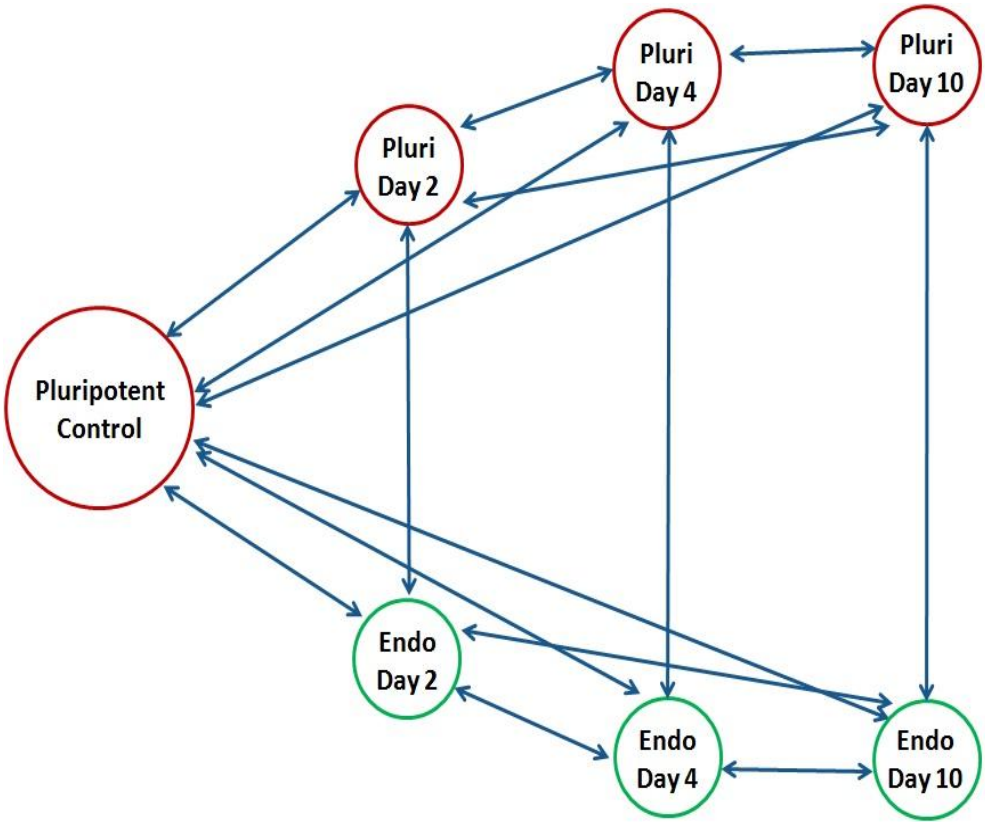


Figure 4.1: Schematic of microarray experimental design.

All SA461 hESC samples were derived from “Pluripotent Control”. SA461 hESC were differentiated in “endothelial differentiation media” for 2, 4 and 10 days with cells maintained in pluripotency harvested at the same timepoints. Comparisons between groups are indicated by arrows.

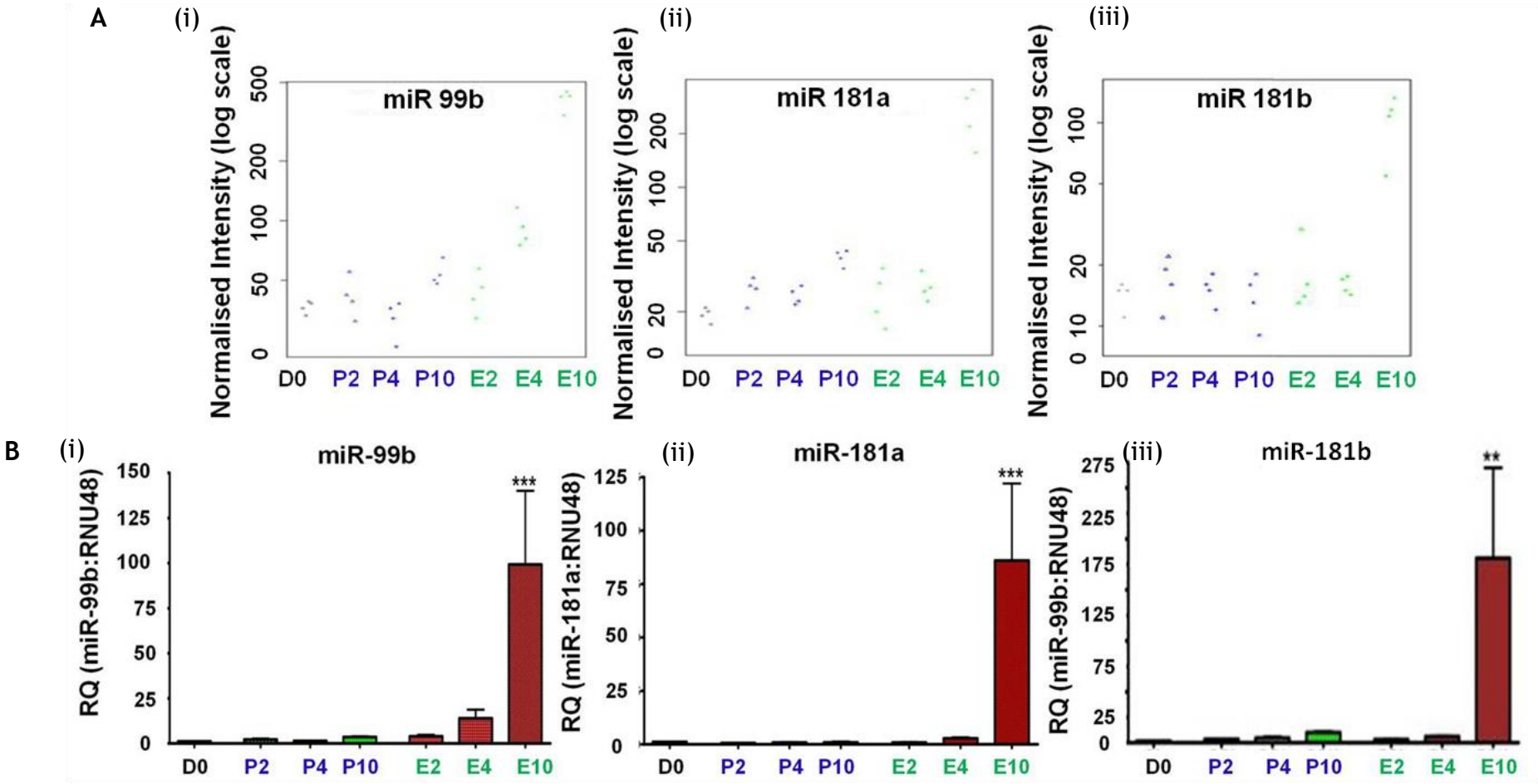


Figure 4.2: MicroRNA characterisation of hESC derived ECs.

Panel A depicts the normalised intensity log ratio from a LC Sciences microRNAs microarray of miR-99b (i), miR-181a (ii) and miR-181b (iii) in pluripotent Day 0 samples, pluripotent Day 2 samples labelled Pluripotent 1, pluripotent day 4 samples labelled Pluripotent 2, pluripotent day 10 samples labelled Pluripotent 3, endothelial differentiation day 2 samples labelled Endothelial 1, endothelial differentiation day 4 samples labelled Endothelial 2 and endothelial differentiation day 10 samples labelled Endothelial 3. Panel B shows validation of the microarray results by TaqMan qRT-PCR for miR-99b (i), miR-181a (ii) and miR-181b (iii) with D0 representing pluripotent day 0, P D2 representing pluripotent day 2, P D4 representing pluripotent day 4, P D10 representing pluripotent day 10, E D2 representing endothelial differentiation day 2, E D4 representing endothelial differentiation day 4 and E D10 representing endothelial differentiation day 10. All data normalised to pluripotent D0. ** = $p<0.01$ vs D0, *** = $p<0.001$ vs D0

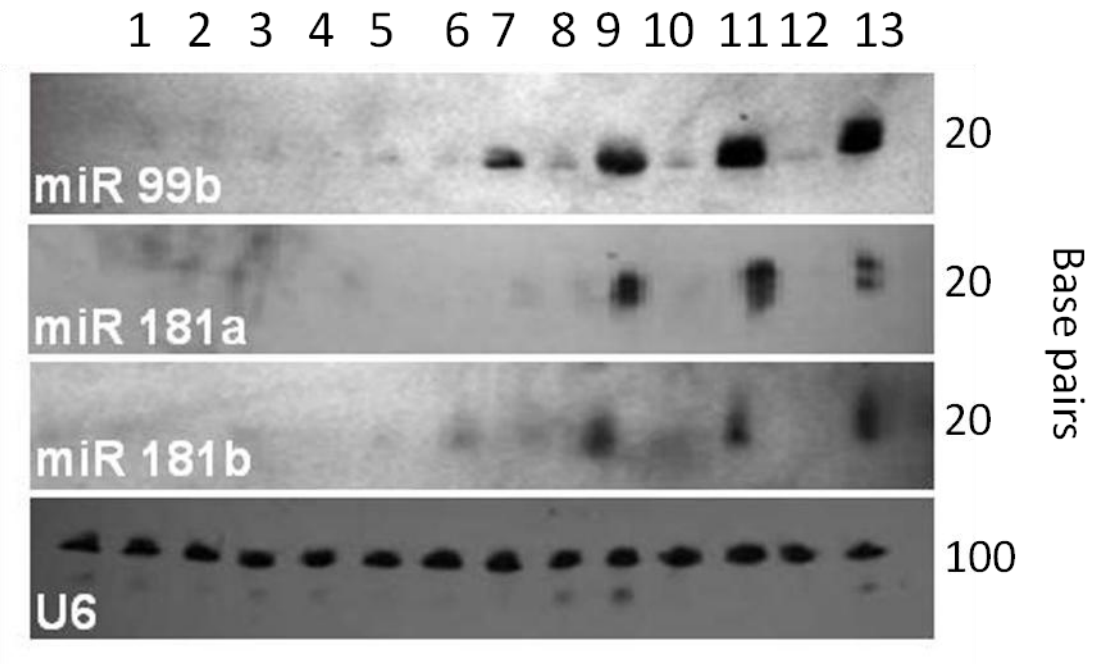


Figure 4.3: Northern Blotting validation of miRNA in hESC-EC.

Northern blots for miR-99b, miR-181a, miR-181b and control U6. Lanes are numbered 1 to 13, with 1 being Pluripotent Day 0, 2 being pluripotent day 2, 3 being endothelial differentiation day 2, 4 being pluripotent day 4, 5 being endothelial day 4, 6 being pluripotent day 7, 7 being endothelial day 7, 8 being pluripotent day 10, 9 being endothelial day 10, 10 pluripotent day 14, 11 being endothelial day 14, 12 being pluripotent day 21 and 13 being endothelial day 21.

4.2.2 Expression of novel "endothelial-associated miRNA" in other mesoderm cell types

To further investigate the expression of the novel endothelial-associated miRNA, miR-99b, -181a and -181b were analysed in a selection of other mesodermal cell types including primary human vascular cells and H1 hESC subjected to a defined haematopoetic differentiation protocol. These were compared to SA461 hESC-EC and H1 hESC-EC by TaqMan qRT-PCR (Figure 4.4). Compared to day 10 hESC-EC from both SA461 and H1, expression of novel endothelial associated miRNAs were at the highest levels in human saphenous vein endothelial Cells (HSVEC) from both patients undergoing elective varicose vein stripping and considered otherwise healthy (Control) and HSVEC from patients undergoing saphenous vein dissection for coronary artery bypass graft as a vascular disease phenotype (CABG). Substantial levels of miR-99b, miR-181a and miR-181b expression compared to H1 and SA461 pluripotent day 0 and hESC-EC day 10 were observed, with miR-181b expression reaching the highest relative levels (Figure 4.4C)($p<0.01$). There was a small but statistically significant difference in expression of miR-181b between control HSVEC and CABG HSVEC by ANOVA ($p<0.05$) (Figure 4.4).

Expression of novel endothelial associated miRNAs in human saphenous vein smooth muscle cells (HSVSMC) from control patients compared to H1 and SA461 day 0 and day 10 hESC-EC samples was also investigated. For miR-99b, expression was slightly higher in HSVSMC than in day 0 samples, however, this did not attain significance (Figure 4.4A). For miR-181a, expression was significantly higher in HSVSMC than in hESC day 0 ($p<0.05$) (Figure 4.4B). For miR-181b, expression was also significantly higher in HSVSMC compared to pluripotent H1 hESC ($p<0.05$) (Figure 4.4C). Additionally, miRNA expression data was collected from human pulmonary artery endothelial cells (HPAEC). Expression of miR-99b in HPAEC appears to be increased compared to pluripotent day 0 H1 hESC, but this trend did not reach significance (Figure 4.4A). MiR-181a expression in HPAEC was significantly higher than H1 hESC as was miR-181b expression ($p<0.05$) (Figure 4.4B and C). In H1 hESC subjected to haematopoetic differentiation, miR-99b was significantly expressed in day 10 samples compared to day 0 samples ($p<0.05$) (Figure 4.4A). MiR-181a and miR-

181b expression in day 10 hESC-haem compared to day 0 was also significant ($p<0.001$) (Figure 4.4B and C).

4.2.3 MiR-99b, miR-181a and miR-181b appear to be mesoderm-specific

In order to investigate whether these miRNA were specific to the mesoderm lineage, miRNA expression was assessed in representative cell types of the other germ layers, ectoderm and endoderm. To investigate the ectoderm lineage, H1 hESC were subjected to a defined, neuronal differentiation protocol as described (Gerrard et al., 2005) and samples collected for analysis at 0, 4, 7, 10 and 14 days post initiation of differentiation (Figure 4.5). TaqMan qRT-PCR was subsequently performed to quantify miRNA expression levels. Expression of miR-99b, miR-181a or miR-181b did not reach levels of significance compared to day 0 pluripotent control ($n=2$, $p>0.05$).

To ascertain expression of miR-99b, miR-181a and miR-181b in cells from the endoderm lineage, we required the generation of endodermal cells from hESC or access to endodermal tissue. Neither of these options were available to this study. Instead, liver hepatocellular carcinoma HepG2 cells were used. There was no significant expression of miR-99b, miR-181a or miR-181b from the HepG2 cells tested (Figure 4.5)($n=3$, $p>0.05$).

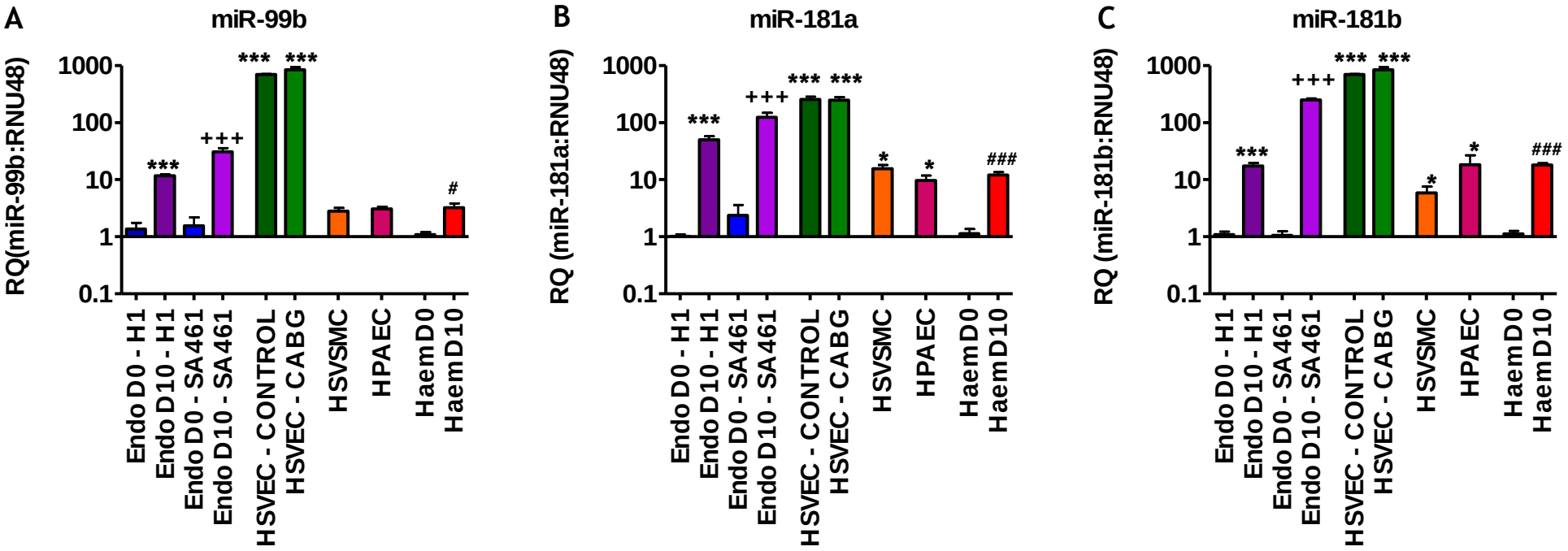


Figure 4.4: Expression of miR-99b, miR-181a and miR-181b in other mesodermal cell types.

TaqMan® qRT-PCR results of miR-99b, miR-181a and miR-181b expression in H1 hESC at day 0 and day 10 endothelial differentiation, SA461 hESC at day 0 and day 10 endothelial differentiation, control HSVEC from 5 age and sex-matched patients, CABG HSVEC from 5 age and sex-matched patients, HSVSMC from 3 age and sex-matched control patients, pooled HPAEC from healthy patients and H1 hESC at day 0 and day 10 haematopoetic differentiation. HESC data normalised to appropriate day 0 population; HSVEC, HSVSMC and HPAEC data normalised to H1 hESC-EC day 0. * = $p<0.05$ vs H1 hESC-EC D0, *** = $p<0.001$ vs H1 hESC-EC D0, +++ = $p<0.001$ vs SA461 hESC-EC D0, # = $p<0.05$ vs H1 hESC-haem D0 and ### = $p<0.001$ vs hESC-haem D0.

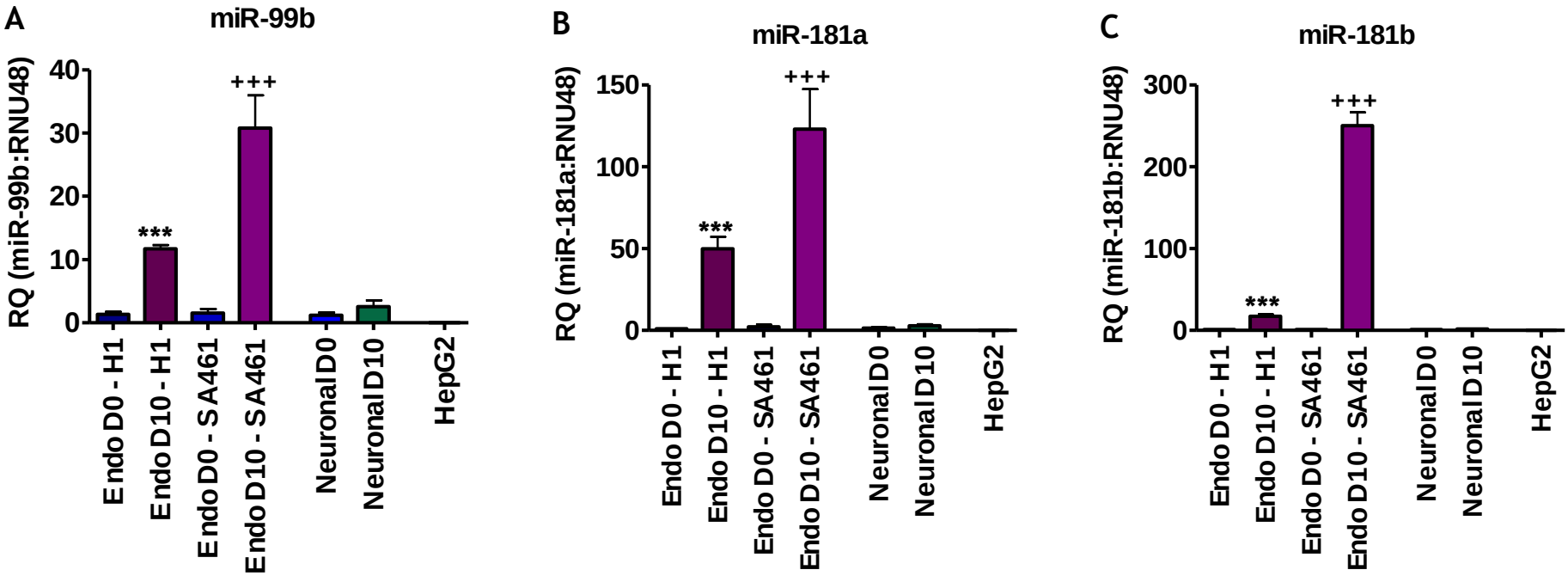


Figure 4.5: Expression of miR-99b, miR-181a and miR181b in ectoderm and endoderm cell types.

QRT-PCR results of miR-99b, miR-181a and miR-181b expression in H1 hESC at day 0 and day 10 endothelial differentiation, SA461 hESC at day 0 and day 10 endothelial differentiation, H1 hESC at day 0 and day 10 neuronal differentiation (ectoderm) and HepG2 cells (endoderm). *** = $p<0.001$ vs H1 hESC-EC D0, +++ = $p<0.001$ vs SA461 hESC-EC D0.

4.2.4 MiR-99b, miR-181a and miR-181b are not altered by "vascular disease stimuli" in vitro

In an attempt to dissect the regulation of novel endothelial associated miRNAs, HSVEC were subjected to a range of physiologically relevant agents to mimic human endothelial disease. Angiotensin II was used at a final concentration of 1 μM, as it had been previously shown to inhibit the growth and migration of endothelial cells through the AT2 receptor at this concentration (Stoll et al., 1995, Benndorf et al., 2003), carbachol was used to stimulate nitric oxide production at a final concentration of 10 μM as had been shown previously to effectively induce a measurable response using ELISA (Kane et al., 2010a), Endothelin-1 is a potent vasoconstrictor and also induces nitric oxide production at a final concentration of 1 μM (Tsukahara et al., 1994, Calo et al., 1996), glucose at a final concentration of 25 mM to mimic diabetes as had previously been shown to elicit modulation in miRNA levels in endothelial cells (Caporali et al., 2011), L-NAME is an inhibitor of endothelial nitric oxide synthase (eNOS) at a final concentration of 100 μM (Pfeiffer et al., 1996) and ox-LDL at a final concentration of 50 μg/ml and 100 μg/ml to cause superoxide production measureable by fluorometric probes as had been previously reported (Cominacini et al., 2000). Samples were collected at 24, 48 and 72 h and total RNA extracted. Expression of miR-99b (Figure 4.6A), miR-181a (Figure 4.6B) and miR-181b (Figure 4.6C) were ascertained by TaqMan qRT-PCR. Angiotensin II, carbachol, endothelin-1, glucose, L-NAME or oxidised-LDL treatment did not elicit any statistically significant difference in miR-99b, miR-181a or miR-181b expression at any of the time-points studied (Figure 4.6).

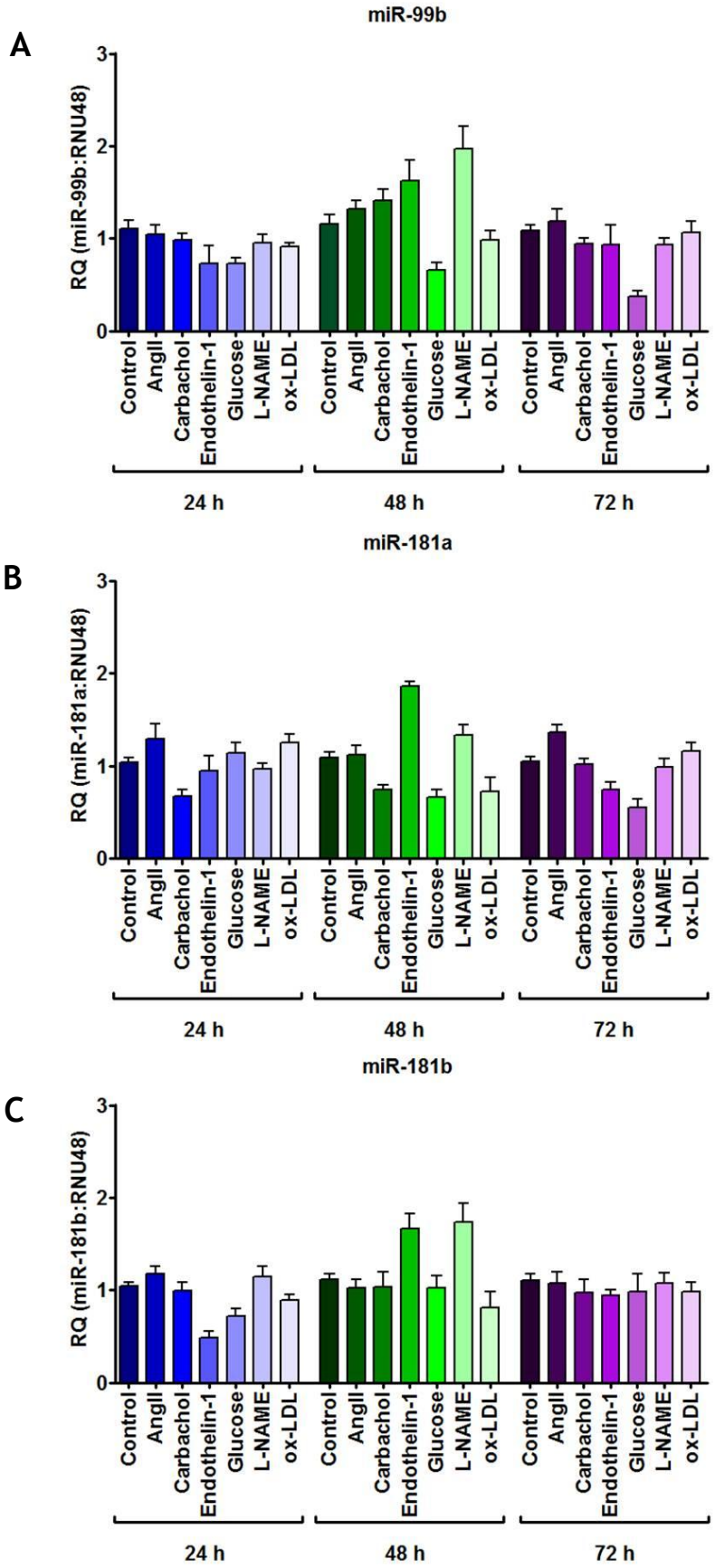


Figure 4.6: MicroRNA expression in HSVEC in response to pathophysiological stimuli.

HSVEC were plated and exposed for 24, 48 or 72 h to normal media (Control), angiotensin II (AngII) at 1µM, carbachol at 10µM, endothelin-1 at 1µM, glucose at 25µM, L-NAME at 100µM and ox-LDL at 100µg/ml and expression of miR-99b(A), miR-181a(B) or miR-181b(C) relative to timematched control and RNU48 ascertained. There were no significant changes in expression of miRNAs tested.

4.2.5 Illumina Gene expression analysis of endothelial differentiation

In order to interrogate dynamic gene expression changes, cells were subjected to directed differentiation using the endothelial protocol (Kane et al., 2010a) and an Illumina whole genome expression microarray was employed. SA461 hESC subjected to endothelial-like differentiation were collected at day 0, 2, 4 and 10 as per sample collection for the LC Sciences microRNA microarray and analysed by Illumina gene expression microarray (Figure 4.1). In parallel HSVEC samples were also analysed to act as a positive control.

Principle Component Analysis (PCA) is a mathematical method to transform several closely related variables into uncorrelated variables termed principal components. This transformation allows the first principle component to account for the highest degree of variance which accounts for as much of the variability in the data as possible. The following components are assigned the next highest variance, orthogonal to the last component. Information provided allowed the visualisation of sample groups clustered together (Figure 4.7) and was used to build the ANOVA model. The PCA analysis showed acceptable separation of the groups, with primary HSVEC cells distinct from everything hESC-derived (Figure 4.7A). HESC-EC day 4 and day 10 also appear separate from all other samples derived from hESC (Figure 4.7B). Analyses of datasets have shown a large number of significantly differentially expressed probes at each time point: Day 2 of endothelial differentiation compared to Day 0 pluripotent control showed 1040 (of which IPA mapped 974) significant differentially expressed probe changes, Day 4 of endothelial differentiation compared to Day 0 pluripotent control showed 2400 (of which IPA mapped 2264) significant differentially expressed probe changes and Day 10 of endothelial differentiation compared to Day 0 pluripotent control showed 2157 (of which IPA mapped 2023) significant differentially expressed probe changes (all FDR<0.05) (Figure 4.8B). Comparison of HSVEC with hESC-EC Day 10 reveals 6133 significantly differentially expressed probes (FDR <0.05) suggesting that day 10 hESC-EC remain markedly different compared to primary HSVEC (Figure 4.8A).

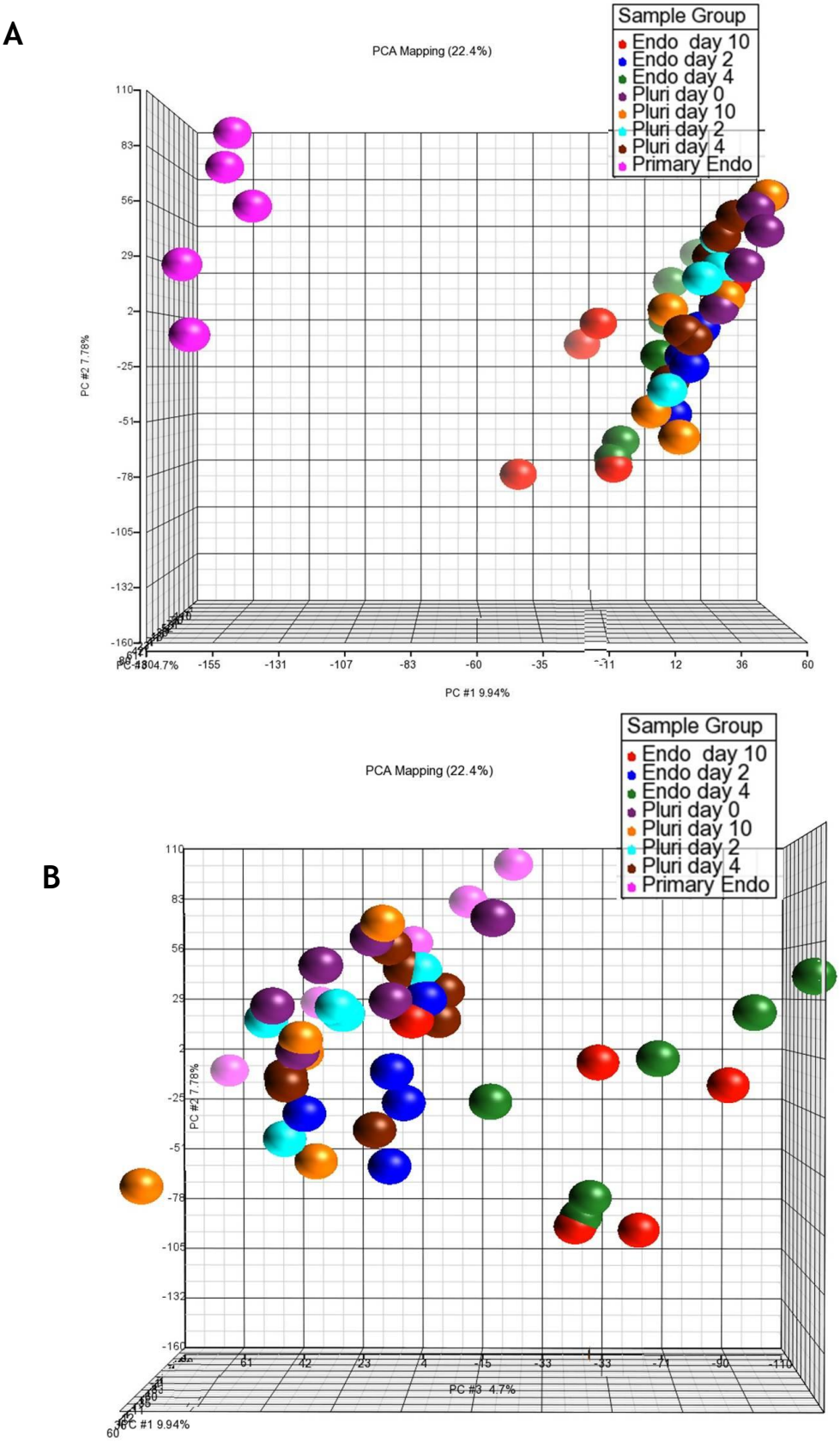


Figure 4.7: Principle component analysis.

Principle component analysis of Illumina microarray. (A) HSVEC (pink) were distinct from hESC and hESC-EC. (B) HESC-EC day 4 (green) and 10 (red) were separate from hESC-EC day 2 (deep blue) and pluripotent time-matched samples at day 0 (purple), day 2 (light blue), day 4 (brown) and day 10 (gold).

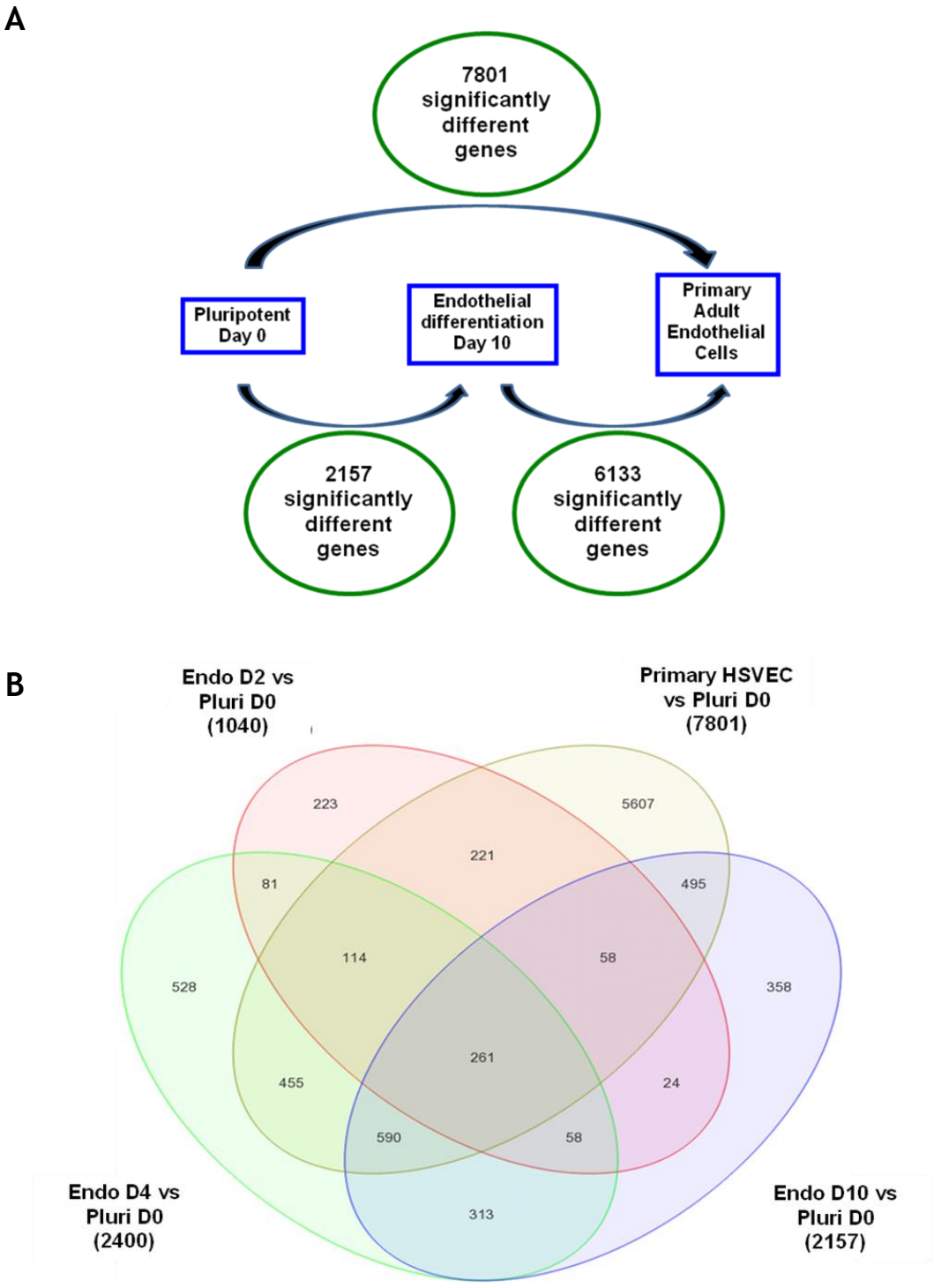


Figure 4.8: Summary of significantly differentially expressed probes from Illumina microarray.

Summary schematic detailing key significant probe expression comparisons. There were 2157 significantly different expressed probes between hESC-EC day 0 (Pluripotent Day 0) and hESC-EC day 10 (Endothelial differentiation Day 10). There were 6133 significantly differentially expressed probes between hESC-EC day 10 (Endothelial differentiation Day 10) and HSVEC (Primary Adult endothelial cells). Between hESC-EC day 0 (Endothelial differentiation Day 10) and HSVEC (Primary Adult endothelial cells) there were 7801 significantly differentially expressed probes (all FDR<0.05) (A). Venn diagram of significantly different probe expression changes, all corrected to hESC-EC day 0 (Pluri D0) (FDR<0.05) (B).

4.2.6 Analyses of hESC-EC day 10 compared to primary endothelial cells

As the latest time-point investigated, day 10 hESC-EC from pluripotent day 0 hESC had 2157 significantly differentially expressed probes, however between HSVEC and hESC-EC day 10 there were 6133 statistically significant probe expression changes (FDR <0.05)(Figure 4.8A). In order to visualise which endothelial associated genes present in adult HSVEC remain at lower levels in day 10 hESC-EC, IPA was employed. Levels of key endothelial genes chemokine (C-X-C motif) receptor 4 (CXCR4), intercellular adhesion molecule 1 (ICAM1), selectin E (SELE), fms-related tyrosine kinase 4 (FLT4), C-type lectin domain family 11, member A (CLEC11A), catenin (cadherin-associated protein), beta 1 (CTNNB1), T, brachyury homolog (T), Kruppel-like factor 6 (KLF6), melanoma cell adhesion molecule (MCAM), intercellular adhesion molecule 2 (ICAM2), cadherin 5, type 2 (vascular endothelium) (CDH5), bone morphogenetic protein 2 (BMP2), CD34 molecule (CD34), selectin P (SELP), fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) (FLT1), von Willebrand factor (VWF), kinase insert domain receptor (KDR), TEK tyrosine kinase, endothelial (TEK), fibroblast growth factor (Fgf) and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) in hESC-EC day 10 compared to day 0 samples were studied and significant changes observed (Figure 4.9). HESC-EC day 10 compared to HSVEC were also studied (Figure 4.10). KLF6, FGF9 and TEK were the only probes statistically significantly upregulated by hESC-EC day 10 compared to day 0 (Figure 4.9), whereas CXCR4, SELE, CLEC11A, KLF6, MCAM, ICAM2, CDH5, BMP2, CD34, VWF, KDR and TEK were statistically significantly expressed in HSVEC compared to hESC-EC day 10 (FDR <0.05) (Figure 4.10). Pluripotency markers such as Oct4 and Sox2 were significantly downregulated in hESC-EC day 10 compared to hESC-EC day 0 (Figure 4.11) however levels were significantly lower in HSVEC compared to hESC-EC day 10 (Figure 4.12).

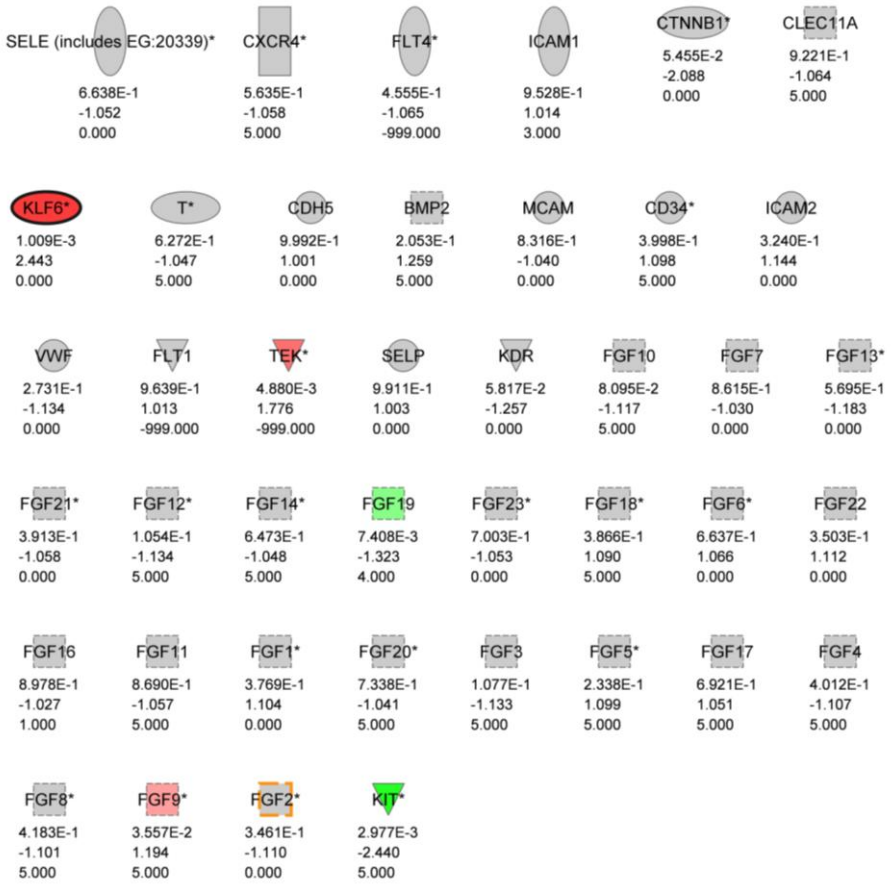


Figure 4.9: Significant expression of endothelial associated probes in hESC-EC day 10 compared to hESC-EC day 0.

Expression of endothelial associated genes in hESC-EC day 10 vs hESC-EC day 0. Red denotes higher expression in hESC-EC day 10 compared to hESC-EC day 0 and green denotes lower expression in hESC-EC day 10 compared to hESC-EC day 0. Numbers underneath genes correspond to FDR, fold-change and prediction of bivalency.

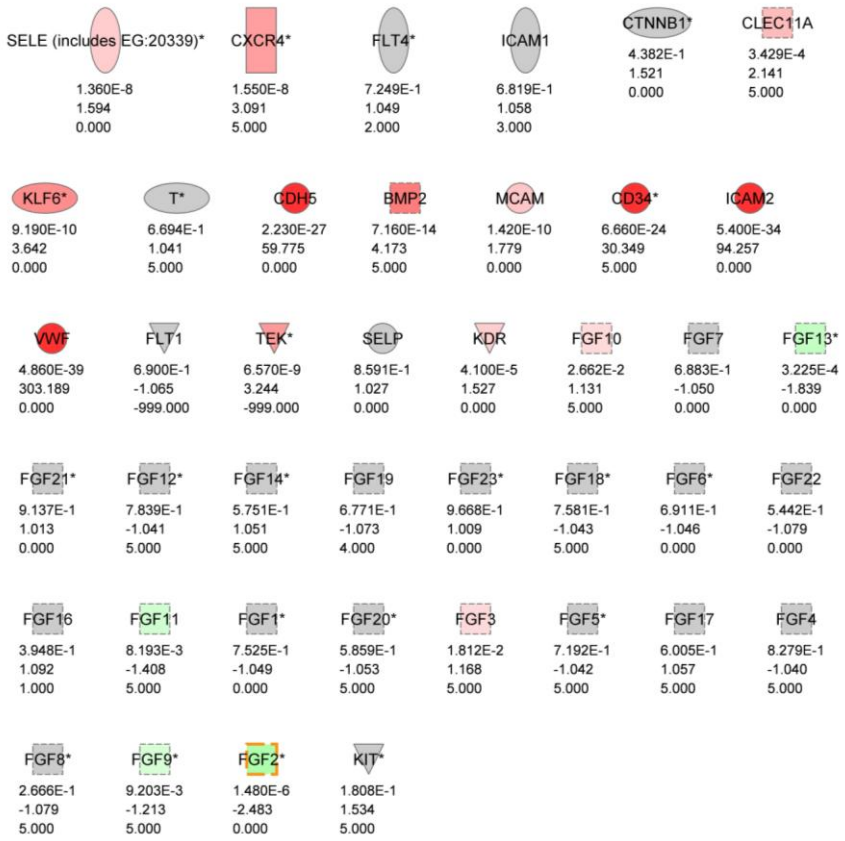


Figure 4.10: Significant expression of endothelial associated probes in primary HSVEC compared to hESC-EC.

Expression of endothelial associated genes in HSVEC vs hESC-EC day 10. Red denotes higher expression in HSVEC compared to hESC-EC day 10 and green denotes lower expression in HSVEC compared to hESC-EC day 10. Numbers underneath genes correspond to FDR, fold-change and prediction of bivalency.

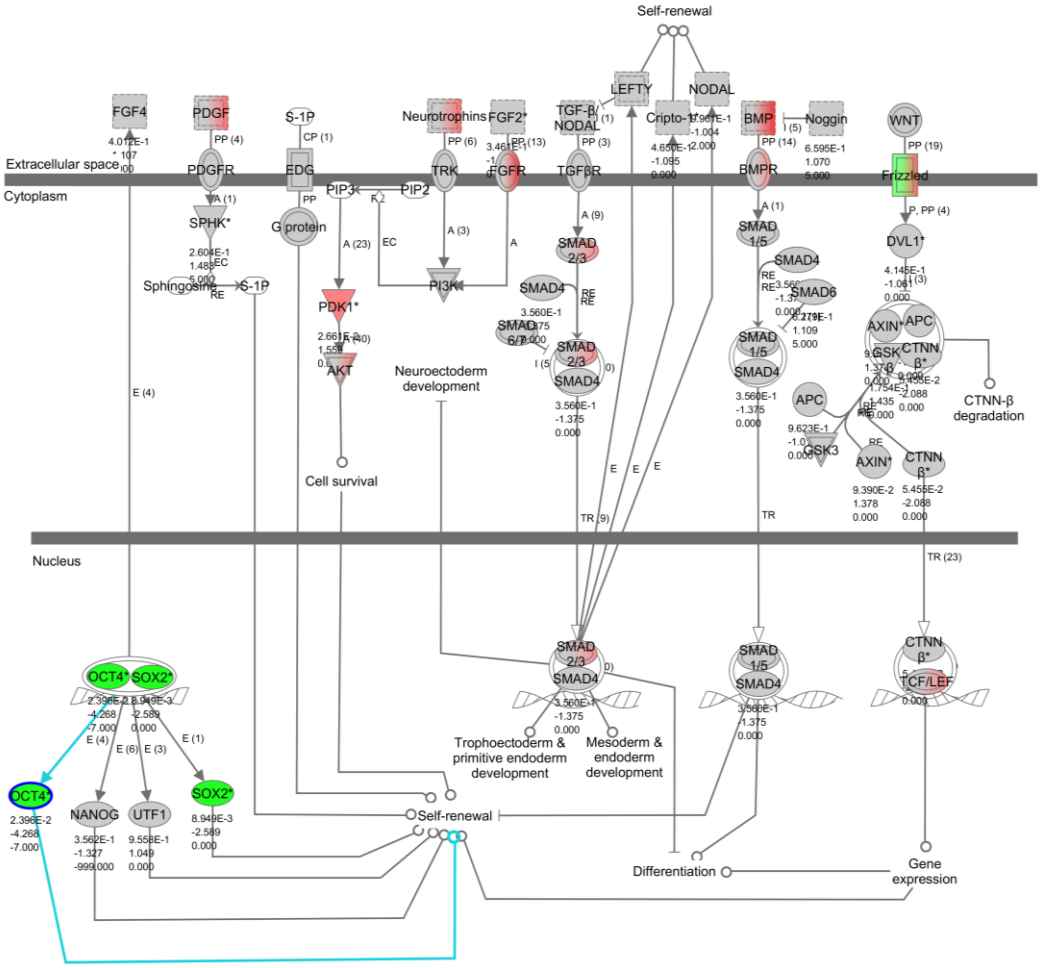


Figure 4.11: Expression of pluripotency associated genes in hESC-EC day 10.

IPA pathway with expression data of pluripotency associated probe levels compared to hESC-EC day 0.

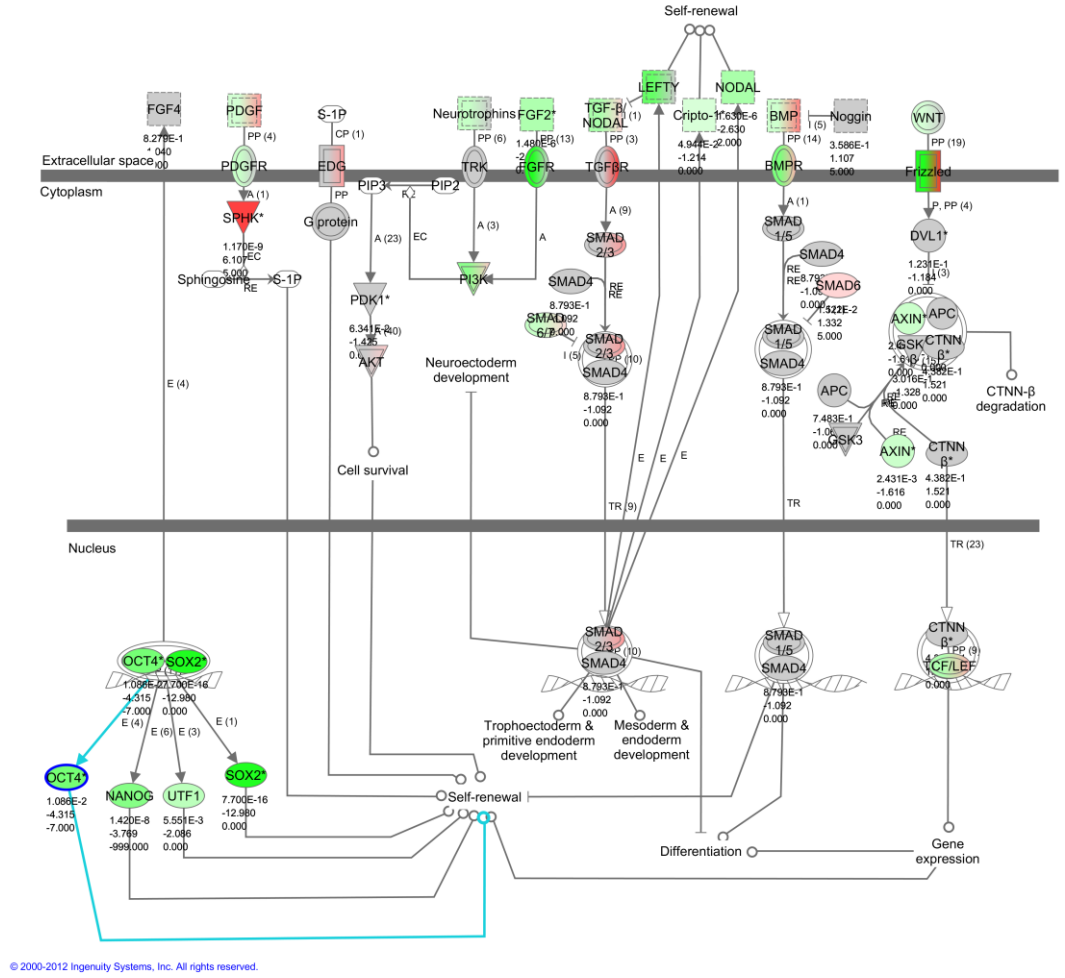


Figure 4.12: Expression of pluripotency associated genes in HSVEC compared to hESC-EC day 10.

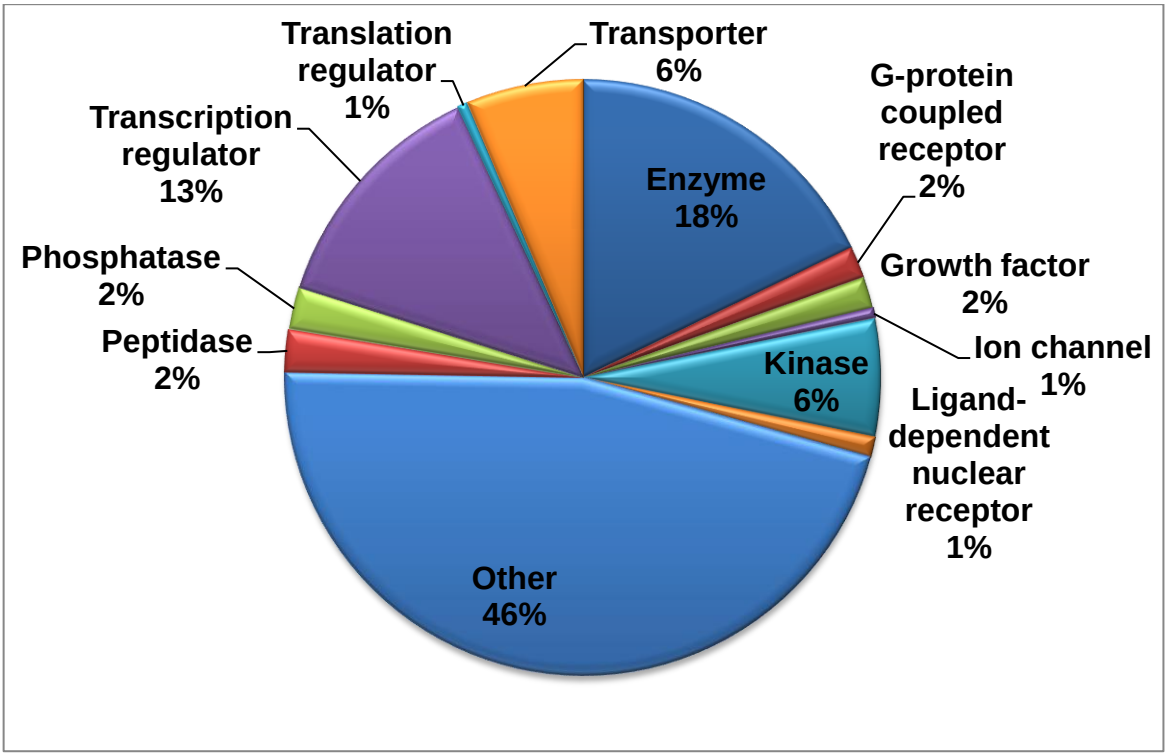
IPA pathway with expression data of pluripotency associated probe levels in HSVEC compared to hESC-EC day 10.

4.2.7 Transient transcription factor expression observed at day 2 hESC-EC differentiation

Ingenuity Pathway Analysis was employed to process the data into the key biological comparisons from the Illumina microarray. It facilitates the interrogation of large datasets for related genes and pathways represented in the expression data. It subsequently displays each probe as an individual symbol based upon the known function. It also displays by colour whether a probe is statistically significantly up (red) or down-regulated (green) compared to another dataset, including the false discovery rate (top number) and fold-change information (middle number). In addition, information from a chip-SEQ dataset identifying bivalent domains on ~2500 genes in H9 hESC (Ku et al., 2008) was also integrated to the SA461 hESC datasets to give an *in silico* prediction of bivalent status, which is detailed as the bottom number.

When hESC-EC day 2 was investigated further, it was noted from the Venn diagram that hESC-EC day had 223 unique significant probe-set expression changes (Figure 4.8). Upon further analysis using IPA, a transient induction of several transcription factors unique to endothelial differentiation day 2 were observed, accounting for approximately 13% of expression changes specific to this time point (Figure 4.13A and B).

A



B

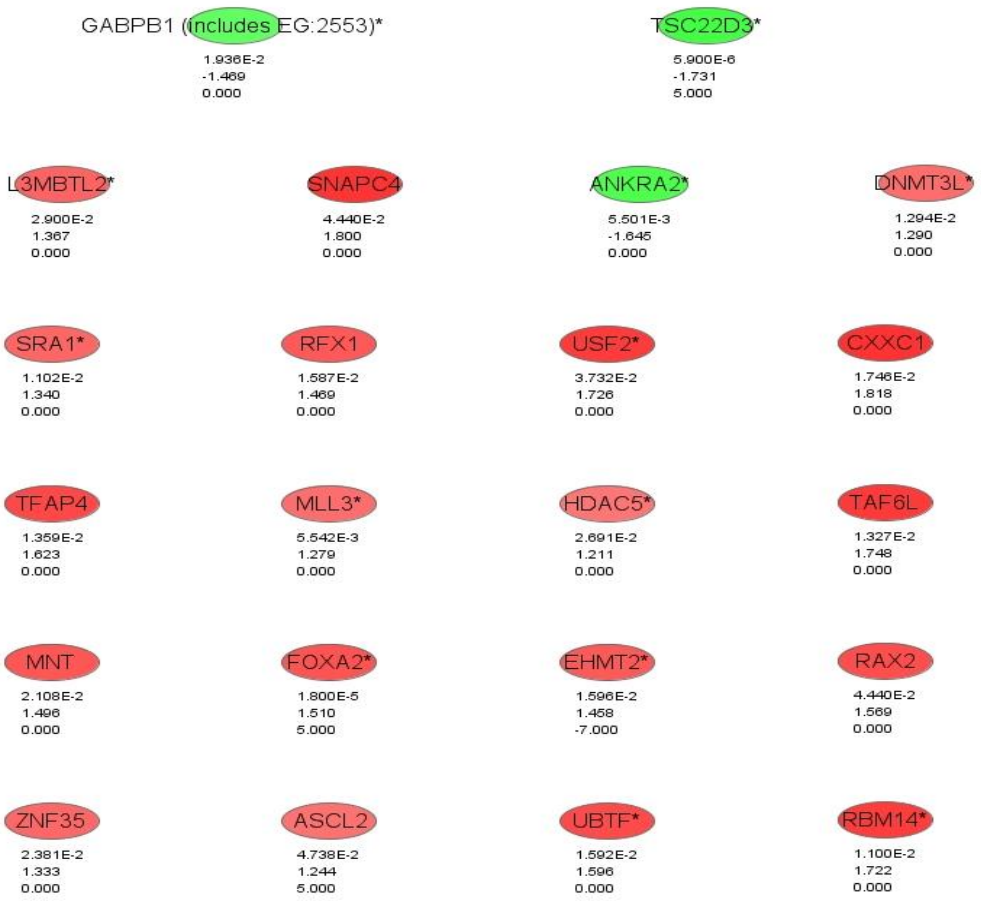


Figure 4.13: HESC-EC day 2 specific genes.

Pie chart of gene ontology classification of significantly differentially expressed genes specific to hESC-EC day 2 (FDR<0.05) (A). IPA output of significant transcription factor expression unique to hESC-EC day 2 (B).

Table 4.1: Transcription Factors specific to hESC-EC day 2.

List of transcription factors unique to hESC-EC day 2, with FDR, fold-change and Illumina probe ID.

Symbol	Fold Change	FDR	Illumina Probe ID
Ankyrin repeat, family A (RFXANK-like), 2 (ANKRA2)	-1.64468	0.00550105	ILMN_1687351
Achaete-scute complex homolog 2 (Drosophila) (ASCL2)	1.24413	0.0473781	ILMN_1723412
CXXC finger protein 1 (CXXC1)	1.81781	0.0174628	ILMN_1691276
DNA (cytosine-5-)-methyltransferase 3-like (DNMT3L)	1.29018	0.0129366	ILMN_2406634
Euchromatic histone-lysine N-methyltransferase 2 (EHMT2)	1.45772	0.0159592	ILMN_1751081
Forkhead box A2 (FOXA2)	1.50997	1.80E-05	ILMN_1668052
GA binding protein transcription factor, beta subunit 1 (GABPB1)	-1.46947	0.0193597	ILMN_1761147
Histone deacetylase 5 (HDAC5)	1.21127	0.0269061	ILMN_1810856
L(3)mbt-like 2 (Drosophila) (L3MBTL2)	1.36717	0.0289989	ILMN_2336109
Myeloid/lymphoid or mixed-lineage leukemia 3 (MLL3)	1.2788	0.00554164	ILMN_1725300
MAX binding protein (MNT)	1.49585	0.0210798	ILMN_1792910
Retina and anterior neural fold homeobox 2 (human) (RAX2)	1.569	0.0443982	ILMN_1653412
RNA binding motif protein 14 (RBM14)	1.72171	0.011002	ILMN_1700604
Regulatory factor X, 1 (influences HLA class II expression) (RFX1 (includes EG:100038773))	1.46898	0.0158731	ILMN_1725787
Small nuclear RNA activating complex, polypeptide 4, 190kDa (SNAPC4)	1.7998	0.0443982	ILMN_1677484
Steroid receptor RNA activator 1 (SRA1)	1.34046	0.0110232	ILMN_2062754
TAF6-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa (TAF6L)	1.74775	0.0132732	ILMN_1727281
Transcription factor AP-4 (activating enhancer binding protein 4) (TFAP4)	1.62316	0.0135871	ILMN_1814657
Upstream binding transcription factor, RNA polymerase I (UBTF)	1.59561	0.015919	ILMN_1806946
Upstream transcription factor 2, c-fos interacting (USF2)	1.7263	0.037322	ILMN_1756696
Zinc finger protein 35 (ZNF35)	1.33306	0.0238139	ILMN_1692100

4.2.7.1 QRT-PCR validation of transient transcription factor expression observed at day 2 hESC-EC differentiation

To further confirm gene expression of the transcription factors listed in Table 4.1, we performed TaqMan qRT-PCR. Of these 24 transcription factors, 12 validated significantly by TaqMan qRT-PCR when subjected to one way analysis of variance (ANOVA) ($p<0.05$); CXXC1, EHMT2, FOXA2, L3MBTL2, MLL3, RBM14, TAF6L, TFAP4, TSC22D3, UBTF, USF2 and ZNF35 (Table 4.1). When included in a list for analysis by IPA, among the top functions published for these transcription factors was cell cycle.

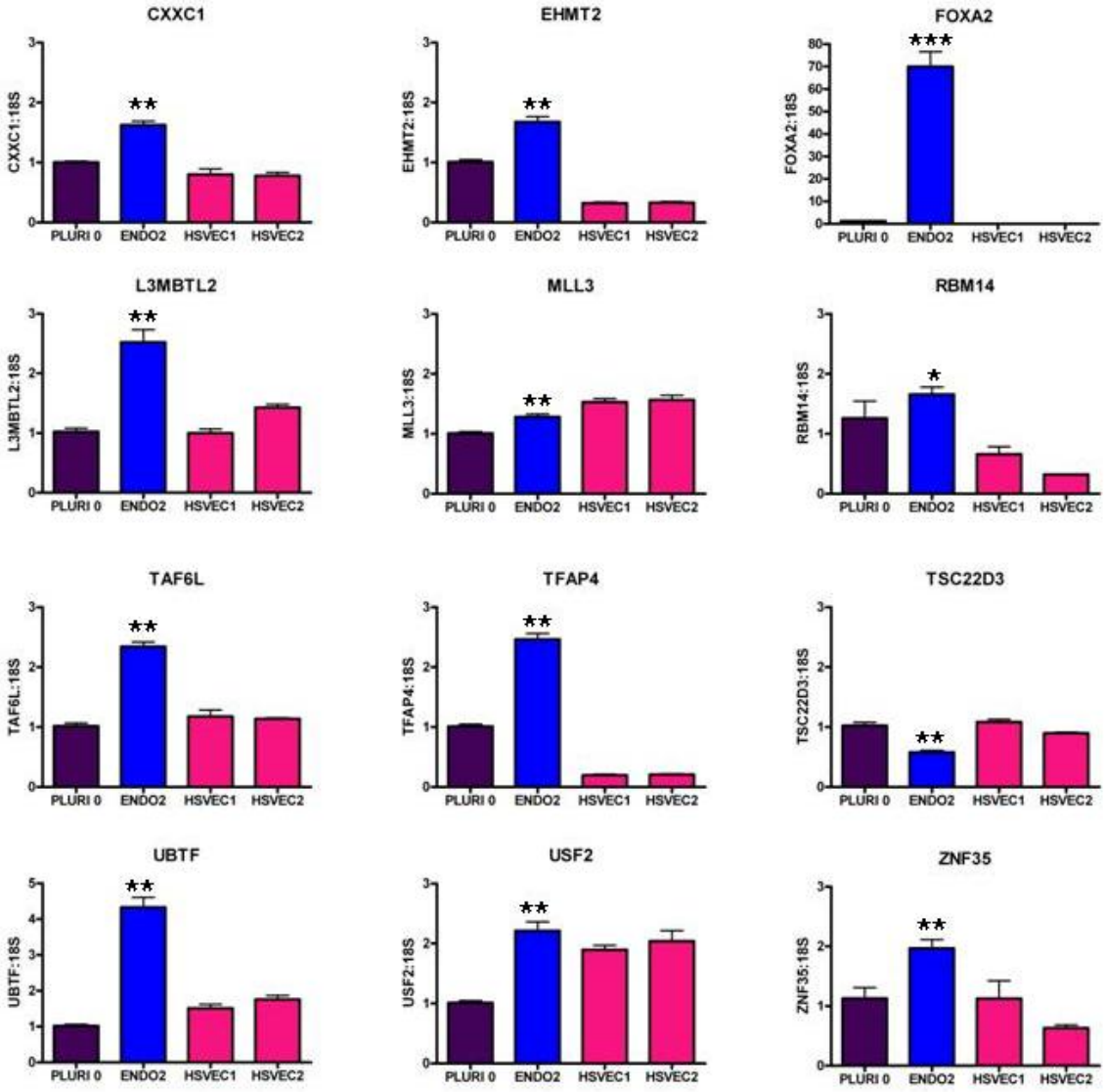


Figure 4.14: TaqMan qRT-PCR validation of hESC-EC day 2 specific transcription factors.

Validative qRT-PCR for hESC-EC day 2 specific transcription factors performed in hESC-EC day 0 (Pluri 0), hESC-EC day 2 (Endo 2) and in HSVEC from 2 patients (HSVEC1, HSVEC2). All data normalised to hESC-EC day 0 and 18S ribosomal control (*=p<0.05 vs hESC-EC day 0, **=p<0.01 vs hESC-EC day 0, ***=p<0.001 vs hESC-EC day 0).

4.2.7.2 FOXA2 protein is upregulated at hESC-EC differentiation day 2

To confirm expression of transcription factors at day 2 at the protein level, immunocytochemistry (ICC) was performed for Oct4, FOXA2, L3MBTL2, TFAP4, TSC22D3 and UBTF in SA461, H1 and RC10 hESC lines (Figure 4.15, Figure 4.16, Figure 4.17). Pluripotent SA461, H1 and RC10 hESC-EC day 0 maintained Oct4 expression as expected, which was reduced by hESC-EC day 2 (Figure 4.15, Figure 4.16, Figure 4.17). FOXA2, although present in a small subset of cells at pluripotent day 0, appeared more abundant at day 2 of endothelial-like differentiation, and there did not appear to be any co-localisation of Oct4 and FOXA2 as evidenced by the merge images (Figure 4.15, Figure 4.16, Figure 4.17). The remaining antibodies did not stain successfully under the conditions tested.

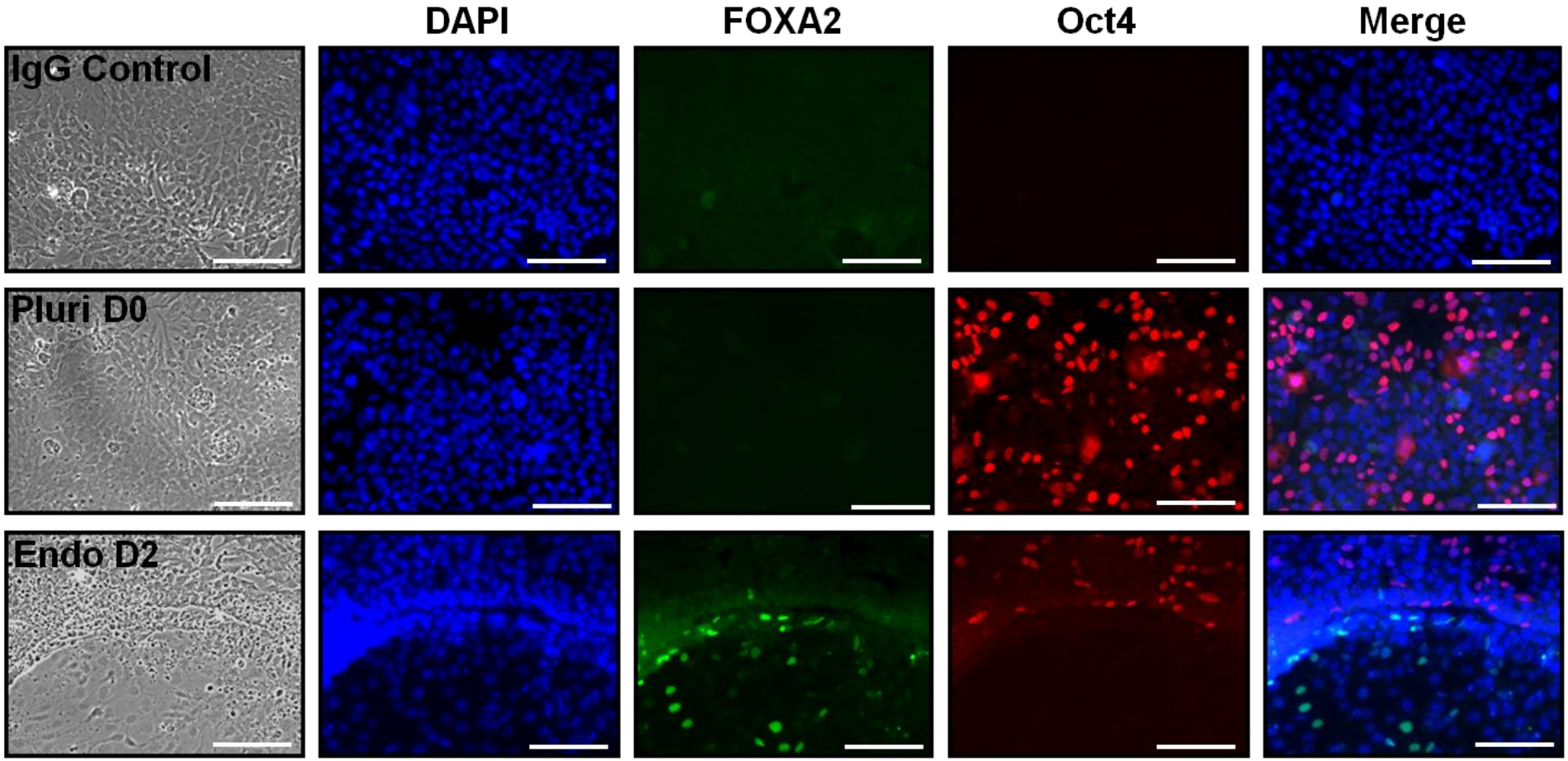


Figure 4.15: Immunofluorescent validation of FOXA2 and Oct4 in SA461 hESC-EC day 2.

ICC performed on hESC-EC day 0 (Pluri D0) and hESC-EC day 2 (Endo D2) for DAPI nuclear stain in blue, FOXA2 in green and Oct 4 in red. Goat (green) and mouse (red) IgG controls also shown. Scale bars represent 20 µm,

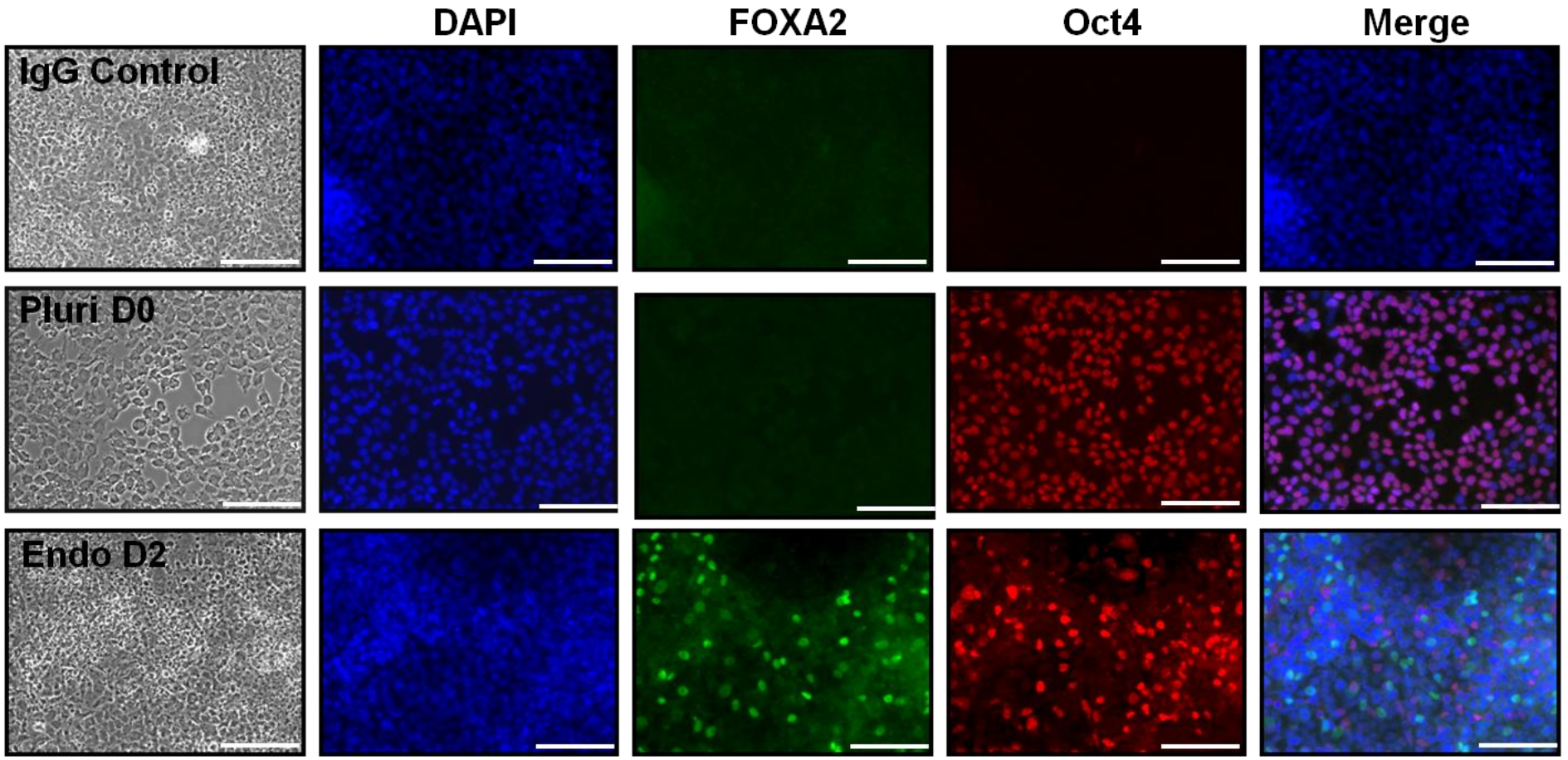


Figure 4.16: Immunofluorescent validation of FOXA2 and Oct4 in H1 hESC-EC day 2.

ICC performed on hESC-EC day 0 (Pluri D0) and hESC-EC day 2 (Endo D2) for DAPI nuclear stain in blue, FOXA2 in green and Oct 4 in red. Goat (green) and mouse (red) IgG controls also shown. Scale bars represent 20 µm.

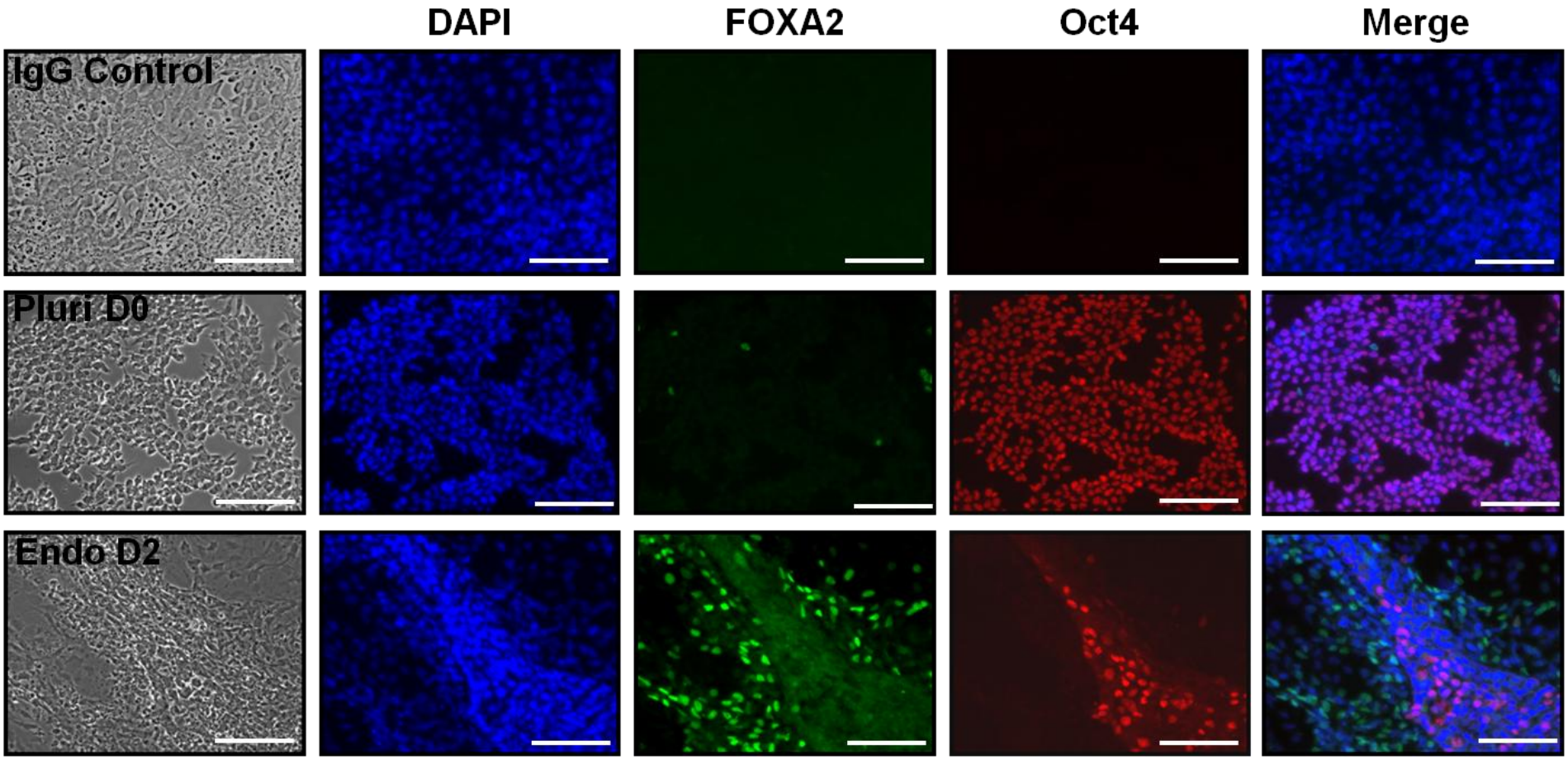


Figure 4.17: Immunofluorescent validation of FOXA2 and Oct4 in RC10 hESC-EC day 2.

ICC performed on hESC-EC day 0 (Pluri D0) and hESC-EC day 2 (Endo D2) for DAPI nuclear stain in blue, FOXA2 in green and Oct 4 in red. Goat (green) and mouse (red) IgG controls also shown. Scale bars represent 20 µm.

4.2.7.3 The transcriptional start site of FOXA2 is bivalently marked

Using data from a ChIP-SEQ experiment performed in H9 hESC by Ku *et al*. a prediction was made on bivalent status for every gene, and displayed in IPA (Ku et al., 2008) (Figure 4.13B). In pluripotent H9 hESC, FOXA2 was predicted to have both H3K4me3 and H3K27me3 histone modifications at its transcriptional start site (TSS) allowing rapid activation and repression of the gene (Figure 4.18). To validate these findings in our culture conditions for both H9 and SA461 hESC, ChIP-PCR was performed. Using the information within the UCSC genome browser, primer pairs were designed to span the TSS (Figure 4.19) (Table 2.5). Immunoprecipitations (IPs) with negative control rabbit anti-mouse IgG antibody, H3K4me3 antibody, H3K27me3 antibody and positive control total H3 antibody were set up with chromatin prepared from pluripotent H9 and SA461 hESC. SYBR green qPCR with the designed primer pairs 2, 7, 10 and 17 (Figure 4.19) was then performed on the eluate from the IPs along with a 2% chromatin input which had not been subjected to IP and a NTC. Data were normalised to IgG negative control and are displayed as fold enrichment. The FOXA2 TSS had both H3K4me3 activation histone modification and H3K27me3 repression histone modification at all genomic regions tested in both H9 hESC and in SA461 hESC (Figure 4.20). The highest fold enrichment was observed in regions 7 and 10 (Figure 4.20)

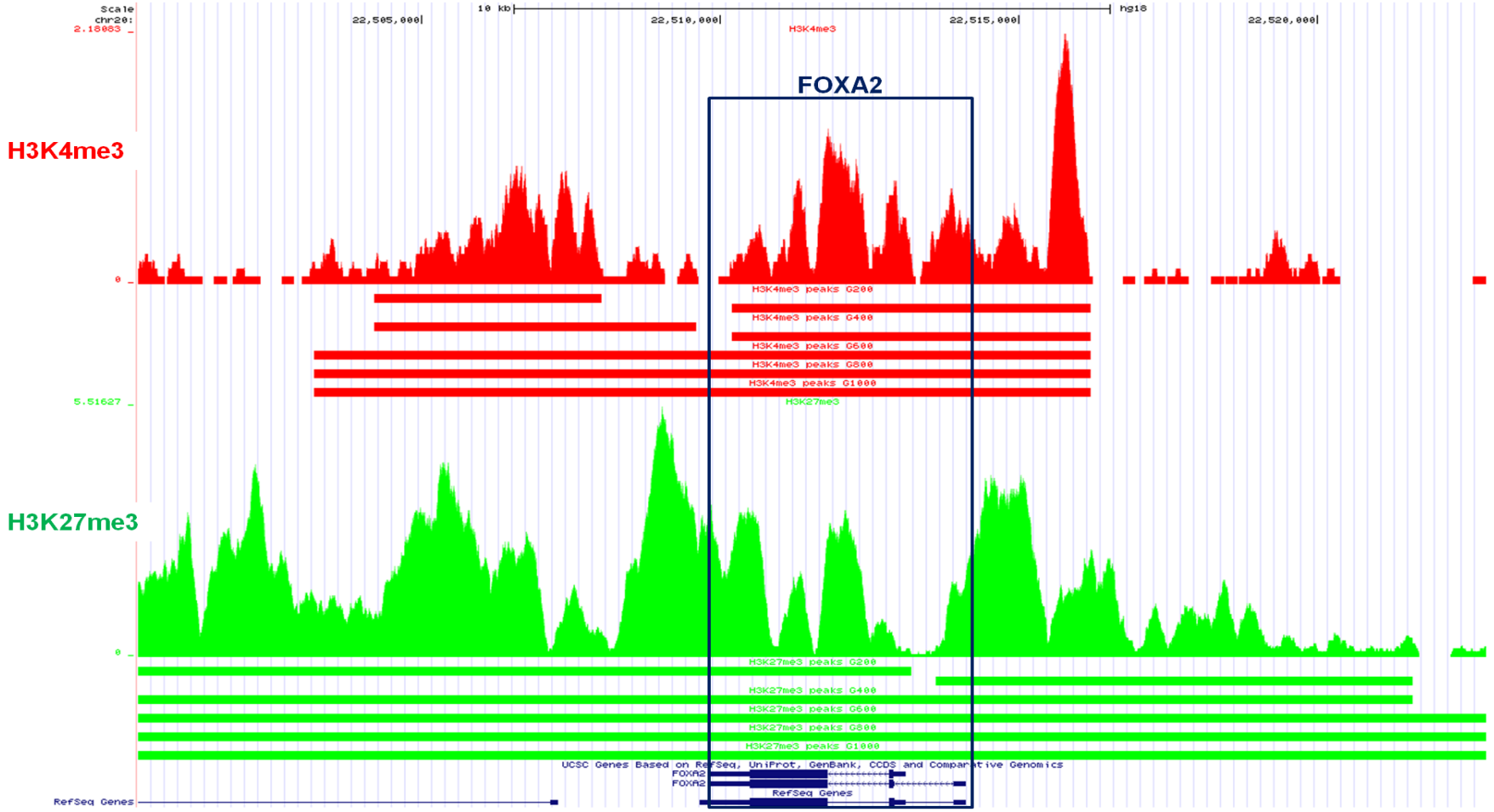


Figure 4.18: UCSC genome browser visualisation of H3K4me3 and H3K27me3 Chip-SEQ performed in H9 hESC.

Genome browser output from FOXA2 genomic location showing presence of H3K4me3 and H3K27me3 at the transcriptional start site from Chip-SEQ performed on pluripotent H9 hESC (Ku et al., 2008).

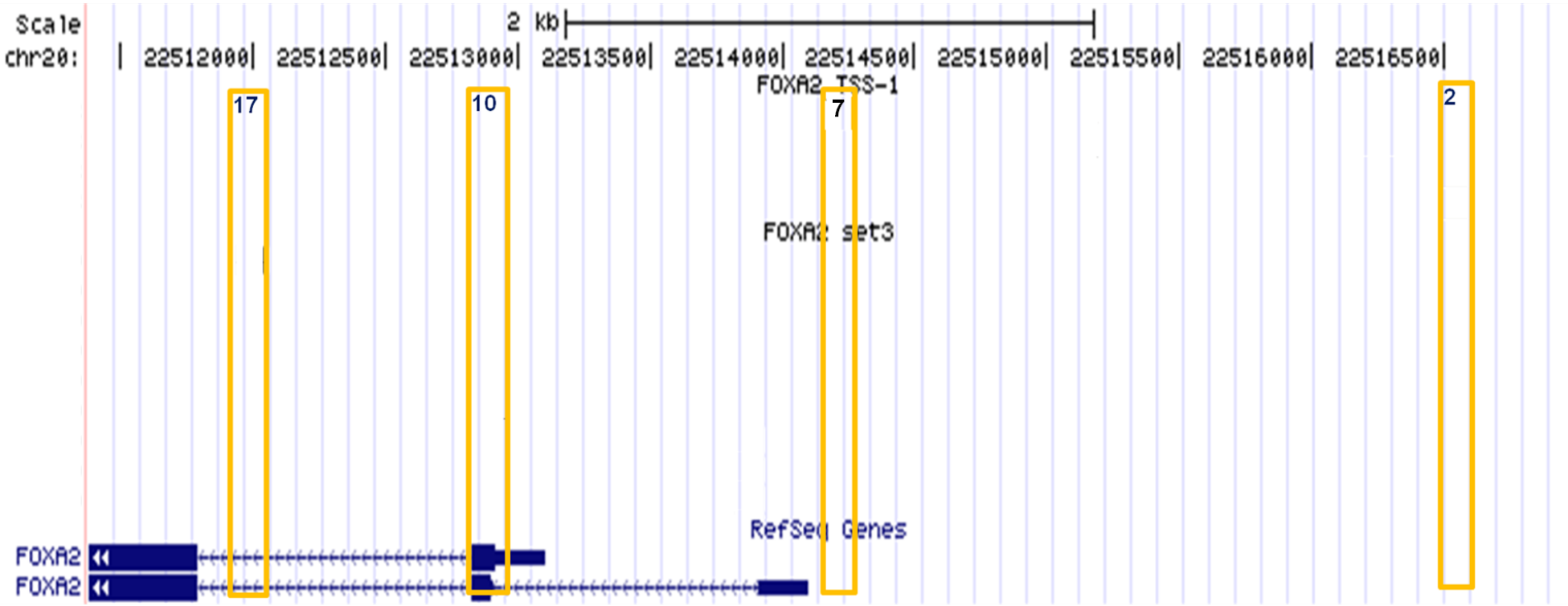


Figure 4.19: Location of primer pairs designed for FOXA2 ChIP.

UCSC output showing the genomic location of the optimised primer pairs designed for the FOXA2 TSS.

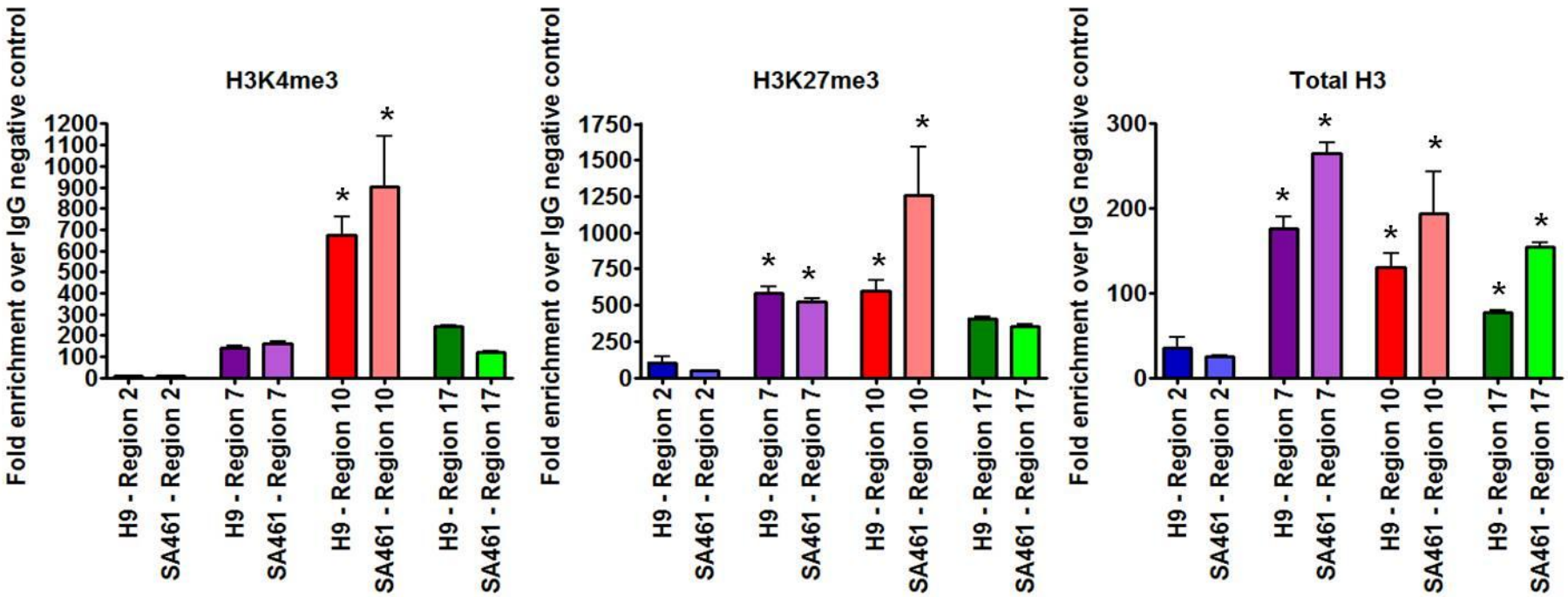


Figure 4.20: Chromatin ImmunoPrecipitation for H3K4me3, H3K27me3 and Total H3 at FOXA2 transcriptional start site.

Enrichment of H3K4me3, H3K27me3 and control Total H3 histone modifications at each primer site within the TSS of FOXA2. * = $p<0.05$ vs IgG control antibody**.**

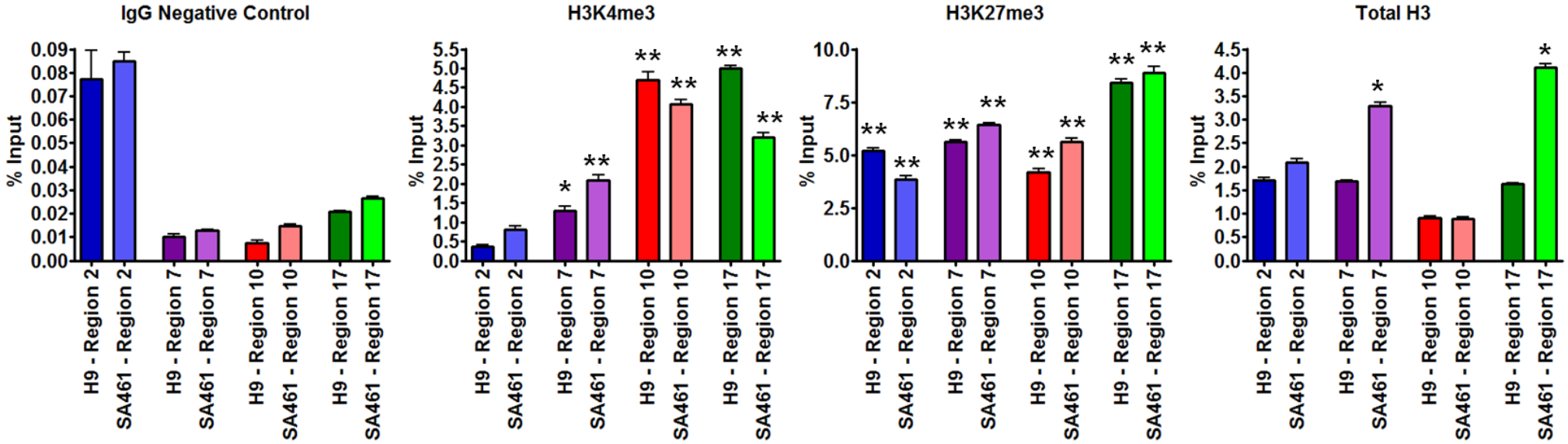


Figure 4.21: Chromatin ImmunoPrecipitation for H3K4me3, H3K27me3 and Total H3 normalised to percentage input chromatin at FOXA2 transcriptional start site.

Enrichment of H3K4me3, H3K27me3 and control Total H3 histone modifications at each primer site within the TSS of FOXA2. * = p<0.05 vs 2% input control, ** = p<0.01 vs 2% input control and *** = p<0.001 vs 2% input control

.

4.2.8 Target analysis of miR-99b, miR-181a and miR-181b

To identify a list of potential gene targets for miR-99b, miR-181a and miR-181b, the 3' UTR of mRNAs were screened for seeds of the miRNAs of interest. The seed can be defined as consecutive Watson-Crick base pairing between miRNA and mRNA at nucleotide positions 2-7 from the 5' end. Previously demonstrated using an unbiased computational approach applied to high-throughput pulsed SILAC data sets, the seed sequence in the 3' UTR correlates with translational repression or mRNA degradation and seed-based target prediction has the highest degree of overlap with data from pulsed SILAC experiments (Grimson et al., 2008). Adding the filter of evolutionary conservation improves likelihood of the prediction being biologically accurate, although the false discovery rate remains fairly substantial at 40%. The extent of miRNA regulation upon a mRNA depends on the number of seeds and the distance between seeds for the miRNA of interest to bind. Seed sequences with less than 40 nucleotides between them appear to act synergistically (Selbach et al., 2008, Grimson et al., 2008). Furthermore, seed regions for different miRNAs that are within an optimal distance appear to cooperatively regulate 1 target (Grimson et al., 2008). The target prediction method used is unique in this respect and was used to search for mRNAs that have multiple seeds for each miRNA within a short stretch of the 3' UTR of mRNA.

To explore which targets of miR-99b, miR-181a and miR-181b were differentially regulated as SA461 hESC were undergoing directed differentiation to an endothelial-like lineage, lists of targets were individually uploaded to IPA, added to a new "my pathway" and overlaid with gene expression data for hESC-EC day 10 from the Illumina gene expression microarray. Targets of miR-99b which were significantly downregulated by day 10 hESC-EC can are listed inTable 4.2. Further analysis of these targets using IPA to find common pathways or processes they are involved in suggests they have involvement in cell cycle and hepatogenesis. MiR-181a and miR-181b share the same seed sequence, therefore their targets are the same. Targets of miR-181a and miR-181b that are downregulated in hESC-EC day 10 are detailed in Table 4.3.

Table 4.2: Significantly downregulated miR-99b targets.

MiR-99b targets that were significantly downregulated at hESC-EC day 10

Target mRNA	Fold Change	FDR
Acetoacetyl-CoA synthetase (AACS)	-1.81778	6.01E-05
ATPase, Na+/K+ transporting, alpha 2 polypeptide (ATP1A2)	-1.27601	0.0301848
Autism susceptibility candidate 2 (AUTS2)	-1.67213	0.0086478
Coenzyme Q2 homolog, prenyltransferase (COQ2)	-1.26927	0.0316319
DCN1, defective in cullin neddylation 1, domain containing 5 (DCUN1D5)	-2.33961	0.0296054
ELOVL family member 6, elongation of long chain fatty acids (ELOVL6)	-1.70739	0.0100094
Frequently rearranged in advanced T-cell lymphomas (FRAT1)	-1.33519	5.48E-04
Golgi to ER traffic protein 4 homolog (GET4)	-1.51798	0.0381108
HEN1 methyltransferase homolog 1 (HEN1MT)	-2.63823	3.08E-04
Huntingtin interacting protein 1 (HIP1)	-1.64945	0.0204874
Major histocompatibility complex, class II, DO alpha (HLA-DOA)	-1.30813	0.031312
Heterogeneous nuclear ribonucleoprotein D-like (HNRPDL)	-1.59162	0.0207067
Opsin 3 (OPN3)	-1.30713	0.0467024
Proprotein convertase subtilisin/kexin type 9 (PCSK9)	-2.13913	0.0010091
Pleckstrin homology domain containing, family B (evectins) member 2 (PLEKHB2)	-1.91322	0.0404824
RAB3A interacting protein (rabin3) (RAB3IP)	-4.44999	1.47E-04
Ras association (RalGDS/AF-6) domain family member 4 (RASSF4)	-1.16883	0.0177361
Sodium channel, nonvoltage-gated 1, gamma (SCNN1G)	-1.38647	6.19E-04
Solute carrier family 39 (zinc transporter), member 1 (SLC39A1)	-1.39436	0.0198011
Sphingomyelin phosphodiesterase, acid-like 3B (SMPDL3B)	-2.0343	0.0395251
Suppression of tumorigenicity 5 (ST5)	-1.65595	0.0230849
Stromal antigen 3-like 1 (STAG3L1)	-1.5186	0.0119033
VAMP (vesicle-associated membrane protein)-associated protein A (VAPA)	-1.35521	0.0338819
Zinc finger protein 483 (ZNF483)	-1.34779	0.0076592

Table 4.3: Significantly downregulated targets of miR-181a and miR-181b.

MiR-181a and miR-181b targets that were significantly downregulated at hESC-EC day 10.

Target mRNA	Fold Change	FDR
Acid phosphatase, prostate (ACPP)	-1.15533	0.0313494
Activin A receptor, type IIA (ACVR2A)	-1.24481	0.00860618
A kinase (PRKA) anchor protein 7 (AKAP7)	-2.11528	2.96E-04
Adaptor-related protein complex 1, gamma 1 subunit (AP1G1)	-1.62345	0.0437767
Rho GTPase activating protein 19 (ARHGAP19)	-1.59107	0.021598
Armadillo repeat containing 10 (ARMC10)	-1.28609	0.0470022
Arrestin domain containing 4 (ARRDC4)	-1.52098	4.86E-05
ATP synthase mitochondrial F1 complex assembly factor 1 (ATPAF1)	-1.27153	0.0209369
B-cell CLL/lymphoma 11A (zinc finger protein) (BCL11A)	-1.46098	0.00345701
B-cell CLL/lymphoma 6 (BCL6)	-1.37126	0.0265635
BCL2-associated transcription factor 1 (BCLAF1)	-1.47122	0.0195685
Biogenesis of lysosomal organelles complex-1, subunit 2 (BLOC1S2)	-1.22854	0.0142713
Chromosome 1 open reading frame 21 (C1orf21)	-1.28984	0.0144568
Chromosome 3 open reading frame 23 (C3orf23)	-1.16191	0.00911217
Cache domain containing 1 (CACHD1)	-1.52894	0.0140239
Cell cycle associated protein 1 (CAPRIN1)	-1.72943	0.0187887
Cathepsin C (CTSC)	-1.78854	0.00486354
Chemokine (C-X-C motif) ligand 5 (CXCL5)	-1.53466	3.34E-05
DDB1 and CUL4 associated factor 12-like 1 (DCAF12L1)	-1.17744	0.0330199

DEK oncogene (DEK)	-1.96101	0.0126222
Eukaryotic translation initiation factor 5 (EIF5)	-3.16452	0.0397181
Ethanolamine kinase 1 (ETNK1)	-1.39447	9.02E-04
Frequently rearranged in advanced T-cell lymphomas 2 (FRAT2)	-1.74418	6.12E-05
GTPase activating protein (SH3 domain) binding protein 2 (G3BP2)	-3.88471	0.00496549
Glycine amidinotransferase (L-arginine:glycine amidinotransferase) (GATM)	-1.29949	0.0306098
GC-rich promoter binding protein 1 (GPBP1)	-2.39719	0.0463725
Hairy/enhancer-of-split related with YRPW motif 2 (HEY2)	-2.03688	0.00831369
Heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3)	-2.00609	0.0165194
Hermansky-Pudlak syndrome 3 (HPS3)	-2.14548	0.00365148
V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian) (MAF)	-1.28121	0.0114694
Multiple EGF-like-domains 10 (MEGF10)	-1.27902	0.00878033
N-ethylmaleimide-sensitive factor attachment protein, beta (NAPB)	-1.31213	0.00481907
Neurocalcin delta (NCALD)	-1.34046	0.00953114
Pallidin homolog (mouse) (PLDN)	-1.60905	0.0201241
Proteolipid protein 1 (PLP1)	-1.58748	6.35E-04
Peroxiredoxin 3 (PRDX3)	-5.70488	0.0375135
RAB3A interacting protein (rabin3) (RAB3IP)	-4.44999	1.47E-04
RAN, member RAS oncogene family (RAN)	-1.73039	0.0033028
Factor C (activator 1) 3 (RFC3)	-1.61046	0.0104135
RecQ-mediated genome instability protein 2 (RMI2)	-1.82678	0.0103454
Reticulon 1 (RTN1)	-1.17173	0.042165
SAM and SH3 domain containing 1 (SASH1)	-1.77383	0.0305972

SUMO1/sentrin/SMT3 specific peptidase 2 (SENP2)	-1.73987	0.0147492
TatD DNase domain containing 3 (TATDN3)	-2.04778	3.54E-04
TRNA aspartic acid methyltransferase 1 (TRDMT1)	-1.16991	0.0355577
Vasohibin 2 (VASH2)	-1.62406	0.016792
Zinc finger, AN1-type domain 6 (ZFAND6)	-2.61869	0.0338819
Zinc finger protein 138 (ZNF138)	-1.44575	0.0126937
Zinc finger protein 483 (ZNF483)	-1.34779	0.00765915
Zinc finger protein 589 (ZNF589)	-1.76052	0.0103845
Zinc finger protein 682 (ZNF682)	-1.7067	0.00370472

4.3 Discussion

In order to commit to a specified lineage, a pluripotent cell must undergo radical transcriptional change. Profiling these changes during cell commitment to a mesoderm and endothelial-like fate was the purpose of the body of research reported in this chapter. MiRNA profiling in hESC-EC, mesodermal cell type, ectodermal cell types and endodermal cell types revealed a distinct expression pattern of miR-99b, miR-181a and miR-181b largely unique to the mesodermal lineage. Expression of miR-99b, miR-181a and miR-181b did not appear to be affected by physiological stimuli used to mimic endothelial disease. Gene expression studies carried out in early EC differentiation captured a transcriptional shift during lineage commitment over time, which resulted in large numbers of significant gene expression changes at each timepoint and compared to primary adult EC cells. HESC-EC day 10 show a downregulation of pluripotency associated genes, but few endothelial associated genes are present at this early time point. Early changes at hESC-EC day 2 included an over-representation of transcription factors including the transient expression of FOXA2, a traditionally endoderm-associated transcription factor. FOXA2 regulation was hypothesised to be down to epigenetic modification at the TSS, and when investigated by ChIP FOXA2 was found to have both the activating H3K4me3 mark and H3K27me3 repressive mark. The FOXA2 gene is therefore bivalent in both SA461 and H9 hESC at pluripotency, marking it as a potential master regulator of lineage commitment. Finally, several predicted targets of miR-99b, -181a and -181b are shown to be downregulated in hESC-EC day 10 compared to hESC-EC day 0, but how these downregulated targets help refine differentiation to EC remains unclear.

Currently, very little is known about miRNA involvement in hESC differentiation to endothelial-like cells. MiRNA control of endothelial differentiation remains a hot topic for research, with miRNAs implicated in EC function and angiogenesis but not in the differentiation of mammalian ESC in vitro. We have recently published data suggesting a role for miRNAs in EC commitment (Kane et al., 2012). It is therefore likely miRNAs have a role in the process of lineage commitment to endothelial cells, and further dissection of these pathways

involved would elucidate the extent of miRNA involvement and governance of these biological processes.

Recently, our group reported an EC directed differentiation protocol which was capable of generating cells phenotypically and genotypically similar to endothelial cells without the need for embryoid body formation (Kane et al., 2010a). Cells seeded in monolayer differentiated in a uniform and highly specific manner, allowing extensive characterisation of cells without the requirement of isolation of the cell type of interest. During the course of the endothelial differentiation, loss of pluripotency associated miRNA clusters miR-302 and miR-372/373 (Kane et al., 2012) an induction of miR-99b, miR-181a and miR-181b was observed by hESC-EC day 10 (Kane et al., 2012). Our hypothesis was that lineage specific miRNAs must play a role in the process of endothelial differentiation, especially in early lineage commitment. Pinpointing the role of miRNA during differentiation of hESC to EC had not previously been studied to the best of our knowledge at the time these studies began. It had been previously reported that knockdown of Dicer abrogated the production of nitric oxide (NO) in endothelial cells (Suárez et al., 2007). For the first time knockdown of Dicer has been shown to result in suppression of NO production from hESC-EC and therefore successful endothelial differentiation (Kane et al., 2012). It had been previously shown that NO was produced as hESC differentiated using our feeder-free system and derived cells showed increased production of NO in response to carbachol (Kane et al., 2010a).

The description of miR-99b, miR-181a and miR-181b expression in SA461 hESC cells differentiating to endothelial lineages is consistent with previous miRNA characterisation studies performed in EC, but not in pluripotent cells differentiating to endothelial lineage (Anand et al., 2010, Kazenwadel et al., 2010, Suárez et al., 2008). MiR-181b has also been reported to be present on CD34+ haematopoetic cells derived from hESC (Huang et al., 2011). ECs and other mesodermal cell types are also positive for CD34 (Flamme and Risau, 1992), therefore this would suggest a role for miR-181b in mesodermal commitment. During the profiled early timepoints of directed-differentiation in SA461 hESC, the induction of expression of miR-99b, miR-181a and miR-181b was observed by LC Sciences microarray, TaqMan qRT-PCR and Northern blotting, reaching statistical significance by day 10. Expression of these miRNAs during

directed differentiation has also been in observed in hESC lines H1 and H9, reaching statistical significance by day 10 (Kane et al., 2012). MiR-99b is an intergenic miRNA found in cluster with let-7e and miR-125a on chromosome 19. MiR-181a and miR-181b can be transcribed from different chromosomal locations; miR-181a-1 and miR-181b-1 are co-localised on chromosome 1, within the RP11-31E23.1 coding region whereas miR-181a-2 and miR-181b-2 are found together on chromosome 9, within the MIR181A2HG coding region. The genes in which these miRNA are located as yet have no known function.

In order to investigate the expression of miR-99b, miR-181a and miR-181b in other mesodermal cell types, further characterisation of their expression was performed in vascular cell types HSVEC, HSVSMC, HPAEC, and H1 hESC directed to haematopoetic lineage (hESC-Haem) by a defined protocol. Expression of miR-99b was seen to be expressed at significant levels in both control and CABG HSVEC compared to day 0 and and similar levels to day 10 hESC-EC, at lower levels in HSVSMC, HPAEC and day 10 hESC-Haem differentiated cells. MiR-181a was expressed at lower levels in HSVSMC, HPAEC and day 10 hESC-Haem differentiated cells. MiR-181b expression is abundant in both control and CABG hESC with a significant difference between patient cohorts. Expression can also be found in HSVSMC, HPAEC and day 10 hESC-Haem differentiated cells. The significant difference between miR-181b expression in HSVEC from control patients and patients undergoing CABG suggested disease status could play a role in differential miRNA expression.

To ascertain whether these miRNAs were specific to the mesodermal lineage, miRNA qRT-PCR was again employed in representative cells from the ectoderm and endoderm germ layers. H1 hESC underwent a defined neuronal differentiation to represent the ectodermal lineage in the absence of access to primary human neuronal cells. There was no significant expression of miR-99b, miR-181a and miR-181b from any of the neuronal differentiated samples tested. However, it has recently been reported that miR-99b is expressed in ectodermal mammary duct carcinoma cell line HCC193 (Tanic et al., 2011), so expression in this lineage could not be ruled out. Unfortunately, we were unable to obtain primary human endodermal cells and no protocol for generation of endoderm from hESC had been optimised for our culture system. As such, HepG2 liver hepatocellular carcinoma cells were the closest cell type to-the endodermal

lineage, and were tested for expression of miR-99b, miR-181a and miR-181b. Expression of miR-99b, miR-181a and miR-181b was below the detection limit in HepG2. This supports the hypothesis that miR-99b, miR-181a and miR-181b are largely mesoderm specific miRNAs, however the use of the HepG2 cell line is a limitation of this study, and this requires future validation in primary endodermal cell types. It is likely that these miRs are expressed as cells differentiate through a common mesodermal progenitor (de Bruijn et al., 2002) which would also account for miR-181b expression being present in haematopetic differentiated hESC (Huang et al., 2011) and in HSVSMC.

We further interrogated the hypothesis that the novel endothelial associated miRNAs were differentially regulated in patients with different health status by exposing HSVEC to various physiologically relevant agents in an attempt to mimic endothelial disease in a cell culture assay. Changes in miRNA expression have been previously reported in a number of diseases including heart disease (van Rooij et al., 2006, Cheng et al., 2007, Ikeda et al., 2007, Naga Prasad et al., 2009, Olivieri et al., 2012) diabetes (Chen et al., 2008a, Caporali et al., 2011) and coronary artery disease (Fichtlscherer et al., 2010, Hoekstra et al., 2010). It had been previously shown that miR-503 expression in cultured EC changes during *in vitro* recapitulation of diabetes and ischaemia (Caporali et al., 2011) therefore we investigated if miR-99b, miR-181a and/or miR-181b could be differentially regulated in HSVEC in response to disease stimuli. Samples were collected at 24, 48 and 72 h post exposure and total RNA extract samples extracted. None of the treatments elicited a significant difference in miRNA expression in n=3 experiments. The difference in expression of miRNA in EC from patients with coronary artery disease and patients without appears to be unrelated to diseased endothelium, or possibly these differences are not able to be mimicked *in vitro*. The mechanism of regulation of miR-99b, miR-181a and miR-181b remains unknown.

To create a gene expression profile of SA461 hESC undergoing directed differentiation to EC, an Illumina human whole genome expression microarray was carried out. HESC were differentiated to EC and samples collected at day 0, 2, 4 and 10, as samples were collected for the LC Sciences microarray. In addition, HSVEC were added to the array to give a gene expression profile of adult EC for comparison. PCA showed the clustering of similar samples in a 3-

dimensonal graph, and samples showed good separation between sample groups. Adult HSVEC remained separated from all other groups plotted on the graph, which is unsurprising considering there are 6133 statistically significant differential expressed genes between hESC-EC 10 and HSVEC. Large numbers of statistically significant gene expression changes were found between sample comparisons. Numbers of genes upregulated versus those downregulated are roughly equal suggesting a complex transcriptional program at work. As there was such a large difference in number of statistically significant differentially expressed genes between hESC-EC day 10 and HSVEC, the expression of a number of key endothelial and mesodermal genes were investigated with the datasets overlayed to give a colour indication of statistically significantly upregulated (red) or statistically significantly downregulated (green), along with FDR, fold-change and predicted bivalent status in H9 hESC. There were no datasets on bivalency in HSVEC that could be added to those data, so HSVEC do not display bivalency prediction. HESC-EC day 10 versus day 0 showed that most of the endothelial associated genes were not yet present at levels of significance. Previously, our group showed hESC-EC day 10 cells derived from the same hESC line and culture protocol effectively improved foot perfusion after injection into a mouse model of hindlimb ischaemia (Kane et al., 2010a), therefore it would appear despite lacking the expected endothelial gene profile, the cells remain biologically and clinically relevant. The level of maturation at which cells are transplanted appears to be critical to clinical improvement – mature adult cells such as hMVEC (Kalka et al., 2000) and immature cell types such as (VEGF) R^2-positive Tra-1–60–positive differentiating hESC cells (Sone et al., 2007) do not result in improved local blood flow *in vivo*.

Furthermore, when pluripotency pathways were analysed in the context of hESC-EC day 10 vs hESC-EC day 0, pluripotency genes were seen to drop significantly by day 10. However, when the level of pluripotency markers in HSVEC was compared to hESC-EC day 10, they were significantly lower in HSVEC, indicating that although expression of Oct4 and Sox2 was reduced there is still much more present in hESC-EC day 10 cells than adult EC. This could be due to a subpopulation of cells remaining pluripotent within the endothelial differentiation, therefore negatively sorting cells for pluripotency markers

before microarray analysis would have been a useful addition to the experimental design to eliminate this possibility.

When day 2 of EC differentiation was investigated further, transcription factors unique to this timepoint were observed, accounting for 13% of the gene expression changes to this timepoint. Ingenuity Pathway Analysis was employed to discover the known functions of these transcription factors, although there was no direct link to endothelial differentiation although cell cycle was a top related result. Of these 24 transcription factors found to be significant, CXXC1, EHMT2, FOXA2, L3MBTL2, MLL3, RBM14, TAF6L, TFAP4, TSC22D3, UBTF, USF2 and ZNF35 validated successfully by TaqMan qRT-PCR (ANOVA $p<0.05$), with FOXA2 being the most dramatic change. To investigate whether these transcription factors are present at hESC-EC day 2 in other hESC lines, ICC was performed with antibodies for Oct4, FOXA2, L3MBTL2, TFAP4, TSC22D3 and UBTF in SA461, H1 and RC10 hESC lines. Oct4 expression can be detected in pluripotent SA461, H1 and RC10 hESC with expression less abundant at day 2 of EC differentiation. However, in SA461 the levels of Oct4 expression in pluripotent cells appeared considerably lower than that of the other lines tested. Of the antibodies for the transcription factors tested, FOXA2 can be seen to be more abundant at day 2 EC differentiation, although some can be detected in pluripotent hESC. The remaining antibodies tested did not stain successfully in tested samples, and further optimisation is required in order to investigate their role in hESC-EC commitment.

FOXA2 is a hepatocyte nuclear factor and is a “winged helix” member of the forkhead class of DNA binding proteins. FOXA2 is reported to be involved in the embryonic development of the axial mesoderm from the primitive streak and node in day 6.5 mouse embryos (Sasaki and Hogan, 1993, Monaghan et al., 1993). Interestingly, FOXA2 has been shown to be able to bind directly and open compacted chromatin at histones H3 and H4, suggesting its importance as a “pioneer” transcription factor in early development (Cirillo et al., 2002). It has been linked to endoderm development with FOXA2-deficient mouse embryos lacking hepatic specification (Lee et al., 2005) and displaying abnormal mesoderm formation lacking notochord although lateral mesoderm, from which the blood vessels are derived, appeared largely normal (Ang and Rossant, 1994). FOXA2 had not previously been observed in hESC-EC differentiation at the time

of writing. In the developing embryo, it is thought that endoderm and mesoderm are derived from a common progenitor which could suggest a role for FOXA2 in early EC committment from hESC (Lickert et al., 2002, Kubo et al., 2004). In order to elucidate the role of FOXA2 in endothelial differentiation further, knockdown using RNA interference and overexpression studies during differentiation would allow documentation of the role it plays during EC commitment and are currently underway.

Bivalent genes have been suggested to be master regulators of lineage commitment due to their transcriptionally poised status and ablity to undergo rapid up and downregulation (Bernstein et al., 2006). ChIP-SEQ data integrated to the Illumina microarray dataset allowed a prediction of bivalency to be made in hESC, and predicted FOXA2 to be bivalently marked in pluripotent H9 hESC (Ku et al., 2008). It was hypothesised that epigenetic modification was responsible for the transient expression of FOXA2 seen at hESC-EC day 2. To test this hypothesis, ChIP was performed using antibodies for H3K4me3 active chromatin state and H3K27me3 repressive chromatin state and qPCR primer pairs directed against the TSS of FOXA2. It can be seen for both H9 and SA461 hESC maintained in our feeder-free pluripotent culture system, that FOXA2 is indeed bivalently marked at hESC-EC day 0, lending weight to the possibility of epigenetic changes illiciting its temporal gene expression. Due to time constraints, we were unable to test whether the loss of the repressive H3K27me3 at the FOXA2 TSS was found at hESC-EC day 2, but this will be investigated in the near future by performing ChIP on both hESC-EC day 0 and hESC-EC day 2 samples.

Finding targets for the novel endothelial associated miRNAs miR-99b, miR-181a and miR-181b by integrating data from the LC Sciences miRNA microarray and data from the Illumina whole genome expression microarray proved to be challenging due to the large amount of data generated. Targets listed in Table 4.2 and Table 4.3 were generated (Grimson et al., 2008) and those with significantly lower expression in hESC-EC day 10 compared with hESC-EC day 0 displayed. Further confirmation of these targets by checking protein expression, overexpression and knockdown of miR-99b, miR-181a and miR-181b and generation of luciferase target constructs remain to be performed to fully elucidate the role of these miRs in EC differentiation. Previous publications have

suggested a role for miR-181a in regulating EC from a lymphatic to a blood vascular phenotype by targeting Prox1 (Kazenwadel et al., 2010). Overexpression of miR-181a during our directed differentiation protocol has been shown to reduce Prox1 levels and cause differential expression of EphrinB1, EphrinB2, Jagged2 and Hey2 (Kane et al., 2012) which are involved in arterial specification (dela Paz and D'Amore, 2009). Overexpression of miR-99b, and miR-181b in hESC-EC transplanted into a mouse model of hindlimb ischaemia showed significant improvement of neoangiogenesis, whereas overexpression of miR-181a did not elicit the same effect (Kane et al., 2012).

In order to refine approaches to creating a viable cell therapy product for the treatment of ischaemia, a thorough dissection of miRNA expression, gene expression and epigenetics is required in EC differentiation but remains highly challenging.

Chapter 5: General Discussion

This thesis contains studies concerned with the transcriptional control of lineage commitment in hESC differentiating to cardiovascular lineages. Cardiac regeneration, involving generation of new functional cardiomyoctes and therapeutic angiogenesis using “endothelial-like” cells remains a key goal in cell therapy for vascular and cardiovascular diseases. Limited improvements using progenitor cell types have been shown in diseases such as chronic angina, acute myocardial infarction and heart failure, however these therapies are by no means optimal at this juncture (Menasché et al., 2008, Britten et al., 2003, Wollert et al., Lunde et al., 2006, Janssens et al., 2006, Zimmet et al., 2011, Assmus et al., 2010, Strauer et al., 2010, Murry et al., 2004b). There remains an unmet clinical need for new therapies to alleviate the global burden caused by these diseases. In order for hESC therapies to progress to the clinic, more understanding of the transcriptional control of lineage commitment programs is therefore warranted.

Although improvements in the scalability of cardiac differentiation have recently been reported (Lecina et al., 2010), an interest in improving yield still remains. Alternative methods to refine cardiomyocte differentiation from hESC could provide some solution to the issue of scalability in producing large enough numbers of cells for clinical benefit in humans. MiRNA modulation by lentivirus has previously been shown to potentiate cardiomyocyte differentiation using an embryoid body protocol (Ivey et al., 2008a). The body of work contained in chapter 3 attempted to dissect the role miR-1 and miR-133 had, if any, on monolayer cardiac differentiation using feeder-free hESC, therefore offering an opportunity to enhance production of these cells more directly. We began by optimising lentiviral infection of hESC and showing that transduction did not have an effect on the expression of Oct 4 or the ability of the cells to undergo embryoid body formation. Next, it was observed that the CMV promoter was silenced in SA461 hESC, consistent with previous findings (Xia et al., 2007, Liew et al., 2007). The SFFV promoter was found to be highly active in pluripotency, passive differentiation, cardiac and endothelial conditions. Previous studies had shown SFFV to be a strong promoter for transducing haematopoetic stem cells (Demaison et al., 2002b, Yam et al., 2002). As the miRNA overexpression constructs gifted from Reuvin Agami were pri-miR inserts encoding not only the miRNA of interest but other miRNAs as well, we redesigned the miRNA

overexpression strategy to a pre-miR format maintaining the native stem loop structure of the pre-miR in an effort to ensure the correct endogenous processing. It was noted at this point that miR-1 expression was substantially lower than miR-133 expression, although the experimental conditions were controlled. Following this, optimisation of cardiac differentiation was performed in SA461 hESC and H1 hESC, and it was observed that SA461 were incapable of generating beating cells regardless of time in cardiac differentiation culture conditions. The decision was taken to find an alternative hESC line to work with for cardiac differentiation, and it had previously been reported that H1 hESC could successfully differentiate to form beating cells with a cardiomyocyte phenotype (Laflamme et al., 2007). H1 hESC were subjected to a modified Laflamme protocol in monolayer which resulted in beating clusters by day 9 and by day 14 ICC staining for troponin T, tropomyosin, NKX2.5 and α-actinin. This result reinforced the reported existence of lineage bias in hESC lines, and could lend weight to the idea of different lines banked for different tissues in a cell therapy settinng (Hong et al., 2011, Alva et al., 2011). With the cardiac differentiation protocol optimised, an investigation of the role of miR-1 and miR-133 in cardiac specification using overexpression lentiviruses was initiated. On day 9 of cardiac differentiation culture, spontaneous beating clusters could be observed, with the control LNT-SFFV-GFP transduced samples showing the highest prevalence of beating cells. When analysed by qRT-PCR for cardiac marker genes, it was found that those samples transduced with LNT-SFFV-GFP virus displayed significantly higher levels of ACTN2 and NKX2.5 than no virus control samples subjected to cardiac conditions. There have been no previously reported incidences where transduction of cells with control virus alone resulted in a potentiation of differentiation to any lineage. However, our group previously reported the induction of induced pluripotent stem cells (iPSC) upon transduction with control LNT-SFFV-GFP virus (Kane et al., 2010b). Contrary to what was previously reported (Ivey et al., 2008a), samples transduced with LNT-SFFV-miR-1 had no statistically significant changes in cardiac gene expression compared with either of the control samples. In samples transduced with LNT-SFFV-miR-133 there were no significant differences in cardiac gene expression when compared to the untreated cardiac differentiation, however, when compared with LNT-SFFV-GFP a significant reduction in ACTN2, NKX2.5, and TNNT2 was observed. This is consistent with previous findings published by Ivey

et al. 2008 however, the difference between the no virus control and the control LNT-SFFV-GFP transduced samples does raise some questions about the safety and off target effects of using integrating lentiviral vectors in cells destined for cell therapy in patients. It cannot be ruled out that lentiviruses can potentiate cardiac differentiation simply by integrating randomly into the host cell DNA. It has been shown previously that lentiviruses preferably integrate into active genes (Schröder et al., 2002). To understand this effect in the future, integration site analysis of cells could be performed. To exclude the unpredictability of using an integrating lentiviral vector, these studies could be repeated using a non-integrating lentiviral vector system, although at the deficit of long lasting transgene expression (Apolonia et al., 2007).

The investigation of approaches for refinement of cardiac differentiation from hESC remains a clinically relevant pursuit. Recent publications which have attempted to address this goal include Dixon *et al*. who reported the overexpression of GATA4, TBX5, NKX2.5 and GTNB to generate beating cells which were cTnI/α-actinin double positive cells while under pluripotent culture conditions (Dixon et al., 2011). In addition, it was observed that overexpression of miR-499 in hESC differentiation to ventricular cardiomyocyte phenotype improved efficiency from 48% to 72% (Fu et al., 2011). It was also observed in this study that lentiviral overexpression of miR-1 did not increase the yield of cardiomyocytes from differentiation, but rather changed the gene expression profile of the cells derived to a more adult phenotype (Fu et al., 2011). Furthermore, it has been reported that miR-1 transfected mESC were transplanted into an MI model in mice which resulted in enhanced differentiation and engraftment compared to mESC alone (Glass and Singla, 2011). However this study is limited in that no hESC were used, and the group did not differentiate cells before transplantation but injected pluripotent mESC. Realistically, this would be unlikely to be translational due to the risk of teratoma formation from injecting pluripotent cells. Recently, a study performed to assess the safety of using lentivirus-transduced cells for transplant in Wiscott Aldrich Syndrome showed an improved safety profile and is currently undergoing phase I/II clinical testing (Scaramuzza et al., 2012) suggesting that lentiviral transduced cell products may be suitable for clinical use in certain circumstances.

The work contained within chapter 4 focused on differentiation to the vascular endothelial cell lineage, and the main aims were to profile miRNA expression, gene expression and epigenetic status of master regulating genes in hESC-EC. Generation of endothelial cells suitable for transplantation in ischaemic diseases would play a major role in reduction of cardiomyocyte death after MI, and could offer a preferred solution to PAD in critical limb ischaemia, circumventing the need for long term blood thinning drugs and much improving quality of life for the patient.

It had previously been shown that injection of hESC-EC day 10 cells in a mouse model of hindlimb ischaemia could stimulate neoangiogenesis and restore perfusion to the limb (Kane et al., 2010a). Studies began with the miRNA microarray profiling of SA461 hESC subjected to a feeder-free monolayer endothelial differentiation protocol as previously published (Kane et al., 2010a). After statistical analyses, miR-99b, miR-181a and miR-181b were discovered to be significantly upregulated over time in hESC-EC differentiation. In addition, this induction of significant miRNA expression had also been observed in H1 and H9 hESC lines during endothelial differentiation (Kane et al., 2012). Investigation into the expression of these miRNAs was then carried out in alternative mesodermal lineage cells; HSVEC, HSVSMC, HPAEC and hESC subjected to a haematopoetic differentiation method as previously reported (Yung et al., 2011). Expression of miR-99b, miR-181a and miR-181b could be found in all of the cells tested, with the most substantial levels being in HSVEC. There was a small but statistically significant difference in miR-181b expression between control HSVEC and CABG HSVEC, which suggested endothelial disease perhaps had an impact on miRNA expression. Expression of all 3 miRNAs was statistically significant in hESC-haem day 10 compared to both hESC-EC day 0 and hESC-haem day 0. This is consistent with a previous report that showed that miR-181b was significantly expressed in hESC derived haematopoetic cells (Huang et al., 2011).

Next, expression in non-mesodermal cells of miR-99b, miR-181a and miR-181b was assessed. H1 hESC were subjected to a published and well validated neuronal differentiation protocol (Gerrard et al., 2005) as human neuronal tissue was not available to this study to represent ectoderm. In addition, HepG2 liver hepatocarcinoma cells were used to represent endoderm, despite the obvious limitations of using a cancer cell line. Time constraints meant that no hESC

differentiation protocol for generation of endodermal cell types was attempted, although this would have been a more appropriate approach. No samples tested reached statistical significance, which suggested that miR-99b, miR-181a and miR-181b were mesodermal associated miRNAs. It seems likely that these miRNAs are expressed as cells differentiate through a common mesodermal progenitor (de Bruijn et al., 2002) which would also account for miR-181b expression being present in haematopetic differentiated hESC (Huang et al., 2011) and in HSVSMC. However, it has recently been reported that miR-99b is expressed in ectodermal mammary duct carcinoma cell line HCC1937 (Tanic et al., 2011), which would highlight the benefit of further miRNA expression profiling in a much wider variety of cell types than investigated here.

To investigate the significant difference observed in miR-181b expression between control and CABG patients HSVEC, HSVEC were exposed to a range of physiological agents designed to cause endothelial stress and mimic vascular disease, and expression of miR-99b, miR-181a and miR-181b ascertained by qRT-PCR on samples from 24, 48 and 72 hours. Surprisingly, none of the treatments elicited any significant change in miRNA expression in experiments performed on three independant occasions. This could have been due to an inability to mimic endothelial disease in this way, or a confounding factor in the patients tested for miRNA expression which caused the significant difference. Little is known on the differences in miRNA expression profile in healthy patients, with most studies concentrating on using miRNA expression as a biomarker to differentiate between disease modalities (Karaayvaz et al., 2012, Slaby et al., 2012, Hennessey et al., 2012, Liu et al., 2012). It has been recently reported that miR-181b regulates NF-κB mediated EC activation and vascular inflammation (Sun et al., 2012), however the samples were taken at 1, 4, 8 and 24 hour timepoints whereas the earliest sample taken in this study was 24 hours. This could represent a limitation and warrants further investigation into the regulation of miR-99b, miR-181a and miR-181b at earlier timepoints.

Next, we created a gene expression profile of hESC-EC differentiation in order to understand more about early lineage commitment and allow integration of the miRNA data with gene expression data to refine target gene selection. Previous studies have attempted to map key gene expression changes during differentiation of hESC to mesodermal lineages (Evseenko et al., 2010), however

no studies specific to the endothelial cell lineage were published at the time of writing. Samples were taken at hESC-EC day 0, day 2, day 4 and day 10 with pluripotent time matched controls and primary HSVEC ran in parallel as a positive control. Priniciple component analysis was used to see clustering of the sample groups. HSVEC were distinct from everything else analysed, which can be accounted for by 6133 significantly differentially expressed genes between hESC-EC day 10 and HSVEC. When the hESC groups were further investigated, it was observed that hESC-EC day 4 and hESC-EC day 10 appeared distinct from everything else hESC derived. When samples were grouped into pairwise comparisons and statistically analysed, it could be observed that there were 1040 statistically significant differential gene expression changes between hESC-EC day 2 and hESC-EC day 0, 2400 statistically significant differential gene expression changes between hESC-EC day 4 and hESC-EC day 0 and 2157 statistically significant differential gene expression changes between hESC-EC day 10 and hESC-EC day 0. Further analyses of hESC-EC day 2 were carried out, and it was observed that there were 221 statistically significant gene changes unique to hESC-EC day 2. Previous studies have analysed day 3.5 of mesoderm differentiation by embryoid body, however there were no similarities in the gene expression profile reported when compared to samples from the endothelial differentiation protocol used (Evseenko et al., 2010). When day 2 changes were analysed further, it was noted that 13% of the genes were transcription factors suggesting high transcriptional activity within the cells at this early commitment stage. To validate these changes qRT-PCR was employed, and CXXC1, EHMT2, FOXA2, L3MBTL2, MLL3, RBM14, TAF6L, TFAP4, TSC22D3, UBTF, USF2 and ZNF35 were found to be significant by this method also, without being significantly expressed in HSVEC, suggesting that these TF's to have no role in an adult EC or the venous lineage. Of these transcription factors the most dramatic fold change by qRT-PCR was FOXA2. FOXA2 is a hepatocyte nuclear factor and is a member of the forkhead class of DNA binding proteins, and has been reported to activate expression of liver-specific genes including albumin. We further validated in SA461, H1 and RC10 hESC the most highly upregulated TF's by ICC in hESC-EC day 0 and hESC-EC day 2 in addition to Oct 4 expression. Oct 4 expression was clearly detected at hESC-EC day 0 with a marked reduction in staining by hESC-EC day 2. It was also noted that Oct 4 expression was markedly reduced in pluripotent day 0 SA461 hESC when compared to H1 or RC10 hESC. Further

studies in our laboratory have continued without using the SA461 hESC line for this reason. There was markedly more expression of FOXA2 in hESC-EC day 2 compared to hESC-EC day 0. Interestingly, even at hESC-EC day 0, there appeared to be no co-localisation of Oct 4 and FOXA2, and this became more apparent at hESC—(Lickert et al., 2002)EC day 2. A recent publication described FOXA2 overexpression to refine differentiation of hepatocyte-like cells from hESC (Takayama et al., 2012), it was therefore surprising to see FOXA2 in the context of early endothelial differentiation. Following these results, we were interested to understand more about FOXA2 and its regulation following such a marked but transient induction at hESC-EC day 2. In addition to having gene expression data from the Illumina microarray, we also integrated ChIP-SEQ data from Ku *et al*. which was performed on pluripotent H9 hESC and gave a numerical indication of whether a gene was bivalently marked and therefore transcriptionally "poised" (Ku et al., 2008). We sought to confirm this in our feeder-free monolayer pluripotency culture systems for H9 and SA461 hESC. ChIP qPCR primers were designed for the FOXA2 TSS area, and chromatin subjected to IP using antibodies for both histone modifications; H3K4me3 activation mark and H3K27me3 repressive mark in addition to appropriate positive and negative controls. It was found in our culture system that both H9 and SA461 hESC the FOXA2 TSS was bivalently marked with both modifications. We hypothesised that histone modification might be responsible for the hESC-EC day 2 specific expression of FOXA2, perhaps by loss of the repressive H3K27me3 from the TSS, however time constraints meant that at the time of submission of this thesis, we had not tested this hypothesis. In the near future, ChIP will be performed on hESC-EC day 2 samples compared to hESC-EC day 0 in order to address this question. Previous literature reporting a common progenitor cell type giving rise to both mesoderm and endoderm during embryonic development and stem cell differentiation may explain why an endodermal transcription factor was expressed in early endothelial commitment (Lickert et al., 2002, Kubo et al., 2004).

In order to interrogate key gene changes in the Illumina dataset, genes of interest were uploaded to IPA to allow visualisation of expression levels. A list of endothelial cell associated genes was uploaded in this fashion, and when hESC-EC day 10 samples were compared to hESC-EC day 0 samples it was observed

that the only significantlly upregulated genes were TEK, KLF6 and FGF9. TEK is reported to be one of the earliest markers of endothelial cells, which would explain its presence in the hESC-EC samples examined from early endothelial differentiation (Yamaguchi et al., 1993). The lack of endothelial specific genes can perhaps be explained due to the early stage of differentiation at which the samples were harvested. When pluripotency genes were examined, it was observed that Oct 4, Nanog, and Sox 2 were significantly downregulated compared to hESC-EC day 0. This indicated a loss of pluripotency was taking place, however to confirm this hypothesis would require to inject cells into an immunodeficient mouse to check for teratoma formation. When HSVEC were compared with hESC-EC day 10 it was found that many of the endothelial list were significantly expressed, and pluripotency genes were significantly downregulated compared to hESC-EC day 10. This could have been due to hESC-EC day 10 still expressing a higher level of pluripotency markers when compared to an adult cell type, or due to a residual population of pluripotent cells within the sample, skewing the result. This question could have been eliminated by the inclusion of a negative selection for pluripotency markers in a cell sorting stage prior to microarray analysis, utilising MACS or FACS technology to remove those pluripotent cells as has been previously described (Hewitt et al., 2006). Finally, computational prediction of mRNA targets of miR-99b, miR-181a and miR-181b produced large gene lists, some of which may never be expressed in pluripotent or endothelial cells, therefore we sought a way to refine targets of interest which were regulated between hESC-EC day 0 and hESC-EC day 10. Lists of predicted targets were uploaded to IPA, and overlayed with gene expression data from hESC-EC day 10 versus hESC-EC day 0. Further analysis past this stage was again limited by time when this thesis was submitted, and ideally these relavent targets require to be validated in independant replicates, as well as luciferase target constructs generated to prove miRNA targeting of these genes directly. Target genes found to be statistically significantly downregulated were subjected to analyses to find any common pathways or function relating to differentiation or suppression of alternate lineages linking them together, but no obvious link was clearly identifiable.

5.1 Conclusion

This thesis shows that the effect of miR-1 and miR-133 overexpression in cardiac differentiation differs between protocols used. It also describes the expression profile of miRNAs only recently linked to endothelial differentiation and mesodermal cell types, as well as their absence from cell types of the other germ layers. Their stability of expression regardless of several pharmacological stimuli added to EC was also shown. Gene expression microarray profiling of a novel feeder and serum free endothelial differentiation protocol at early timepoints revealed previously unreported transcriptional changes and a potential novel role of the endodermal transcription factor FOXA2 in commitment to endothelial cells. It has also been shown that FOXA2 is bivalently marked in a feeder-free pluripotency maintenance system in H9 and SA461 hESC, indicating it as a master regulator for hESC differentiation. Taken together, these studies have added to existing knowledge on the master control of hESC differentiation to cardiovascular lineages, and provide further rational for the refinement of differentiation approaches toward clinically viable cell products.

List of References

2009. World's first clinical trial of human embryonic stem cell therapy cleared. *Regen Med,* 4**,** 161.

ABDEL-LATIF, A., BOLLI, R., TLEYJEH, I. M., MONTORI, V. M., PERIN, E. C., HORNUNG, C. A., ZUBA-SURMA, E. K., AL-MALLAH, M. & DAWN, B. 2007. Adult Bone Marrow-Derived Cells for Cardiac Repair: A Systematic Review and Meta-analysis. *Arch Intern Med,* 167**,** 989-997.

ADAM YOUNG, D., DEQUACH, J. A. & CHRISTMAN, K. L. 2011. Human cardiomyogenesis and the need for systems biology analysis. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine,* 3**,** 666-680.

ALLEGRUCCI, C. & YOUNG, L. E. 2007. Differences between human embryonic stem cell lines. *Human Reproduction Update,* 13**,** 103-120.

ALVA, J. A., LEE, G. E., ESCOBAR, E. E. & PYLE, A. D. 2011. Phosphatase and Tensin Homolog Regulates the Pluripotent State and Lineage Fate Choice in Human Embryonic Stem Cells. *Stem Cells,* 29**,** 1952-1962.

AMANN, B., LUEDEMANN, C., RATEI, R. & SCHMIDT-LUCKE, J. A. 2009. Autologous Bone Marrow Cell Transplantation Increases Leg Perfusion and Reduces Amputations in Patients With Advanced Critical Limb Ischemia Due to Peripheral Artery Disease. *Cell Transplantation,* 18**,** 371-380.

ANAND, S., MAJETI, B. K., ACEVEDO, L. M., MURPHY, E. A., MUKTHAVARAM, R., SCHEPPKE, L., HUANG, M., SHIELDS, D. J., LINDQUIST, J. N., LAPINSKI, P. E., KING, P. D., WEIS, S. M. & CHERESH, D. A. 2010. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. *Nat Med,* 16**,** 909-914.

ANG, S.-L. & ROSSANT, J. 1994. HNF-3β is essential for node and notochord formation in mouse development. *Cell,* 78**,** 561-574.

APOLONIA, L., WADDINGTON, S. N., FERNANDES, C., WARD, N. J., BOUMA, G., BLUNDELL, M. P., THRASHER, A. J., COLLINS, M. K. & PHILPOTT, N. J. 2007. Stable Gene Transfer to Muscle Using Non-integrating Lentiviral Vectors. *Mol Ther,* 15**,** 1947-1954.

ASAHARA, T., MUROHARA, T., SULLIVAN, A., SILVER, M., VAN DER ZEE, R., LI, T., WITZENBICHLER, B., SCHATTEMAN, G. & ISNER, J. M. 1997. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science,* 275**,** 964-966.

ASSMUS, B., ROLF, A., ERBS, S., ELSÄSSER, A., HABERBOSCH, W., HAMBRECHT, R., TILLMANNS, H., YU, J., CORTI, R., MATHEY, D. G., HAMM, C. W., SÜSELBECK, T., TONN, T., DIMMELER, S., DILL, T., ZEIHER, A. M. & SCHÄCHINGER, V. 2010. Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction. *Circulation: Heart Failure,* 3**,** 89-96.

AVILION, A. A., NICOLIS, S. K., PEVNY, L. H., PEREZ, L., VIVIAN, N. & LOVELL-BADGE, R. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & Development,* 17**,** 126-140.

AZUARA, V., PERRY, P., SAUER, S., SPIVAKOV, M., JORGENSEN, H. F., JOHN, R. M., GOUTI, M., CASANOVA, M., WARNES, G., MERKENSCHLAGER, M. & FISHER, A. G. 2006. Chromatin signatures of pluripotent cell lines. *Nat Cell Biol,* 8**,** 532-538.

BALSAM, L. B., WAGERS, A. J., CHRISTENSEN, J. L., KOFIDIS, T., WEISSMAN, I. L. & ROBBINS, R. C. 2004. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature,* 428**,** 668-673.

BARROSO-DEL JESUS, A., LUCENA-AGUILAR, G. & MENENDEZ, P. 2009. The miR-302-367 cluster as a potential stemness regulator in ESCs. *Cell Cycle,* 8**,** 394-398.

BARTSCH, T., FALKE, T., BREHM, M., ZEUS, T., KOGLER, G., WERNET, P. & STRAUER, B. E. 2006. [Transplantation of autologous adult bone marrow stem cells in patients with severe peripheral arterial occlusion disease]. *Med Klin (Munich),* 101 Suppl 1**,** 195-7.

BEARZI, C., ROTA, M., HOSODA, T., TILLMANNS, J., NASCIMBENE, A., DE ANGELIS, A., YASUZAWA-AMANO, S., TROFIMOVA, I., SIGGINS, R. W., LECAPITAINE, N., CASCAPERA, S., BELTRAMI, A. P., D'ALESSANDRO, D. A., ZIAS, E., QUAINI, F., URBANEK, K., MICHLER, R. E., BOLLI, R., KAJSTURA, J., LERI, A. & ANVERSA, P. 2007. Human cardiac stem cells. *Proceedings of the National Academy of Sciences,* 104**,** 14068-14073.

BECKER, K. A., STEIN, J. L., LIAN, J. B., VAN WIJNEN, A. J. & STEIN, G. S. 2007. Establishment of histone gene regulation and cell cycle checkpoint control in human embryonic stem cells. *Journal of Cellular Physiology,* 210**,** 517-526.

BELTRAMI, A. P., BARLUCCHI, L., TORELLA, D., BAKER, M., LIMANA, F., CHIMENTI, S., KASAHARA, H., ROTA, M., MUSSO, E., URBANEK, K., LERI, A., KAJSTURA, J., NADAL-GINARD, B. & ANVERSA, P. 2003. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell,* 114**,** 763-76.

BENETTI, R., GONZALO, S., JACO, I., MUNOZ, P., GONZALEZ, S., SCHOEFTNER, S., MURCHISON, E., ANDL, T., CHEN, T., KLATT, P., LI, E., SERRANO, M., MILLAR, S., HANNON, G. & BLASCO, M. A. 2008. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat Struct Mol Biol,* 15**,** 998-998.

BENNDORF, R., BÖGER, R. H., ERGÜN, S., STEENPASS, A. & WIELAND, T. 2003. Angiotensin II Type 2 Receptor Inhibits Vascular Endothelial Growth Factor–Induced Migration and In Vitro Tube Formation of Human Endothelial Cells. *Circulation Research,* 93**,** 438-447.

BERGER, S. L. 2007. The complex language of chromatin regulation during transcription. *Nature,* 447**,** 407-412.

BERGMANN, O., BHARDWAJ, R. D., BERNARD, S., ZDUNEK, S., BARNABÉ-HEIDER, F., WALSH, S., ZUPICICH, J., ALKASS, K., BUCHHOLZ, B. A., DRUID, H., JOVINGE, S. & FRISÉN, J. 2009. Evidence for Cardiomyocyte Renewal in Humans. *Science,* 324**,** 98-102.

BERNSTEIN, B. E., MIKKELSEN, T. S., XIE, X., KAMAL, M., HUEBERT, D. J., CUFF, J., FRY, B., MEISSNER, A., WERNIG, M., PLATH, K., JAENISCH, R., WAGSCHAL, A., FEIL, R., SCHREIBER, S. L. & LANDER, E. S. 2006. A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell,* 125**,** 315-326.

BIFFI, A., BARTOLOMAE, C. C., CESANA, D., CARTIER, N., AUBOURG, P., RANZANI, M., CESANI, M., BENEDICENTI, F., PLATI, T., RUBAGOTTI, E., MERELLA, S., CAPOTONDO, A., SGUALDINO, J., ZANETTI, G., VON KALLE, C., SCHMIDT, M., NALDINI, L. & MONTINI, E. 2011. Lentiviral vector common integration sites in preclinical models and a clinical trial reflect a benign integration bias and not oncogenic selection. *Blood,* 117**,** 5332-5339.

BOLLI, R., CHUGH, A. R., D'AMARIO, D., LOUGHRAN, J. H., STODDARD, M. F., IKRAM, S., BEACHE, G. M., WAGNER, S. G., LERI, A., HOSODA, T., SANADA, F., ELMORE, J. B., GOICHBERG, P., CAPPETTA, D., SOLANKHI, N. K., FAHSAH, I., ROKOSH, D. G., SLAUGHTER, M. S., KAJSTURA, J. & ANVERSA, P. 2011. Cardiac stem cells in patients with ischaemic cardiomyopathy

(SCIPIO): initial results of a randomised phase 1 trial. *The Lancet,* 378**,** 1847-1857.

BONAUER, A., CARMONA, G., IWASAKI, M., MIONE, M., KOYANAGI, M., FISCHER, A., BURCHFIELD, J., FOX, H., DOEBELE, C., OHTANI, K., CHAVAKIS, E., POTENTE, M., TJWA, M., URBICH, C., ZEIHER, A. M. & DIMMELER, S. 2009. MicroRNA-92a Controls Angiogenesis and Functional Recovery of Ischemic Tissues in Mice. *Science,* 324**,** 1710-1713.

BOYER, L. A., LEE, T. I., COLE, M. F., JOHNSTONE, S. E., LEVINE, S. S., ZUCKER, J. P., GUENTHER, M. G., KUMAR, R. M., MURRAY, H. L., JENNER, R. G., GIFFORD, D. K., MELTON, D. A., JAENISCH, R. & YOUNG, R. A. 2005. Core Transcriptional Regulatory Circuitry in Human Embryonic Stem Cells. *Cell,* 122**,** 947-956.

BRADBURY, A. W., RUCKLEY, C. V., FOWKES, F. G. R., FORBES, J. F., GILLESPIE, I., ADAM, D. J., BEARD, J. D., CLEVELAND, T., BELL, J., RAAB, G., STORKEY, H. & PARTICIPANTS, B. T. 2005. Bypass versus angioplasty in severe ischaemia of the leg (BASIL): multicentre, randomised controlled trial. *Lancet,* 366**,** 1925-1934.

BREITLING, R., ARMENGAUD, P., AMTMANN, A. & HERZYK, P. 2004. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Letters,* 573**,** 83-92.

BRITTEN, M. B., ABOLMAALI, N. D., ASSMUS, B., LEHMANN, R., HONOLD, J., SCHMITT, J., VOGL, T. J., MARTIN, H., SCHÄCHINGER, V., DIMMELER, S. & ZEIHER, A. M. 2003. Infarct Remodeling After Intracoronary Progenitor Cell Treatment in Patients With Acute Myocardial Infarction (TOPCARE-AMI). *Circulation,* 108**,** 2212-2218.

BRODIE, B. R., STONE, G. W., MORICE, M.-C., COX, D. A., GARCIA, E., MATTOS, L. A., BOURA, J., O'NEILL, W. W., STUCKEY, T. D., MILKS, S., LANSKY, A. J. & GRINES, C. L. 2001. Importance of time to reperfusion on outcomes with primary coronary angioplasty for acute myocardial infarction (results from the Stent Primary Angioplasty in Myocardial Infarction Trial). *The American Journal of Cardiology,* 88**,** 1085-1090.

BUCKLEY, S. M. K., HOWE, S. J., SHEARD, V., WARD, N. J., COUTELLE, C., THRASHER, A. J., WADDINGTON, S. N. & MCKAY, T. R. 2008. Lentiviral transduction of the murine lung provides efficient pseudotype and developmental stage-dependent cell-specific transgene expression. *Gene Ther,* 15**,** 1167-1175.

BURRIDGE, P. W., ANDERSON, D., PRIDDLE, H., BARBADILLO MUÑOZ, M. D., CHAMBERLAIN, S., ALLEGRUCCI, C., YOUNG, L. E. & DENNING, C. 2007. Improved Human Embryonic Stem Cell Embryoid Body Homogeneity and Cardiomyocyte Differentiation from a Novel V-96 Plate Aggregation System Highlights Interline Variability. *Stem Cells,* 25**,** 929-938.

BUTLER, S. L., HANSEN, M. S. & BUSHMAN, F. D. 2001. A quantitative assay for HIV DNA integration in vivo. *Nat Med,* 7**,** 631-4.

CALO, G., GRATTON, J. P., TELEMAQUE, S., D'ORLEANS-JUSTE, P. & REGOLI, D. 1996. Pharmacology of endothelins: vascular preparations for studying ETA and ETB receptors. *Mol Cell Biochem,* 154**,** 31-7.

CAPORALI, A., MELONI, M., VÖLLENKLE, C., BONCI, D., SALA-NEWBY, G. B., ADDIS, R., SPINETTI, G., LOSA, S., MASSON, R., BAKER, A. H., AGAMI, R., LE SAGE, C., CONDORELLI, G., MADEDDU, P., MARTELLI, F. & EMANUELI, C. 2011. Deregulation of microRNA-503 Contributes to Diabetes Mellitus-Induced Impairment of Endothelial Function and Reparative Angiogenesis After Limb Ischemia. *Circulation.*

CARE, A., CATALUCCI, D., FELICETTI, F., BONCI, D., ADDARIO, A., GALLO, P., BANG, M.-L., SEGNALINI, P., GU, Y., DALTON, N. D., ELIA, L., LATRONICO, M. V. G., HOYDAL, M., AUTORE, C., RUSSO, M. A., DORN, G. W., ELLINGSEN, O., RUIZ-LOZANO, P., PETERSON, K. L., CROCE, C. M., PESCHLE, C. & CONDORELLI, G. 2007. MicroRNA-133 controls cardiac hypertrophy. *Nat Med,* 13**,** 613-618.

CARPENTER, M. K., INOKUMA, M. S., DENHAM, J., MUJTABA, T., CHIU, C.-P. & RAO, M. S. 2001. Enrichment of Neurons and Neural Precursors from Human Embryonic Stem Cells. *Experimental Neurology,* 172**,** 383-397.

CARUSO, P., MACLEAN, M. R., KHANIN, R., MCCLURE, J., SOON, E., SOUTHGATE, M., MACDONALD, R. A., GREIG, J. A., ROBERTSON, K. E., MASSON, R., DENBY, L., DEMPSIE, Y., LONG, L., MORRELL, N. W. & BAKER, A. H. 2010. Dynamic Changes in Lung MicroRNA Profiles During the Development of Pulmonary Hypertension due to Chronic Hypoxia and Monocrotaline. *Arterioscler Thromb Vasc Biol,* 30**,** 716-723.

CASE, J., MEAD, L. E., BESSLER, W. K., PRATER, D., WHITE, H. A., SAADATZADEH, M. R., BHAVSAR, J. R., YODER, M. C., HANELINE, L. S. & INGRAM, D. A. 2007. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Experimental Hematology,* 35**,** 1109-1118.

CAVAZZANA-CALVO, M., PAYEN, E., NEGRE, O., WANG, G., HEHIR, K., FUSIL, F., DOWN, J., DENARO, M., BRADY, T., WESTERMAN, K., CAVALLESCO, R., GILLET-LEGRAND, B., CACCAVELLI, L., SGARRA, R., MAOUCHE-CHRETIEN, L., BERNAUDIN, F., GIROT, R., DORAZIO, R., MULDER, G.-J., POLACK, A., BANK, A., SOULIER, J., LARGHERO, J., KABBARA, N., DALLE, B., GOURMEL, B., SOCIE, G., CHRETIEN, S., CARTIER, N., AUBOURG, P., FISCHER, A., CORNETTA, K., GALACTEROS, F., BEUZARD, Y., GLUCKMAN, E., BUSHMAN, F., HACEIN-BEY-ABINA, S. & LEBOULCH, P. 2010. Transfusion independence and HMGA2 activation after gene therapy of human [bgr]-thalassaemia. *Nature,* 467**,** 318-322.

CHALFIE, M., HORVITZ, H. R. & SULSTON, J. E. 1981. Mutations that lead to reiterations in the cell lineages of C. elegans. *Cell,* 24**,** 59-69.

CHAMBERS, I., SILVA, J., COLBY, D., NICHOLS, J., NIJMEIJER, B., ROBERTSON, M., VRANA, J., JONES, K., GROTEWOLD, L. & SMITH, A. 2007. Nanog safeguards pluripotency and mediates germline development. *Nature,* 450**,** 1230-1234.

CHANG, T.-C., ZEITELS, L. R., HWANG, H.-W., CHIVUKULA, R. R., WENTZEL, E. A., DEWS, M., JUNG, J., GAO, P., DANG, C. V., BEER, M. A., THOMAS-TIKHONENKO, A. & MENDELL, J. T. 2009. Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proceedings of the National Academy of Sciences,* 106**,** 3384-3389.

CHEN, J.-F., MANDEL, E. M., THOMSON, J. M., WU, Q., CALLIS, T. E., HAMMOND, S. M., CONLON, F. L. & WANG, D.-Z. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet,* 38**,** 228-233.

CHEN, X., BA, Y., MA, L., CAI, X., YIN, Y., WANG, K., GUO, J., ZHANG, Y., CHEN, J., GUO, X., LI, Q., LI, X., WANG, W., ZHANG, Y., WANG, J., JIANG, X., XIANG, Y., XU, C., ZHENG, P., ZHANG, J., LI, R., ZHANG, H., SHANG, X., GONG, T., NING, G., WANG, J., ZEN, K., ZHANG, J. & ZHANG, C.-Y. 2008a. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res,* 18**,** 997-1006.

CHEN, X., XU, H., YUAN, P., FANG, F., HUSS, M., VEGA, V. B., WONG, E., ORLOV, Y. L., ZHANG, W., JIANG, J., LOH, Y.-H., YEO, H. C., YEO, Z. X., NARANG, V., GOVINDARAJAN, K. R., LEONG, B., SHAHAB, A., RUAN, Y., BOURQUE, G., SUNG, W.-K., CLARKE, N. D., WEI, C.-L. & NG, H.-H. 2008b. Integration of External Signaling Pathways with the Core Transcriptional Network in Embryonic Stem Cells. *Cell,* 133**,** 1106-1117.

CHEN, Y. & GORSKI, D. H. 2008. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood,* 111**,** 1217-1226.

CHENG, Y., JI, R., YUE, J., YANG, J., LIU, X., CHEN, H., DEAN, D. B. & ZHANG, C. 2007. MicroRNAs Are Aberrantly Expressed in Hypertrophic Heart: Do They Play a Role in Cardiac Hypertrophy? *The American Journal of Pathology,* 170**,** 1831-1840.

CHIMENTI, I., SMITH, R. R., LI, T.-S., GERSTENBLITH, G., MESSINA, E., GIACOMELLO, A. & MARBÁN, E. 2010. Relative Roles of Direct Regeneration Versus Paracrine Effects of Human Cardiosphere-Derived Cells Transplanted Into Infarcted Mice. *Circulation Research,* 106**,** 971-980.

CHO, S.-W., MOON, S.-H., LEE, S.-H., KANG, S.-W., KIM, J., LIM, J. M., KIM, H.-S., KIM, B.-S. & CHUNG, H.-M. 2007. Improvement of Postnatal Neovascularization by Human Embryonic Stem Cell–Derived Endothelial-Like Cell Transplantation in a Mouse Model of Hindlimb Ischemia. *Circulation,* 116**,** 2409-2419.

CHOCHOLA, M., PYTLIK, R., KOBYLKA, P., SKALICKA, L., KIDERYOVA, L., BERAN, S., VAREJKA, P., JIRAT, S., KOIVANEK, J., ASCHERMANN, M. & LINHART, A. 2008. Autologous intra-arterial infusion of bone marrow mononuclear cells in patients with critical leg ischemia. *Int Angiol,* 27**,** 281-90.

CIRILLO, L. A., LIN, F. R., CUESTA, I., FRIEDMAN, D., JARNIK, M. & ZARET, K. S. 2002. Opening of Compacted Chromatin by Early Developmental Transcription Factors HNF3 (FoxA) and GATA-4. *Molecular Cell,* 9**,** 279-289.

COCHRANE, A. W., CHEN, C. H. & ROSEN, C. A. 1990. Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. *Proceedings of the National Academy of Sciences,* 87**,** 1198-1202.

COCKRELL, A. & KAFRI, T. 2007. Gene delivery by lentivirus vectors. *Molecular Biotechnology,* 36**,** 184-204.

COMINACINI, L., PASINI, A. F., GARBIN, U., DAVOLI, A., TOSETTI, M. L., CAMPAGNOLA, M., RIGONI, A., PASTORINO, A. M., LO CASCIO, V. & SAWAMURA, T. 2000. Oxidized Low Density Lipoprotein (ox-LDL) Binding to ox-LDL Receptor-1 in Endothelial Cells Induces the Activation of NF-κB through an Increased Production of Intracellular Reactive Oxygen Species. *Journal of Biological Chemistry,* 275**,** 12633-12638.

CONDORELLI, G., LATRONICO, M. V. G. & DORN, G. W. 2010. microRNAs in heart disease: putative novel therapeutic targets? *European Heart Journal,* 31**,** 649-658.

D'AMARIO, D., FIORINI, C., CAMPBELL, P. M., GOICHBERG, P., SANADA, F., ZHENG, H., HOSODA, T., ROTA, M., CONNELL, J. M., GALLEGOS, R. P., WELT, F. G., GIVERTZ, M. M., MITCHELL, R. N., LERI, A., KAJSTURA, J., PFEFFER, M. A. & ANVERSA, P. 2011. Functionally Competent Cardiac Stem Cells Can Be Isolated From Endomyocardial Biopsies of Patients With Advanced Cardiomyopathies / Novelty and Significance. *Circulation Research,* 108**,** 857-861.

DE BRUIJN, M. F. T. R., MA, X., ROBIN, C., OTTERSBACH, K., SANCHEZ, M.-J. & DZIERZAK, E. 2002. Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta. *Immunity,* 16**,** 673-683.

DELA PAZ, N. G. & D'AMORE, P. A. 2009. Arterial versus venous endothelial cells. *Cell Tissue Res,* 335**,** 5-16.

DELAVAL, K. & FEIL, R. 2004. Epigenetic regulation of mammalian genomic imprinting. *Current Opinion in Genetics & Development,* 14**,** 188-195.

DEMAISON, C., PARSLEY, K., BROUNS, G., SCHERR, M., BATTMER, K., KINNON, C., GREZ, M. & THRASHER, A. J. 2002a. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Hum Gene Ther,* 13**,** 803-13.

DEMAISON, C., PARSLEY, K., BROUNS, G., SCHERR, M., BATTMER, K., KINNON, C., GREZ, M. & THRASHER, A. J. 2002b. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Human Gene Therapy,* 13**,** 803-13.

DENLI, A. M., TOPS, B. B. J., PLASTERK, R. H. A., KETTING, R. F. & HANNON, G. J. 2004. Processing of primary microRNAs by the Microprocessor complex. *Nature,* 432**,** 231-235.

DEWS, M., HOMAYOUNI, A., YU, D., MURPHY, D., SEVIGNANI, C., WENTZEL, E., FURTH, E. E., LEE, W. M., ENDERS, G. H., MENDELL, J. T. & THOMAS-TIKHONENKO, A. 2006. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet,* 38**,** 1060-1065.

DIXON, J. E., DICK, E., RAJAMOHAN, D., SHAKESHEFF, K. M. & DENNING, C. 2011. Directed Differentiation of Human Embryonic Stem Cells to Interrogate the Cardiac Gene Regulatory Network. *Mol Ther,* 19**,** 1695-1703.

DOHMANN, H. F., SILVA, S. A., SOUSA, A. L., BRAGA, A. M., BRANCO, R. V., HADDAD, A. F., OLIVEIRA, M. A., MOREIRA, R. C., TUCHE, F. A., PEIXOTO, C. M., TURA, B. R., BOROJEVIC, R., RIBEIRO, J. P., NICOLAU, J. C., NOBREGA, A. C. & CARVALHO, A. C. 2008. Multicenter double blind trial of autologous bone marrow mononuclear cell transplantation through intracoronary injection post acute myocardium infarction - MiHeart/AMI study. *Trials,* 9**,** 41.

DULL, T., ZUFFEREY, R., KELLY, M., MANDEL, R. J., NGUYEN, M., TRONO, D. & NALDINI, L. 1998. A Third-Generation Lentivirus Vector with a Conditional Packaging System. *Journal of Virology,* 72**,** 8463-8471.

DURDU, S., AKAR, A. R., ARAT, M., SANCAK, T., EREN, N. T. & OZYURDA, U. 2006. Autologous bone-marrow mononuclear cell implantation for patients with Rutherford grade II-III thromboangiitis obliterans. *Journal of Vascular Surgery,* 44**,** 732-739.

ELIA, L., CONTU, R., QUINTAVALLE, M., VARRONE, F., CHIMENTI, C., RUSSO, M. A., CIMINO, V., DE MARINIS, L., FRUSTACI, A., CATALUCCI, D. & CONDORELLI, G. 2009. Reciprocal Regulation of MicroRNA-1 and Insulin-Like Growth Factor-1 Signal Transduction Cascade in Cardiac and Skeletal Muscle in Physiological and Pathological Conditions. *Circulation,* 120**,** 2377-2385.

ESATO, K., HAMANO, K., LI, T. S., FURUTANI, A., SEYAMA, A., TAKENAKA, H. & ZEMPO, N. 2002. Neovascularization Induced by Autologous Bone Marrow

Cell Implantation in Peripheral Arterial Disease. *Cell Transplantation,* 11**,** 747-752.

EVANS, M. J. & KAUFMAN, M. H. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature,* 292**,** 154-156.

EVSEENKO, D., ZHU, Y., SCHENKE-LAYLAND, K., KUO, J., LATOUR, B., GE, S., SCHOLES, J., DRAVID, G., LI, X., MACLELLAN, W. R. & CROOKS, G. M. 2010. Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells. *Proceedings of the National Academy of Sciences,* 107**,** 13742-13747.

FASANARO, P., D'ALESSANDRA, Y., DI STEFANO, V., MELCHIONNA, R., ROMANI, S., POMPILIO, G., CAPOGROSSI, M. C. & MARTELLI, F. 2008. MicroRNA-210 Modulates Endothelial Cell Response to Hypoxia and Inhibits the Receptor Tyrosine Kinase Ligand Ephrin-A3. *Journal of Biological Chemistry,* 283**,** 15878-15883.

FERGUSON, E. L., STERNBERG, P. W. & HORVITZ, H. R. 1987. A genetic pathway for the specification of the vulval cell lineages of Caenorhabditis elegans. *Nature,* 326**,** 259-267.

FICHTLSCHERER, S., DE ROSA, S., FOX, H., SCHWIETZ, T., FISCHER, A., LIEBETRAU, C., WEBER, M., HAMM, C. W., RÖXE, T., MÜLLER-ARDOGAN, M., BONAUER, A., ZEIHER, A. M. & DIMMELER, S. 2010. Circulating MicroRNAs in Patients With Coronary Artery Disease / Novelty and Significance. *Circulation Research,* 107**,** 677-684.

FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature,* 391**,** 806-811.

FISH, J. E., SANTORO, M. M., MORTON, S. U., YU, S., YEH, R.-F., WYTHE, J. D., IVEY, K. N., BRUNEAU, B. G., STAINIER, D. Y. R. & SRIVASTAVA, D. 2008. miR-126 Regulates Angiogenic Signaling and Vascular Integrity. *Developmental Cell,* 15**,** 272-284.

FLAMME, I. & RISAU, W. 1992. Induction of vasculogenesis and hematopoiesis in vitro. *Development,* 116**,** 435-439.

FRANCIS, N. J., KINGSTON, R. E. & WOODCOCK, C. L. 2004. Chromatin Compaction by a Polycomb Group Protein Complex. *Science,* 306**,** 1574-1577.

FRIEDRICH, E. B., WALENTA, K., SCHARLAU, J., NICKENIG, G. & WERNER, N. 2006. CD34−/CD133+/VEGFR-2+ Endothelial Progenitor Cell Subpopulation With Potent Vasoregenerative Capacities. *Circulation Research,* 98**,** e20-e25.

FU, J.-D., RUSHING, S. N., LIEU, D. K., CHAN, C. W., KONG, C.-W., GENG, L., WILSON, K. D., CHIAMVIMONVAT, N., BOHELER, K. R., WU, J. C., KELLER, G., HAJJAR, R. J. & LI, R. A. 2011. Distinct Roles of MicroRNA-1 and -499 in Ventricular Specification and Functional Maturation of Human Embryonic Stem Cell-Derived Cardiomyocytes. *PLoS ONE,* 6**,** e27417.

GANGARAJU, V. K. & LIN, H. 2009. MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol,* 10**,** 116-125.

GEHLING, U. M., ERGÜN, S., SCHUMACHER, U., WAGENER, C., PANTEL, K., OTTE, M., SCHUCH, G., SCHAFHAUSEN, P., MENDE, T., KILIC, N., KLUGE, K., SCHÄFER, B., HOSSFELD, D. K. & FIEDLER, W. 2000. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood,* 95**,** 3106-3112.

GERRARD, L., RODGERS, L. & CUI, W. 2005. Differentiation of Human Embryonic Stem Cells to Neural Lineages in Adherent Culture by Blocking Bone Morphogenetic Protein Signaling. *Stem Cells,* 23**,** 1234-1241.

GIANNOTTI, G., DOERRIES, C., MOCHARLA, P. S., MUELLER, M. F., BAHLMANN, F. H., HORVÀTH, T., JIANG, H., SORRENTINO, S. A., STEENKEN, N., MANES, C., MARZILLI, M., RUDOLPH, K. L., LÜSCHER, T. F., DREXLER, H. & LANDMESSER, U. 2010. Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Prehypertension. *Hypertension,* 55**,** 1389-1397.

GIRALDEZ, A. J., CINALLI, R. M., GLASNER, M. E., ENRIGHT, A. J., THOMSON, J. M., BASKERVILLE, S., HAMMOND, S. M., BARTEL, D. P. & SCHIER, A. F. 2005. MicroRNAs Regulate Brain Morphogenesis in Zebrafish. *Science,* 308**,** 833-838.

GLASS, C. & SINGLA, D. K. 2011. MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. *American Journal of Physiology - Heart and Circulatory Physiology,* 301**,** H2038-H2049.

GNECCHI, M., HE, H., NOISEUX, N., LIANG, O. D., ZHANG, L., MORELLO, F., MU, H., MELO, L. G., PRATT, R. E., INGWALL, J. S. & DZAU, V. J. 2006. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *The FASEB Journal,* 20**,** 661-669.

GRAICHEN, R., XU, X., BRAAM, S. R., BALAKRISHNAN, T., NORFIZA, S., SIEH, S., SOO, S. Y., THAM, S. C., MUMMERY, C., COLMAN, A., ZWEIGERDT, R. & DAVIDSON, B. P. 2008. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. *Differentiation,* 76**,** 357-370.

GRAU, D. J., CHAPMAN, B. A., GARLICK, J. D., BOROWSKY, M., FRANCIS, N. J. & KINGSTON, R. E. 2011. Compaction of chromatin by diverse Polycomb group proteins requires localized regions of high charge. *Genes & Development,* 25**,** 2210-2221.

GREER CARD, D. A., HEBBAR, P. B., LI, L., TROTTER, K. W., KOMATSU, Y., MISHINA, Y. & ARCHER, T. K. 2008. Oct4/Sox2-Regulated miR-302 Targets Cyclin D1 in Human Embryonic Stem Cells. *Mol. Cell. Biol.,* 28**,** 6426-6438.

GREGORY, R. I., YAN, K.-P., AMUTHAN, G., CHENDRIMADA, T., DORATOTAJ, B., COOCH, N. & SHIEKHATTAR, R. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature,* 432**,** 235-240.

GRIFFITHS-JONES, S. 2004. The microRNA Registry. *Nucleic Acids Research,* 32**,** D109-D111.

GRIMSON, A., SRIVASTAVA, M., FAHEY, B., WOODCROFT, B. J., CHIANG, H. R., KING, N., DEGNAN, B. M., ROKHSAR, D. S. & BARTEL, D. P. 2008. Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature,* 455**,** 1193-1197.

GU, Y. Q., ZHANG, J., GUO, L. R., QI, L. X., ZHANG, S. W., XU, J., LI, J. X., LUO, T., JI, B. X., LI, X. F., YU, H. X., CUI, S. J. & WANG, Z. G. 2008. Transplantation of autologous bone marrow mononuclear cells for patients with lower limb ischemia. *Chin Med J (Engl),* 121**,** 963-7.

HARRIS, T. A., YAMAKUCHI, M., FERLITO, M., MENDELL, J. T. & LOWENSTEIN, C. J. 2008. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proceedings of the National Academy of Sciences,* 105**,** 1516-1521.

HE, L. & HANNON, G. J. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet,* 5**,** 522-531.

HENNESSEY, P. T., SANFORD, T., CHOUDHARY, A., MYDLARZ, W. W., BROWN, D., ADAI, A. T., OCHS, M. F., AHRENDT, S. A., MAMBO, E. & CALIFANO, J. A.

2012. Serum microRNA Biomarkers for Detection of Non-Small Cell Lung Cancer. *PLoS ONE,* 7**,** e32307.

HERNÁNDEZ, P., CORTINA, L., ARTAZA, H., POL, N., LAM, R. M., DORTICÓS, E., MACÍAS, C., HERNÁNDEZ, C., DEL VALLE, L., BLANCO, A., MARTÍNEZ, A. & DÍAZ, F. 2007. Autologous bone-marrow mononuclear cell implantation in patients with severe lower limb ischaemia: A comparison of using blood cell separator and Ficoll density gradient centrifugation. *Atherosclerosis,* 194**,** e52-e56.

HERSHEY, J. C., BASKIN, E. P., GLASS, J. D., HARTMAN, H. A., GILBERTO, D. B., ROGERS, I. T. & COOK, J. J. 2001. Revascularization in the rabbit hindlimb: dissociation between capillary sprouting and arteriogenesis. *Cardiovasc Res,* 49**,** 618-25.

HEWITT, Z., FORSYTH, N. R., WATERFALL, M., WOJTACHA, D., THOMSON, A. J. & MCWHIR, J. 2006. Fluorescence-activated single cell sorting of human embryonic stem cells. *Cloning Stem Cells,* 8**,** 225-34.

HIGASHI, Y., KIMURA, M., HARA, K., NOMA, K., JITSUIKI, D., NAKAGAWA, K., OSHIMA, T., CHAYAMA, K., SUEDA, T., GOTO, C., MATSUBARA, H., MUROHARA, T. & YOSHIZUMI, M. 2004. Autologous Bone-Marrow Mononuclear Cell Implantation Improves Endothelium-Dependent Vasodilation in Patients With Limb Ischemia. *Circulation,* 109**,** 1215-1218.

HIRSCH, A. T., HASKAL, Z. J., HERTZER, N. R., BAKAL, C. W., CREAGER, M. A., HALPERIN, J. L., HIRATZKA, L. F., MURPHY, W. R., OLIN, J. W., PUSCHETT, J. B., ROSENFIELD, K. A., SACKS, D., STANLEY, J. C., TAYLOR, L. M., JR., WHITE, C. J., WHITE, J., WHITE, R. A., ANTMAN, E. M., SMITH, S. C., JR., ADAMS, C. D., ANDERSON, J. L., FAXON, D. P., FUSTER, V., GIBBONS, R. J., HUNT, S. A., JACOBS, A. K., NISHIMURA, R., ORNATO, J. P., PAGE, R. L. & RIEGEL, B. 2006. ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic): a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation,* 113**,** e463-654.

HOEKSTRA, M., VAN DER LANS, C. A. C., HALVORSEN, B., GULLESTAD, L., KUIPER, J., AUKRUST, P., VAN BERKEL, T. J. C. & BIESSEN, E. A. L. 2010. The peripheral blood mononuclear cell microRNA signature of coronary artery disease. *Biochemical and Biophysical Research Communications,* 394**,** 792-797.

HONG, S.-H., RAMPALLI, S., LEE, JUNG B., MCNICOL, J., COLLINS, T., DRAPER, JONATHAN S. & BHATIA, M. 2011. Cell Fate Potential of Human Pluripotent Stem Cells Is Encoded by Histone Modifications. *Cell Stem Cell,* 9**,** 24-36.

HOVATTA, O. 2011. Infectious problems associated with transplantation of cells differentiated from pluripotent stem cells. *Semin Immunopathol,* 33**,** 627-30.

HSIEH, P. C. H., SEGERS, V. F. M., DAVIS, M. E., MACGILLIVRAY, C., GANNON, J., MOLKENTIN, J. D., ROBBINS, J. & LEE, R. T. 2007. Evidence from a genetic

fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med,* 13**,** 970-974.

HUANG, P. P., YANG, X. F., LI, S. Z., WEN, J. C., ZHANG, Y. & HAN, Z. C. 2007. Randomised comparison of G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow-mononuclear cells for the treatment of patients with lower limb arteriosclerosis obliterans. *Thromb Haemost,* 98**,** 1335-42.

HUANG, X., GSCHWENG, E., VAN HANDEL, B., CHENG, D., MIKKOLA, H. K. A. & WITTE, O. N. 2011. Regulated expression of microRNAs-126/126* inhibits erythropoiesis from human embryonic stem cells. *Blood,* 117**,** 2157-2165.

HUNTZINGER, E. & IZAURRALDE, E. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet,* 12**,** 99-110.

HURST, D. R., EDMONDS, M. D., SCOTT, G. K., BENZ, C. C., VAIDYA, K. S. & WELCH, D. R. 2009. Breast Cancer Metastasis Suppressor 1 Up-regulates miR-146, Which Suppresses Breast Cancer Metastasis. *Cancer Research,* 69**,** 1279-1283.

IKEDA, S., KONG, S. W., LU, J., BISPING, E., ZHANG, H., ALLEN, P. D., GOLUB, T. R., PIESKE, B. & PU, W. T. 2007. Altered microRNA expression in human heart disease. *Physiological Genomics,* 31**,** 367-373.

IVEY, K. N., MUTH, A., ARNOLD, J., KING, F. W., YEH, R.-F., FISH, J. E., HSIAO, E. C., SCHWARTZ, R. J., CONKLIN, B. R., BERNSTEIN, H. S. & SRIVASTAVA, D. 2008a. MicroRNA Regulation of Cell Lineages in Mouse and Human Embryonic Stem Cells. *Cell Stem Cell,* 2**,** 219-229.

IVEY, K. N., MUTH, A., ARNOLD, J., KING, F. W., YEH, R. F., FISH, J. E., HSIAO, E. C., SCHWARTZ, R. J., CONKLIN, B. R., BERNSTEIN, H. S. & SRIVASTAVA, D. 2008b. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell,* 2**,** 219-29.

JACKSON, K. A., MAJKA, S. M., WANG, H., POCIUS, J., HARTLEY, C. J., MAJESKY, M. W., ENTMAN, M. L., MICHAEL, L. H., HIRSCHI, K. K. & GOODELL, M. A. 2001. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *The Journal of Clinical Investigation,* 107**,** 1395-1402.

JAENISCH, R. & YOUNG, R. 2008. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell,* 132**,** 567-82.

JAMES, D., NAM, H.-S., SEANDEL, M., NOLAN, D., JANOVITZ, T., TOMISHIMA, M., STUDER, L., LEE, G., LYDEN, D., BENEZRA, R., ZANINOVIC, N., ROSENWAKS, Z., RABBANY, S. Y. & RAFII, S. 2010. Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGF[beta] inhibition is Id1 dependent. *Nat Biotech,* 28**,** 161-166.

JANSSENS, S., DUBOIS, C., BOGAERT, J., THEUNISSEN, K., DEROOSE, C., DESMET, W., KALANTZI, M., HERBOTS, L., SINNAEVE, P., DENS, J., MAERTENS, J., RADEMAKERS, F., DYMARKOWSKI, S., GHEYSENS, O., VAN CLEEMPUT, J., BORMANS, G., NUYTS, J., BELMANS, A., MORTELMANS, L., BOOGAERTS, M. & VAN DE WERF, F. 2006. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: Double-blind, randomised controlled trial. *Lancet,* 367**,** 113-121.

JAYAWARDENA, T. M., EGEMNAZAROV, B., FINCH, E. A., ZHANG, L., PAYNE, J. A., PANDYA, K., ZHANG, Z., ROSENBERG, P., MIROTSOU, M. & DZAU, V. J. 2012. MicroRNA-Mediated In Vitro and In Vivo Direct Reprogramming of Cardiac Fibroblasts to Cardiomyocytes. *Circulation Research*.

JENUWEIN, T. & ALLIS, C. D. 2001. Translating the Histone Code. *Science,* 293**,** 1074-1080.

JOPLING, C., SLEEP, E., RAYA, M., MARTI, M., RAYA, A. & BELMONTE, J. C. I. 2010. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature,* 464**,** 606-609.

KAJIGUCHI, M., KONDO, T., IZAWA, H., KOBAYASHI, M., YAMAMOTO, K., SHINTANI, S., NUMAGUCHI, Y., NAOE, T., TAKAMATSU, J., KOMORI, K. & MUROHARA, T. 2007. Safety and Efficacy of Autologous Progenitor Cell Transplantation for Therapeutic Angiogenesis in Patients With Critical Limb Ischemia. *Circulation Journal,* 71**,** 196-201.

KALKA, C., MASUDA, H., TAKAHASHI, T., KALKA-MOLL, W. M., SILVER, M., KEARNEY, M., LI, T., ISNER, J. M. & ASAHARA, T. 2000. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proceedings of the National Academy of Sciences,* 97**,** 3422-3427.

KANE, N. M., HOWARD, L., DESCAMPS, B., MELONI, M., MCCLURE, J. D., LU, R., MCCAHILL, A., BREEN, C., MACKENZIE, R. M., DELLES, C., MOUNTFORD, J. C., MILLIGAN, G., EMANUELI, C. & BAKER, A. H. 2012. A Role for microRNAs 99b, 181a and 181b in the Differentiation to Vascular Endothelial Cells from Human Embryonic Stem Cells. *Stem Cells***,** N/A-N/A.

KANE, N. M., MELONI, M., SPENCER, H. L., CRAIG, M. A., STREHL, R., MILLIGAN, G., HOUSLAY, M. D., MOUNTFORD, J. C., EMANUELI, C. & BAKER, A. H. 2010a. Derivation of Endothelial Cells From Human Embryonic Stem Cells by Directed Differentiation: Analysis of MicroRNA and Angiogenesis In Vitro and In Vivo. *Arterioscler Thromb Vasc Biol,* 30**,** 1389-1397.

KANE, N. M., NOWROUZI, A., MUKHERJEE, S., BLUNDELL, M. P., GREIG, J. A., LEE, W. K., HOUSLAY, M. D., MILLIGAN, G., MOUNTFORD, J. C., VON KALLE, C., SCHMIDT, M., THRASHER, A. J. & BAKER, A. H. 2010b. Lentivirus-mediated Reprogramming of Somatic Cells in the Absence of Transgenic Transcription Factors. *Mol Ther,* 18**,** 2139-2145.

KANELLOPOULOU, C., MULJO, S. A., KUNG, A. L., GANESAN, S., DRAPKIN, R., JENUWEIN, T., LIVINGSTON, D. M. & RAJEWSKY, K. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev,* 19**,** 489-501.

KANG, H. J., KIM, M. K., KIM, M. G., CHOI, D. J., YOON, J. H., PARK, Y. B. & KIM, H. S. 2011. A multicenter, prospective, randomized, controlled trial evaluating the safety and efficacy of intracoronary cell infusion mobilized with granulocyte colony-stimulating factor and darbepoetin after acute myocardial infarction: study design and rationale of the 'MAGIC cell-5-combination cytokine trial'. *Trials,* 12**,** 33.

KARAAYVAZ, M., ZHANG, C., LIANG, S., SHROYER, K. R. & JU, J. 2012. Prognostic Significance of miR-205 in Endometrial Cancer. *PLoS ONE,* 7**,** e35158.

KASHYAP, V., REZENDE, N. C., SCOTLAND, K. B., SHAFFER, S. M., PERSSON, J. L., GUDAS, L. J. & MONGAN, N. P. 2009. Regulation of Stem Cell Pluripotency and Differentiation Involves a Mutual Regulatory Circuit of the Nanog, OCT4, and SOX2 Pluripotency Transcription Factors With Polycomb Repressive Complexes and Stem Cell microRNAs. *Stem Cells and Development,* 18**,** 1093-1108.

KAWAMOTO, A., IWASAKI, H., KUSANO, K., MURAYAMA, T., OYAMADA, A., SILVER, M., HULBERT, C., GAVIN, M., HANLEY, A., MA, H., KEARNEY, M., ZAK, V., ASAHARA, T. & LOSORDO, D. W. 2006. CD34-Positive Cells Exhibit Increased Potency and Safety for Therapeutic Neovascularization After Myocardial Infarction Compared With Total Mononuclear Cells. *Circulation,* 114**,** 2163-2169.

KAZENWADEL, J., MICHAEL, M. Z. & HARVEY, N. L. 2010. Prox1 expression is negatively regulated by miR-181 in endothelial cells. *Blood,* 116**,** 2395-2401.

KEHAT, I., KENYAGIN-KARSENTI, D., SNIR, M., SEGEV, H., AMIT, M., GEPSTEIN, A., LIVNE, E., BINAH, O., ITSKOVITZ-ELDOR, J. & GEPSTEIN, L. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *The Journal of Clinical Investigation,* 108**,** 407-414.

KIKUCHI, K., HOLDWAY, J. E., WERDICH, A. A., ANDERSON, R. M., FANG, Y., EGNACZYK, G. F., EVANS, T., MACRAE, C. A., STAINIER, D. Y. R. & POSS, K. D. 2010. Primary contribution to zebrafish heart regeneration by gata4+ cardiomyocytes. *Nature,* 464**,** 601-605.

KIM, S. 2009. A study of microRNAs in silico and in vivo. *FEBS Journal,* 276**,** 2139-2139.

KNIGHT, S. W. & BASS, B. L. 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science,* 293**,** 2269-71.

KOCHER, A. A., SCHUSTER, M. D., SZABOLCS, M. J., TAKUMA, S., BURKHOFF, D., WANG, J., HOMMA, S., EDWARDS, N. M. & ITESCU, S. 2001. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med,* 7**,** 430-436.

KORF-KLINGEBIEL, M., KEMPF, T., SAUER, T., BRINKMANN, E., FISCHER, P., MEYER, G. P., GANSER, A., DREXLER, H. & WOLLERT, K. C. 2008. Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. *European Heart Journal,* 29**,** 2851-2858.

KOZAK, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Research,* 15**,** 8125-8148.

KU, M., KOCHE, R. P., RHEINBAY, E., MENDENHALL, E. M., ENDOH, M., MIKKELSEN, T. S., PRESSER, A., NUSBAUM, C., XIE, X., CHI, A. S., ADLI, M., KASIF, S., PTASZEK, L. M., COWAN, C. A., LANDER, E. S., KOSEKI, H. & BERNSTEIN, B. E. 2008. Genomewide Analysis of PRC1 and PRC2 Occupancy Identifies Two Classes of Bivalent Domains. *PLoS Genet,* 4**,** e1000242.

KUBO, A., SHINOZAKI, K., SHANNON, J. M., KOUSKOFF, V., KENNEDY, M., WOO, S., FEHLING, H. J. & KELLER, G. 2004. Development of definitive endoderm from embryonic stem cells in culture. *Development,* 131**,** 1651-1662.

KWON, C., HAN, Z., OLSON, E. N. & SRIVASTAVA, D. 2005a. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 18986-18991.

KWON, C., HAN, Z., OLSON, E. N. & SRIVASTAVA, D. 2005b. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc Natl Acad Sci U S A,* 102**,** 18986-91.

LADD, A. N., YATSKIEVYCH, T. A. & ANTIN, P. B. 1998. Regulation of Avian Cardiac Myogenesis by Activin/TGFß and Bone Morphogenetic Proteins. *Developmental Biology,* 204**,** 407-419.

LAFLAMME, M. A., CHEN, K. Y., NAUMOVA, A. V., MUSKHELI, V., FUGATE, J. A., DUPRAS, S. K., REINECKE, H., XU, C., HASSANIPOUR, M., POLICE, S., O'SULLIVAN, C., COLLINS, L., CHEN, Y., MINAMI, E., GILL, E. A., UENO, S., YUAN, C., GOLD, J. & MURRY, C. E. 2007. Cardiomyocytes derived from

human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotech,* 25**,** 1015-1024.

LAFLAMME, M. A. & MURRY, C. E. 2005. Regenerating the heart. *Nat Biotech,* 23**,** 845-856.

LAFLAMME, M. A. & MURRY, C. E. 2011. Heart regeneration. *Nature,* 473**,** 326-335.

LAGARKOVA, M. A., VOLCHKOV, P. Y., PHILONENKO, E. S. & KISELEV, S. L. 2008. Efficient differentiation of hESCs into endothelial cells in vitro is secured by epigenetic changes. *Cell Cycle,* 7**,** 2929-2935.

LAI, M., WENDY-YEO, W., RAMASAMY, R., NORDIN, N., ROSLI, R., VEERAKUMARASIVAM, A. & ABDULLAH, S. 2011. Advancements in reprogramming strategies for the generation of induced pluripotent stem cells. *Journal of Assisted Reproduction and Genetics*, 1-11.

LAKSHMIPATHY, U., LOVE, B., GOFF, L. A., JÖRNSTEN, R., GRAICHEN, R., HART, R. P. & CHESNUT, J. D. 2007. MicroRNA Expression Pattern of Undifferentiated and Differentiated Human Embryonic Stem Cells. *Stem Cells and Development,* 16**,** 1003-1016.

LANDMESSER, U., ENGBERDING, N., BAHLMANN, F. H., SCHAEFER, A., WIENCKE, A., HEINEKE, A., SPIEKERMANN, S., HILFIKER-KLEINER, D., TEMPLIN, C., KOTLARZ, D., MUELLER, M., FUCHS, M., HORNIG, B., HALLER, H. & DREXLER, H. 2004. Statin-Induced Improvement of Endothelial Progenitor Cell Mobilization, Myocardial Neovascularization, Left Ventricular Function, and Survival After Experimental Myocardial Infarction Requires Endothelial Nitric Oxide Synthase. *Circulation,* 110**,** 1933-1939.

LAWALL, H., BRAMLAGE, P. & AMANN, B. 2010. Stem cell and progenitor cell therapy in peripheral artery disease. A critical appraisal. *Thromb Haemost,* 103**,** 696-709.

LECINA, M., TING, S., CHOO, A., REUVENY, S. & OH, S. 2010. Scalable platform for human embryonic stem cell differentiation to cardiomyocytes in suspended microcarrier cultures. *Tissue Eng Part C Methods,* 16**,** 1609-19.

LEE, C. S., FRIEDMAN, J. R., FULMER, J. T. & KAESTNER, K. H. 2005. The initiation of liver development is dependent on Foxa transcription factors. *Nature,* 435**,** 944-947.

LEE, D. Y., DENG, Z., WANG, C.-H. & YANG, B. B. 2007. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proceedings of the National Academy of Sciences,* 104**,** 20350-20355.

LEE, R., FEINBAUM, R. & AMBROS, V. 2004. A short history of a short RNA. *Cell,* 116, Supplement 2**,** S89-S92.

LEE, R. C., FEINBAUM, R. L. & AMBROS, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75**,** 843-854.

LEE, Y., AHN, C., HAN, J., CHOI, H., KIM, J., YIM, J., LEE, J., PROVOST, P., RADMARK, O., KIM, S. & KIM, V. N. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature,* 425**,** 415-9.

LEVENBERG, S., GOLUB, J. S., AMIT, M., ITSKOVITZ-ELDOR, J. & LANGER, R. 2002. Endothelial cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences,* 99**,** 4391-4396.

LI, Z., WILSON, K. D., SMITH, B., KRAFT, D. L., JIA, F., HUANG, M., XIE, X., ROBBINS, R. C., GAMBHIR, S. S., WEISSMAN, I. L. & WU, J. C. 2009. Functional and Transcriptional Characterization of Human Embryonic Stem Cell-Derived Endothelial Cells for Treatment of Myocardial Infarction. *PLoS ONE,* 4**,** e8443.

LICKERT, H., KUTSCH, S., KANZLER, B. T., TAMAI, Y., TAKETO, M. M. & KEMLER, R. 2002. Formation of Multiple Hearts in Mice following Deletion of ß-catenin in the Embryonic Endoderm. *Developmental Cell,* 3**,** 171-181.

LIEW, C. G., DRAPER, J. S., WALSH, J., MOORE, H. & ANDREWS, P. W. 2007. Transient and stable transgene expression in human embryonic stem cells. *Stem Cells,* 25**,** 1521-8.

LIN, C.-H., JACKSON, A. L., GUO, J., LINSLEY, P. S. & EISENMAN, R. N. 2009. Myc-regulated microRNAs attenuate embryonic stem cell differentiation. *EMBO J,* 28**,** 3157-3170.

LIN, S.-L., CHANG, D. C., LIN, C.-H., YING, S.-Y., LEU, D. & WU, D. T. S. 2011. Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Research,* 39**,** 1054-1065.

LIU, N., BEZPROZVANNAYA, S., WILLIAMS, A. H., QI, X., RICHARDSON, J. A., BASSEL-DUBY, R. & OLSON, E. N. 2008. microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Genes & Development,* 22**,** 3242-3254.

LIU, R., CHEN, X., DU, Y., YAO, W., SHEN, L., WANG, C., HU, Z., ZHUANG, R., NING, G., ZHANG, C., YUAN, Y., LI, Z., ZEN, K., BA, Y. & ZHANG, C.-Y. 2012. Serum MicroRNA Expression Profile as a Biomarker in the Diagnosis and Prognosis of Pancreatic Cancer. *Clinical Chemistry,* 58**,** 610-618.

LIU, Y., SONG, Z., ZHAO, Y., QIN, H., CAI, J., ZHANG, H., YU, T., JIANG, S., WANG, G., DING, M. & DENG, H. 2006. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. *Biochem Biophys Res Commun,* 346**,** 131-9.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.),* 25**,** 402-8.

LLOYD-JONES, D. M. 2001. The risk of congestive heart failure: sobering lessons from the Framingham Heart Study. *Curr Cardiol Rep,* 3**,** 184-90.

LOGAN, A. C., NIGHTINGALE, S. J., HAAS, D. L., CHO, G. J., PEPPER, K. A. & KOHN, D. B. 2004. Factors influencing the titer and infectivity of lentiviral vectors. *Human Gene Therapy,* 15**,** 976-988.

LU, S.-J., FENG, Q., CABALLERO, S., CHEN, Y., MOORE, M. A. S., GRANT, M. B. & LANZA, R. 2007. Generation of functional hemangioblasts from human embryonic stem cells. *Nat Meth,* 4**,** 501-509.

LUND, E., GUTTINGER, S., CALADO, A., DAHLBERG, J. E. & KUTAY, U. 2004. Nuclear export of microRNA precursors. *Science,* 303**,** 95-8.

LUNDE, K., SOLHEIM, S., AAKHUS, S., ARNESEN, H., ABDELNOOR, M., EGELAND, T., ENDRESEN, K., ILEBEKK, A., MANGSCHAU, A., FJELD, J. G., SMITH, H. J., TARALDSRUD, E., GRØGAARD, H. K., BJØRNERHEIM, R., BREKKE, M., MÜLLER, C., HOPP, E., RAGNARSSON, A., BRINCHMANN, J. E. & FORFANG, K. 2006. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *New England Journal of Medicine,* 355**,** 1199-1209.

MADEDDU, P., EMANUELI, C., PELOSI, E., SALIS, M. B., CERIO, A. M., BONANNO, G., PATTI, M., STASSI, G., CONDORELLI, G. & PESCHLE, C. 2004. Transplantation of low dose CD34+Kdr+ cells promotes vascular and muscular regeneration in ischemic limbs. *The FASEB Journal*.

MAKKAR, R. R., SMITH, R. R., CHENG, K., MALLIARAS, K., THOMSON, L. E., BERMAN, D., CZER, L. S., MARBAN, L., MENDIZABAL, A., JOHNSTON, P. V., RUSSELL, S. D., SCHULERI, K. H., LARDO, A. C., GERSTENBLITH, G. & MARBAN, E. 2012. Intracoronary cardiosphere-derived cells for heart

regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet,* 379**,** 895-904.

MARGUERON, R., TROJER, P. & REINBERG, D. 2005. The key to development: interpreting the histone code? *Current Opinion in Genetics & Development,* 15**,** 163-176.

MARSON, A., LEVINE, S. S., COLE, M. F., FRAMPTON, G. M., BRAMBRINK, T., JOHNSTONE, S., GUENTHER, M. G., JOHNSTON, W. K., WERNIG, M., NEWMAN, J., CALABRESE, J. M., DENNIS, L. M., VOLKERT, T. L., GUPTA, S., LOVE, J., HANNETT, N., SHARP, P. A., BARTEL, D. P., JAENISCH, R. & YOUNG, R. A. 2008. Connecting microRNA Genes to the Core Transcriptional Regulatory Circuitry of Embryonic Stem Cells. *Cell,* 134**,** 521-533.

MASON, C. & DUNNILL, P. 2007. A brief definition of regenerative medicine. *Regenerative Medicine,* 3**,** 1-5.

MASUDA, H., ALEV, C., AKIMARU, H., ITO, R., SHIZUNO, T., KOBORI, M., HORII, M., ISHIHARA, T., ISOBE, K., ISOZAKI, M., ITOH, J., ITOH, Y., OKADA, Y., MCINTYRE, B. A. S., KATO, S. & ASAHARA, T. 2011. Methodological Development of a Clonogenic Assay to Determine Endothelial Progenitor Cell Potential / Novelty and Significance. *Circulation Research,* 109**,** 20-37.

MCDERMOTT, M. M., LIU, K., FERRUCCI, L., TIAN, L., GURALNIK, J. M., LIAO, Y. & CRIQUI, M. H. 2011. Decline in Functional Performance Predicts Later Increased Mobility Loss and Mortality in Peripheral Arterial Disease. *J Am Coll Cardiol,* 57**,** 962-970.

MEILINGER, D., FELLINGER, K., BULTMANN, S., ROTHBAUER, U., BONAPACE, I. M., KLINKERT, W. E. F., SPADA, F. & LEONHARDT, H. 2009. Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. *EMBO Rep,* 10**,** 1259-1264.

MELTON, C. & BLELLOCH, R. 2010. MicroRNA Regulation of Embryonic Stem Cell Self-Renewal and Differentiation. *Adv Exp Med Biol,* 695**,** 105-17.

MEMBERS, W. C., ANTMAN, E. M., ANBE, D. T., ARMSTRONG, P. W., BATES, E. R., GREEN, L. A., HAND, M., HOCHMAN, J. S., KRUMHOLZ, H. M., KUSHNER, F. G., LAMAS, G. A., MULLANY, C. J., ORNATO, J. P., PEARLE, D. L., SLOAN, M. A., SMITH, S. C., ALPERT, J. S., ANDERSON, J. L., FAXON, D. P., FUSTER, V., GIBBONS, R. J., GREGORATOS, G., HALPERIN, J. L., HIRATZKA, L. F., HUNT, S. A. & JACOBS, A. K. 2004. ACC/AHA Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction—Executive Summary. *Circulation,* 110**,** 588-636.

MENASCHE, P. 2007. Skeletal myoblasts as a therapeutic agent. *Progress in Cardiovascular Diseases,* 50**,** 7-17.

MENASCHÉ, P., ALFIERI, O., JANSSENS, S., MCKENNA, W., REICHENSPURNER, H., TRINQUART, L., VILQUIN, J.-T., MAROLLEAU, J.-P., SEYMOUR, B., LARGHERO, J., LAKE, S., CHATELLIER, G., SOLOMON, S., DESNOS, M. & HAGÈGE, A. A. 2008. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) Trial. *Circulation,* 117**,** 1189-1200.

MENASCHÉ, P., HAGÈGE, A. A., VILQUIN, J.-T., DESNOS, M., ABERGEL, E., POUZET, B., BEL, A., SARATEANU, S., SCORSIN, M., SCHWARTZ, K., BRUNEVAL, P., BENBUNAN, M., MAROLLEAU, J.-P. & DUBOC, D. 2003. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *Journal of the American College of Cardiology,* 41**,** 1078-1083.

MENDELL, J. T. 2005. MicroRNAs: Critical Regulators of Development, Cellular Physiology and Malignancy. *Cell Cycle,* 4**,** 1179-1184.

MIYAMOTO, K., NISHIGAMI, K., NAGAYA, N., AKUTSU, K., CHIKU, M., KAMEI, M., SOMA, T., MIYATA, S., HIGASHI, M., TANAKA, R., NAKATANI, T., NONOGI, H. & TAKESHITA, S. 2006. Unblinded Pilot Study of Autologous Transplantation of Bone Marrow Mononuclear Cells in Patients With Thromboangiitis Obliterans. *Circulation,* 114**,** 2679-2684.

MIYAMOTO, M., YASUTAKE, M., TAKANO, H., TAKAGI, H., TAKAGI, G., MIZUNO, H., KUMITA, S. & TAKANO, T. 2004. Therapeutic angiogenesis by autologous bone marrow cell implantation for refractory chronic peripheral arterial disease using assessment of neovascularization by 99mTc-tetrofosmin (TF) perfusion scintigraphy. *Cell Transplant,* 13**,** 429-37.

MODLICH, U., NAVARRO, S., ZYCHLINSKI, D., MAETZIG, T., KNOESS, S., BRUGMAN, M. H., SCHAMBACH, A., CHARRIER, S., GALY, A., THRASHER, A. J., BUEREN, J. & BAUM, C. 2009. Insertional Transformation of Hematopoietic Cells by Self-inactivating Lentiviral and Gammaretroviral Vectors. *Mol Ther,* 17**,** 1919-1928.

MONAGHAN, A. P., KAESTNER, K. H., GRAU, E. & SCHUTZ, G. 1993. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development,* 119**,** 567-578.

MOORE, J. C., FU, J., CHAN, Y.-C., LIN, D., TRAN, H., TSE, H.-F. & LI, R. A. 2008. Distinct cardiogenic preferences of two human embryonic stem cell (hESC) lines are imprinted in their proteomes in the pluripotent state. *Biochemical and Biophysical Research Communications,* 372**,** 553-558.

MUMMERY, C., WARD-VAN OOSTWAARD, D., DOEVENDANS, P., SPIJKER, R., VAN DEN BRINK, S., HASSINK, R., VAN DER HEYDEN, M., OPTHOF, T., PERA, M., DE LA RIVIERE, A. B., PASSIER, R. & TERTOOLEN, L. 2003. Differentiation of Human Embryonic Stem Cells to Cardiomyocytes. *Circulation,* 107**,** 2733-2740.

MUMMERY, C. L., VAN ACHTERBERG, T. A. E., VAN DEN EIJNDEN-VAN RAAIJ, A. J. M., VAN HAASTER, L., WILLEMSE, A., DE LAAT, S. W. & PIERSMA, A. H. 1991. Visceral-endoderm-like cell lines induce differentiation of murine P19 embryonal carcinoma cells. *Differentiation,* 46**,** 51-60.

MURRY, C. E. & KELLER, G. 2008. Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. *Cell,* 132**,** 661-680.

MURRY, C. E., REINECKE, H. & PABON, L. M. 2006. Regeneration Gaps: Observations on Stem Cells and Cardiac Repair. *Journal of the American College of Cardiology,* 47**,** 1777-1785.

MURRY, C. E., SOONPAA, M. H., REINECKE, H., NAKAJIMA, H., NAKAJIMA, H. O., RUBART, M., PASUMARTHI, K. B. S., ISMAIL VIRAG, J., BARTELMEZ, S. H., POPPA, V., BRADFORD, G., DOWELL, J. D., WILLIAMS, D. A. & FIELD, L. J. 2004a. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature,* 428**,** 664-668.

MURRY, C. E., SOONPAA, M. H., REINECKE, H., NAKAJIMA, H., NAKAJIMA, H. O., RUBART, M., PASUMARTHI, K. B. S., VIRAG, J. I., BARTELMEZ, S. H., POPPA, V., BRADFORD, G., DOWELL, J. D., WILLIAMS, D. A. & FIELD, L. J. 2004b. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature,* 428**,** 664-668.

NAGA PRASAD, S. V., DUAN, Z.-H., GUPTA, M. K., SURAMPUDI, V. S. K., VOLINIA, S., CALIN, G. A., LIU, C.-G., KOTWAL, A., MORAVEC, C. S., STARLING, R. C., PEREZ, D. M., SEN, S., WU, Q., PLOW, E. F., CROCE, C. M. & KARNIK, S. 2009. Unique MicroRNA Profile in End-stage Heart Failure Indicates Alterations in Specific Cardiovascular Signaling Networks. *Journal of Biological Chemistry,* 284**,** 27487-27499.

NAKATAKE, Y., FUKUI, N., IWAMATSU, Y., MASUI, S., TAKAHASHI, K., YAGI, R., YAGI, K., MIYAZAKI, J.-I., MATOBA, R., KO, M. S. H. & NIWA, H. 2006. Klf4 Cooperates with Oct3/4 and Sox2 To Activate the Lefty1 Core Promoter in Embryonic Stem Cells. *Mol. Cell. Biol.,* 26**,** 7772-7782.

NALDINI, L., BLÖMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F. H., VERMA, I. M. & TRONO, D. 1996. In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. *Science,* 272**,** 263-267.

NARMONEVA, D. A., VUKMIROVIC, R., DAVIS, M. E., KAMM, R. D. & LEE, R. T. 2004. Endothelial Cells Promote Cardiac Myocyte Survival and Spatial Reorganization. *Circulation,* 110**,** 962-968.

NICHOLS, J., ZEVNIK, B., ANASTASSIADIS, K., NIWA, H., KLEWE-NEBENIUS, D., CHAMBERS, I., SCHÖLER, H. & SMITH, A. 1998. Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4. *Cell,* 95**,** 379-391.

NICKLIN, S. A., BUENING, H., DISHART, K. L., DE ALWIS, M., GIROD, A., HACKER, U., THRASHER, A. J., ALI, R. R., HALLEK, M. & BAKER, A. H. 2001. Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. *Mol Ther,* 4**,** 174-81.

NICOLI, S., STANDLEY, C., WALKER, P., HURLSTONE, A., FOGARTY, K. E. & LAWSON, N. D. 2010. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. *Nature,* 464**,** 1196-1200.

NIZANKOWSKI, R., PETRICZEK, T., SKOTNICKI, A. & SZCZEKLIK, A. 2005. The treatment of advanced chronic lower limb ischaemia with marrow stem cell autotransplantation. *Kardiol Pol,* 63**,** 351-60; discussion 361.

NOURSE, M. B., HALPIN, D. E., SCATENA, M., MORTISEN, D. J., TULLOCH, N. L., HAUCH, K. D., TOROK-STORB, B., RATNER, B. D., PABON, L. & MURRY, C. E. 2010. VEGF Induces Differentiation of Functional Endothelium From Human Embryonic Stem Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 30**,** 80-89.

O'DONNELL, K. A., WENTZEL, E. A., ZELLER, K. I., DANG, C. V. & MENDELL, J. T. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature,* 435**,** 839-843.

OH, H., CHI, X., BRADFUTE, S. B., MISHINA, Y., POCIUS, J., MICHAEL, L. H., BEHRINGER, R. R., SCHWARTZ, R. J., ENTMAN, M. L. & SCHNEIDER, M. D. 2004. Cardiac Muscle Plasticity in Adult and Embryo by Heart-Derived Progenitor Cells. *Annals of the New York Academy of Sciences,* 1015**,** 182-189.

OHTANI, K., VLACHOJANNIS, G. J., KOYANAGI, M., BOECKEL, J.-N., URBICH, C., FARCAS, R., BONIG, H., MARQUEZ, V. E., ZEIHER, A. M. & DIMMELER, S. 2011. Epigenetic Regulation of Endothelial Lineage Committed Genes in Pro-Angiogenic Hematopoietic and Endothelial Progenitor Cells / Novelty and Significance. *Circulation Research,* 109**,** 1219-1229.

OHTSUKA, S. & DALTON, S. 2008. Molecular and biological properties of pluripotent embryonic stem cells. *Gene Ther,* 15**,** 74-81.

OLIVIERI, F., ANTONICELLI, R., LORENZI, M., D'ALESSANDRA, Y., LAZZARINI, R., SANTINI, G., SPAZZAFUMO, L., LISA, R., LA SALA, L., GALEAZZI, R.,

RECCHIONI, R., TESTA, R., POMPILIO, G., CAPOGROSSI, M. C. & PROCOPIO, A. D. 2012. Diagnostic potential of circulating miR-499-5p in elderly patients with acute non ST-elevation myocardial infarction. *Int J Cardiol*.

ORLIC, D., KAJSTURA, J., CHIMENTI, S., JAKONIUK, I., ANDERSON, S. M., LI, B., PICKEL, J., MCKAY, R., NADAL-GINARD, B., BODINE, D. M., LERI, A. & ANVERSA, P. 2001. Bone marrow cells regenerate infarcted myocardium. *Nature,* 410**,** 701-705.

PAIGE, S. L., OSUGI, T., AFANASIEV, O. K., PABON, L., REINECKE, H. & MURRY, C. E. 2010. Endogenous Wnt/β-Catenin Signaling Is Required for Cardiac Differentiation in Human Embryonic Stem Cells. *PLoS ONE,* 5**,** e11134.

PAL, R., TOTEY, S., MAMIDI, M. K., BHAT, V. S. & TOTEY, S. 2009. Propensity of Human Embryonic Stem Cell Lines During Early Stage of Lineage Specification Controls Their Terminal Differentiation into Mature Cell Types. *Experimental Biology and Medicine,* 234**,** 1230-1243.

PARK, Y. J., HERMAN, H., GAO, Y., LINDROTH, A. M., HU, B. Y., MURPHY, P. J., PUTNAM, J. R. & SOLOWAY, P. D. 2012. Sequences Sufficient for Programming Imprinted Germline DNA Methylation Defined. *PLoS ONE,* 7**,** e33024.

PAUL, C. P., GOOD, P. D., WINER, I. & ENGELKE, D. R. 2002. Effective expression of small interfering RNA in human cells. *Nat Biotech,* 20**,** 505-508.

PENG, L., XIE, D. Y., LIN, B. L., LIU, J., ZHU, H. P., XIE, C., ZHENG, Y. B. & GAO, Z. L. 2011. Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. *Hepatology,* 54**,** 820-8.

PFEIFFER, S., LEOPOLD, E., SCHMIDT, K., BRUNNER, F. & MAYER, B. 1996. Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. *Br J Pharmacol,* 118**,** 1433-40.

PFISTER, O., OIKONOMOPOULOS, A., SERETI, K.-I., SOHN, R. L., CULLEN, D., FINE, G. C., MOUQUET, F., WESTERMAN, K. & LIAO, R. 2008. Role of the ATP-Binding Cassette Transporter Abcg2 in the Phenotype and Function of Cardiac Side Population Cells. *Circulation Research,* 103**,** 825-835.

PORRELLO, E. R., MAHMOUD, A. I., SIMPSON, E., HILL, J. A., RICHARDSON, J. A., OLSON, E. N. & SADEK, H. A. 2011. Transient Regenerative Potential of the Neonatal Mouse Heart. *Science,* 331**,** 1078-1080.

POSS, K. D., WILSON, L. G. & KEATING, M. T. 2002. Heart Regeneration in Zebrafish. *Science,* 298**,** 2188-2190.

POZZOLI, O., VELLA, P., IAFFALDANO, G., PARENTE, V., DEVANNA, P., LACOVICH, M., LAMIA, C. L., FASCIO, U., LONGONI, D., COTELLI, F., CAPOGROSSI, M. C. & PESCE, M. 2011. Endothelial Fate and Angiogenic Properties of Human CD34+ Progenitor Cells in Zebrafish. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 31**,** 1589-1597.

PRADO-LOPEZ, S., CONESA, A., ARMIÑÁN, A., MARTÍNEZ-LOSA, M., ESCOBEDO-LUCEA, C., GANDIA, C., TARAZONA, S., MELGUIZO, D., BLESA, D., MONTANER, D., SANZ-GONZÁLEZ, S., SEPÚLVEDA, P., GÖTZ, S., O'CONNOR, J. E., MORENO, R., DOPAZO, J., BURKS, D. J. & STOJKOVIC, M. 2010. Hypoxia Promotes Efficient Differentiation of Human Embryonic Stem Cells to Functional Endothelium. *Stem Cells,* 28**,** 407-418.

PRAY-GRANT, M. G., DANIEL, J. A., SCHIELTZ, D., YATES, J. R. & GRANT, P. A. 2005. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature,* 433**,** 434-438.

PROCHAZKA, V., GUMULEC, J., CHMELOVA, J., KLEMENT, P., KLEMENT, G. L., JONSZTA, T., CZERNY, D. & KRAJCA, J. 2009. Autologous bone marrow stem cell transplantation in patients with end-stage chronical critical limb ischemia and diabetic foot. *Vnitr Lek,* 55**,** 173-8.

QI, J., YU, J. Y., SHCHERBATA, H. R., MATHIEU, J., WANG, A. J., SEAL, S., ZHOU, W., STADLER, B. M., BOURGIN, D., WANG, L., NELSON, A., WARE, C., RAYMOND, C., LIM, L. P., MAGNUS, J., IVANOVSKA, I., DIAZ, R., BALL, A., CLEARY, M. A. & RUOHOLA-BAKER, H. 2009. microRNAs regulate human embryonic stem cell division. *Cell Cycle,* 8**,** 3729-41.

QIU, C., MA, Y., WANG, J., PENG, S. & HUANG, Y. 2010. Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells. *Nucleic Acids Research,* 38**,** 1240-1248.

RAO, P. K., KUMAR, R. M., FARKHONDEH, M., BASKERVILLE, S. & LODISH, H. F. 2006. Myogenic factors that regulate expression of muscle-specific microRNAs. *Proceedings of the National Academy of Sciences,* 103**,** 8721-8726.

REUBINOFF, B. E., ITSYKSON, P., TURETSKY, T., PERA, M. F., REINHARTZ, E., ITZIK, A. & BEN-HUR, T. 2001. Neural progenitors from human embryonic stem cells. *Nat Biotech,* 19**,** 1134-1140.

REUBINOFF, B. E., PERA, M. F., FONG, C.-Y., TROUNSON, A. & BONGSO, A. 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotech,* 18**,** 399-404.

RINGROSE, L., EHRET, H. & PARO, R. 2004. Distinct Contributions of Histone H3 Lysine 9 and 27 Methylation to Locus-Specific Stability of Polycomb Complexes. *Molecular Cell,* 16**,** 641-653.

RINGROSE, L. & PARO, R. 2004. EPIGENETIC REGULATION OF CELLULAR MEMORY BY THE POLYCOMB AND TRITHORAX GROUP PROTEINS. *Annual Review of Genetics,* 38**,** 413-443.

ROBLESS, P., MIKHAILIDIS, D. P. & STANSBY, G. P. 2007. Cilostazol for peripheral arterial disease. *Cochrane Database Syst Rev***,** CD003748.

RODDA, D. J., CHEW, J.-L., LIM, L.-H., LOH, Y.-H., WANG, B., NG, H.-H. & ROBSON, P. 2005. Transcriptional Regulation of Nanog by OCT4 and SOX2. *Journal of Biological Chemistry,* 280**,** 24731-24737.

ROONEY, G. E., NISTOR, G. I., BARRY, F. B. & KEIRSTEAD, H. S. 2010. In vitro differentiation potential of human embryonic versus adult stem cells. *Regenerative Medicine,* 5**,** 365-379.

RUBART, M. & FIELD, L. J. 2006. CARDIAC REGENERATION: Repopulating the Heart. *Annual Review of Physiology,* 68**,** 29-49.

RUO, B., LIU, K., TIAN, L., TAN, J., FERRUCCI, L., GURALNIK, J. M. & MCDERMOTT, M. M. 2007. Persistent Depressive Symptoms and Functional Decline Among Patients With Peripheral Arterial Disease. *Psychosomatic Medicine,* 69**,** 415-424.

RYBAK, A., FUCHS, H., SMIRNOVA, L., BRANDT, C., POHL, E. E., NITSCH, R. & WULCZYN, F. G. 2008. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat Cell Biol,* 10**,** 987-993.

SAIGAWA, T., KATO, K., OZAWA, T., TOBA, K., MAKIYAMA, Y., MINAGAWA, S., HASHIMOTO, S., FURUKAWA, T., NAKAMURA, Y., HANAWA, H., KODAMA, M., YOSHIMURA, N., FUJIWARA, H., NAMURA, O., SOGAWA, M., HAYASHI, J.-I. & AIZAWA, Y. 2004. Clinical Application of Bone Marrow Implantation in Patients With Arteriosclerosis Obliterans, and the Association Between Efficacy and the Number of Implanted Bone Marrow Cells. *Circulation Journal,* 68**,** 1189-1193.

SANTOS-ROSA, H., SCHNEIDER, R., BERNSTEIN, B. E., KARABETSOU, N., MORILLON, A., WEISE, C., SCHREIBER, S. L., MELLOR, J. & KOUZARIDES, T. 2003. Methylation of Histone H3 K4 Mediates Association of the Isw1p ATPase with Chromatin. *Molecular Cell,* 12**,** 1325-1332.

SASAKI, H. & HOGAN, B. L. 1993. Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development,* 118**,** 47-59.

SCARAMUZZA, S., BIASCO, L., RIPAMONTI, A., CASTIELLO, M. C., LOPERFIDO, M., DRAGHICI, E., HERNANDEZ, R. J., BENEDICENTI, F., RADRIZZANI, M., SALOMONI, M., RANZANI, M., BARTHOLOMAE, C. C., VICENZI, E., FINOCCHI, A., BREDIUS, R., BOSTICARDO, M., SCHMIDT, M., VON KALLE, C., MONTINI, E., BIFFI, A., RONCAROLO, M. G., NALDINI, L., VILLA, A. & AIUTI, A. 2012. Preclinical Safety and Efficacy of Human CD34+ Cells Transduced With Lentiviral Vector for the Treatment of Wiskott-Aldrich Syndrome. *Mol Ther*.

SCHRÖDER, A. R. W., SHINN, P., CHEN, H., BERRY, C., ECKER, J. R. & BUSHMAN, F. 2002. HIV-1 Integration in the Human Genome Favors Active Genes and Local Hotspots. *Cell,* 110**,** 521-529.

SCHULTHEISS, T. M., BURCH, J. B. & LASSAR, A. B. 1997. A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes & Development,* 11**,** 451-462.

SCHWARTZ, S. D., HUBSCHMAN, J. P., HEILWELL, G., FRANCO-CARDENAS, V., PAN, C. K., OSTRICK, R. M., MICKUNAS, E., GAY, R., KLIMANSKAYA, I. & LANZA, R. 2012. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet,* 379**,** 713-20.

SCOTT, C. & HUGGETT, B. 2012. Geron's quixotic fate. *Nat Biotech,* 30**,** 497-497.

SCOUMANNE, A., KALAMATI, T., MOSS, J., POWELL, J. T., GOSLING, M. & CAREY, N. 2002. Generation and characterisation of human saphenous vein endothelial cell lines. *Atherosclerosis,* 160**,** 59-67.

SEGANTI, L., SUPERTI, F., GIRMENIA, C., MELUCCI, L. & ORSI, N. 1986. Study of Receptors for Vesicular Stomatitis-Virus in Vertebrate and Invertebrate Cells. *Microbiologica,* 9**,** 259-267.

SEGERS, V. F. & LEE, R. T. 2008. Stem-cell therapy for cardiac disease. *Nature,* 451**,** 937-42.

SELBACH, M., SCHWANHAUSSER, B., THIERFELDER, N., FANG, Z., KHANIN, R. & RAJEWSKY, N. 2008. Widespread changes in protein synthesis induced by microRNAs. *Nature,* 455**,** 58-63.

SENGUPTA, S., NIE, J., WAGNER, R. J., YANG, C., STEWART, R. & THOMSON, J. A. 2009. MicroRNA 92b Controls the G1/S Checkpoint Gene p57 in Human Embryonic Stem Cells. *Stem Cells,* 27**,** 1524-1528.

SHAMBLOTT, M. J., AXELMAN, J., WANG, S., BUGG, E. M., LITTLEFIELD, J. W., DONOVAN, P. J., BLUMENTHAL, P. D., HUGGINS, G. R. & GEARHART, J. D. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proceedings of the National Academy of Sciences,* 95**,** 13726-13731.

SHOWELL, C., BINDER, O. & CONLON, F. L. 2004. T-box genes in early embryogenesis. *Developmental Dynamics,* 229**,** 201-218.

SIDHU, K. S., WALKE, S. & TUCH, B. E. 2008. Derivation and propagation of hESC under a therapeutic environment. *Curr Protoc Stem Cell Biol,* Chapter 1**,** Unit 1A 4.

SILVA, J., NICHOLS, J., THEUNISSEN, T. W., GUO, G., VAN OOSTEN, A. L., BARRANDON, O., WRAY, J., YAMANAKA, S., CHAMBERS, I. & SMITH, A.

2009. Nanog Is the Gateway to the Pluripotent Ground State. *Cell,* 138**,** 722-737.

SIMS, R. J., CHEN, C.-F., SANTOS-ROSA, H., KOUZARIDES, T., PATEL, S. S. & REINBERG, D. 2005. Human but Not Yeast CHD1 Binds Directly and Selectively to Histone H3 Methylated at Lysine 4 via Its Tandem Chromodomains. *Journal of Biological Chemistry,* 280**,** 41789-41792.

SINKKONEN, L., HUGENSCHMIDT, T., BERNINGER, P., GAIDATZIS, D., MOHN, F., ARTUS-REVEL, C. G., ZAVOLAN, M., SVOBODA, P. & FILIPOWICZ, W. 2008. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat Struct Mol Biol,* 15**,** 259-267.

SINN, P. L., SAUTER, S. L. & MCCRAY, P. B., JR. 2005. Gene Therapy Progress and Prospects: Development of improved lentiviral and retroviral vectors - design, biosafety, and production. *Gene Ther,* 12**,** 1089-1098.

SLABY, O., REDOVA, M., POPRACH, A., NEKVINDOVA, J., ILIEV, R., RADOVA, L., LAKOMY, R., SVOBODA, M. & VYZULA, R. 2012. Identification of MicroRNAs associated with early relapse after nephrectomy in renal cell carcinoma patients. *Genes, Chromosomes and Cancer,* 51**,** 707-716.

SLUIJTER, J. P. G., VAN MIL, A., VAN VLIET, P., METZ, C. H. G., LIU, J., DOEVENDANS, P. A. & GOUMANS, M.-J. 2010. MicroRNA-1 and -499 Regulate Differentiation and Proliferation in Human-Derived Cardiomyocyte Progenitor Cells. *Arterioscler Thromb Vasc Biol,* 30**,** 859-868.

SMALE, S. T. 2010. Pioneer factors in embryonic stem cells and differentiation. *Current Opinion in Genetics & Development,* 20**,** 519-526.

SMITH, K. N., SINGH, A. M. & DALTON, S. 2010. Myc Represses Primitive Endoderm Differentiation in Pluripotent Stem Cells. *Cell Stem Cell,* 7**,** 343-354.

SONE, M., ITOH, H., YAMAHARA, K., YAMASHITA, J. K., YURUGI-KOBAYASHI, T., NONOGUCHI, A., SUZUKI, Y., CHAO, T.-H., SAWADA, N., FUKUNAGA, Y., MIYASHITA, K., PARK, K., OYAMADA, N., SAWADA, N., TAURA, D., TAMURA, N., KONDO, Y., NITO, S., SUEMORI, H., NAKATSUJI, N., NISHIKAWA, S.-I. & NAKAO, K. 2007. Pathway for Differentiation of Human Embryonic Stem Cells to Vascular Cell Components and Their Potential for Vascular Regeneration. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 27**,** 2127-2134.

SOONPAA, M. H. & FIELD, L. J. 1998. Survey of Studies Examining Mammalian Cardiomyocyte DNA Synthesis. *Circulation Research,* 83**,** 15-26.

SORRENTINO, S. A., BAHLMANN, F. H., BESLER, C., MÜLLER, M., SCHULZ, S., KIRCHHOFF, N., DOERRIES, C., HORVÁTH, T., LIMBOURG, A., LIMBOURG, F., FLISER, D., HALLER, H., DREXLER, H. & LANDMESSER, U. 2007. Oxidant Stress Impairs In Vivo Reendothelialization Capacity of Endothelial Progenitor Cells From Patients With Type 2 Diabetes Mellitus. *Circulation,* 116**,** 163-173.

SPINETTI, G., KRAENKEL, N., EMANUELI, C. & MADEDDU, P. 2008. Diabetes and vessel wall remodelling: from mechanistic insights to regenerative therapies. *Cardiovascular Research,* 78**,** 265-273.

STADLER, B., IVANOVSKA, I., MEHTA, K., SONG, S., NELSON, A., TAN, Y., MATHIEU, J., DARBY, C., BLAU, C. A., WARE, C., PETERS, G., MILLER, D. G., SHEN, L., CLEARY, M. A. & RUOHOLA-BAKER, H. 2010. Characterization of microRNAs Involved in Embryonic Stem Cell States. *Stem Cells and Development,* 19**,** 935-950.

STAMM, C., KLEINE, H.-D., CHOI, Y.-H., DUNKELMANN, S., LAUFFS, J.-A., LORENZEN, B., DAVID, A., LIEBOLD, A., NIENABER, C., ZURAKOWSKI, D., FREUND, M. & STEINHOFF, G. 2007. Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: Safety and efficacy studies. *The Journal of thoracic and cardiovascular surgery,* 133**,** 717-725.e5.

STEINHAUSER, M. L. & LEE, R. T. 2011. Regeneration of the heart. *EMBO Molecular Medicine,* 3**,** 701-712.

STOLL, M., STECKELINGS, U. M., PAUL, M., BOTTARI, S. P., METZGER, R. & UNGER, T. 1995. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *The Journal of Clinical Investigation,* 95**,** 651-657.

STRAHL, B. D. & ALLIS, C. D. 2000. The language of covalent histone modifications. *Nature,* 403**,** 41-45.

STRAUER, B. E., YOUSEF, M. & SCHANNWELL, C. M. 2010. The acute and long-term effects of intracoronary Stem cell Transplantation in 191 patients with chronic heARt failure: The STAR-heart study. *European Journal of Heart Failure,* 12**,** 721-729.

SUAREZ, Y., FERNANDEZ-HERNANDO, C., POBER, J. S. & SESSA, W. C. 2007. Dicer Dependent MicroRNAs Regulate Gene Expression and Functions in Human Endothelial Cells. *Circ Res,* 100**,** 1164-1173.

SUH, M.-R., LEE, Y., KIM, J. Y., KIM, S.-K., MOON, S.-H., LEE, J. Y., CHA, K.-Y., CHUNG, H. M., YOON, H. S., MOON, S. Y., KIM, V. N. & KIM, K.-S. 2004. Human embryonic stem cells express a unique set of microRNAs. *Developmental Biology,* 270**,** 488-498.

SUN, X., ICLI, B., WARA, A. K., BELKIN, N., HE, S., KOBZIK, L., HUNNINGHAKE, G. M., VERA, M. P., BLACKWELL, T. S., BARON, R. M. & FEINBERG, M. W. 2012. MicroRNA-181b regulates NF-κB–mediated vascular inflammation. *The Journal of Clinical Investigation,* 122**,** 1973-1990.

SUÁREZ, Y., FERNÁNDEZ-HERNANDO, C., POBER, J. S. & SESSA, W. C. 2007. Dicer Dependent MicroRNAs Regulate Gene Expression and Functions in Human Endothelial Cells. *Circulation Research,* 100**,** 1164-1173.

SUÁREZ, Y., FERNÁNDEZ-HERNANDO, C., YU, J., GERBER, S. A., HARRISON, K. D., POBER, J. S., IRUELA-ARISPE, M. L., MERKENSCHLAGER, M. & SESSA, W. C. 2008. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proceedings of the National Academy of Sciences,* 105**,** 14082-14087.

SÜRDER, D., SCHWITTER, J., MOCCETTI, T., ASTORI, G., RUFIBACH, K., PLEIN, S., CICERO, V. L., SONCIN, S., WINDECKER, S., MOSCHOVITIS, A., WAHL, A., ERNE, P., JAMSHIDI, P., AUF DER MAUR, C., MANKA, R., SOLDATI, G., BÜHLER, I., WYSS, C., LANDMESSER, U., LÜSCHER, T. F. & CORTI, R. 2010. Cell-based therapy for myocardial repair in patients with acute myocardial infarction: Rationale and study design of the SWiss multicenter Intracoronary Stem cells Study in Acute Myocardial Infarction (SWISS-AMI). *American Heart Journal,* 160**,** 58-64.

TAGUCHI, A., YANAGISAWA, K., TANAKA, M., CAO, K., MATSUYAMA, Y., GOTO, H. & TAKAHASHI, T. 2008. Identification of Hypoxia-Inducible Factor-1α as a Novel Target for miR-17-92 MicroRNA Cluster. *Cancer Research,* 68**,** 5540-5545.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell,* 131**,** 861-872.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell,* 126**,** 663-676.

TAKAYA, T., ONO, K., KAWAMURA, T., TAKANABE, R., KAICHI, S., MORIMOTO, T., WADA, H., KITA, T., SHIMATSU, A. & HASEGAWA, K. 2009a. MicroRNA-1 and MicroRNA-133 in Spontaneous Myocardial Differentiation of Mouse Embryonic Stem Cells. *Circulation Journal,* 73**,** 1492-1497.

TAKAYA, T., ONO, K., KAWAMURA, T., TAKANABE, R., KAICHI, S., MORIMOTO, T., WADA, H., KITA, T., SHIMATSU, A. & HASEGAWA, K. 2009b. MicroRNA-1 and MicroRNA-133 in spontaneous myocardial differentiation of mouse embryonic stem cells. *Circulation journal : official journal of the Japanese Circulation Society,* 73**,** 1492-7.

TAKAYAMA, K., INAMURA, M., KAWABATA, K., SUGAWARA, M., KIKUCHI, K., HIGUCHI, M., NAGAMOTO, Y., WATANABE, H., TASHIRO, K., SAKURAI, F., HAYAKAWA, T., FURUE, M. K. & MIZUGUCHI, H. 2012. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1alpha transduction. *J Hepatol*.

TALLINI, Y. N., GREENE, K. S., CRAVEN, M., SPEALMAN, A., BREITBACH, M., SMITH, J., FISHER, P. J., STEFFEY, M., HESSE, M., DORAN, R. M., WOODS, A., SINGH, B., YEN, A., FLEISCHMANN, B. K. & KOTLIKOFF, M. I. 2009. c-kit expression identifies cardiovascular precursors in the neonatal heart. *Proceedings of the National Academy of Sciences*.

TANG, C., LEE, A. S., VOLKMER, J. P., SAHOO, D., NAG, D., MOSLEY, A. R., INLAY, M. A., ARDEHALI, R., CHAVEZ, S. L., PERA, R. R., BEHR, B., WU, J. C., WEISSMAN, I. L. & DRUKKER, M. 2011. An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat Biotechnol,* 29**,** 829-34.

TANIC, M., ZAJAC, M., GOMEZ-LOPEZ, G., BENITEZ, J. & MARTINEZ-DELGADO, B. 2011. Integration of BRCA1-mediated miRNA and mRNA profiles reveals microRNA regulation of TRAF2 and NFkappaB pathway. *Breast Cancer Res Treat*.

TATEISHI-YUYAMA, E., MATSUBARA, H., MUROHARA, T., IKEDA, U., SHINTANI, S., MASAKI, H., AMANO, K., KISHIMOTO, Y., YOSHIMOTO, K., AKASHI, H., SHIMADA, K., IWASAKA, T. & IMAIZUMI, T. 2002. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *The Lancet,* 360**,** 427-435.

TAY, Y., ZHANG, J., THOMSON, A. M., LIM, B. & RIGOUTSOS, I. 2008. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature,* 455**,** 1124-1128.

THOMSON, J. A., ITSKOVITZ-ELDOR, J., SHAPIRO, S. S., WAKNITZ, M. A., SWIERGIEL, J. J., MARSHALL, V. S. & JONES, J. M. 1998. Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science,* 282**,** 1145-1147.

TRACHTENBERG, B., VELAZQUEZ, D. L., WILLIAMS, A. R., MCNIECE, I., FISHMAN, J., NGUYEN, K., ROUY, D., ALTMAN, P., SCHWARZ, R., MENDIZABAL, A., OSKOUEI, B., BYRNES, J., SOTO, V., TRACY, M., ZAMBRANO, J. P., HELDMAN, A. W. & HARE, J. M. 2011. Rationale and design of the Transendocardial Injection of Autologous Human Cells (bone marrow or mesenchymal) in Chronic Ischemic Left Ventricular Dysfunction and Heart Failure Secondary to Myocardial Infarction (TAC-HFT) trial: A randomized, double-blind, placebo-controlled study of safety and efficacy. *American Heart Journal,* 161**,** 487-493.

TRAN, T. H., WANG, X., BROWNE, C., ZHANG, Y., SCHINKE, M., IZUMO, S. & BURCIN, M. 2009. Wnt3a-Induced Mesoderm Formation and Cardiomyogenesis in Human Embryonic Stem Cells. *Stem Cells,* 27**,** 1869-1878.

TRAVERSE, J. H., HENRY, T. D., VAUGHN, D. E., ELLIS, S. G., PEPINE, C. J., WILLERSON, J. T., ZHAO, D. X. M., PILLER, L. B., PENN, M. S., BYRNE, B. J., PERIN, E. C., GEE, A. P., HATZOPOULOS, A. K., MCKENNA, D. H., FORDER, J. R., TAYLOR, D. A., COGLE, C. R., OLSON, R. E., JORGENSON, B. C., SAYRE, S. L., VOJVODIC, R. W., GORDON, D. J., SKARLATOS, S. I., MOYE, L. A. & SIMARI, R. D. 2009. Rationale and design for TIME: A phase II, randomized, double-blind, placebo-controlled pilot trial evaluating the safety and effect of timing of administration of bone marrow mononuclear cells after acute myocardial infarction. *American Heart Journal,* 158**,** 356-363.

TRAVERSE JH, H. T. D. E. S. G. & ET AL. 2011. Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: The latetime randomized trial. *JAMA: The Journal of the American Medical Association,* 306**,** 2110-2119.

TRAVERSE, J. H., MCKENNA, D. H., HARVEY, K., JORGENSO, B. C., OLSON, R. E., BOSTROM, N., KADIDLO, D., LESSER, J. R., JAGADEESAN, V., GARBERICH, R. & HENRY, T. D. 2010. Results of a phase 1, randomized, double-blind, placebo-controlled trial of bone marrow mononuclear stem cell administration in patients following ST-elevation myocardial infarction. *American Heart Journal,* 160**,** 428-434.

TSUKAHARA, H., ENDE, H., MAGAZINE, H. I., BAHOU, W. F. & GOLIGORSKY, M. S. 1994. Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *Journal of Biological Chemistry,* 269**,** 21778-21785.

TZUR, G., LEVY, A., MEIRI, E., BARAD, O., SPECTOR, Y., BENTWICH, Z., MIZRAHI, L., KATZENELLENBOGEN, M., BEN-SHUSHAN, E., REUBINOFF, B. E. & GALUN, E. 2008. MicroRNA Expression Patterns and Function in Endodermal Differentiation of Human Embryonic Stem Cells. *PLoS ONE,* 3**,** e3726.

VAN GELDER, R. N., VON ZASTROW, M. E., YOOL, A., DEMENT, W. C., BARCHAS, J. D. & EBERWINE, J. H. 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proceedings of the National Academy of Sciences,* 87**,** 1663-1667.

VAN ROOIJ, E. & OLSON, E. N. 2007. MicroRNAs: powerful new regulators of heart disease and provocative therapeutic targets. *J Clin Invest,* 117**,** 2369-76.

VAN ROOIJ, E., SUTHERLAND, L. B., LIU, N., WILLIAMS, A. H., MCANALLY, J., GERARD, R. D., RICHARDSON, J. A. & OLSON, E. N. 2006. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proceedings of the National Academy of Sciences,* 103**,** 18255-18260.

VAN ROOIJ, E., SUTHERLAND, L. B., QI, X., RICHARDSON, J. A., HILL, J. & OLSON, E. N. 2007. Control of Stress-Dependent Cardiac Growth and Gene Expression by a MicroRNA. *Science,* 316**,** 575-579.

VAN ROYEN, N., PIEK, J. J., BUSCHMANN, I., HOEFER, I., VOSKUIL, M. & SCHAPER, W. 2001. Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. *Cardiovasc Res,* 49**,** 543-53.

VAN TONGEREN, R. B., HAMMING, J. F., FIBBE, W. E., VAN WEEL, V., FRERICHS, S. J., STIGGELBOUT, A. M., VAN BOCKEL, J. H. & LINDEMAN, J. H. 2008. Intramuscular or combined intramuscular/intra-arterial administration of bone marrow mononuclear cells: a clinical trial in patients with advanced limb ischemia. *J Cardiovasc Surg (Torino),* 49**,** 51-8.

VANDENDRIESSCHE, T., THORREZ, L., NALDINI, L., FOLLENZI, A., MOONS, L., BERNEMAN, Z., COLLEN, D. & CHUAH, M. K. L. 2002. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood,* 100**,** 813-822.

VENTURA, A., YOUNG, A. G., WINSLOW, M. M., LINTAULT, L., MEISSNER, A., ERKELAND, S. J., NEWMAN, J., BRONSON, R. T., CROWLEY, D., STONE, J. R., JAENISCH, R., SHARP, P. A. & JACKS, T. 2008. Targeted Deletion Reveals Essential and Overlapping Functions of the miR-17~92 Family of miRNA Clusters. *Cell,* 132**,** 875-886.

VISWANATHAN, S. R., DALEY, G. Q. & GREGORY, R. I. 2008. Selective Blockade of MicroRNA Processing by Lin28. *Science,* 320**,** 97-100.

VRIESE, A. S. D., BILLIET, J., DROOGENBROECK, J. V., GHEKIERE, J. & LETTER, J. A. D. 2008. Autologous transplantation of bone marrow mononuclear cells for limb ischemia in a caucasian population with atherosclerosis obliterans. *Journal of Internal Medicine,* 263**,** 395-403.

WAKIOKA, T., SASAKI, A., KATO, R., SHOUDA, T., MATSUMOTO, A., MIYOSHI, K., TSUNEOKA, M., KOMIYA, S., BARON, R. & YOSHIMURA, A. 2001. Spred is a Sprouty-related suppressor of Ras signalling. *Nature,* 412**,** 647-651.

WANG, L., LI, L., SHOJAEI, F., LEVAC, K., CERDAN, C., MENENDEZ, P., MARTIN, T., ROULEAU, A. & BHATIA, M. 2004. Endothelial and Hematopoietic Cell Fate of Human Embryonic Stem Cells Originates from Primitive Endothelium with Hemangioblastic Properties. *Immunity,* 21**,** 31-41.

WANG, S., AURORA, A. B., JOHNSON, B. A., QI, X., MCANALLY, J., HILL, J. A., RICHARDSON, J. A., BASSEL-DUBY, R. & OLSON, E. N. 2008a. The Endothelial-Specific MicroRNA miR-126 Governs Vascular Integrity and Angiogenesis. *Developmental Cell,* 15**,** 261-271.

WANG, Y., BASKERVILLE, S., SHENOY, A., BABIARZ, J. E., BAEHNER, L. & BLELLOCH, R. 2008b. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nat Genet,* 40**,** 1478-1483.

WANG, Y., MEDVID, R., MELTON, C., JAENISCH, R. & BLELLOCH, R. 2007a. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet,* 39**,** 380-5.

WANG, Z. Z., AU, P., CHEN, T., SHAO, Y., DAHERON, L. M., BAI, H., ARZIGIAN, M., FUKUMURA, D., JAIN, R. K. & SCADDEN, D. T. 2007b. Endothelial cells derived from human embryonic stem cells form durable blood vessels in vivo. *Nat Biotech,* 25**,** 317-318.

WEBER, F., TERESI, R. E., BROELSCH, C. E., FRILLING, A. & ENG, C. 2006. A Limited Set of Human MicroRNA Is Deregulated in Follicular Thyroid Carcinoma. *J Clin Endocrinol Metab,* 91**,** 3584-3591.

WELLNER, U., SCHUBERT, J., BURK, U. C., SCHMALHOFER, O., ZHU, F., SONNTAG, A., WALDVOGEL, B., VANNIER, C., DARLING, D., HAUSEN, A. Z., BRUNTON, V. G., MORTON, J., SANSOM, O., SCHULER, J., STEMMLER, M. P., HERZBERGER, C., HOPT, U., KECK, T., BRABLETZ, S. & BRABLETZ, T. 2009. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol,* 11**,** 1487-1495.

WESTER, T., JORGENSEN, J. J., STRANDEN, E., SANDBAEK, G., TJONNFJORD, G., BAY, D., KOLLEROS, D., KROESE, A. J. & BRINCHMANN, J. E. 2008. Treatment with autologous bone marrow mononuclear cells in patients with critical lower limb ischaemia. A pilot study. *Scand J Surg,* 97**,** 56-62.

WIGHTMAN, B., HA, I. & RUVKUN, G. 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell,* 75**,** 855-862.

WILKINSON, D. G., BHATT, S. & HERRMANN, B. G. 1990. Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature,* 343**,** 657-659.

WILLERSON, J. T., PERIN, E. C., ELLIS, S. G., PEPINE, C. J., HENRY, T. D., ZHAO, D. X. M., LAI, D., PENN, M. S., BYRNE, B. J., SILVA, G., GEE, A., TRAVERSE, J. H., HATZOPOULOS, A. K., FORDER, J. R., MARTIN, D., KRONENBERG, M., TAYLOR, D. A., COGLE, C. R., BARANIUK, S., WESTBROOK, L., SAYRE, S. L., VOJVODIC, R. W., GORDON, D. J., SKARLATOS, S. I., MOYÉ, L. A. & SIMARI, R. D. 2010. Intramyocardial injection of autologous bone marrow mononuclear cells for patients with chronic ischemic heart disease and left ventricular dysfunction (First Mononuclear Cells injected in the US [FOCUS]): Rationale and design. *American Heart Journal,* 160**,** 215-223.

WILSON, K. D., HU, S., VENKATASUBRAHMANYAM, S., FU, J.-D., SUN, N., ABILEZ, O. J., BAUGH, J. J. A., JIA, F., GHOSH, Z., LI, R. A., BUTTE, A. J. & WU, J. C. 2010. Dynamic MicroRNA Expression Programs During Cardiac Differentiation of Human Embryonic Stem Cells / Clinical Perspective. *Circulation: Cardiovascular Genetics,* 3**,** 426-435.

WINITSKY, S. O., GOPAL, T. V., HASSANZADEH, S., TAKAHASHI, H., GRYDER, D., ROGAWSKI, M. A., TAKEDA, K., YU, Z. X., XU, Y. H. & EPSTEIN, N. D. 2005. Adult Murine Skeletal Muscle Contains Cells That Can Differentiate into Beating Cardiomyocytes In Vitro. *PLoS Biol,* 3**,** e87.

WOLLERT, K. C., MEYER, G. P., LOTZ, J., RINGES LICHTENBERG, S., LIPPOLT, P., BREIDENBACH, C., FICHTNER, S., KORTE, T., HORNIG, B., MESSINGER, D., ARSENIEV, L., HERTENSTEIN, B., GANSER, A. & DREXLER, H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *The Lancet,* 364**,** 141-148.

WOLLERT, K. C., MEYER, G. P., LOTZ, J., RINGES-LICHTENBERG, S., LIPPOLT, P., BREIDENBACH, C., FICHTNER, S., KORTE, T., HORNIG, B., MESSINGER, D., ARSENIEV, L., HERTENSTEIN, B., GANSER, A. & DREXLER, H. 2004. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet,* 364**,** 141-8.

WU, H., XU, J., PANG, Z. P., GE, W., KIM, K. J., BLANCHI, B., CHEN, C., SÜDHOF, T. C. & SUN, Y. E. 2007. Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines. *Proceedings of the National Academy of Sciences,* 104**,** 13821-13826.

WYSOCKA, J., SWIGUT, T., MILNE, T. A., DOU, Y., ZHANG, X., BURLINGAME, A. L., ROEDER, R. G., BRIVANLOU, A. H. & ALLIS, C. D. 2005. WDR5 Associates with Histone H3 Methylated at K4 and Is Essential for H3 K4 Methylation and Vertebrate Development. *Cell,* 121**,** 859-872.

WÜRDINGER, T., TANNOUS, B. A., SAYDAM, O., SKOG, J., GRAU, S., SOUTSCHEK, J., WEISSLEDER, R., BREAKEFIELD, X. O. & KRICHEVSKY, A. M. 2008. miR-296 Regulates Growth Factor Receptor Overexpression in Angiogenic Endothelial Cells. *Cancer Cell,* 14**,** 382-393.

XIA, X., ZHANG, Y., ZIETH, C. R. & ZHANG, S. C. 2007. Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner. *Stem Cells Dev,* 16**,** 167-76.

XU, C., POLICE, S., HASSANIPOUR, M., LI, Y., CHEN, Y., PRIEST, C., O'SULLIVAN, C., LAFLAMME, M. A., ZHU, W.-Z., VAN BIBER, B., HEGEROVA, L., YANG, J., DELAVAN-BOORSMA, K., DAVIES, A., LEBKOWSKI, J. & GOLD, J. D. 2010. Efficient generation and cryopreservation of cardiomyocytes derived from human embryonic stem cells. *Regenerative Medicine,* 6**,** 53-66.

XU, C., POLICE, S., RAO, N. & CARPENTER, M. K. 2002. Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells. *Circulation Research,* 91**,** 501-508.

XU, N., PAPAGIANNAKOPOULOS, T., PAN, G., THOMSON, J. A. & KOSIK, K. S. 2009. MicroRNA-145 Regulates OCT4, SOX2, and KLF4 and Represses Pluripotency in Human Embryonic Stem Cells. *Cell,* 137**,** 647-658.

YAM, P. Y., LI, S., WU, J., HU, J., ZAIA, J. A. & YEE, J. K. 2002. Design of HIV vectors for efficient gene delivery into human hematopoietic cells. *Mol Ther,* 5**,** 479-84.

YAMAGUCHI, T. P., DUMONT, D. J., CONLON, R. A., BREITMAN, M. L. & ROSSANT, J. 1993. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development,* 118**,** 489-498.

YAMAHARA, K., SONE, M., ITOH, H., YAMASHITA, J. K., YURUGI-KOBAYASHI, T., HOMMA, K., CHAO, T.-H., MIYASHITA, K., PARK, K., OYAMADA, N., SAWADA, N., TAURA, D., FUKUNAGA, Y., TAMURA, N. & NAKAO, K. 2008. Augmentation of Neovascularizaiton in Hindlimb Ischemia by Combined Transplantation of Human Embryonic Stem Cells-Derived Endothelial and Mural Cells. *PLoS ONE,* 3**,** e1666.

YANG, L., SOONPAA, M. H., ADLER, E. D., ROEPKE, T. K., KATTMAN, S. J., KENNEDY, M., HENCKAERTS, E., BONHAM, K., ABBOTT, G. W., LINDEN, R. M., FIELD, L. J. & KELLER, G. M. 2008. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature,* 453**,** 524-528.

YANG, W. J., YANG, D. D., NA, S., SANDUSKY, G. E., ZHANG, Q. & ZHAO, G. 2005. Dicer Is Required for Embryonic Angiogenesis during Mouse Development. *Journal of Biological Chemistry,* 280**,** 9330-9335.

YOUNG, P. P., VAUGHAN, D. E. & HATZOPOULOS, A. K. 2007. Biologic properties of endothelial progenitor cells and their potential for cell therapy. *Progress in Cardiovascular Diseases,* 49**,** 421-429.

YU, J., VODYANIK, M. A., SMUGA-OTTO, K., ANTOSIEWICZ-BOURGET, J., FRANE, J. L., TIAN, S., NIE, J., JONSDOTTIR, G. A., RUOTTI, V., STEWART, R., SLUKVIN, I. I. & THOMSON, J. A. 2007. Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science,* 318**,** 1917-1920.

YUNG, S., LEDRAN, M., MORENO-GIMENO, I., CONESA, A., MONTANER, D., DOPAZO, J., DIMMICK, I., SLATER, N. J., MARENAH, L., REAL, P. J., PARASKEVOPOULOU, I., BISBAL, V., BURKS, D., SANTIBANEZ-KOREF, M., MORENO, R., MOUNTFORD, J., MENENDEZ, P., ARMSTRONG, L. & LAKO, M. 2011. Large-scale transcriptional profiling and functional assays reveal important roles for Rho-GTPase signalling and SCL during haematopoietic differentiation of human embryonic stem cells. *Human Molecular Genetics.*

ZHANG, S.-C., WERNIG, M., DUNCAN, I. D., BRUSTLE, O. & THOMSON, J. A. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotech,* 19**,** 1129-1133.

ZHAO, T., ZHANG, Z.-N., RONG, Z. & XU, Y. 2011. Immunogenicity of induced pluripotent stem cells. *Nature,* 474**,** 212-215.

ZHAO, Y., RANSOM, J. F., LI, A., VEDANTHAM, V., VON DREHLE, M., MUTH, A. N., TSUCHIHASHI, T., MCMANUS, M. T., SCHWARTZ, R. J. & SRIVASTAVA, D. 2007a. Dysregulation of Cardiogenesis, Cardiac Conduction, and Cell Cycle in Mice Lacking miRNA-1-2. *Cell,* 129**,** 303-317.

ZHAO, Y., RANSOM, J. F., LI, A., VEDANTHAM, V., VON DREHLE, M., MUTH, A. N., TSUCHIHASHI, T., MCMANUS, M. T., SCHWARTZ, R. J. & SRIVASTAVA, D. 2007b. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell,* 129**,** 303-17.

ZHAO, Y., SAMAL, E. & SRIVASTAVA, D. 2005. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature,* 436**,** 214-220.

ZIMMET, H., PORAPAKKHAM, P., PORAPAKKHAM, P., SATA, Y., HAAS, S. J., ITESCU, S., FORBES, A. & KRUM, H. 2011. Short- and long-term outcomes of intracoronary and endogenously mobilized bone marrow stem cells in the treatment of ST-segment elevation myocardial infarction: a meta-analysis of randomized control trials. *European Journal of Heart Failure*.

ZUFFEREY, R., DONELLO, J. E., TRONO, D. & HOPE, T. J. 1999. Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. *Journal of Virology,* 73**,** 2886-2892.

ZUFFEREY, R., DULL, T., MANDEL, R. J., BUKOVSKY, A., QUIROZ, D., NALDINI, L. & TRONO, D. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol,* 72**,** 9873-80.

ZUFFEREY, R., NAGY, D., MANDEL, R. J., NALDINI, L. & TRONO, D. 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotech,* 15**,** 871-875.