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**Nanoscale Surfaces for the Long-term Maintenance of
Mesenchymal Stem Cell Phenotype and Multipotency**

Rebecca Jane McMurray

For the degree of Doctor of Philosophy

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University
of Glasgow

Centre for Cell Engineering
College of Medical, Veterinary and Life Sciences
University of Glasgow
UK, G12 8QQ

Abstract

The discovery of stem cells has led to rapid advances in the field of regenerative medicine. Their unique properties, including the ability to self-renew and differentiate make them ideal for the repair/replacement of tissues that have been damaged as a result of disease or injury. Mesenchymal stem cells in particular represent a highly valuable pool of adult stem cells for such regenerative applications due to their accessibility, and potential as an autologous patient derived autologous nature

However current methods for the *in vitro* expansion of *high quality autologous* mesenchymal stem cells results in spontaneous differentiation of the stem cell population and a loss of differentiation capacity over time. *In vivo*, it is the stem cell niche that provides stem cells with the appropriate cues required to maintain stem cell self-renewal. It is proposed that by mimicking these cues using biomaterials, that the self-renewal of mesenchymal stem cells can be controlled *in vitro*. In this study, a novel nanopit topography was investigated for its effects on the maintenance and growth of mesenchymal stem cells *in vitro*.

To investigate this, three main aspects of mesenchymal stem cell state were examined in response to this novel nanotopography: maintenance of the stem cell phenotype over time including expression of stem cell markers and differentiation potential over time, changes in signalling pathways associated with differentiation and lastly, the metabolic profile of stem cells.

As a result of this study we have identified a novel nanopit topography, which in the absence of chemical supplements, provides a substrate that is conducive to the maintenance of mesenchymal stem cells. Small RNAs have also been implicated in the regulation of signalling pathways and the metabolic state of stem cells. Furthermore, the ability to produce nanotopographically-patterned substrates using current standard techniques provides an inexpensive, high throughput method for the production of novel tissue culture plastics suitable for the maintenance of mesenchymal stem cells.

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Author's Declaration

I hereby declare that the research reported within this thesis is my own work, unless otherwise stated, and that at the time of submission is not being considered elsewhere for any other academic qualification.

Rebecca McMurray

21st September 2011

Abstracts and Publications

Original research publications authored by the candidate on work relating to this thesis.

McMurray RJ, Gadegaard N, Tsimbouri PM, Burgess KV, McNamara LE, Tare R, Murawski K, Kingham E, Oreffo ROC, Dalby MJ: Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency. *Nature Materials*. (2011) 10(8):637-44

A list of abstracts accompanying oral presentations given by the candidate.

McMurray RJ, Gadegaard N, Dalby MJ: Nanotopographical Control of Skeletal Stem Cell Differentiation. *Tissue and Cell Engineering Society, Glasgow 2009*.

McMurray RJ, Gadegaard N, Burgess K, Oreffo ROC, Dalby MJ: Nanotopographical Control of Mesenchymal Stem Cells *In Vitro*. *Tissue Engineering and Regenerative Medicine Society, Granada 2011*.

Definitions

α -MEM	alpha minimal essential media
μm	micrometer
μg	microgram
μl	microliter
AFM	atomic force microscopy
ALCAM	activated leukocyte cell adhesion molecule
ALP	alkaline phosphatase
ANOVA	analysis of variance
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
cDNA	complementary deoxyribose nucleic acid
C=C	carbon-carbon
CFU-F	colony forming unit - fibroblast
CO ₂	carbon dioxide
COL	collagen
cRNA	complementary ribonucleic acid
DAPI	4'-6-Diamidino-2-phenylindole
DNA	deoxyribose nucleic acid
EBL	electron beam lithography
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	endothelial growth factor
ERK	extracellular signal-regulated kinases
ESC	embryonic stem cell
FA	focal adhesion
FAK	focal adhesion kinase
FBS	fetal bovine serum
Fig	figure
FITC	fluorescein isothiocyanate
FN	fibronectin
g	acceleration due to gravity
G ₁	growth phase 1
G ₂	growth phase 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
IPA	ingenuity pathway analysis
KEGG	kyoto encyclopedia of genes and genomes
kV	kilovolt
LC-MS	liquid chromatography mass spectrometry
MACS	magnetic activated cell sorting
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
miRNA	microRNA
MSC	mesenchymal stem cell
nm	nanometer (10^{-9})
NSQ	nanopit topography arranged in a square lattice with a random ± 50 nm displacement from centre
OCN	osteocalcin

OPN	osteopontin
OGM	osteogenic media
P	passage
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PC	polycarbonate
PCL	polycaprolactone
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PMMA	polymethylmethacrylate
PPAR- γ	peroxisome proliferator-activated receptor-gamma
PS	polystyrene
p-value	probability value
qPCR	quantitative real time PCR
Ra	average roughness
RGD	arginine-glycine-aspartate motif
RNA	ribonucleic acid
ROCK	Rho-associated kinase
ROH ₂ O	reverse osmosis water
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope (microscopy)
SQ	nanopit topography arranged in a square lattice
SMA	super-mature adhesion
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
SOX	sex determining region Y-like, high mobility group box
S-phase	synthesis-phase
Src	Src family kinase
SSC	saline sodium citrate
STD	standard deviation
STRO-1	stromal cell surface protein
TAB1	transforming growth factor- β activated kinase-1 binding protein
TGF- β	transforming growth factor beta
TGFBR	transforming growth factor beta receptor
tRNA	transfer RNA
UV	ultraviolet
v/v	volume (of solute) per volume (of solvent)
VEGF	vascular endothelial growth factor
Wnt	wingless-type mouse mammary tumor virus integration site family member
w/v	weight (of solute) per volume (of solvent)
w/w	weight per weight

1 Introduction

Injury to tissues can often result in a reduced, or loss of, function associated with that tissue. This is mainly due to the formation of scar tissue at the site of injury that is both structurally and mechanically different. Additionally, degenerative diseases such as Alzheimer's or Parkinson's disease, whereby a loss or abnormal function of tissues results in the gradual decline of a patient's health eventually leading to death can often have far reaching effects, not only for the patient but can also place pressure on a patient's family both emotionally and financially. However, the advent of tissue engineering and regenerative medicine, a rapidly developing field which combines the unique properties of stem cells and biomaterials to repair or regenerate tissues that have undergone damage due to disease or injury, has the potential to provide a revolutionary method for helping to treat such injuries and the many currently incurable degenerative diseases (Langer and Vacanti 1993; Vacanti, Langer et al. 1998; Vacanti and Langer 1999).

Adult stem cells, isolated from tissues such as bone marrow, represent a valuable source of autologous stem cells for such regenerative applications (Friedenstein 1976). However it is the underpinning ability to deliver *large quantities of high quality* stem cells, which remains a significant problem.

In vitro, adult stem cells have a tendency to undergo spontaneous differentiation when cultured on standard tissue culture plastic, losing their multi-lineage potential (Siddappa, Licht et al. 2007). It is understood that standard tissue culture plastic fails to provide stem cells with appropriate biological cues conducive to maintenance of stem cell self-renewal and as a consequence, a heterogeneous cell population develops and ultimately results in a loss of the stem cell population. This is the result of current plastics being designed around fastidious, stable, cell types rather than phenotypically unstable stem cells. One potential mechanism to overcome this problem is the use of stem cell specific biomaterial substrates.

1.1 Biomaterials

Biomaterials, unlike conventional 'bioinert' materials have the ability to interact with cells to invoke an appropriate biological response. In tissue engineering, biomaterials were first shown to influence terminally differentiated cells by affecting a variety of biological processes such as cell growth, adhesion, morphology, and apoptosis (Clark, Connolly et al. 1990; Burrige and Chrzanowska-Wodnicka 1996; Aspenstrom 1999; Bonfield 2002). In regenerative medicine, however, it is desirable to use stem cells to regenerate complex tissues instead of terminally differentiated cells with the ability to form only single tissue types. Excitingly, research from the last decade has shown that biomaterials also have the ability to drive stem cell fate decisions and to target desirable differentiation (Engler, Sen et al. 2006; Dalby, Gadegaard et al. 2007; Kilian, Bugarija et al. 2010).

In vivo, complex tissues can be identified as having unique physical, chemical and topographical features. Bone for example, has a high elastic modulus when compared to tissue such as brain, it also has a complex architectural structure with macro, micro and nano-meter levels of topography in the form of fibrillar structures such as collagen and protein:protein interactions at the nanoscale via cell:ECM interactions (Stevens and George 2005). Lastly, chemical cues such as ECM proteins play a role in bone formation promoting matrix mineralization, whilst glycoproteins, which contain active domains such as the RGD motif, are important for intracellular signalling (Reddi, Gay et al. 1977; Meyer, Alenghat et al. 2000; Stevens and George 2005).

It is the potential for biomaterials to mimic these features *in vitro* and elicit specific cues to control the self-renewal and differentiation of stem cells, which could have profound implications for regenerative medicine. Current biomaterial strategies which have been employed to promote the controlled differentiation of adult stem cells such as mesenchymal stem cells (MSCs), include the use of chemistry, such as RGD motifs (McBeath, Pirone et al. 2004; Curran, Chen et al. 2006; Engler, Sen et al. 2006; Benoit, Schwartz et al. 2008; Kilian, Bugarija et al. 2010), material modulus, e.g. hard/soft substrates (Engler, Sen et al. 2006), and also topography (Dalby, Gadegaard et al. 2007; Yim, Pang et al. 2007), both

at the micro- and nanoscale, for instance the incorporation of grooves or pits into the material surface.

Whilst the ability to demonstrate differentiation *in vitro* using different biomaterial strategies is now well established, the capacity to maintain adult stem cell self-renewal *in vitro* using biomaterials remains limited and is now a major research focus. In the current literature, a study by Gilbert *et al* identified skeletal muscle stem cell self-renewal to be affected by material modulus and further identified that a material modulus similar to that found in native muscle tissue was able to promote the maintenance of the skeletal muscle stem cells *in vitro* (Gilbert, Havenstrite *et al.* 2010). Additionally, Curran *et al* have identified chemically modified surfaces as having the potential to control stem cell behaviour (Curran, Stokes *et al.* 2010). Here, however, we test nanotopography as a potential candidate for the long-term maintenance of MSC self-renewal *in vitro*. This would not only increase the functionally useful life span of the stem cell population for clinical applications but also, critically, allow for growth of the stem cell population to useful numbers *in vitro* over time.

1.2 Stem cells

Two properties of stem cells make them a unique and valuable source for applications in regenerative medicine:

1. Self-renewal - the ability to divide whilst maintaining an undifferentiated cell state ultimately maintaining the stem cell population, and
2. Potency - the potential to differentiate into multiple functionally specialized cell type. Stem cells can either be pluripotent; able to differentiate into all three germ layers, mesoderm, ectoderm and endoderm or they can be multipotent; able to differentiate into multiple cell types but are lineage restricted (Williams, Hilton *et al.* 1988; Keller 1995; Pittenger, Mackay *et al.* 1999; Clarke, Johansson *et al.* 2000).

Currently three broad categories of stem cells have been identified (fig. 1-1):

- Adult stem cells

- Embryonic stem cells
- Induced pluripotent stem cells

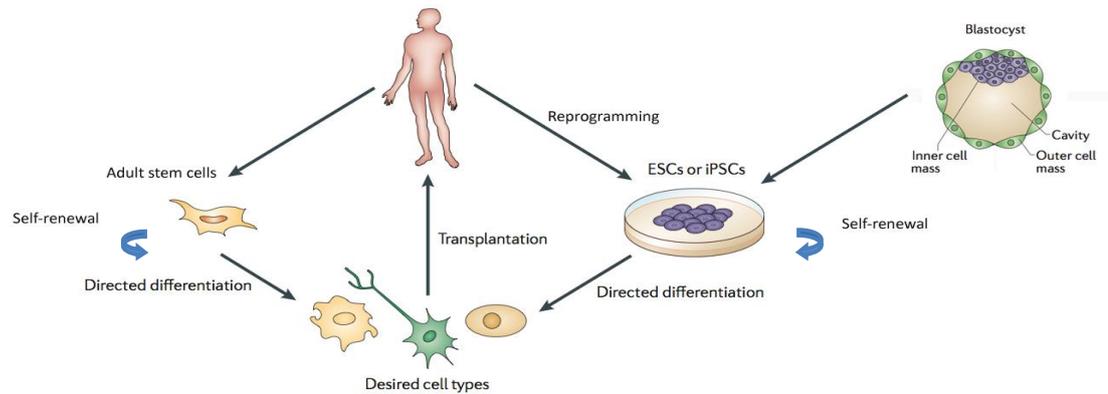


Figure 1-1 Stem cells and regenerative medicine. Autologous stem cells can be acquired either from adult tissues (adult stem cells) or by the reprogramming of somatic cells (induced pluripotent stem cells (iPS cells)). Non-patient-derived stem cells can be found in the blastocyst of an embryo (embryonic stem cells (ESCs)). These stem cells can undergo directed differentiation after which they could be transplanted back into the patient to replace or repair damaged tissue. (Adapted from (O'Connor and Crystal 2006; Cohen and Melton 2011)).

Adult stem cells were first discovered in the bone marrow during the 1960's, primarily with the identification of HSCs and then MSCs (Becker, McCulloch et al. 1963). It is these first discoveries that have led to the current era of regenerative medicine. Table 1-1 identifies the three main types of adult stem cells, their tissues of origin and differentiation potential.

Name	Tissue of Origin	Differentiation
Hematopoietic	Bone marrow	All blood cells Osteoclasts
Mesenchymal	Bone marrow Umbilical Cord Adipose tissue	Bone Cartilage Fat Muscle Connective tissue Nerve
Neural stem cells	Brain	Neurons Astrocytes Oligodendrocytes
Epithelial stem cells	Digestive tract	Absorptive cells Goblet cells Paneth cells Enteroendocrine cells
Endothelial stem cells	Bone marrow Blood	Blood vessels Cardiomyocytes

Table 1-1 The main types of stem cells found in adult tissues, their tissue of origin and differentiation potential.

In 1981, embryonic stem cells were first discovered through research using mouse embryos, a discovery which ultimately led to the isolation of human embryonic stem cells in 1998 (Evans and Kaufman 1981; Martin 1981; Thomson, Itskovitz-Eldor et al. 1998). Then in 2007, a significant breakthrough occurred when Yamanaka *et al* identified four key transcription factors (oct-3/4, sox2, klf-4 and c-myc) that are required for the reprogramming of human somatic cells into stem cells known as induced pluripotent stem cells (iPS) (Takahashi, Tanabe et al. 2007; Yu, Vodyanik et al. 2007). Initially the reprogramming of somatic cells was achievable using viral vectors that incorporate these reprogramming factors into the host-cell genome, a high-risk strategy with the potential to cause mutations and the activation of oncogenes following implantation. Recent studies, however, using direct protein delivery methods have eliminated the use of viral vectors, although these have been found to work with lower efficiency (Kaji, Norrby et al. 2009; Kim, Kim et al. 2009; Woltjen, Michael et al. 2009; Zhou, Wu et al. 2009).

While stem cells are unique, differences exist in their origin, capacity to differentiate and ability to undergo self-renewal, and these ultimately reflect their potential for use in regenerative therapies. The advantages and disadvantages for each stem cell type are discussed in table 1-2.

Overall the ability of ES and iPS cells to generate cell types of all three germ layers, known as pluripotency, makes these stem cells an extremely valuable and potent research tool. However, the risks associated with their use following implantation (e.g. teratoma formation, problems arising from use of viral vectors etc) and also associated ethical problems for ES do not currently make them a viable tool for therapeutic use.

Stem cells	Origin	Potential	Advantages	Disadvantages
Adult stem cells	Bone marrow Adipose tissue Umbilical cord Brain	Multipotent	Accessible Autologous Easy to culture Less ethical issues Immunodulation	Multipotent Spontaneous differentiation
Embryonic Stem Cells	Inner cell mass of a blastocyst	Pluripotent	Pluripotent	Ethical issues Complicated cell culture Tumorigenic Immune rejection
Induced pluripotent	Reprogramming of somatic cells	Pluripotent	Autologous Pluripotent	Reprogrammed using viral vector

stem cells				protein delivery - low efficiency Tumorigenic
------------	--	--	--	---

Table 1-2 The advantages and disadvantages associated with different types of stem cells.

As can be seen from table 1-2 the advantages for the use of adult stem cells, particularly MSCs, over the use of ES or iPS cells include their accessibility, the potential to use patient derived stem cells, eliminating the risk of immune rejection, no feeder layer requirements reducing the need for animal derived products, adult MSCs are also thought to provide an immunomodulatory effect (Uccelli, Moretta et al. 2008), and lastly adult stem cells hold less ethical implications than ES cells in particular. However, in contrast to ES and iPS cells, which readily undergo self-renewal during *in vitro* culture, adult stem cells tend to undergo spontaneous differentiation forming fibroblast-like cells (Sherley 2002; Sarugaser, Hanoun et al. 2009).

1.3 Mesenchymal Stem Cells

Adult MSCs were first identified in the bone marrow along with hematopoietic stem cells (HSCs) by Becker *et al*, and their clonogenic nature was later revealed by Friedenstein and colleagues during the 1970's (Becker, McCulloch et al. 1963; Friedenstein 1976; Owen 1988; Owen and Friedenstein 1988). Further discoveries identified MSCs that reside in adipose tissue, umbilical cord blood and Wharton's jelly of the umbilical cord tissue, however MSC's from the bone marrow are the best characterised (Zuk, Zhu et al. 2001; Lee, Kuo et al. 2004; Wang, Hung et al. 2004).

Characteristically MSC's are large cells with a fibroblastic morphology (fig. 1-2), and they were first separated from HSCs in the bone marrow due by their adherence onto tissue culture plastic, and propensity to form clonogenic colony forming unit-fibroblasts known as CFU-F's.

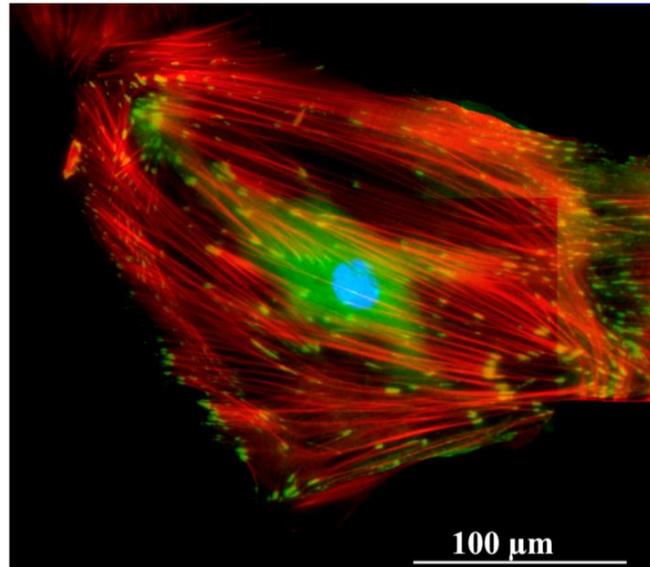


Figure 1-2 Immunofluorescence image of a mesenchymal stem cell. Blue = nucleus, red = actin, green = vinculin. Adapted from (Biggs 2008).

Since the discovery of MSCs there has been considerable effort to identify a definitive marker that can be used to isolate this multipotent population from the bone marrow, however this has so far gone unrewarded and currently there are a range of markers which collectively or individually by their presence and/or absence have been used to identify MSCs. Positive markers include a range of cell surface markers, cell adhesion molecules, and the hematopoietic marker CD44. Other negative markers that have been found to be absent in MSCs include a range of hematopoietic markers and co-stimulatory molecules. Each marker is individually identified in table 1-3 as well as its presence or absence associated with MSCs.

Marker Type	Positive/Negative	Details
Cell surface antigen		
STRO-1 antigen	+	Cell surface trypsin-resistant antigen expressed by CFU-F Antibody: STRO-1
Tissue nonspecific alkaline phosphatase	+	Cell surface glycoprotein associated with osteoblast lineage cells Antibody: STRO-3
CD63	+	Synonyms: Melanoma associated antigen ME491; Tetraspanin 30; Lysosomal-associated membrane protein 3; Antibody: HOP26
CD105	+	Synonym: Endoglin/SH2
CD73	+	Synonym: SH3/4
CD71	+	Synonym: Transferrin receptor protein 1 (TfR1)
CD90	+	Synonym: Thymocyte antigen-1 (Thy-1)

CD49a	+	Synonym: Laminin and collagen receptor VLA- α 1
Cell adhesion molecules		
CD106	+	Synonym: Vascular cell-adhesion molecule-1 (VCAM)
CD166	+	Synonym: Activated leukocyte cell-adhesion molecule (ALCAM) Antibody: SB-10
ICAM-1	+	Intracellular cell-adhesion molecule-1
CD29	+	Synonym: β 1 subunit of the integrin family
CD31	-	Synonym: Platelet/endothelial cell-adhesion molecule-1 (PECAM-1)
CD56	-	Synonym: Neural cell-adhesion molecule-1 (NCAM-1)
CD18	-	Synonym: Leukocyte cell-adhesion molecule, integrin β 2 (LCAMB), Lymphocyte function associated antigen-1 (LAD, LFA-1)
Hematopoietic markers		
CD45	-	Synonym: Leukocyte common antigen Located on hematopoietic cells except erythrocytes and platelets
CD34	-	Identified on hematopoietic progenitors and endothelial progenitors
CD14	-	Preferentially expressed on monocytes/macrophages
CD11	-	Component of various integrins, especially those in which the component is CD18
CD44	+	Expressed by hematopoietic as well as bone marrow mesenchymal stem cells
Co-stimulatory molecules		
CD80	-	Synonym: B7.1, provides co-stimulatory signal necessary for T cell activation and survival
CD86	-	Synonym: B7.2, provides co-stimulatory signal necessary for T cell activation and survival
CD40	-	Expressed by all mature B lymphocytes, monocytes, dendritic, endothelial and epithelial cells

Table 1-3 Cell surface antigen expression profile of human bone marrow-derived mesenchymal stem cells. Adapted from (Tare, Babister et al. 2008).

The most widely used marker of those in table 1-3 is STRO-1 of which positive cells isolated from the bone marrow make up approximately 10% of the total cell population (Simmons and Torok-Storb 1991). In addition, the differentiation potential of a proposed stem cell population has also been used as a method for identification.

1.3.1 Differentiation Potential

MSCs originate from the mesoderm during development, and using chemically defined media, *in vitro* studies have shown MSCs have the capacity to undergo differentiation into a variety of cell types mainly derived from the mesodermal lineages (fig. 1-3) including osteoblasts, adipocytes, chondrocytes, fibroblasts and myoblasts (Pittenger, Mackay et al. 1999; Majumdar, Banks et al. 2000; Gang, Jeong et al. 2004). More recently, and somewhat controversially, MSCs have also been shown to have the ability to transdifferentiate across germ layers to differentiate into cell types of ecto- and endoderm lineages (Woodbury, Schwarz et al. 2000; Jiang, Jahagirdar et al. 2002; Wang, Bunnell et al. 2005; Tropel, Platet et al. 2006) indicating they may have a much broader differentiation potential.

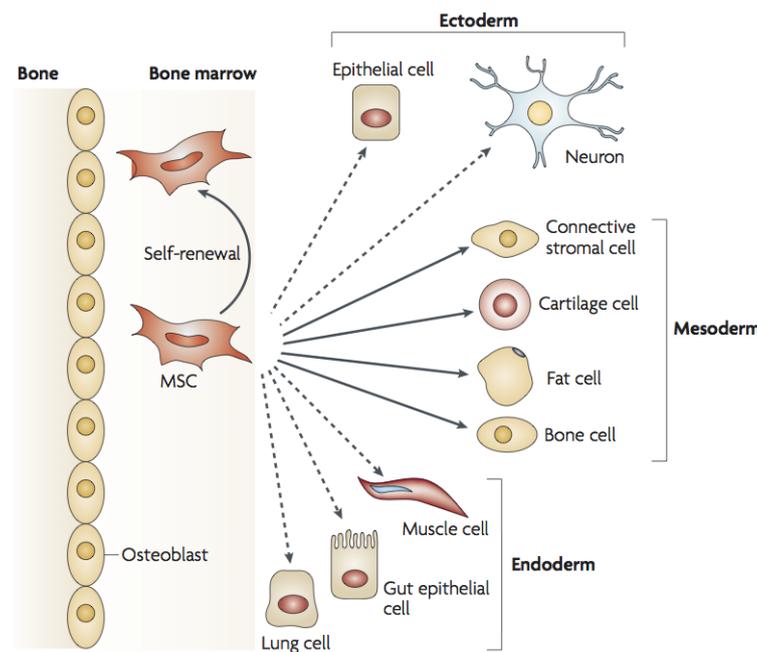


Figure 1-3 Differentiation of MSCs. MSCs from the bone marrow have been shown to differentiate into cell types of the mesoderm such as bone, fat and cartilage. MSCs have also been shown to transdifferentiate across other germ layers *in vitro* to develop into cells of the endoderm and ectoderm, however this occurring *in vivo* is controversial and hence is represented by the dashed line. Image from (Uccelli, Moretta et al. 2008)

1.3.1.1 Osteogenic Differentiation

During osteogenic differentiation MSCs become increasingly committed to the osteogenic lineage, differentiating from a multipotent MSC to a pre-osteoblast and lastly, to an osteoblast. According to early reviews by Stein *et al*, differentiation is coupled with a decrease in cell proliferation and an increase in

extracellular matrix proteins such as fibronectin (FN) and type I collagen (COL), with osteoblast cell markers upregulated over time such as alkaline phosphatase (ALP), and lastly osteopontin (OPN) and osteocalcin (OCN), proteins associated with mineralization of the extracellular matrix (figure 1-4).

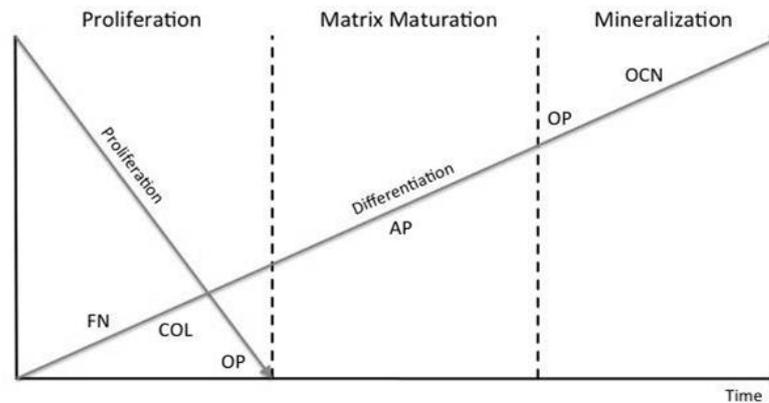


Figure 1-4 Osteogenic differentiation. Differentiation occurs in three stages, the first stage being proliferation. A decrease in proliferation coincides with an increase in markers associated the extracellular matrix. The last stage, mineralization of the extracellular matrix sees an increase in osteogenic markers osteopontin and osteocalcin. (Image adapted from (Stein and Lian 1993))

Further studies have identified key signalling pathways involved in osteogenic differentiation. These include canonical and non-canonical Wnt signalling, although this has been shown to be subject to levels of Wnt expression and in particular Wnt3a is thought to actually repress osteogenic differentiation and promote proliferation (Boland, Perkins et al. 2004; de Boer, Siddappa et al. 2004). Other signalling pathways include ERK/MAPK, TGF- β /BMP, Notch, Hedgehog and FGF signalling pathways (Linkhart, Mohan et al. 1996; Ge, Xiao et al. 2007; Ugarte, Ryser et al. 2009)(Hu, Hilton et al. 2005; Engin, Yao et al. 2008); (Khatiwala, Kim et al. 2009) (Yu, Xu et al. 2003).

1.3.1.2 Adipogenic Differentiation

Adipogenic differentiation is characterised by an increase in PPAR- γ expression, and is found to be regulated by the protein TAZ, a transcriptional regulator known to regulate the interplay between the differentiation of MSCs down an osteogenic or adipogenic lineage (Hong, Hwang et al. 2005). An up-regulation of TAZ is seen to up-regulate expression of runx2-dependent gene expression whilst conversely down-regulating expression of PPAR- γ -dependent gene expression.

Interestingly, adipogenesis is also thought to be negatively regulated by the mitogen-activated ERK pathway, and a study by Jaiswal *et al* showed that suppression of ERK signalling using a dominant negative map kinase kinase, MEK-1, lead to increased adipogenic differentiation and decreased osteogenic differentiation (Jaiswal, Jaiswal et al. 2000).

Further studies identified the role of shape-induced tension in regulating lineage specification during differentiation. McBeath *et al.* found that the cell shape regulates the Rho/ROCK pathway and the generation of myosin induced cytoskeletal tension (McBeath, Pirone et al. 2004). In the study MSCs were shown to undergo either osteogenic or adipogenic differentiation according to the degree of cell spreading. Cells that were allowed to spread, were found to express more active RhoA and underwent osteogenic differentiation, whereas cells that remained rounded expressed lower levels of RhoA and underwent adipogenic differentiation. More recently, Mrksich and co-workers demonstrated further the role geometric cues play in the generation of cell contractility and its importance in directing stem cell fate. Furthermore, the authors implicate myosin contractility in activating key signalling pathways, MAPK and Wnt signalling to promote cellular differentiation (Kilian, Bugarija et al. 2010). This is interesting, as nanotopographic regulation of cellular tension has also been shown to modulate Wnt signalling (Biggs, Richards et al. 2009).

1.4 The Stem Cell Niche

In vivo, adult stem cells are thought to reside in what has been termed 'stem cell niches', and it is the niche which plays a critical role in maintaining the balance between self-renewal and differentiation. The concept of a stem cell niche was first proposed by R. Schofield in 1978, who reported that a stem cell may associate with other cells *in vivo* which help to regulate their behaviour and maintenance (Schofield 1978). Early research first identified a germ-line stem cell niche that exists in *Drosophila melanogaster* (Xie and Spradling 2000). Further research in mammals has lead to the discovery of stem cell niches associated with HSCs (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003), neural stem cells (Doetsch 2003), skin stem cell (Tumbar, Guasch et al. 2004; Tavazoie, Van der Veken et al. 2008), and intestinal stem cells (Bjerknes and Cheng 2001)

amongst others. Whilst identification of a definitive MSC niche in the bone marrow so far remains elusive, research into other stem cell niches has identified key factors which make up the niche and together can act to regulate stem cell self-renewal or differentiation (figure 1-5) (Li and Xie 2005; Scadden 2006). These include both structural and functional components such as:

- A physical interaction with the basement membrane, extracellular matrix and other cell types of the niche.
- Signalling between adjacent or daughter cells within the niche.
- Extrinsic signalling from out with the niche.
- Neural signalling.
- Metabolic changes due to cellular processes.

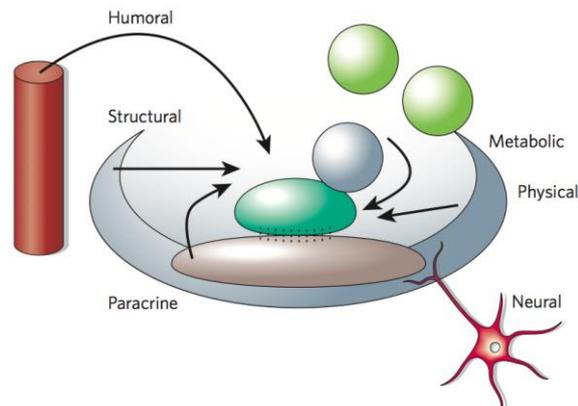


Figure 1-5 Overview of the stem cell niche. The stem cell niche is made up of various factors including chemistry, topography and tissue stiffness, which together regulate stem cell self-renewal or differentiation *in vivo*. (Adapted from (Scadden 2006))

The importance of the stem cell niche in the maintenance of stem cells has been highlighted in several experiments using model organisms *Caenorhabditis elegans* (*C. Elegans*) and *Drosophila melanogaster* (*Drosophila*). In *C. Elegans*, during development repositioning of the stem cell niche results in maintenance of germ stem cells close to the new niche position, whilst in *Drosophila*, it was shown that induced differentiation, followed by subsequent removal of differentiation stimuli, committed stem cells in the niche could revert back to a stem cell-state (Kimble and White 1981; Brawley and Matunis 2004; Kai and Spradling 2004)

1.4.1 Stem Cell Self-renewal

The molecular control of MSC self-renewal is currently not well understood, however using knowledge of both stem cell niche structure and function, and properties which have been identified as important regulators of self renewal in other types of stem cells, it is possible to infer potential mechanisms which may be regulating MSC self-renewal. To date *in vitro* studies have identified a functional link between self-renewal, the cell cycle and cell division.

1.4.1.1 The Cell cycle

The cell cycle consists of four main phases, G₁ phase (growth), S phase (synthesis), G₂ phase (growth) and M phase (mitosis) (fig 1-6). Cells which are not dividing, and are either quiescent or senescent are leave the cell cycle at early G₁ phase and entered into G₀ phase. Control over progression of the cell cycle is highly regulated by intrinsic checkpoints which exist between the G₁ and S phases and G₂ and M phases of the cell cycle ensuring integrity of the genome (Elledge 1996). Progression through G₁ phase however is also regulated by extrinsic signals and creates a restriction point dividing G₁ into early and late phases, with G₁ early phase characterised as mitogen-dependant and G₁ late characterised as mitogen-independent (Foster, Yellen et al. 2010).

During early phase G₁ a class of regulatory molecules known as cyclins and cyclin dependent kinases, combine to form active complexes. During early phase G₁ cyclin D expression is up-regulated following mitogenic signalling, and together with cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) leads to partial inactivation of the retinoblastoma (Rb) protein (Hatakeyama, Brill et al. 1994; Nath, Wang et al. 2003). In its active form Rb forms a complex with the transcription factor E2F and its co-factor DP1 forming the E2F/DP1/Rb complex. When bound together this complex binds to E2F responsive gene, blocking transcription of cyclin E, which is required for the transition from G₁ to S-phase. However, inactivation of Rb causes it to dissociate from this complex leaving E2F able to bind and initiate transcription of cyclin E (Ohtani, DeGregori et al. 1995). Cyclin E then form a complex with cyclin-dependent kinase 2 (CDK2) which leads to hyperphosphorylation of Rb, alongside inactivation of the Rb tumour suppressor protein. Once a threshold level of cyclin E-CDK2 has been

reached, progression through late phase G1 into S phase occurs independent of mitogenic factors (Yanischevsky and Stein 1981; Hatakeyama, Brill et al. 1994).

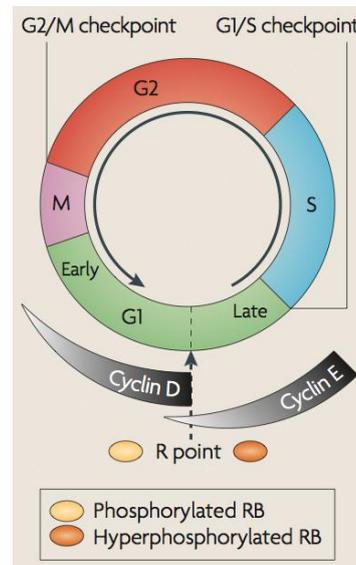


Figure 1-6 The cell cycle is thought to play an important part in self-renewal and differentiation. In particular, in self-renewing stem cells the G1 phase of the cell cycle is particularly critical. This is thought to be due to mitogenic factors, often associated with the initiation of differentiation also being required for progression through early G1 to late G1 phase. Adapted from (Orford and Scadden 2008).

The cell cycle was first thought to play a key role in stem cell self-renewal, with the observation that ES cells have a G1 phase shorter than somatic cells (this is similar in iPS (Savatier, Huang et al. 1994; White, Stead et al. 2005; Becker, Ghule et al. 2006; Ghule, Medina et al. 2011)). Some adult stem cells, such as haematopoietic stem cells, maintain their multipotent state via quiescence, and therefore do not undergo cell division (Cheshier, Morrison et al. 1999; Wilson, Laurenti et al. 2008). Lastly, as seen in figure 1-6, MSCs *in vitro* are seen to undergo proliferation, albeit at a reduced rate, and loss of proliferation is coupled with the onset of differentiation (Stein, Lian et al. 1990; Stein, Stein et al. 1995). Further evidence that the cell cycle and self-renewal are functionally linked then came when iPS cells were shown to also have a shortened G1 phase of the cell cycle similar to that of ES cells when compared to somatic cells (Ghule, Medina et al. 2011). It is thought that the transition from early phase G1 to late phase G1 plays a critical part in the determining of cell fate decisions of stem cells. In particular, the dependence on mitogenic factors for the progression from early G1 to late G1 is thought to be central.

It is the dual actions of mitogen activated protein kinases (MAPKs) in both their requirement for progression through the G1 phase of the cell cycle and in

promotion of differentiation which makes self-renewing stem cells particularly susceptible to differentiation (Jaiswal, Jaiswal et al. 2000; Zhang and Liu 2002). To overcome this as mentioned above, ES cells have developed a characteristically shorter G₁ cell cycle phase due to constitutive expression of cyclin E-CDK2, while the quiescent nature of haematopoietic stem cells means these stem cells evade the G₁ phase all together. In a review by Orford *et al* it is proposed that ESCs and HSCs are either actively or passively preventing differentiation (Orford and Scadden 2008); constitutive expression of the late G₁ phase cyclin E-CDK2 means that the cell cycle can progress independently of mitogenic factors bypassing early phase G₁ enabling these cells to undergo proliferation without differentiation, or passively preventing entry into the cell cycle and thereby evading early G₁, a critical point in determining cell fate decisions (Siminovitch, Till et al. 1964; Cheng, Rodrigues et al. 2000; Stead, White et al. 2002; Filipczyk, Laslett et al. 2007; Wilson, Laurenti et al. 2008). Other factors which affect the cell cycle include mechanical tension-dependent changes caused by changes in cell shape and cytoskeletal arrangement. This will be discussed later in the chapter.

1.4.1.2 Stem Cell Division

Stem cell participation in the cell cycle ultimately results in cell division. *In vivo*, a stem cell within its niche may undergo either symmetrical division, where by a stem cell divides to produce two stem cells or asymmetrical cell division, producing one stem cell that is retained within the niche and one progenitor cell which leaves the niche to undergo differentiation (figure 1-6) (Morrison and Kimble 2006). *In vivo* adult stem cells are thought to mainly undergo asymmetrical cell division stabilizing the stem cell population whilst also maintaining tissue homeostasis. However, following injury or during development when expansion of the stem cell population is critical, symmetrical cell division predominantly occurs (Wilson, Laurenti et al. 2008).

During symmetrical or asymmetrical division it is thought that both intrinsic and extrinsic factors play key roles in regulation. Intrinsic factors include cell polarity, mitotic spindle orientation relative to the niche and the localisation of differentiation factors (Wodarz 2005; Giebel 2008; Kanamori, Inoue et al. 2008; Yamashita 2009; Yamashita, Yuan et al. 2010). Extrinsic factors outwith the

niche may function to establish asymmetry via the exposure of only one daughter cell due to differentiation factors via the relative positioning of both daughter cells to the niche. It is therefore also possible that a stem cell may divide symmetrically but due to extrinsic factors acting on only one daughter cell, the fate of both daughter cells is ultimately different resulting in an overall asymmetric outcome. In particular, integrin binding within the niche is thought to be involved in regulating the plane of cell division and this will be discussed later in the chapter (Kanamori, Inoue et al. 2008; Yamashita 2010).

1.4.1.3 *In vitro* Self-renewal

In vitro, adult MSCs are conventionally cultured on flat polystyrene tissue culture plastic. This results in *in vitro* cultured MSCs predominantly undergoing asymmetric division, commonly termed spontaneous differentiation, becoming mainly fibroblastic cells and resulting in a loss of the stem cell population over time (fig. 1-7) (Banfi, Muraglia et al. 2000; Muraglia, Cancedda et al. 2000; Siddappa, Licht et al. 2007). As a result the clinical potential of mesenchymal stem cells is commonly considered to have declined following successive passaging (Sherley 2002; Siddappa, Licht et al. 2007; Sarugaser, Hanoun et al. 2009).

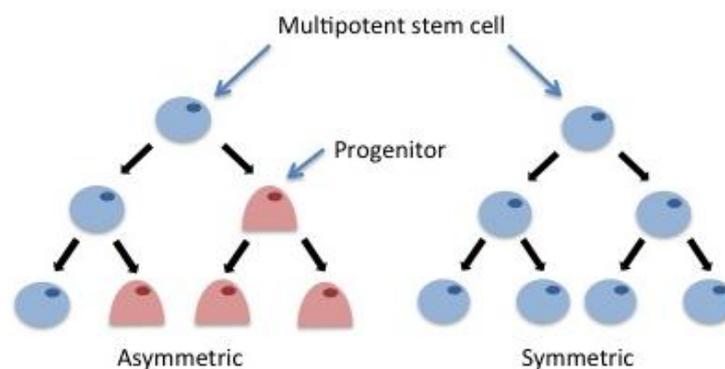


Figure 1-7 Stem cell division. Division can be either symmetrical, expanding the stem cell population or asymmetric generating a stem cells and a progenitor cell. While asymmetric division *in vivo* maintains a stable stem cell population in the niche whilst allowing for differentiation, *in vitro* however, asymmetric cell division leads to loss of the stem cell population.

Symmetrical division, which is the focus of this thesis, results in expansion of the stem cells population. Evidence for the ability of mammalian adult stem cells to undergo symmetrical cell division *in vivo* has mainly been identified in

haematopoietic stem cells following treatment with chemotherapy (Richman, Weiner et al. 1976). However, it is the role of the stem cell niche, particularly the role of the physical environment of the extracellular matrix and adhesion molecules, in regulating symmetrical cell division, which are of particular interest in reference to nanotopography.

1.5 Cell : Extracellular Matrix Adhesions

Adhesion between the extracellular matrix and cells are mediated by the binding of transmembrane integrins to extracellular proteins such as fibronectin and vitronectin (Humphries 1990). The integrin family consist of a heterodimer of α and β subunits. Currently 18 different α and 8 β subunits exist, and due to alternative splicing, variants of each subunit also exist. In general it is thought that approximately 24 different integrins can be generated using unique combinations of the subunits. The makeup of each integrin dictates which extracellular protein it can bind to. The most common are $\alpha_5\beta_1$ which binds to fibronectin and $\alpha_v\beta_3$ found to bind vitronectin (Springer and Wang 2004).

As mentioned previously the integrin family are transmembrane proteins with an extracellular domain capable of binding proteins of the ECM. However, they also contain a short cytoplasmic domain coupled to microfilaments (actin) of the cytoskeleton via integrin-actin linkers such as α -actinin and filamin. Other intracellular proteins present in focal contacts include vinculin (seen in fig 1-8), talin and paxillin (Samuelsson, Luther et al. 1993; Goldmann, Ezzell et al. 1996; Ezzell, Goldmann et al. 1997). These latter proteins, however, do not directly bind integrins but instead bind actin. Many of the proteins involved in focal contacts, however, not only act as linkers to integrins or the cytoskeleton, but also function as enzymes with the potential to initiate signal transduction pathways. Examples include focal adhesion kinase (FAK), integrin linked kinase (ILK) and p21-activated kinase (PAK)(Hannigan, Leung-Hagesteijn et al. 1996; Slack-Davis, Eblen et al. 2003; Schaller 2010).

Due to their role in coupling of the ECM to the intracellular environment, integrins are considered to be the main mediators of interaction between a biomaterial substrate and cells. Adhesions between the extracellular matrix and cells usually fall into one of three categories usually dependent on their size and

location. The most common type of adhesions are focal adhesions, found around the periphery of a cell and commonly between 2-5 μm long. Focal complexes are smaller, approximately 1 μm in length and are found at the leading edge of lamellipodia (Zimmerman, Volberg et al. 2004). Fibrillar adhesions make up the largest adhesions, ranging in size from 1 - 10 μm and are usually found in the central area of cells (Zamir and Geiger 2001). Fibrillar adhesions in particular are thought to play an important role in directing ECM remodelling and architecture producing a favourable environment for MSC osteogenic differentiation and matrix mineralization (Biggs and Dalby 2010).

These results are in accordance with those found by Engler et al who identified matrix stiffness as a direct regulator of MSC differentiation. Interestingly, the authors found increasing cellular tension was associated with an increased size of focal adhesion length, and osteogenic differentiation associated with increasing the matrix stiffness to mimic that of collagenous bone (Engler, Sen et al. 2006).

The adhesion of cells to the ECM via integrins is highly complex and serves two main functions; adhesion to the ECM and secondly, in transmitting signals from the ECM to the cell. Whilst integrin molecules themselves do not have intrinsic kinase activity, the binding of intracellular kinases such as FAK, as shown in figure 1-8 A, leads to the activation of intracellular signalling cascades such as the ERK/MAPK pathway, a pathway known to affect various cellular processes such as proliferation and differentiation (Miyamoto, Teramoto et al. 1995; Zhu and Assoian 1995).

Upon adhesion, initial integrin binding results in changes in conformation and affinity of the integrin molecules leading to integrin clustering (Kawakami, Tatsumi et al. 2001). During these initial cell:ECM contacts only transient focal complexes are formed. The evolution of focal complexes into more mature focal adhesions is thought to be mediated via changes in cellular tension either intracellularly or as a result of the extracellular environment (Chen, Alonso et al. 2003). The linking of cytoskeletal actin filaments provides mechanical support for cells and during adhesion the cell must apply traction forces to the surface to counterbalance the internal forces produced as a result of increased cytoskeletal tension (fig. 1-8 B). As discussed in section 1.3.1.2., cellular tension

has been implicated in directing stem cell differentiation with a high tension state inducing osteogenic differentiation, while a low tension state is conducive to adipogenic differentiation (Thomas, Collier et al. 2002; McBeath, Pirone et al. 2004; Kilian, Bugarija et al. 2010). Current evidence is also indicating that stem cell self-renewal may require an intermediate level of cellular tension (Gilbert, Havenstrite et al. 2010).

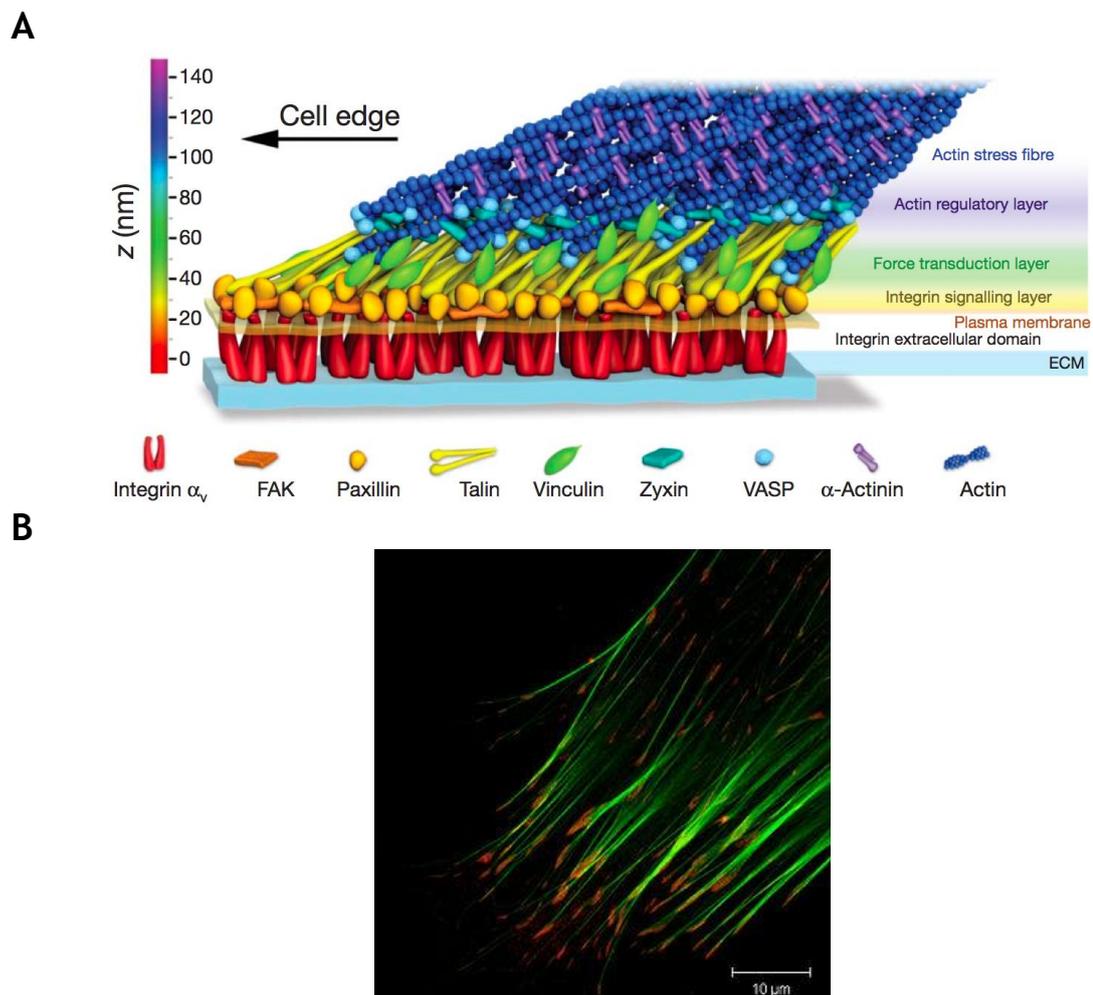


Figure 1-8 Nanoscale architecture of focal adhesions. (A) Schematic diagram of a focal adhesion linking the extra cellular matrix to the intracellular environment. Extracellular proteins of the ECM bind to integrin molecules, whilst adaptor proteins such as talin and paxillin link integrins to the cytoskeletal protein actin. (B) Immunofluorescence of the focal adhesions protein vinculin. Green = actin, red = vinculin. Adapted from (Kanchanawong, Shtengel et al. 2010) and courtesy of Cristoph Moehl respectively.

1.6 Mechanotransduction

Interactions between stem cells and the ECM, such as those mentioned above can have what is known as a direct or indirect effect on cells, otherwise known as mechanotransduction, to elicit changes in gene expression. Direct

mechanotransduction occurs as a consequence of conformational changes in the cell cytoskeleton, which forms a direct link between the extracellular matrix and the nucleus of the cell via proteins called lamins (Wang, Butler et al. 1993; Ingber 1997). Indirect mechanotransduction encompasses the intracellular signalling cascades which result from integrin binding and focal adhesion formation (Wang, Du et al. 2011).

The cytoskeleton is a dynamic structure and as discussed in a review by Fletcher *et al* it fulfils three main roles (Fletcher and Mullins 2010):

1. It provides the spatial organization for the cellular organelles
2. Provides a physical and biochemical link to the extracellular environment
3. Generates forces which enable cell migration and maintain cell shape.

The cytoskeleton consists of three main components:

- Microfilaments

Microfilaments are composed of globular actin subunits, which bind unidirectionally to form filamentous actin (fig. 1-9 c). A microfilament is formed when two actin filaments lie parallel to each other forming a double helix. The microfilaments are responsible for contraction of the cell and play a key role in cell migration, with actin subunits being added and removed from the filament in what is known as treadmilling and leads to movement of the filament.

Microfilaments can also interact with myosin molecules, also known as molecular motors, and together function in muscle, and other, cells to cause contraction (Allison, Davies et al. 1971).

- Microtubules

Microtubules are polymers made up of α and β subunits which bind to form heterodimers (fig 1-9 a). These heterodimers polymerise to form a protofilament. Polarity within the filament generated by the binding of the α and β subunits leads to the generation of (-) and (+) ends respectively, with the (-) end subject to capping leaving the (+) end available for elongation. Bundling of 13 protofilaments produces a hollow microtubule, with polymerization and capping occurring within the microtubule organising centre of the cell.

Microtubules can interact with motor proteins such as dynein to act as mediators of cell transport.

- Intermediate filaments.

Intermediate filaments consist of a family of mainly cytoplasmic proteins which can be divided into six categories based on their amino acid sequence and protein structure (fig. 1-9 b). Type I and II include keratins, type III includes the most abundant intermediate filament found in MSCs; vimentin, type IV and VI include filaments expressed in neurons and neural stem cells respectively, and type V, the lamins, a group of nuclear proteins. Intermediate filaments form a rope-like structure composed of parallel dimmers.

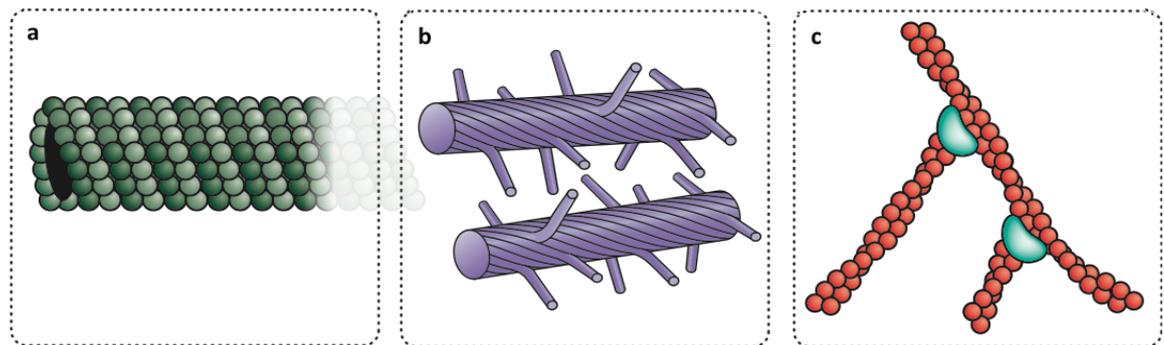


Figure 1-9 The three main cytoskeletal components. (a) microtubule (b) intermediate filament (neurofilament) (c) microfilament (actin). Adapted from (Fletcher and Mullins 2010)

It is postulated that together these cytoskeletal components work to maintain cellular tensegrity (tensional integrity) (Ingber 1997; Ingber 2008). Cellular tensegrity was first proposed by R. Buckminster Fuller, an architect who defined tensegrity structures as systems which maintain their shape due to continuous tension rather than compression. In this context microfilaments and intermediate filaments together bare tensional forces, while microtubules act to balance these forces, resisting compression (fig. 1-10). The ability to propagate signals from the extracellular environment into the cell nucleus, via cytoskeletal filaments is known as percolation. As reviewed by Forgacs, the mechanism is described as being like an insect becoming trapped on a spiders web, where detailed information can be relayed across the web to the spider (Forgacs 1995). Evidence for the ability to transmit these mechanical signals into changes in gene expression were identified in a paper by Bloom et al which showed that when myocytes undergo mechanical stress, the arrangement of lamin-bound intermediate filaments becomes changed. In turn, this leads to changes in the spatial arrangement of lamin-bound chromatin altering chromosome packing or positioning leading to changes in the activation or deactivation of gene

expression (Bloom, Lockard et al. 1996). Furthermore, a seminal study investigating the effect of matrix elasticity's designed to mimic the physiological stiffness associated with bone, muscle, and brain tissue. As a result it was found that MSCs differentiate accordingly into osteogenic, myogenic and neurogenic cells as a result of culture on the corresponding matrices. The role of actin-myosin generated intracellular tension was again implicated in directing differentiation with myosin inhibition studies found to result in a loss of elasticity-directed lineage specification (Engler, Sen et al. 2006).

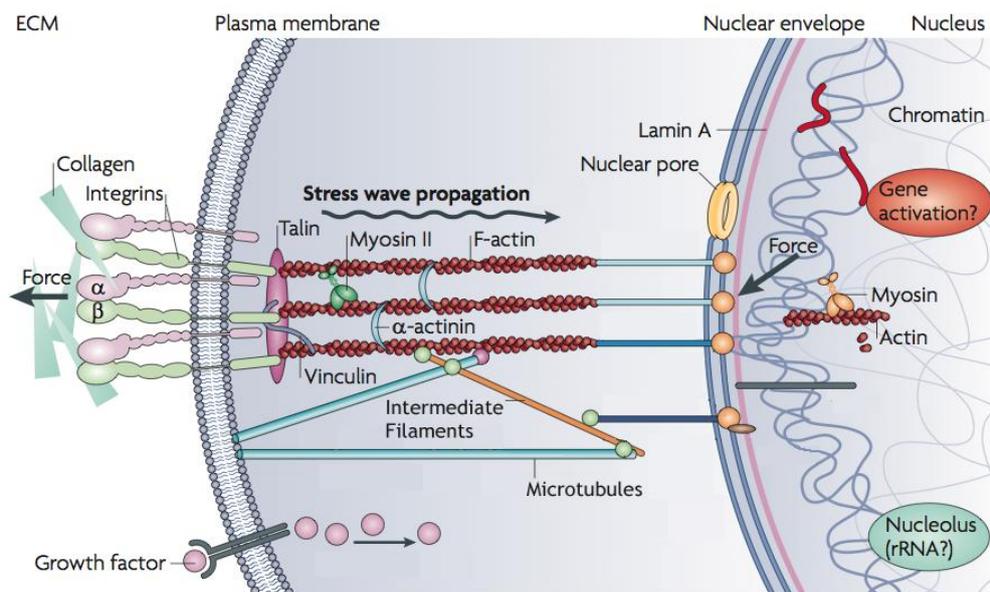


Figure 1-10 Direct Mechanotransduction. Schematic identifying a direct link between the extracellular matrix and the nucleus of the cell. A force applied to integrins via the ECM can be channelled to the nucleus through focal adhesion interaction with the cytoskeletal proteins microfilaments (actin), intermediate filaments (e.g. vimentin) and microtubules. Cytoskeletal interactions with the nucleus can lead to changes in gene expression via chromatin remodelling. Adapted from (Wang, Tytell et al. 2009)

1.7 Integrin Binding within the Stem Cell Niche

Interestingly, one main identifying features of stem cell niches is their high integrin expression (Jones and Wagers 2008). Integrin binding of stem cells within the niche is thought to play a vital role in several aspects of stem cell function including stem cell homing, tethering stem cells to the niche, in development of the niche architecture, regulating proliferation and self-renewal, and finally controlling the orientation of dividing cells (Quesenberry and Becker 1998; Qian, Tryggvason et al. 2006; Tanentzapf, Devenport et al. 2007; Lee, Lee et al. 2011).

The importance of integrin binding in the regulation of key stem cell functions therefore makes them a particularly interesting target for manipulation using biomaterials. In particular the use of topography as a mechanism for controlling the spatial formation and size of focal adhesions holds great potential. The investigation of topography on the function of stem cells has been investigated at both the micro- (10^{-6}) and nano-scale (10^{-9}) (Dalby, Gadegaard et al. 2007; Yim, Pang et al. 2007; Biggs 2008; Tsuruma, Tanaka et al. 2008). However, while topographic features on the micro-scale affect the whole cell, the comparative size of nano-scale features relative to integrins (approximately 10 nm) makes nanotopography a particularly exciting tool for manipulating focal adhesion formation. It is thought that nanotopography can alter integrin clustering and ultimately the formation of focal adhesions leading to changes in cellular tension. As discussed in section 1.5, changes in cellular tension can lead to changes in intracellular signalling and ultimately stem cell function. Using nanopit topographies of different geometries (ordered/disordered), it has previously been identified that nanotopographies which promote the formation of larger focal adhesions (disordered) promote the osteogenic differentiation of MSCs, compared to those which do not (ordered) (Dalby, Gadegaard et al. 2007; Biggs, Richards et al. 2009; Biggs, Richards et al. 2010). These results are exciting as they identify the potential for the regulation of stem cells not only through nano-scale interactions but also via spatial distribution of such features.

Interestingly, an investigation into the spatial distribution (order/disorder) of RGD nanopattern surfaces was also found to also effect integrin-mediated cell adhesion (Arnold, Cavalcanti-Adam et al. 2004; Huang, Grater et al. 2009). Results from the study were in agreement with those of the topographical study, indicating that disordered distribution of the RGD ligand promotes the formation of stable focal adhesions in contrast to an ordered distribution which disrupts adhesion formation. In this study the authors also propose that these cellular responses are due to a critical lateral distance imposed by the molecular size of particular integrin cross-linking proteins (Huang, Grater et al. 2009).

In the context of maintaining the self-renewal properties of MSCs, in particular evidence for the role of integrin interactions within the niche and in driving intracellular tension, coupled with increasing evidence for the role of

nanotopography in manipulating focal adhesion formation presents great scope for the use of nanotopography as a tool for maintaining MSC self-renewal.

1.8 Integrins and the Cell Cycle

As mentioned previously in section 1.4.1., participation in the cell cycle plays a crucial role in stem cell self-renewal and in the determination of cell fate decisions (Wozniak and Chen 2009). Evidence that cellular spreading and intracellular tension play a role in proliferation was first demonstrated during the 1970's through cell spreading studies (Curtis and Seehar 1978; Folkman and Moscona 1978).

The role of cellular spreading and intracellular tension in the control of the cell cycle occurs via adhesion-mediated activation of Rho GTPases and downstream signalling molecules such as ROCK, which work to control actin polymerisation and cytoskeletal tension (Mammoto and Ingber 2009; Parsons, Horwitz et al. 2010). In MSCs, Rho has also been linked to determining cell fate via changes in cell shape, again coupling cellular spreading and tension to determining cell fate decisions (McBeath, Pirone et al. 2004). During adhesion, initial integrin binding has been shown to induce focal adhesion kinase (FAK) signalling, the effect of which is also dependant on the degree of cellular spreading (Kornberg, Earp et al. 1992). Under conditions of increased cellular spreading FAK becomes phosphorylated, stimulating downstream signalling, while in rounded cells, FAK does not become phosphorylated having a two fold effect; downstream signalling events are not activated, while the unphosphorylated FAK also inhibits Rho activity via the activation of p190RhoGAP, a Rho inhibitor (McBeath, Pirone et al. 2004). Rac, another small GTPase of the same family, involved in modulating cytoskeletal remodelling has also been shown to influence the cell cycle via interactions with cyclin D1, a key mediator of cell cycle progression (Welsh, Roovers et al. 2001). Adhesion-mediated induction of Rac leads to increased cyclin D1 transcription, promoting progression through the cell cycle (Baldin, Lukas et al. 1993).

Further evidence supporting a role for nanotopography in mediating changes in stem cell function via alterations in integrin clustering and focal adhesion

formation, were identified following a study examining cellular spreading and focal adhesion formation in response to biofunctionalised RGD nanopatterned surfaces. In the study, the spacing of the RGD ligands were varied leading to alterations of integrin clustering. These changes in integrin clustering were shown to affect both the ability of cells to form stable focal adhesions and the induction of cell spreading (Cavalcanti-Adam, Volberg et al. 2007).

1.9 Integrins and Stem Cell Division

One of the key processes in the regulation of symmetrical versus asymmetrical stem cell division is the establishment of the plane of cell division and the orientation of the mitotic spindle (Toledano and Jones 2008). Key studies have identified integrins and other cell adhesion molecules as modulators of this process (Marthiens, Kazanis et al. 2010).

The mitotic spindle operates during mitosis to dictate the plane of cell division, and according to the Sachs and Hertwig rules the mitotic spindle usually orientates along the long axis of the cell indicating that cell shape plays an important part in its regulation (Inoue 1981; Toyoshima and Nishida 2007). Integrin-mediated cell adhesion, a key regulator of cell shape was shown to play a role in regulating mitotic spindle orientation following a study using fibronectin micro-patterned substrates. The authors showed that when HeLa cells were cultured on this substrate the mitotic spindle formed parallel to the adhesion plane. However, with loss of β_1 integrin following blocking, cells lost the ability to orientate their spindle (Toyoshima and Nishida 2007). It has been proposed that integrin-mediated regulation of spindle orientation may occur via myosin. This protein is found to bind microtubules at the spindle pole, linking it with the cytoplasmic domain of integrins (Toyoshima and Nishida 2007).

Spindle orientation in stem cells however not only dictates the plane of cell division but is important for the regulation of symmetrical and asymmetrical stem cell division (Morin and Bellaiche 2011). Spindle orientation during embryonic development has been shown to be important for directing asymmetrical stem cell division during tissue formation (Siller and Doe 2009; Yamashita 2009).

Integrin binding has been shown to play an important role in switching between asymmetrical and symmetrical cell division. In a study using mouse embryo-derived neural stem cells, Kosodo et al showed that inhibition of integrins was sufficient to generate a small shift in the plane of cell division switching division from asymmetrical to symmetrical (Kosodo, Roper et al. 2004). Recent evidence has also shown that cells have the ability to regulate their mitotic spindle positioning in response to mechanical stimuli (Fink, Carpi et al. 2011).

1.10 Nanotopographical Control of Stem Cells

The role of topographic surface features on the contact guidance of cells was first observed by R. Harrison in 1911 (Harrison 1911). It is his pioneering work which demonstrated the guidance of cells by the fibers of a spider's web, that has fuelled the field of biomaterials as a means to manipulate and direct the development of cells in culture. Further developments however, during the 1980's in techniques traditionally applied in the area of microelectronics made the availability and production of topographically patterned surfaces for the study of cellular response to topography more much more accessible.

In 1964 Curtis and Varde identified the ability of fibroblast cells to orientate themselves in the direction of silica fibers (Curtis and Varde 1964). Since this initial discoveries great focus has been placed on the investigation of micron sized topographic features, however during the last 10 years with further advances in the electronics field focus has been driven towards the study of even smaller topographical features, those on the nanoscale range. The importance of such nanoscale features has been driven by the abundance of nanoscale features within the extra-cellular matrix and the nanoscale interaction of proteins at the cell-material interface, and to which a cells readily comes into contact with within their native environment. To date research has identified nanotopography to have an effect on several cellular aspects including proliferation (Milner and Siedlecki 2007), morphology (Bettinger, Zhang et al. 2008; Lamers, van Horssen et al. 2010), adhesion (Dalby, Gadegaard et al. 2008; Le Guehennec, Lopez-Heredia et al. 2008; Le Guehennec, Martin et al. 2008), and gene expression (Biggs, Richards et al. 2009; Gasiorowski, Liliensiek et al. 2010; Yim, Darling et al. 2010). Importantly

however, current research has also shown that nanotopography has the ability to elicit specific cues and promote the controlled differentiation of stem cells *in vitro*.

1.10.1 MSC Response to Nanotopography

With regard to stem cells, biomaterials have the potential to serve two purposes. Firstly, and the main focus of this thesis, is the need to maintain a population of undifferentiated proliferating stem cells, and secondly, the ability to non-invasively promote the differentiation of stem cells down a specific lineage without the need for chemical supplements. Research into the nanotopographical control of mesenchymal stem cells has mainly focused on a requirement for differentiation, as discussed below, however, in recent years focus has shifted as the need to develop material strategies which promote maintenance of the stem cell population for regenerative applications grows.

As mentioned in section MSCs have been found to undergo differentiation into various cell lineages including bone, fat, cartilage (Owen and Friedenstein 1988; Pittenger, Mackay et al. 1999) and neurons (Song and Tuan 2004; Fu, Zhu et al. 2008) using chemically defined media. Results from several key studies have generated compelling evidence on the effect that substrate topography, especially at the nanoscale, can have on MSCs, and as discussed in section 3 disordered nanopit topography was shown to initiate the osteogenic differentiation of MSCs without the need for osteogenic differentiation supplements. (Dalby, McCloy et al. 2006; Dalby, Gadegaard et al. 2007). In a similar study MSCs were also shown to differentiate down an osteoblast lineage, this time in response to carbon nanotubes (Oh, Brammer et al. 2009). In this case, carbon nanotube diameter was identified as a crucial factor in promoting differentiation, with MSCs cultured on nanotubes of less than 50 nm in diameter producing negligible amounts of osteogenic markers. In addition, the trans-differentiation of MSCs into neuronal-like cells has been shown to occur in response to nanogratings (Yim, Pang et al. 2007). Yim *et al* identified the up-regulation of mature neuronal markers when MSCs were cultured on nanogratings in the absence of differentiation media. Interestingly, the authors went on to report higher levels of neuronal marker expression in

response to the nano-grating topography without differentiation media than chemical induction alone.

Furthermore, applied research has investigated the effect of nanotopographically-patterned metal surfaces on stem cell behaviour, as a pre-emptive step towards orthopaedic clinical applications (Popat, Chatvanichkul et al. 2007; Sjostrom, Dalby et al. 2009; Lavenus, Ricquier et al. 2010; McNamara, Sjostrom et al. 2011).

It is therefore evident that nanotopography can have a huge effect on skeletal stem cell regulation, however the mechanisms which underlie this topographical regulation are only recently beginning to be deciphered. It is hypothesized that a topographic surface primarily affects the ability of a cell to form focal adhesions via altered protein adsorption to the surface, or the disruption of a cells ability to form stable focal adhesions as discussed in section 3 (Yamamoto, Tanaka et al. 2006; Huang, Grater et al. 2009; Oh, Brammer et al. 2009; Scopelliti, Borgonovo et al. 2010). In 2007, it was also demonstrated that stem cell growth on a nanotopographic surface could lead to changes in gene expression between topographically and chemically differentiated MSCs, an indication that topography may work via a distinct mechanism (Dalby, Gadegaard et al. 2007; Dalby, Andar et al. 2008). Additionally, it was later identified that these changes in gene expression correlate with differences in focal adhesion formation on various nanotopographical substrates (Biggs, Richards et al. 2009; Biggs, Richards et al. 2009). In a later study Yim *et al* identified that the disruption of focal adhesion formation results in changes in the mechanical properties of cells, and also identified changes in gene expression (Yim, Darling et al. 2010).

1.11 Aims and Objectives

The ability to deliver high quality autologous MSCs to the clinic for therapeutic applications is of utmost importance. Current materials for *in vitro* culture do not provide stem cells with the appropriate cues necessary for maintenance of self-renewal ultimately leading to a critical loss of the multipotent population. Nanotopographically patterned polymer substrates which have the ability to

elicit an appropriate cellular response without the need for chemical intervention presents an attractive solution.

The aim of this thesis therefore is to examine the effects of nanotopographically patterned substrates on the retained multipotency of MSCs, in the absence of supplements. A flat control was used throughout, in conjunction with two osteogenic controls, a disordered osteogenesis promoting nanotopography and a flat control with osteogenic supplemented media. This will be achieved by the following:

- The identification of MSC maintenance over time in response to ordered nanotopography.
 - An investigation of key stem cell and osteogenic marker expression over a period of four weeks.
 - Long-term maintenance of stem cell markers for up to eight weeks.
 - A quantitative assessment of key stem cell and osteogenic markers using real time quantitative PCR.
 - Assessment of stem cell proliferation.
 - Assessment for continued multipotency following culture over four weeks.
- Assessment of global gene changes in MSCs in response to ordered nanotopography.
 - Microarray and ingenuity pathway analysis of canonical and functional signalling in MSCs in response to nanotopography.
 - Assessment of smallRNA expression in response to nanotopography.
- Examination of the metabolic profile of MSCs in response to nanotopography.

- **Examination of MSC maintenance in response to different polymers (polycarbonate/polystyrene) using the same nanotopographical pattern.**

2 Materials and Methods

2.1 General Introduction

In recent decades, multiple experimental systems have shown the capability of MSCs to differentiate down a wide number of cellular lineages, cementing their potential as ideal candidates for use in tissue engineering and regenerative medicine purposes. Clinically, however, a major limitation in the use of MSCs is their relatively low frequency (MSCs make up only 10% of the adherent cells isolated from the bone marrow) *in vivo*. Additionally, subsequent *in vitro* culture is commonly known to result in a loss of their multipotent phenotype. As a consequence of this, throughout this study MSCs were used at low passage number to ensure their multipotency.

Whilst definitive identification of a single surface antigen that recognizes self-renewing MSCs remains elusive, throughout the literature, the STRO-1 antigen has been identified as a key marker for the identification of this multipotent population within the bone marrow. To isolate the stem cells from the general population, magnetic activated cell sorting (MACS) in combination with a STRO-1 monoclonal antibody has proved a valuable technique. Whilst the role of this trypsin-resistant cell surface antigen in the function of MSCs has not yet been identified, it is widely accepted that use of the STRO-1 antigen as a selection marker for isolating cells from the main stromal cell population results in an adherent, multipotent population, with reduced heterogeneity within the isolated stem cell population (Bianco, Riminucci et al. 2001; Gronthos and Zannettino 2008).

In vitro, MSCs have previously been shown to respond to multiple material strategies, including nanotopography, undergoing differentiation down various cell lineages in response to specific cues (Thomas, Collier et al. 2002; McBeath, Pirone et al. 2004; Dalby, McCloy et al. 2006; Dalby, McCloy et al. 2006; Engler, Sen et al. 2006; Dalby, Gadegaard et al. 2007; Oh, Brammer et al. 2009; Kilian, Bugarija et al. 2010). A study by Gilbert et al, using biomimetic stiffness matching and muscle stem cells, also provides evidence to support the

hypothesis for MSC self-renewal in response to material properties (Gilbert, Havenstrite et al. 2010).

Previously within the lab a study was undertaken to examine the effects of nanopits with varying degrees of order on the differentiation of MSCs down an osteogenic lineage (Dalby, Gadegaard et al. 2007). As a result of this study, not only was osteogenic differentiation on disordered nanopits (120nm pits in a square arrangement with centre-centre spacing of 300nm, but with ± 50 nm offset in pit placement in x and y axes, referred to as near square NSQ) shown to be comparable to that using chemical supplements such as dexamethasone, but a lack of differentiation was also observed on nanopits of the same dimensions but with an offset reduced to zero, forming a near absolute square (SQ) lattice. These empirical observations formed the first basis of our hypothesis (Dalby, Gadegaard et al. 2007).

2.2 STRO-1⁺ MSCs

STRO-1⁺ MSCs were provided by Prof Richard Oreffo, University of Southampton. STRO-1⁺ cells were isolated from bone marrow samples obtained from haematologically normal patients undergoing routine total hip replacement surgery and with the approval of the Southampton General Hospital Ethics Committee. Only tissue that would have been discarded was used.

Marrow cells were aspirated from trabecular bone marrow samples and collected in minimal essential medium - α modification (α -MEM). Cells underwent centrifugation at 250 g for 4 minutes at 4°C to form a pellet. Following pellet resuspension in α -MEM the solution was passed through a nylon mesh (70 μ m pore size; *Becton-Dickinson, Franklin Lakes, NJ*).

Using a lymphoprep solution (*Robins scientific, Solihull, UK*), red blood cells were removed by centrifugation. Cells from the buffy layer were resuspended at 1×10^8 cells in 10 ml blocking solution (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution (HBSS) with 5% volume per volume (v/v) FCS, 5% (v/v) human normal serum, and 1% weight per volume (w/v) bovine serum albumin (BSA) fraction volume) and subsequently

incubated with a STRO-1 antibody in a hybridoma supernatant (*hybridoma was provided by Dr. J. Beresford, University of Bath*). Cells were washed with MACS buffer (HBSS containing 1% fraction volume BSA) to remove any excess STRO-1 antibody before incubation with MACS anti-immunoglobulin M beads (*Miltenyi Biotech, Bisley, UK*). As a control, labelled cells were added to an unmagnetised column and collected as control unsorted cells. Separated cells were collected as follows; the antibody incubated cell suspension was added to a column within the magnet and the elutant was collected making up the STRO-1 negative fraction. The column was then washed, and in the absence of the magnet, 1 ml MACS buffer was added. The resultant cell population was eluted as the STRO-1 positive fraction. For each fraction, five cell counts were performed using a fast read disposable counting chamber (*ISL, Paignton, UK*).

2.2.1 Cell culture

MSCs were maintained in basal media (10% FBS/ α MEM) (PAA, UK) at 37°C with 5% CO₂ in humid conditions. Cells are seeded onto the nanopatterned and flat materials of 1cm² in size at 5,000 cells/cm² and the media changed twice a week. Osteogenic media (OGM) was used as a control for induced differentiation containing dexamethasone (10nm) and L-ascorbic acid (150 μ g ml⁻¹). Cells were used at passages P0 - P2 throughout - this allows for not only expansion, but also preservation of multipotency. Cells from a large number of patients were used over the course of the study which shows the robustness of the data.

2.3 Experimental and Control Substrate Preparation

Electron beam lithography (EBL), a technique routinely used within the electronics industry (e.g. in the production of nano-scale electronics (e.g. transistors, gates etc) and optics), has made it possible to produce precise, reproducible nano-scale topographies (Gadegaard, Thoms et al. 2003). Continuing advances within the field, particularly regarding EBL, have now made it possible to generate topographical features down to 3-5nm (Vieu, Carcenac et al. 2000). Whilst the fabrication process involving EBL is generally regarded to be slow, replication techniques such as hot embossing (low throughput) and injection moulding (high throughput) into polymers provide an inexpensive

method for the replication of such topographies. Replication of topographies into a polymer substrate also provides consistency, and ensures that only changes in topographical features, not changes in chemistry are observed. Such approaches where a nano-scale master is used to produce many polymeric replicas (i.e. amenable to mass production) are often used in e.g. DVD and BluRay manufacture.

Throughout this study three types of polymers were used as both as control and experimental substrates: polycaprolactone (PCL), polycarbonate (PC), and polystyrene (PS), the gold standard polymer used in tissue culture plastic, to observe any changes in stem cell phenotype which may be contributed to polymer chemistry and to test how facile the nanotopographies are in terms of translation of cell response on different base substrates. Table 2-1 identifies key characteristics of the polymers used throughout the study.

	Melting temperature (T _m)	Young's Modulus (E)	Transparent	FDA approved
Polycaprolactone	60°C		No	Yes
Polycarbonate	267°C	2.0 - 2.4 GPa	Yes	Some types
Polystyrene	240°C	3.0 - 3.6 GPa	Yes	Yes

Table 2-1 Properties of the three polymers used throughout the study.

Materials were made in a three-step process of electron beam lithography, nickel die fabrication and replication.

2.3.1 Electron Beam Lithography

Electron beam lithography is based on the same principles that underlie a scanning electron microscope (SEM). As with SEM, electrons from an electron source undergo acceleration, with the electron beam focused using electrostatic lenses. According to the de Broglie relations, the wavelength of a particle is inversely proportional to the momentum of the particle (De Broglie 1924).

Therefore using this technique, the high-speed acceleration of electrons produces an extremely short wavelength relative to light, whereby narrow spots of only 3 - 5 nm can be achieved (Vieu, Carcenac et al. 2000; Gadegaard, Thoms et al. 2003).

EBL uses a pre-programmed, computer controlled electron gun to steer the beam of electrons and an electron sensitive resist (fig. 2-1). Whilst other techniques such as photolithography use a mask to define and therefore a resist can be exposed uniformly, EBL requires consecutive, or serial exposure of the resist from the electron source increasing production time (Ng and et al. 2002).

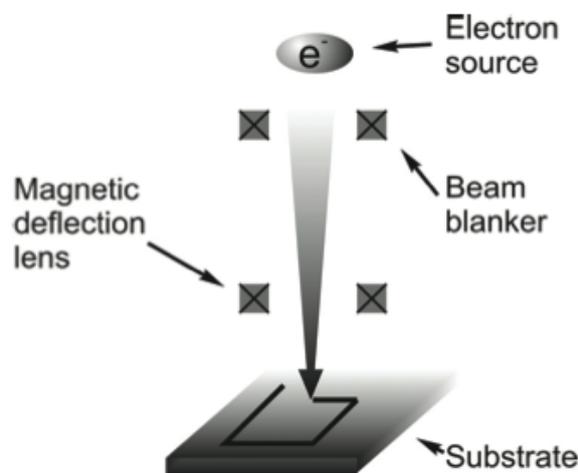


Figure 2-1 Electron beam lithography. Electrons are produced from an electron source, which undergo focusing onto a narrow spot. Image courtesy of (McMurray, Dalby et al. 2011)

The process of EBL begins with the choice of substrate, which must be conducting (typically silicon) to prevent the build-up of electrical charge as a result of electron bombardment (fig. 2-2). A resist is then applied to the substrate, which must be electron sensitive as mentioned previously. A resist can take one of two forms, positive or negative tone, depending on whether the exposed area breaks down or becomes cross-linked. Positive tone resists make up the former with exposed areas breaking down following exposure and subsequent development, while negative tone resists make up the latter with exposed areas becoming insoluble. Following development of the resist to reveal the final pattern, etching is required to transfer that pattern into the underlying substrate. During the etching process the resist acts as a mask protecting the substrate while the exposed substrate undergoes etching (Gadegaard, Thoms et al. 2003; Norman and Desai 2006).

Initial substrate fabrication was carried out by Dr. Nikolaj Gadegaard at the James Watt Nanofabrication Centre, University of Glasgow. Firstly, silicon wafers were cleaned for 5 minutes using acetone in an ultrasonic bath. The wafers were subsequently rinsed in reverse osmosis water (ROH₂O) and blown dry with a filter-fitted air gun. Next, they were spun with primer for 30 seconds at 4000 revolutions per minute (rpm), then spun with a positive tone resist, ZEP 520 (*Nippon Zeon, Tokyo, Japan*) for 30 seconds at 4000 rpm, and baked for 30 minutes at 90°C. The resulting layer was measured to be 1.8 μm thick. A Leica EBPG5-HR using an 80 nm spot size beam was used to write the pattern at 50 kV. The exposed samples were developed in o-xylene at 23°C for 60 seconds and rinsed in iso-2-propanol. Write fields were stitched together by mechanical movement of the stage.

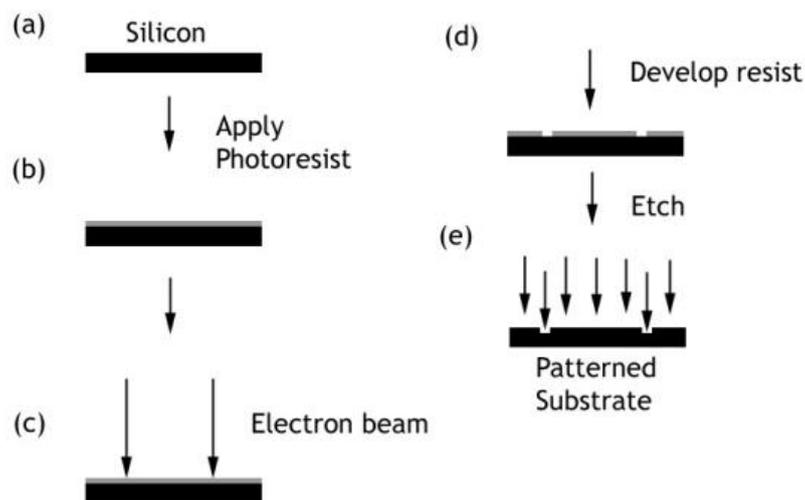


Figure 2-2 The process of electron beam lithography. An electron sensitive resist is applied to the substrate (b), which is then exposed to an electron beam (c). Development of the resist (in this case a positive tone resist) reveals the final pattern (d), which is then transferred into a conducting substrate such as silicon (a) via an etching process (e). Adapted from (Dalby, Riehle et al. 2004).

2.3.2 Nickel Die Fabrication

As silicon is a brittle material, fabrication of a nickel shim is ideal. The nickel shim was kindly produced by John Pederson (SDC Dandisc A/S, Denmark). This can be achieved using a process of nickel die fabrication from the original ZEP 520 EBL produced master. Initially a thin layer of Ni-V (approximately 50 nm with 7% vanadium) is sputter coated onto the sample, and this subsequently acts as an electrode during electroplating of nickel onto the Ni-V to thickness of 300 μm. Cleaning of the nickel shims occurs by stripping the protective polyurethane layer using chloroform in an ultrasound bath for 10-15 mins. Silicon residues

were also removed by wet etching in 25% potassium hydroxide for 1 hr at 80°C. Removal of resist and silicon residues reveals the nano-pattern in a negative (inverse) format. Lastly, the shim was rinsed in ROH₂O, air-gun dried and checked using AFM (Gadegaard, Mosler et al. 2003).

2.3.3 Master Substrate

Master substrates were fabricated to form an array of 120 nm diameter pits of 100 nm depth and 300 nm pitch in a square (SQ) arrangement (fig. 2-3 A). Arrays of dots were also fabricated with near square (NSQ) order, having a random displacement of ± 50 nm, and maintaining an average 300 nm pitch (fig. 2-3 B).

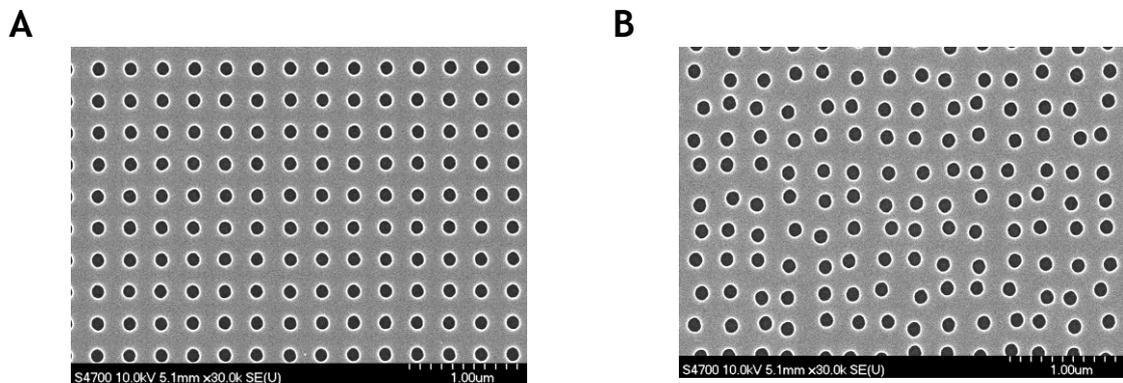


Figure 2-3 SEM images of the master substrates produced by EBL (A) Nanopits with 120 nm diameter, 100 nm depth and 300 nm pitch in a square arrangement (SQ) (B) Nanopits with 120 nm diameter, 100 nm depth, with a random displacement of ± 50 nm, however maintaining an average 300 nm pitch (NSQ).

A previous study investigating the effect of nanopit geometry on the differentiation of MSCs, identified that MSCs were induced to undergo osteogenic differentiation, in the absence of induction media, as a result of culture on nanopits with a random displacement of 50 nm (NSQ), as shown in fig. 2-3 B (Dalby, Gadegaard et al. 2007). As a result of this study, the NSQ nanotopography was used as a topographical osteogenic control throughout this thesis.

2.3.4 Replication

As the nickel shim is a hard, negative, of the master pattern, it can be used to imprint with into polymer materials very easily. This allows samples to be produced with high fidelity in sufficient numbers for e.g. cell culture

experiments. Replication also makes substrate fabrication cost efficient and rapid.

2.3.4.1 Hot Embossing

Hot embossing although a relatively low throughput technique, is one of the most common methods for imprinting samples required for research purposes as it does not require specialist equipment or knowledge (fig. 2-3 A). The technique of hot embossing is straightforward and utilises the low melting temperature of polymers such as PCL (60°C). A hot plate is used to reach the melting temperature of the polymer, pressure can then be applied to imprint the pattern of the master shim into the polymer (fig. 2-3 B). The polymer and master shim are then left to cool, after which the imprinted polymer can be released from the master shim (Gadegaard, Thoms et al. 2003; Mills, Martinez et al. 2005).

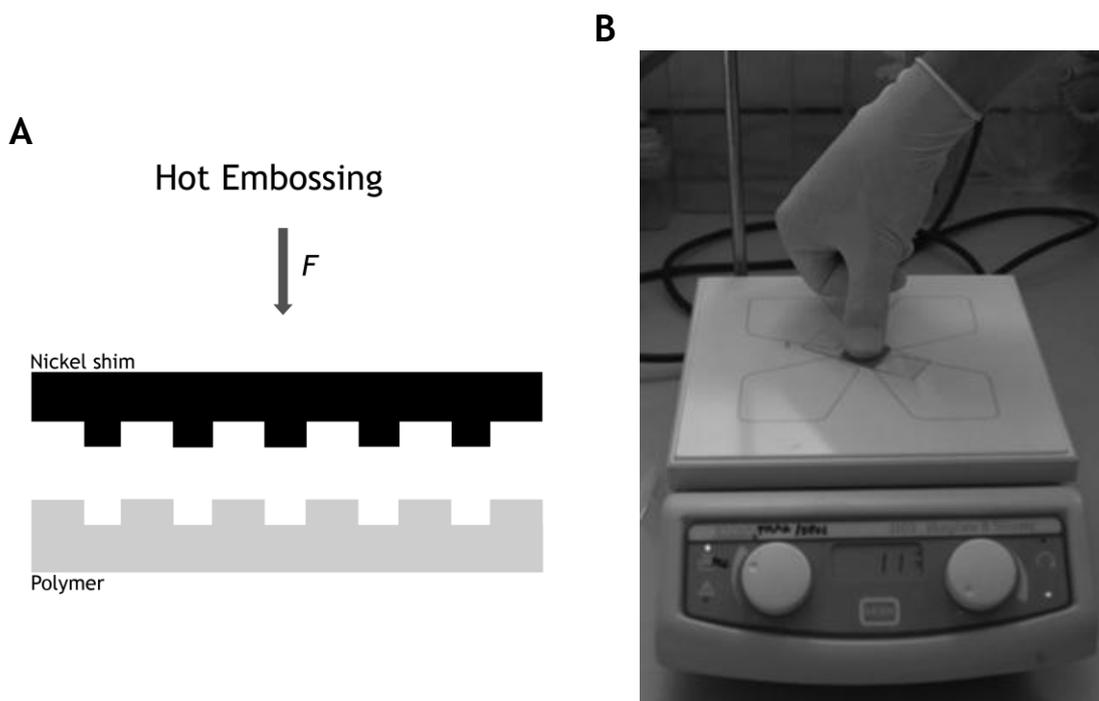


Figure 2-4 (a) Hot embossing is used to imprint into polymer substrates, (b) a typical setup using a hot plate for embossing. Images adapted from (McMurray, Dalby et al. 2011).

2.3.4.2 Injection moulding

In an industrial setting injection moulding is *the* method of choice for substrate replication, and is at present already employed in the manufacture of standard tissue culture plastics as well as the mass production of a number of products

associated with the electronics industry, such as DVD and Blu-Ray discs. In contrast to hot embossing, an injection moulder has the ability to produce thousands of replicates, however, this type of production requires specialist machinery and knowledge (fig 2-5 A).

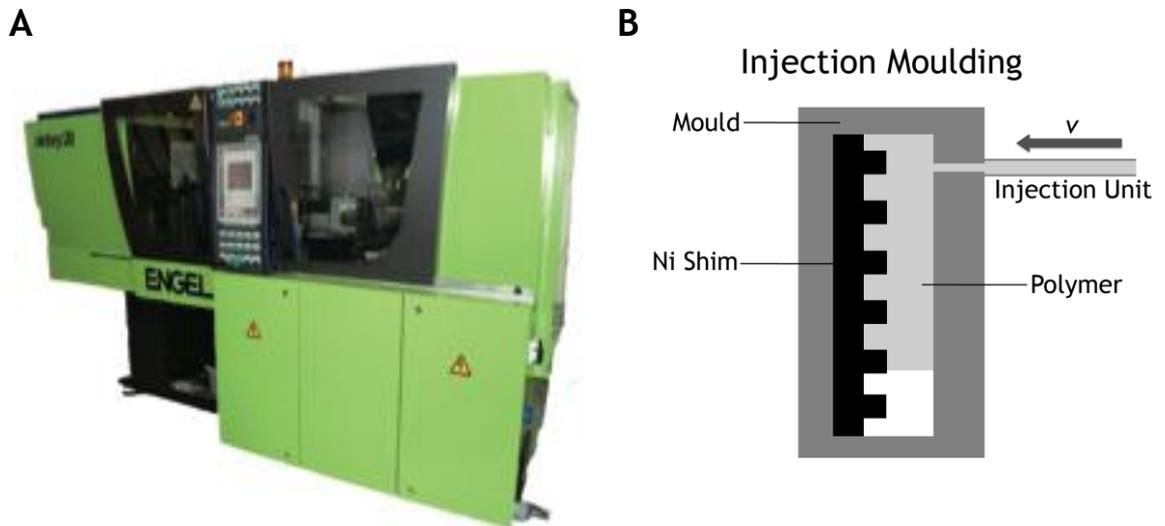


Figure 2-5 (A) injection moulder, (B) Basic principle behind injection moulding. Images adapted from (McMurray, Dalby et al. 2011).

The basic principle of injection moulding involves injecting a molten polymer into a mould which is kept at a lower temperature than the glass transition temperature of the particular polymer. This ensures that the polymer cools rapidly during the process cycle, allowing for quick removal from the cavity and the retention of shape after removal (fig 2-5 B) (Gadegaard, Mosler et al. 2003).

2.3.5 Polymer replication

Imprints of the nickel shims into PCL were achieved by hot-embossing (fig 2-6 A & B). The resulting imprints, SQ and NSQ, were trimmed for use in tissue culture well plates and planar PCL (Ra of 1.17 nm over 10 μm measured using AFM) was used as a control substrate. In alternative experiments the nanotopographies were imprinted into PC or PS using an injection moulder (Engel Victory Tech 28) (fig. 2-6 C & D). Planar PC/PS was also used as a control. PC and PS samples were given a 2 and 10 second air plasma treatment respectively, to allow for cell attachment (Harrick Plasma, USA). Plasma treatment was carried out on the lowest setting (7.16W, with a total pressure of 0.27 mbar)

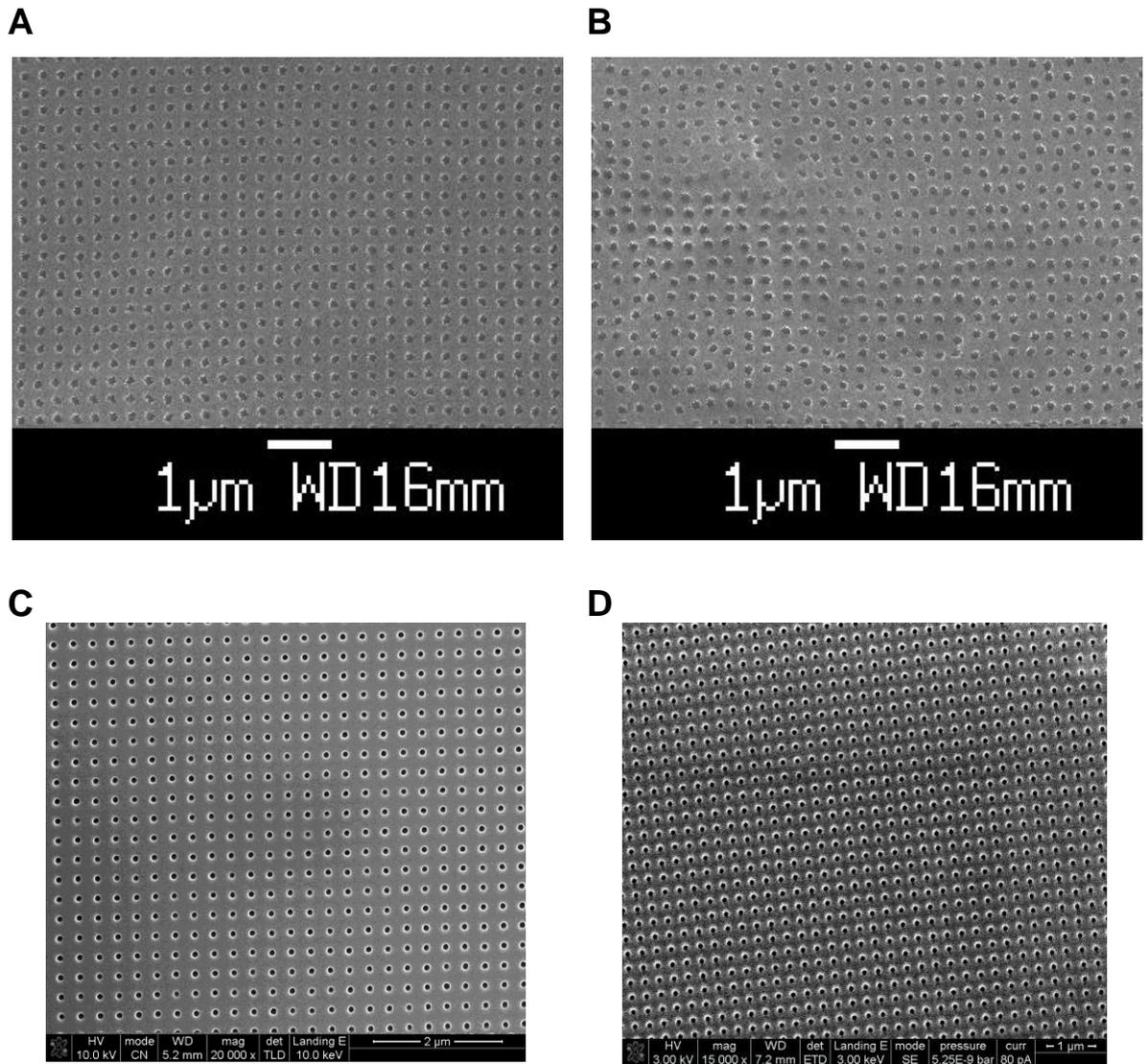


Figure 2-6 SEM images of nanopatterned PCL, PC and PS substrates produced by hot embossing (A) SQ (B) NSQ and injection moulding (C) PC and (D) PS.

2.3.6 Water Contact Angle Measurements

Throughout the study the NSQ nanopattern will be used as a positive nanopatterned control for osteogenic differentiation. In order to determine that changes in stem cell phenotype and function can be attributed to actual changes in nanopatterned geometry and not changes in surface chemistry imparted as a result of the nanopattern, the water contact angle of embossed PCL was examined to study the influence on the wettability of the substrates when nano-patterns were imprinted onto the surface.

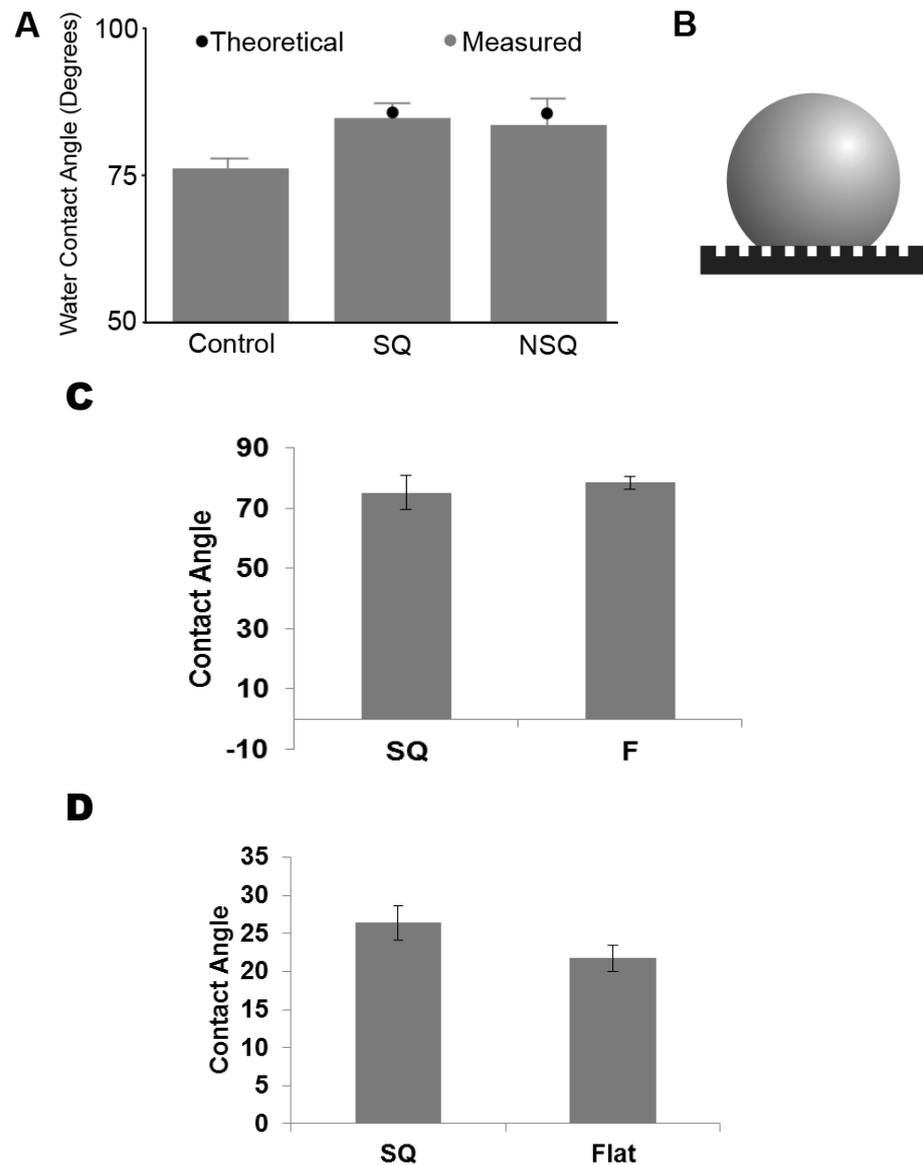


Figure 2-7 Water contact angle analysis for flat and nano-patterned (A) the water contact angle measured for the flat control and both nanotopographies embossed into PCL. (B) In the Cassie-Baxter state, a drop of water will not penetrate the structure. (C) Contact angle measurement for flat and nanopatterned polycarbonate (D) contact angle measurement for flat and nanopatterned polystyrene.

It is well known that surface roughening or surface topography influences surface energy and thus changes the contact angle with which a drop of water forms with the surface. This change in contact angle was formulated by Cassie-Baxter $\cos \theta_{CB} = rf \cos \theta_Y + f - 1$ as, where θ_{CB} is the predicted contact angle on a roughened or structured surface, r is the roughness, θ_Y is the contact angle on an (ideal) flat surface and f is the area fraction of the projected wet area (fig. 2-7 B) (Cassie and Baxter 1944). The nanopits, in both cases (SQ and NSQ), cover 10% of the surface area ($f=0.9$) assumed on an ideally uniform surface ($r=1$). The water contact angle was measured on a flat PCL surface

($\theta_Y=75.5\pm 2.5^\circ$). According to the Cassie-Baxter formulation, the disorder in the pattern will not change the apparent contact angle as the surface coverage for both patterns is ca. 10%. In both cases we measured the water contact angle to be 81-82° (fig. 2-7 A). The Cassie-Baxter predicted contact angle is 83° which is in good agreement with the measurements. Furthermore, there is no significant difference between SQ and NSQ but there is the expected difference to the flat control. Thus, we can be sure that changes in cell response to SQ and NSQ are due to nano-scale topography alone.

2.4 Discussion

Throughout the study, three polymers were used to assess the effect of nanotopography on self-renewal of MSCs. Initially in the study PCL was used due to its low melting temperature, making it a viable polymer for hot embossing. For later experiments, after the acquisition of an injection moulder, cell testing of PC and PS imprinted substrates could be used to test the strength of the topographical effects in different base polymers.

PC and PS have the advantage that they are both transparent enabling better visualization of *in vitro* culture, but PS is also the current gold standard polymer in the production of tissue culture plasticware, allowing a direct comparison between the industrial standard flat PS and topographically patterned PS. Oxygen plasma treatment of both PC and PS substrates was used to overcome the slightly hydrophobic nature of both polymers, and is common practice for industrial produced tissue culture plastics.

As the main aim of the study is to investigate the self-renewal of MSCs *in vitro* when cultured on nanotopographies in comparison to both a flat control and osteogenic controls, it is therefore critical to identify the multipotent stem cell population within the bone marrow stroma. To ensure multipotency throughout the study a well-established selection technique, MACs was used in combination with the STRO-1 marker, to provide robust, reliable isolation of the multipotent population. During *in vitro* culture on standard tissue culture flasks MSCs were used at passages 1-2 to ensure there is still a largely homogenous multipotent stem cell population.

3 Multipotency and Differentiation

3.1 General Introduction

When culturing stem cells for clinical applications it is vital that during expansion of the stem cell population *in vitro*, MSCs retain their stem cell phenotype and multi-lineage potential. As stated previously, MSCs undergo spontaneous differentiation into fibroblast-like cells during *in vitro* culture on standard tissue culture plastic, progressively losing their multi-lineage potential (Banfi, Muraglia et al. 2000; Sherley 2002; Siddappa, Licht et al. 2007). It is therefore crucial to investigate any phenotypic changes that occur in MSCs over time in response to the test nanotopography and controls. Throughout this chapter various phenotypic markers have been used to identify both the stem cell population and the up-regulation of markers associated with osteogenic or adipogenic differentiation in response to all treatments.

STRO-1 is a widely accepted marker for the positive selection of a subset of bone marrow cells, identified to isolate a largely homogenous multipotent MSC population by Gronthos and co-workers (Simmons and Torok-Storb 1991; Gronthos and Zannettino 2008). Throughout this study STRO-1 was used to isolate the initial population of cells, therefore STRO-1 has been used as a marker to detect continued phenotypic maintenance of the multipotent population. In addition, another marker, ALCAM, was used in combination with STRO-1. ALCAM is known to be less selective and has been shown to identify a less primitive progenitor population than STRO-1 (Stewart, Monk et al. 2003). Importantly, loss of both STRO-1 and ALCAM expression has been shown to correlate with differentiation (Simmons and Torok-Storb 1991; Bruder, Jaiswal et al. 1997; Arai, Ohneda et al. 2002).

The osteogenic differentiation of MSCs is marked by the expression of key genes and transcription factors at distinct time points. These can be classed as either early or late depending on when they become expressed during differentiation (Stein, Lian et al. 1990). To induce osteogenic differentiation a combination of ascorbic acid and dexamethasone can be added to supplement the media (Pittenger, Mackay et al. 1999). Ascorbic acid, more commonly known as vitamin

C, increases collagen type I and III ECM production, and leads to the up-regulation of genes associated with maturation and mineralization of the matrix (Schwarz 1985; Schwarz, Kleinman et al. 1987; Aronow, Gerstenfeld et al. 1990). Additionally, dexamethasone, a synthetic glucocorticoid, up-regulates the expression of alkaline phosphatase to promote osteogenic differentiation (McCulloch and Tenenbaum 1986; Rickard, Sullivan et al. 1994; Bruder and Jaiswal 1995; Mikami, Asano et al. 2010). A key marker of early osteogenic differentiation is the transcription factor RUNX2. RUNX2 regulates key osteoblast-specific gene expressions such as OPN and OCN, late stage osteogenic markers (Ducy, Zhang et al. 1997; Harada, Tagashira et al. 1999; Komori 2002; Hong, Hwang et al. 2005).

To demonstrate their multipotent nature, MSCs can also be induced to undergo adipogenesis using a combination of insulin, indomethacin and isobutylmethylxanthine, known to induce expression of a key early time point adipogenic marker, PPAR- γ (Hung, Chang et al. 2004). PPAR- γ is a transcription factor which acts to regulate the expression of genes involved in lipid uptake into the cells (Lowell 1999). Lipid uptake into the cells leads to the accumulation of lipid vacuoles, another marker for adipogenic differentiation (Lowell 1999; Rosen, Sarraf et al. 1999). Therefore, using a combination both stem cell/progenitor and differentiation markers it is possible to visualise the maintenance or differentiation of the MSCs on different substrates.

In addition to maintenance of the stem cell phenotype, the ability to maintain expansion of the stem cell population is critical. Not only has proliferation been coupled to self-renewal as discussed in section 1.4.1., but a loss of proliferation has been shown to indicate the onset of differentiation (Stein, Lian et al. 1990; Stein and Lian 1993). Using a thymidine analogue, bromodeoxyuridine (BrdU), incorporation of the synthetic nucleoside during S-phase into the DNA can be used in conjunction with immunohistochemistry to detect proliferating cells. This enables us to make sure that we can expand the multipotent cell population rather than just producing quiescence cells that retain certain markers.

Using topographical substrates as a means to provide large quantities of stem cells requires the application of conventional cell culture techniques such as

passaging, a technique routinely used to maintain optimum cell density whilst expanding the cell population. Passaging of adherent cells such as MSCs requires the cells to be detached from the substrate using trypsin, a serine protease. Passaging is often used as an estimate of the number of cell divisions a cell has undergone *in vitro*. Within the field it is generally accepted that successive passaging of MSCs leads to a loss of multilineage potential, therefore it is important to establish that maintenance of the stem cell phenotype occurs following serial passaging (Banfi, Muraglia et al. 2000; Sherley 2002; Siddappa, Licht et al. 2007).

The aims of this chapter therefore are three fold, firstly, to identify phenotypic markers which demonstrate continued maintenance of the MSC phenotype on the SQ nanotopography. Secondly, to demonstrate the multipotency potential of MSCs cultured on the SQ nanotopography over time by carrying out differentiation studies, and lastly, to demonstrate continued proliferation of MSCs cultured on the SQ nanotopography.

3.2 Materials and Methods

3.2.1 Polymer replication

Imprints of the nickel substrate into PCL (M_w 65,000 Sigma Aldrich, UK) was achieved by hot-embossing as described in section 2.3.4.1.. The resulting imprints, SQ and NSQ, were trimmed to fit in tissue culture well plates and flat PCL (Ra of 1.17 nm over 10 μ m measured using AFM) was used as a control substrate.

3.2.2 Cell culture

STRO-1 MSCs were provided by Prof. Richard Oreffo at the University of Southampton. STRO-1 cells were isolated as described in section 2.2 from bone marrow samples obtained from haematologically normal patients undergoing routine total hip replacement surgery and with the approval of the Southampton General Hospital Ethics Committee. Only tissue that would have been discarded was used. In addition, commercially available MSCs (skeletal and adipose-

derived) were purchased from Promocell (*Promocell, Germany*) and used at passage 2; these were also cultured in basal media (10% FBS/ α MEM) or for osteogenic differentiation basal media was supplemented with dexamethasone (10nm) (*Sigma, UK*) and L-ascorbic acid ($150 \mu\text{g ml}^{-1}$) (*Sigma, UK*).

3.2.3 Immunofluorescence

MSCs were cultured for 7, 14, 21 and 28 days in basal media (10% FBS/ α MEM) (PAA, UK) at 37°C with 5% CO₂ in humid conditions. Cells are seeded onto the materials at 1×10^4 cells / ml and the media changed twice a week. Osteogenic media was used as a control for induced differentiation containing dexamethasone (10nm) and L-ascorbic acid ($150 \mu\text{g ml}^{-1}$). At the relevant time points MSCs were fixed in 4% formaldehyde for 15 mins, followed by 5 minutes permeabilisation in permeabilising buffer. Then samples were blocked in 1% PBS/BSA for 5 mins, and subsequently stained for osteogenic markers using mouse-monoclonal OCN/OPN antibodies (1:50) (*Santa Cruz Biotechnology*) and stem cell markers using mouse-monoclonal STRO-1/ALCAM antibodies (1:50) (*Santa Cruz Biotechnology*) in conjunction with rhodamine-phalloidin (1:50) (*Sigma*) for 1 hr at 37°C. Samples were then washed three times in PBS/Tween 20 to remove the primary antibody. An anti-mouse secondary biotinylated (1:50) (*Sigma*) was then added and incubated at 37°C for 1 hr. Following this samples were again washed three times in PBS/Tween 20 to remove the secondary antibody. A tertiary FITC-labelled streptavidin antibody (1:50) (*Sigma*) was added and samples incubated at 4°C for 30 mins. Samples were then washed again three times in PBS/Tween 20 and mounted using mounting medium containing DAPI to stain the cell nucleus. Similarly, after 8 weeks culture cells were fixed and stained for STRO-1 and ALCAM.

For quantification, images (x 10 magnification) were taken with similar background and exported to Image J (free download from NIH). In image J, the images were thresholded to select positively stained areas and mean greyscale and area calculated. DAPI staining was used to allow counting of cell numbers. Integrated intensity (arbitrary units) was calculated by multiplying mean greyscale intensity by feature area. These values were totalled for each

substrate and normalized to cell number to give intensity per cell. ANOVA was used to compare samples (approx 900 cells were considered for each treatment).

3.2.4 RNA isolation

STRO-1+ cells were seeded onto nanotopographical and control substrates at a density of 1×10^4 cells per ml. Cells were maintained at 37°C with a 5% CO₂ atmosphere in α -MEM (*Invitrogen, UK*) containing 10% FBS, which was replaced twice weekly. Cells were then lysed and total RNA extracted using a Qiagen RNeasy Micro Kit according to manufacturer's protocols (*Qiagen, West Sussex, UK*).

Briefly, STRO-1+ MSC lysate was homogenised by vortexing and subsequent passing the lysate through a 20-gauge syringe needle. Equal volumes of 70% ethanol were added to the homogenized lysate, mixed thoroughly and added to the spin column and centrifuged at 8,000 g for 15 seconds. 350 μ l of buffer RW1 containing guanidine thiocyanate was added to the spin column to denature proteins, such as RNases. The spin column was again centrifuged at 8,000 g for 15 seconds. DNA was then denatured for 15 minutes using a solution of DNase I and buffer RDD. Buffer RW1 was added again to remove the DNase I and the spin column centrifuged for 15 seconds at 8,000 g. Following washing all filtrate was discarded. A further wash was carried out using buffer RPE followed by 80% ethanol to precipitate the RNA. The filter was then centrifuged at full speed for 2 minutes to remove any remaining ethanol. The dried filter was then transferred to a 1 ml Eppendorf tube and 14 μ l RNase-free water added. The tube and filter were subsequently centrifuged at full speed for 1 minute. Measurement of extracted RNA yields was performed with a NanoDrop® ND-1000 UV- Vis Spectrophotometer at ratios of 230/260 nm and 260/280 nm.

3.2.5 cDNA synthesis

Total RNA was reverse transcribed using the Applied Biosystems High Capacity RNA-to-cDNA kit (*Applied Biosystems, UK*). Firstly a reverse transcription master mix was prepared to a total volume of 10 μ l:

10x RT buffer	2.0 ul
25x dNTP Mix (100mM)	0.8 ul
10x RT Random primers	2.0 ul
Reverse Transcriptase	1.0 ul
RNase Inhibitor	1.0 ul
Nuclease-free H ₂ O	3.2 ul

Following this, prepare a 10 µl solution of RNA containing equal concentrations of RNA (make up with H₂O).

Finally, mix the master Mix and RNA sample gently and put on ice until ready to load the thermal cycler. The following programme was used to run the thermal cycler:

25°C	10 min
37°C	120 min
85°C	5 min
4°C	forever

3.2.6 Quantitative real time PCR.

MSCs were cultured on materials for 28 days (4 replicas for each materials). Following this cells were lysed and total RNA extracted using a Qiagen RNeasy micro kit (*Qiagen, UK*) as described in section 3.1.3.. Following this, RNA samples were reverse transcribed using the Applied Biosystems High Capacity RNA to cDNA kit (*Applied Biosystems, UK*).

Real-time qPCR was carried out using the 7500 Real Time PCR system from Applied Biosystems. β -actin served as the housekeeping gene, and expression for the genes of interest was normalized to β -actin expression. As the SYBR green method was used, primer sequences for the genes was validated by dissociation curve/meltcurve analysis. QPCR was carried out by Dr Emma-Jayne Kingham at the University of Southampton. The comparative cycle-threshold method was used for quantification of gene expression. The relative transcript levels were expressed as mean \pm s.d. for plotting as graphs. Statistical analysis was carried out used the Tukey-Kramer multiple comparisons post-test analysis of variance (ANOVA).

Gene	Forward Primer	Reverse Primer
β -actin	GGCATCCTCACCTGAAGTA	GGTGTGGTGCCAGATTTTC
ALCAM	ACGATGAGGCAGACGAGATAAGT	CAGCAAGGAGGAACCAACAAC
CD63	GCCCTTGGAATTGCTTTTGTCG	CATCACCTCGTAGCCACTTCTG

Table 3-1 QPCR primers.

3.2.7 BrdU labelling

Cells were cultured for 3 and 7 days in basal media (10%FBS/ α MEM) (PAA, UK) at 37°C with 5% CO₂ in humid conditions. Cells are seeded onto the materials at 1 x 10⁴ cells / ml and the media changed twice a week. Osteogenic media was used as a control for induced differentiation containing dexamethasone (10nm) and L-ascorbic acid (150 μ g ml⁻¹). At the relevant time points cells were washed in HEPES saline twice to remove any residual α MEM medium before the addition of 100 μ m BrdU/DMEM medium for 1.5 hours at 37°C. Following this MSCs were washed again in HEPES saline and fixed in 4% formaldehyde/PBS for 15 mins at 37°C. Cells were permeabilised and blocked as described in section 3.1.1.. Following this cells were incubated with mouse monoclonal anti-BrdU/DNase (1:100) (GE Healthcare) in conjunction with rhodamine-phalloidin (Sigma). Cells were then washed and incubated with an anti-mouse secondary biotin-labelled antibody (Sigma) and streptavidin-labelled tertiary antibody (Sigma) respectively as also described in section 3.1.1.. Mounting medium containing DAPI was also used to mount the samples and for visualization of the cell nucleus.

3.2.8 Cell Passaging

MSCs were primarily seeded onto 4 replicas of the SQ topography at 1x 10⁴ cells/ml and cultured using basal media (10% FBS/ α MEM) (PAA, UK) at 37°C. Cells were passaged once a week, with half of the replicas being reseeded again onto 4 SQ replicas at a ratio of 1:2. The remaining replicas were fixed at the current passage and stained for STRO-1 using a mouse-monoclonal antibody as in section 3.1.1. (Santa Cruz Biotechnology). Cells were cultured up to passage 6.

3.2.9 Osteogenic/Adipogenic Differentiation

MSCs were cultured on both the SQ and NSQ nanotopographies with flat PCL used as a control. Cells were cultured in basal media (10%FBS/ α MEM) (PAA, UK) for a period of 28 days. Following which, 2 strategies were undertaken:

1. MSCs were maintained on all substrates, however basal media was replaced with either adipogenic (1 μ M dexamethasone, 10 μ g/ml human insulin, 100 μ M indomethacin and 500 μ M isobutylmethylxanthine) or osteogenic induction media, as previously described in section 2.2.1. for 14 days. After 14 days culture in differentiation media cells were fixed and stained for OPN and PPAR- γ (mouse-monoclonal and goat-polyclonal respectively (*Insight Biotechnology*)) as described in section 3.1.1..
2. MSCs were trypsinised off all substrates and reseeded onto glass coverslips. Basal media was replaced with either adipogenic (1 μ M DEX, 10 μ g/ml human insulin, 100 μ M indomethacin and 500 μ M isobutylmethylxanthine) or osteogenic induction media (dexamethasone (10nm) and L-ascorbic acid (150 μ g ml⁻¹)). Cells were cultured in differentiation media for either 1, 3 or 14 days. Samples were subsequently fixed and stained as described in section 3.1.1. with an osteogenic or adipogenic marker; RUNX2 or PPAR- γ at day 1 and 3 (mouse monoclonal and goat polyclonal respectively (*Insight Biotechnology, UK*)) and OPN or PPAR- γ at day 14 (mouse monoclonal and goat polyclonal respectively (*Insight Biotechnology, UK*)).

3.3 Results

3.3.1 Multipotency/Differentiation

Initial immunofluorescent staining of MSCs cultured for 7 days on the SQ and NSQ nanotopographies as well as the planar and OGM controls identified both stem cell markers, STRO-1 and ALCAM, to be present on all substrates, while OPN and OCN bone cell markers showed negligible expression at this time point (fig. 3-1). Expression of only ALCAM and STRO-1 at this time point demonstrates the homogenous nature of the STRO-1 selected cell population.

At day 14, expression of STRO-1 and ALCAM were seen to decrease at this time point on the NSQ osteogenic topography as well as both the planar and OGM control, whilst expression remained high on the SQ nanotopography. Low levels of OPN expression were evident on the NSQ nanotopography (fig 3-2).

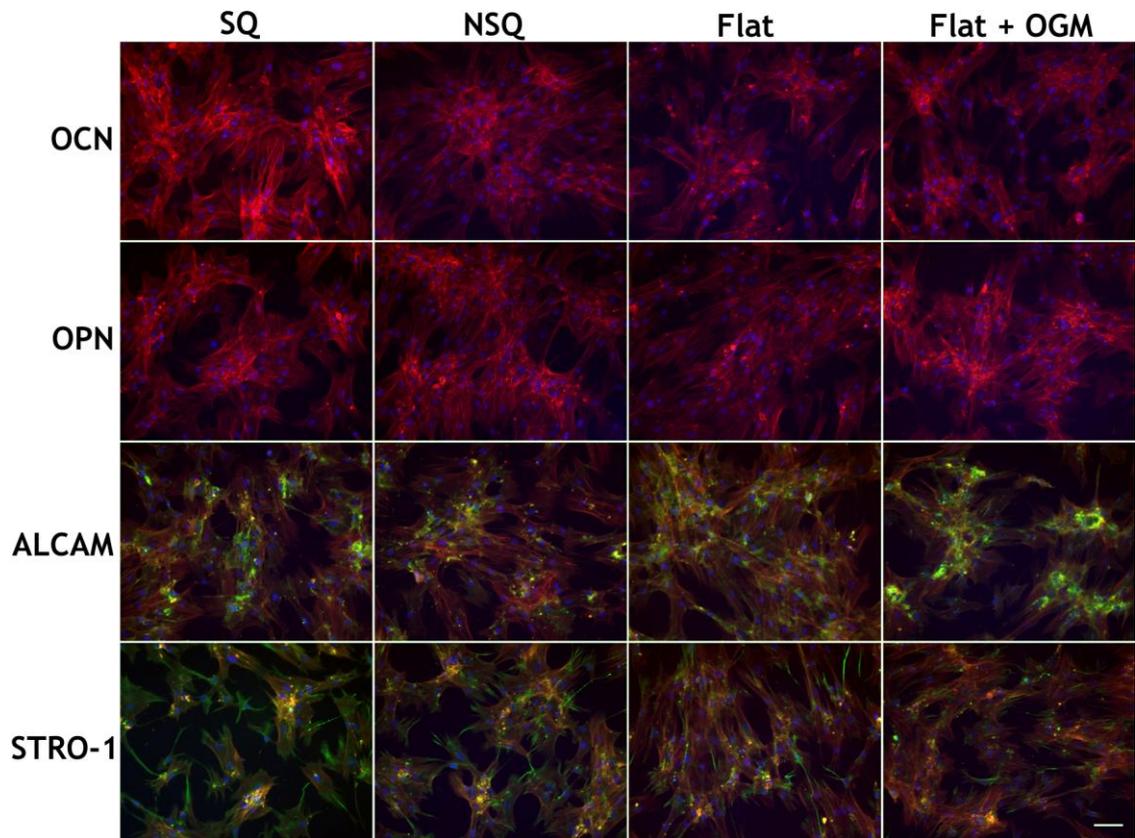


Figure 3-1. Phenotypic staining at day 7 in MSCs cultured on the SQ and NSQ topographies and on planar control and planar control with OGM. High levels of STRO-1 and ALCAM in all samples at this early time point demonstrate the enriched homogeneous STRO-1 nature of the MSC population at the start. Red=actin, blue = nucleus, green= STRO-1, ALCAM, OCN or OPN. Scale bar = 100 μ m.

By day 21, OPN expression was visible on the NSQ topography, and planar and OGM control, whilst expression of ALCAM and STRO-1 had diminished. Low levels of ALCAM and STRO-1 expression, in addition to slight OPN expression on the planar control indicates the formation of a heterogeneous population. In contrast however, MSCs cultured on the SQ topography continued to express high levels of ALCAM and STRO-1 with negligible levels of OPN and OCN expression at this time point (fig. 3-3).

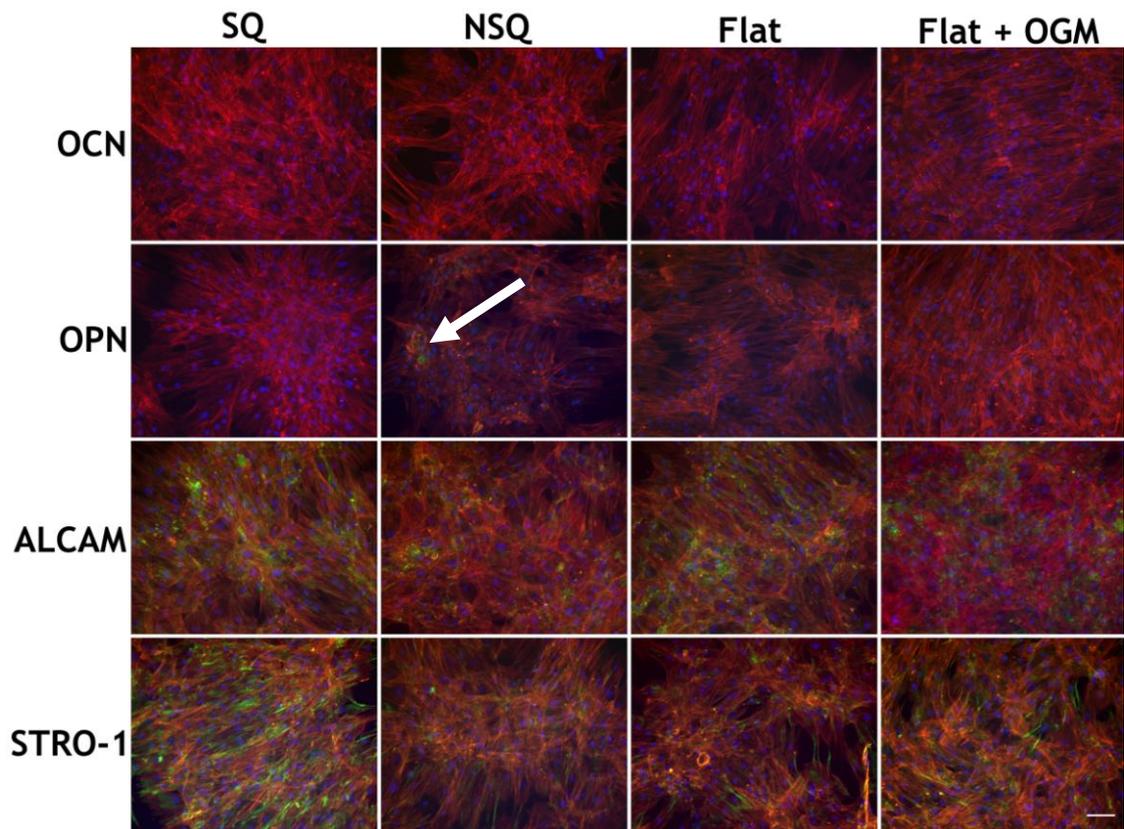


Figure 3-2. Phenotypic staining at day 14 in MSCs cultured on the SQ and NSQ topographies and on planar control and planar control with OGM. At this time point levels of STRO-1 and ALCAM are seen to decrease on the NSQ topography as well as the planar and osteogenic controls, whilst high levels of STRO-1 and ALCAM are still expressed on the SQ topography. Low levels of OPN expression can be seen on the NSQ topography. Red = actin, blue = nucleus, green = STRO-1, ALCAM, OCN or OPN. Scale bar = 100 μ m.

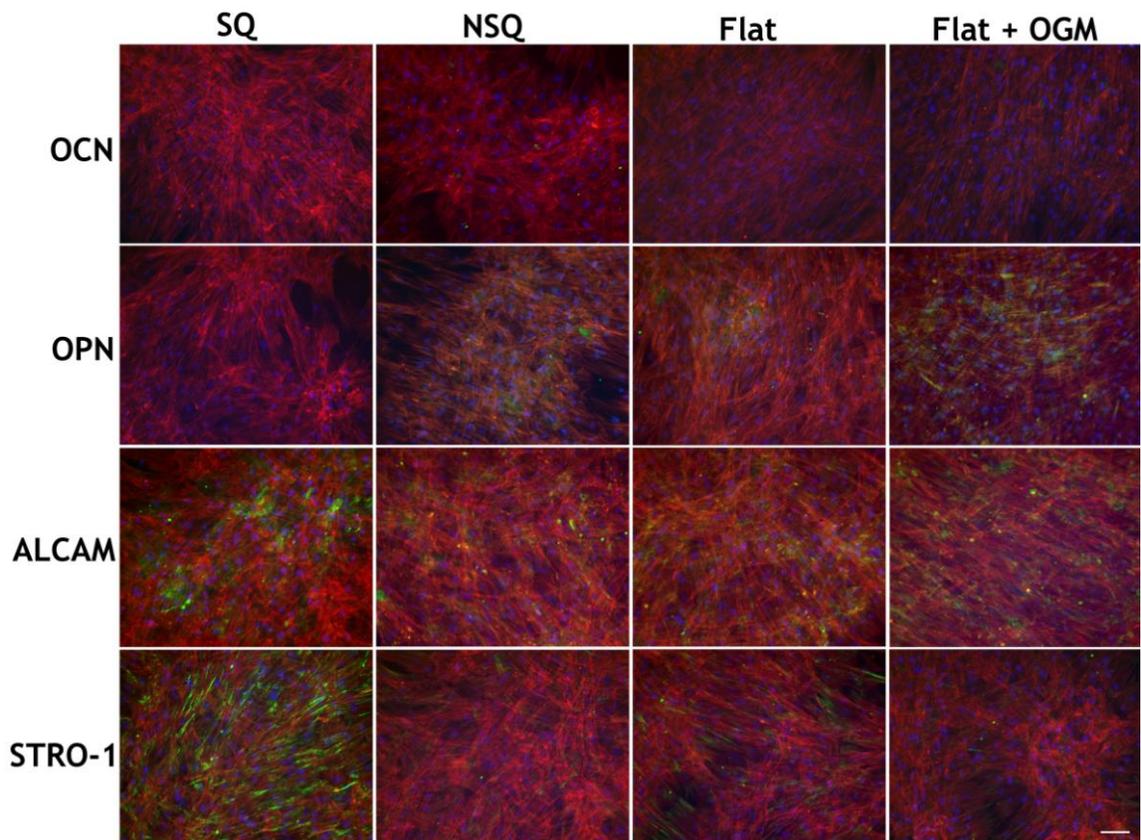


Figure 3-3 Phenotypic staining at day 21 in MSCs cultured on the SQ and NSQ topographies and on planar control and planar control with OGM. At this time point expression levels of STRO-1 and ALCAM have visually decreased on the NSQ topography and osteogenic controls, whilst increases in OPN are visible. On the flat control, low levels of STRO-1, ALCAM and OPN indicate a heterogeneous cell population. In contrast, STRO-1 and ALCAM are still expressed on the SQ topography with no expression of OPN or OCN. Red = actin, blue = nucleus, green = STRO-1, ALCAM, OCN or OPN. Scale bar = 100 μ m.

Finally, by day 28, OPN expression had visually increased on the NSQ sample and OGM control. In addition, low levels of OCN expression could be noted on these samples. Similar results to day 21 were seen on the planar control with low levels of OPN, ALCAM and STRO-1 still visible. On the SQ nanotopography however, expression of ALCAM and STRO-1 remained high with no notable expression of OPN or OCN (fig. 3-4).

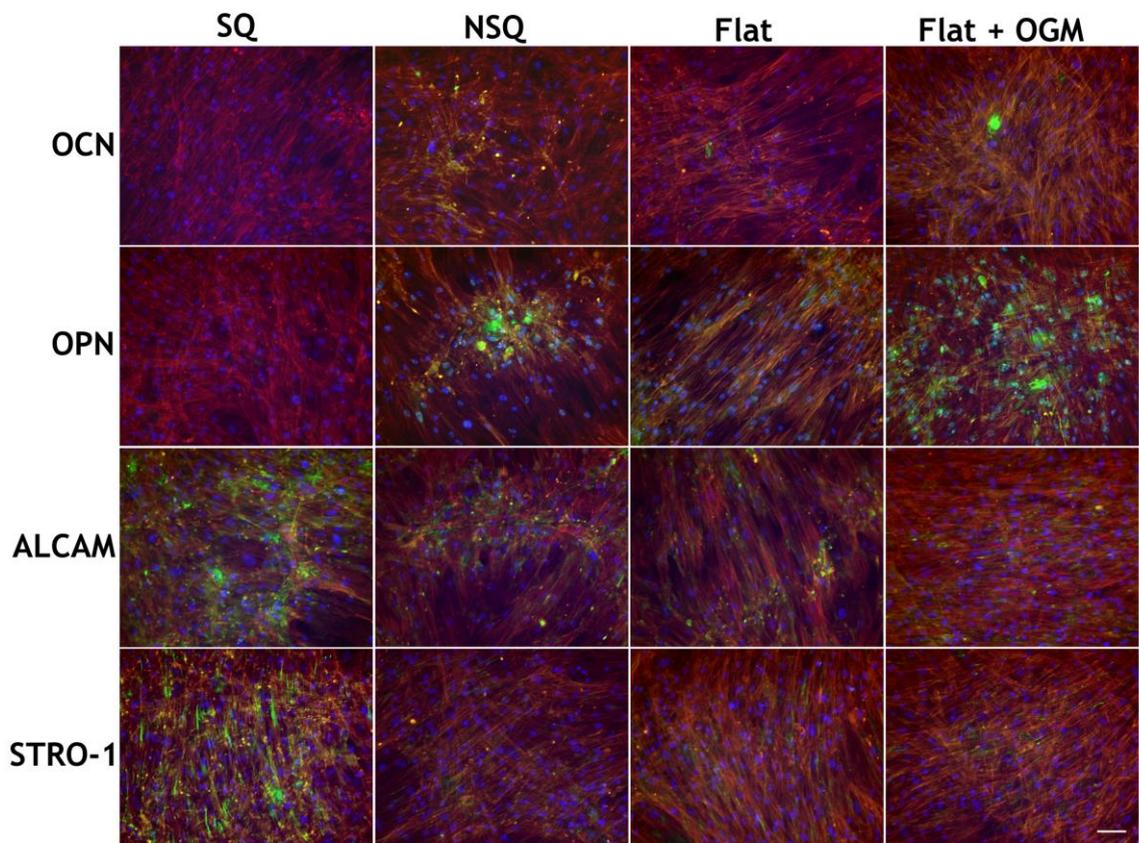


Figure 3-4 Phenotypic staining at day 28 in MSCs cultured on the SQ and NSQ topographies and on planar control and planar control with OGM. At this time point expression levels of STRO-1 and ALCAM have visually decreased on the NSQ topography and osteogenic controls, whilst increases in OPN are visible. On the flat control, low levels of STRO-1, ALCAM and OPN again indicate a heterogeneous cell population. In contrast, STRO-1 and ALCAM are still expressed on the SQ topography with no expression of OPN or OCN. Red = actin, blue = nucleus, green = STRO-1, ALCAM, OCN or OPN. Scale bar = 100 μ m.

3.3.2 Quantitative Analysis of Marker Expression

Quantitative analysis of immunofluorescent data at day 7 identified STRO-1 expression on all substrates, and these results were comparable to the flat control. In contrast, there was no evidence of OPN expression on any of the substrates (fig. 3-5 A). At day 21, STRO-1 expression on the SQ nanotopography is approximately 10 times higher than expression on the other substrates, with STRO-1 expression the NSQ and OGM control comparable to the flat control. This suggests that expression had been significantly lost from cells in the other treatments. For OPN expression, however, the converse was apparent with MSCs expressing high levels of OPN when cultured on the NSQ and OGM control. In contrast no OPN expression was detectable on the SQ nanotopography (fig. 3-5 B). Analysis at day 28 identified STRO-1 expression on the SQ nanotopography to be approximately 100 fold higher with significantly decreased expression in cells on the other treatments. Expression of OPN was found to be negligible on the SQ topography at this time point and levels of expression on the NSQ and OGM control where higher but more comparable to the flat control (fig. 3-5 C). It is noted that the standard deviations are large. This is because the cells were predominantly confluent and the proteins could be expressed in groups of cells (a large expression area) or in individual cells (a small expression area, thus the results are bimodal); this is especially the case with osteogenesis as the cells naturally clump to form osteoid nodules. Results are mean \pm SD, *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ by ANOVA.

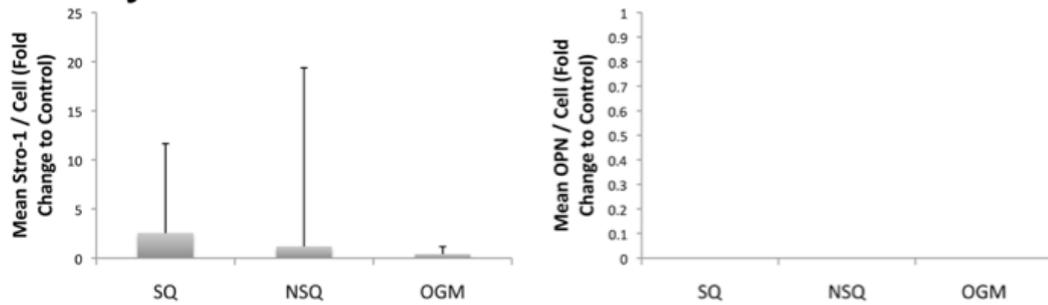
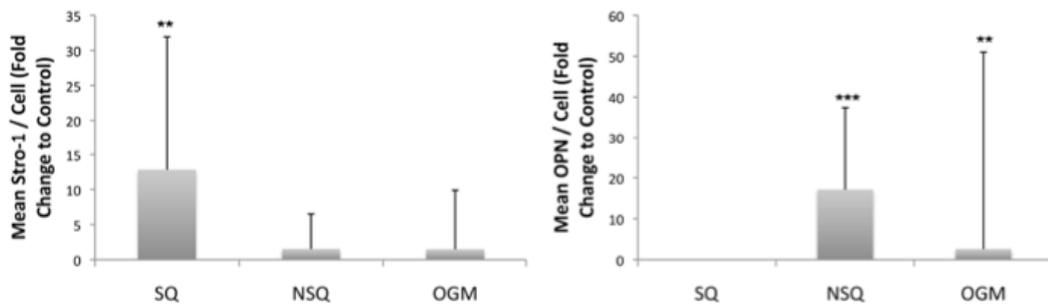
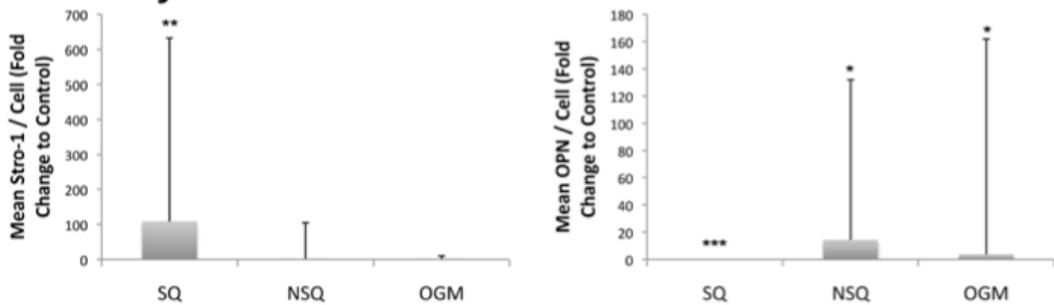
A - 7 days**B - 21 days****C - 28 days**

Figure 3-5 Time course study for expression of STRO-1 and OPN in MSCs on flat control, test nanotopographies and with OGM. (A) At day 7 all surfaces express STRO-1 similarly and thus are comparable to control. For OPN, no expression was detected on any surface. (B) At day 21 MSCs cultured on NSQ and with OGM displayed similar STRO-1 expression to control, however, MSCs on the SQ topography expressed approximately 10 times more STRO-1. For OPN expression the results were the opposite, cells on NSQ and with OGM expressed high levels of OPN but none was yet detectable in MSCs on SQ or control. (C) By 28 days MSCs on SQ expressed approximately 100 fold higher expression. For OPN, MSCs on NSQ and with OGM still expressed more OPN than on control, however, cells on control expressed a degree of OPN. Negligible expression was noted by MSCs on SQ surfaces.

3.3.3 MSC Maintenance Following Long-term Culture

MSCs were cultured on both the SQ nanotopography and flat control substrate for 8 weeks. Immunofluorescent staining identified that both stem cells markers, STRO-1 and ALCAM were expressed at 8 weeks following culture on the SQ nanotopography. MSCs cultured on the flat control however were identified to only express ALCAM, a less stringent marker for multipotent MSCs at this time point (fig. 3-6).

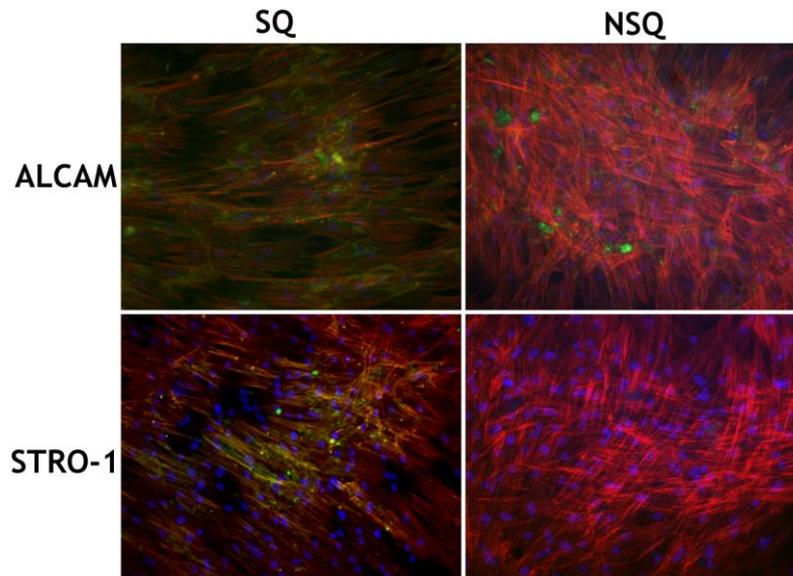


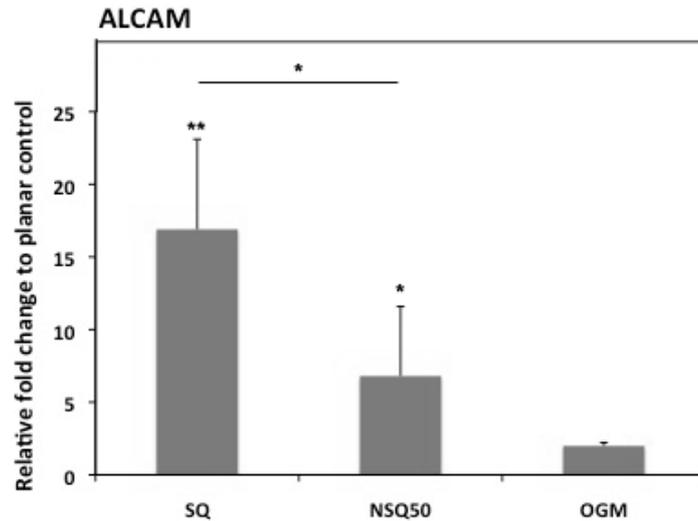
Figure 3-6. Stem cell/progenitor expression following 8 weeks in culture. High levels of ALCAM and STRO-1 were seen in MSCs cultured on SQ compared with cells cultured on the osteogenic NSQ. It is noted that STRO-1 is a more stringent marker for MSCs than ALCAM, which is expressed by both stem cells and progenitor cells. In all images, green = phenotypic marker as indicated by the images, red = actin, blue = nucleus.

3.3.4 Quantitative Real Time PCR

QPCR was used to examine the expression of two progenitor markers, ALCAM and CD63 after four weeks culture. The results identified highly significant up-regulation of ALCAM on the SQ nanotopography compared to the flat control. Significant expression of ALCAM was identified on the NSQ topography compared to flat, however, there was also a significantly higher level of expression in MSCs cultured on the SQ when compared to the NSQ. Levels of ALCAM expression in MSCs cultured with osteogenic media were shown to be similar to the flat control (fig. 3-7 A).

Similarly, CD63 expression was shown to be significantly up-regulated in MSCs cultured on the SQ topography in comparison to those cultured on the flat control and NSQ topography. In contrast, MSCs cultured on the NSQ and osteogenic controls were identified to have expression levels similar to the flat control (fig. 3-7 B).

A



B

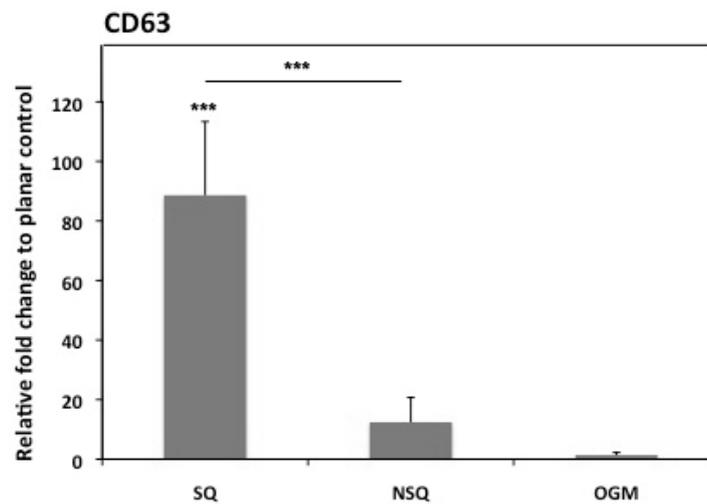


Figure 3-7 Quantitative, real time (q) PCR at four weeks for progenitor markers ALCAM (A) and CD63 (B) showed a statistically significant increase in expression between SQ and planar control, SQ and NSQ, and SQ and OGM. Graphs show mean \pm s.d., comparison by ANOVA— * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$. Note: markings above the error bars denote comparison to flat control; comparison between SQ and NSQ is denoted by a line.

3.3.5 Multipotency/Differentiation of Commercial MSCs

Bone marrow MSCs (*Promocell, UK*) were shown to maintain high levels of STRO-1 and ALCAM expression when cultured on the SQ topography, with negligible expression of OPN or OCN bone markers visible. ALCAM expression was visible on the NSQ topography and both controls, however STRO-1 expression was visibly diminished following 28 days of culture. On both the NSQ topography and osteogenic control high levels of both bone markers, OPN and OCN were visible. On the flat control OPN expression was only visible without expression of OCN, again indicating the presence of a heterogeneous population (fig. 3-8).

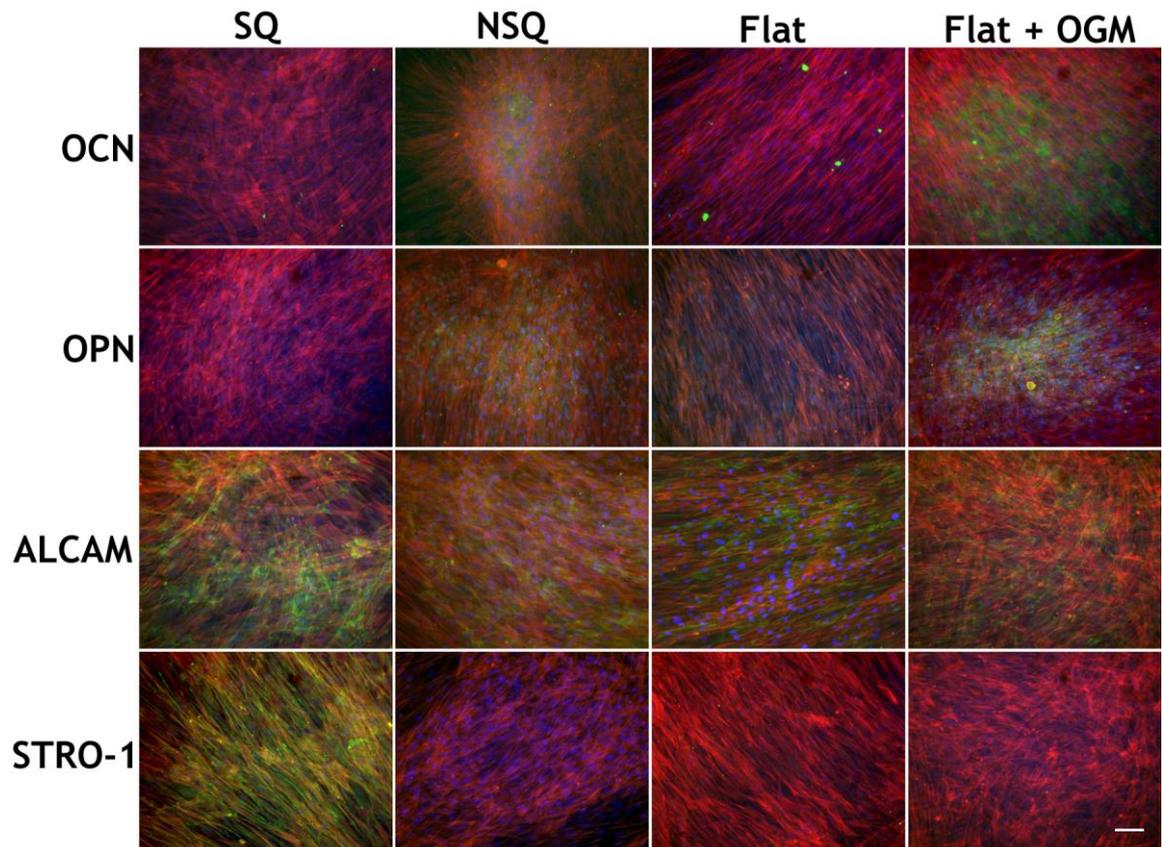


Figure 3-8 Commercial skeletal MSCs cultured for 28 days undergo similar differentiation / phenotype maintenance to STRO-1 selected skeletal MSCs in response to nanotopography. Only MSCs cultured on the SQ surface expressed STRO-1 at 28 days of culture. The commercial skeletal MSCs cultured on NSQ and with osteogenic media displayed loss of ALCAM expression with expression of the bone markers OCN and OPN. Red = actin, blue = nucleus, green= STRO-1, ALCAM, OCN or OPN. This result demonstrates that skeletal MSCs isolated by different protocols respond in a similar fashion. Scale bar = 100 μ m.

3.3.6 Adipose-derived MSCs

Following 28 days culture adipose derived MSCs (*Promocell, UK*) cultured on the NSQ and with OGM are both seen to express osteogenic markers, OPN and OCN with no STRO-1 expression observed. MSCs cultured on the flat control are seen to express ALCAM without expression of OPN, OCN or STRO-1. In contrast, expression of ALCAM and STRO-1 was only visible in MSCs cultured on the SQ topography, with no expression of OPN or OCN (fig. 3-9).

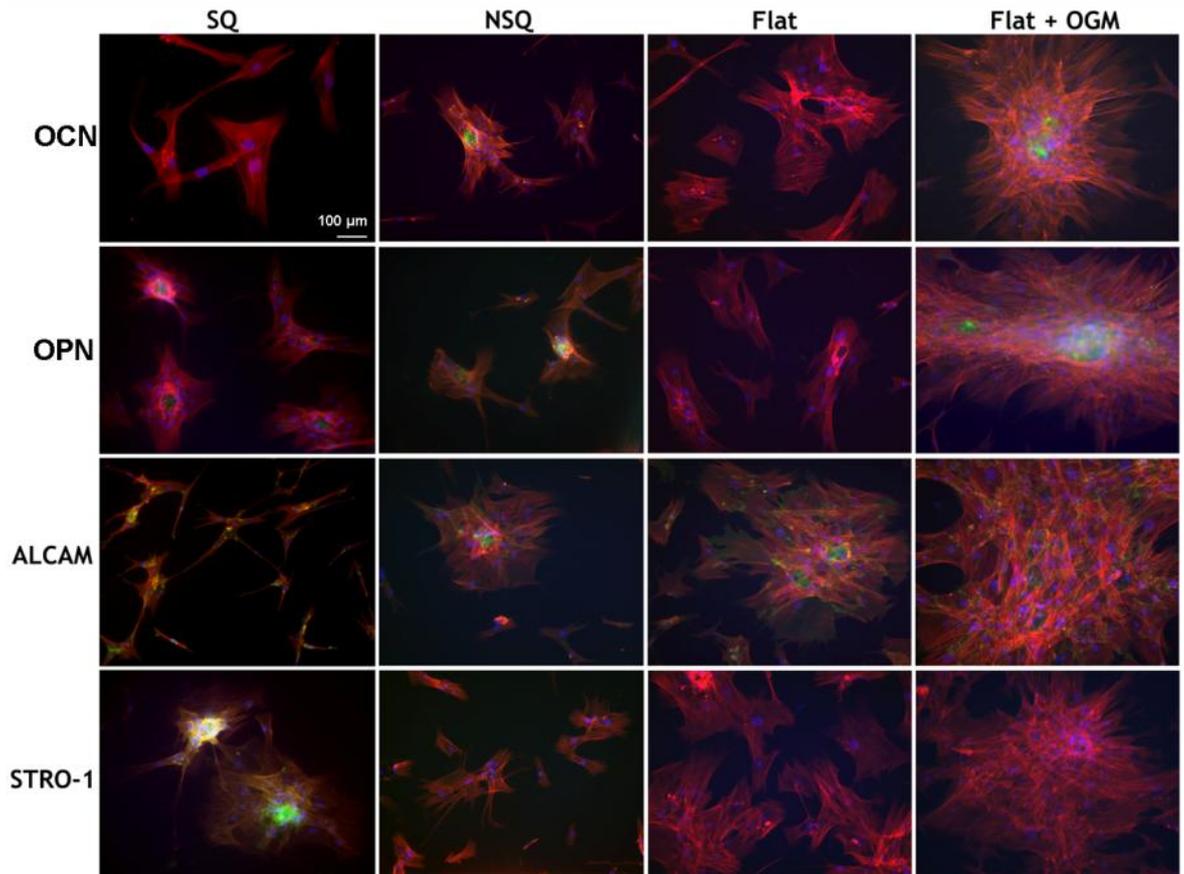


Figure 3-9 Adipose derived MSCs cultured for 28 days undergo similar differentiation / phenotype maintenance to skeletal MSCs (STRO-1 selected or Promocell) in response to nanotopography. Only adipose-derived MSCs cultured on the SQ surface still expressed STRO-1 at 28 days of culture. Adipose-derived MSCs grown on NSQ and cultured with osteogenic medium displayed signs of OCN and OPN expression at this time point. Red=actin, green= STRO-1, ALCAM, OCN or OPN.

3.3.7 Induced Differentiation Study

MSCs were cultured on the SQ and flat control PCL substrates for 28 days, after which the cells were trypsinised, disaggregated and re-cultured on glass coverslips (fig. 3-10 A) or cell remained on substrates and differentiation media was added *in situ* (fig. 3-10 B). Following trypsinisation from the SQ topography and re-culturing on glass coverslips, MSCs were shown to undergo osteogenic and adipogenic differentiation in response to differentiation media. MSCs cultured in osteogenic differentiation media were shown to express RUNX2 following 24 and 72 hours of culture. PPAR- γ was also shown to be expressed at 24 and 72 hours of culture in response to adipogenic differentiation media.

In fig. 3-10 B, MSCs maintained on the original SQ topography showed localised PPAR- γ expression in aggregated cell groups, whilst OPN expression is visible in clusters of cells. In this case therefore, when MSCs are left on the SQ

nanotopography and induced to undergo differentiation, neither osteogenesis nor adipogenesis is complete in contrast to when MSCs undergo disaggregation and re-culturing on glass coverslips. This suggests a competitive effect between the retention properties of the nanotopography and chemistry.

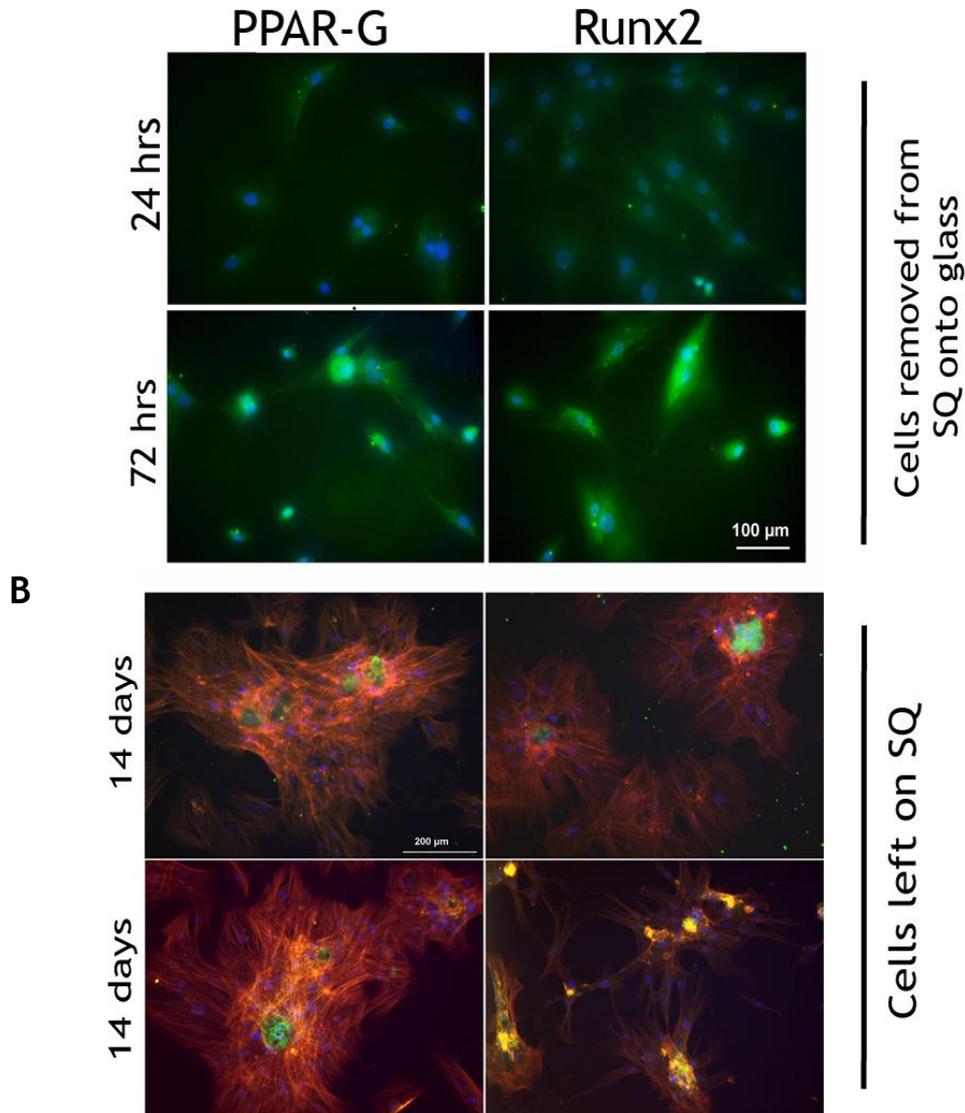


Figure 3-10. MSC multipotency after prolonged culture on the SQ topography. (A) After 28 days of culture on SQ, cells were trypsinised, disaggregated and re-cultured on glass coverslips. The cells were then cultured with adipogenic or osteogenic media for 24 and 72 hours before immunostaining for RUNX2 or PPARG. After 24 hours cells could be seen to express the adipogenic and osteogenic transcription factors and this was intensified after 72 hours. (B) After 28 days of culture on SQ, adipogenic or osteogenic media was added *in situ* with the MSCs still on the SQ pattern and the cells cultured for a further 14 days before staining for PPARG or OPN. In response to adipogenic media, the MSCs clustered and did express some localised PPARG, however not to the same extent as when disaggregated. In response to osteogenic media, the MSCs formed a limited number of bone-nodule-like clusters positive for OPN. Blue = nucleus, Red=actin, green = PPARG, RUNX2 or OPN.

3.3.8 Passaging

MSCs cultured on the SQ topography were subject to passaging, a typical cell culture technique when cultured on PCL. Fig. 3-11 shows that during weekly passaging, MSCs cultured on the SQ topography maintained expression of STRO-1 over a 6-week period.

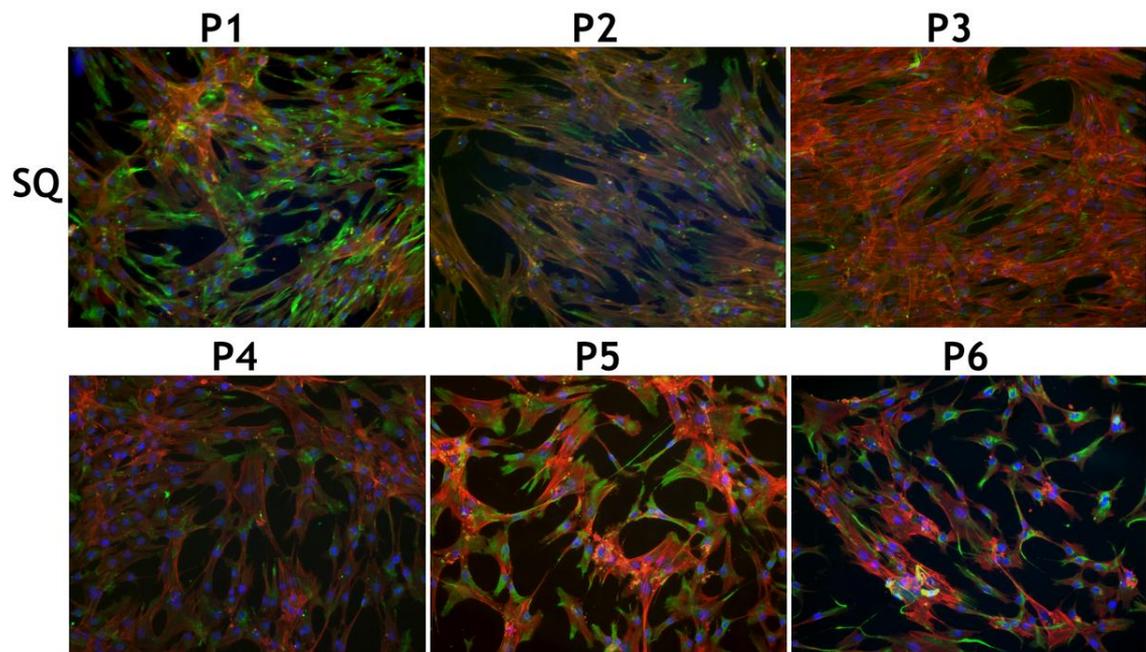


Figure 3-11 Study of STRO-1 selected MSCs after passaging. MSCs cultured for 6 passages (trypsin detachment). MSCs were passaged once per week for 6 weeks and stained for STRO-1. The MSCs demonstrated STRO-1 expression at each time sub-culture.

3.3.9 Proliferation

MSCs were observed to undergo proliferation rates comparable to the flat control at both day 7 and day 14. No significant difference was observed at either time point (fig. 3-12 A&B).

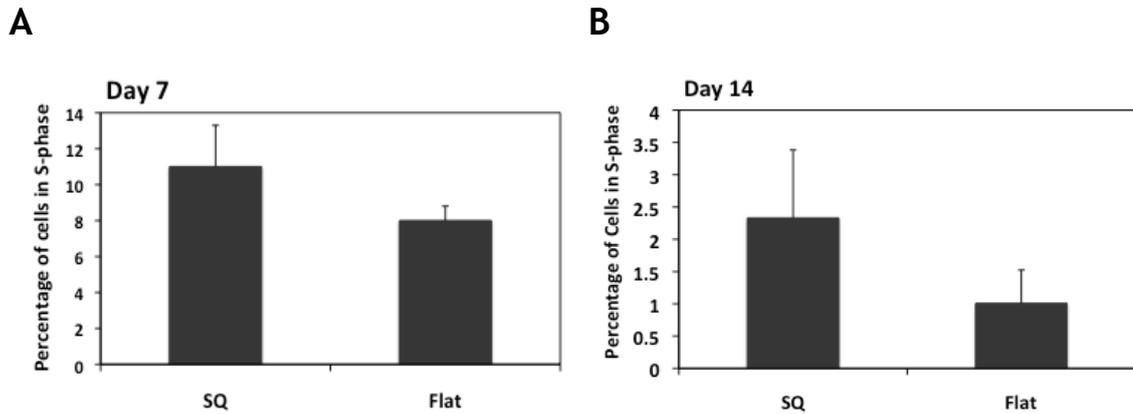


Figure 3-12 Proliferation of MSCs on the SQ topography and flat control at day 7 (A) and day 14 (B). Cells in S-phase were identified using BrdU. At both days 7 and 14 the percentage of cells in S-phase on the SQ was comparable to the flat and no significant difference was observed.

3.4 Discussion

This chapter demonstrates the effect of the SQ nanotopography on the maintenance of MSC phenotype and multipotency. Analysis of key osteogenic and stem/progenitor markers in MSCs over a period of four weeks, across all substrates allows for the profiling of MSCs phenotype in response to both test topographies and controls. Evidence of only stem/progenitor markers at day 7 indicates the presence of a STRO-1 enriched homogeneous population at the start of the study, whilst an up-regulation of osteogenic markers concomitant with the down-regulation of both stem/progenitor markers, demonstrates the osteoinductive properties of both the NSQ nanotopography, and OGM control as previously reported (Dalby, Gadegaard et al. 2007). Evidence for the generation of a heterogeneous cell population with continued expression of low levels of STRO-1 and ALCAM expression in response to the flat control are again conclusive with previous research indicating that MSCs undergo spontaneous differentiation in response to culture on a flat substrate.

In contrast, however, MSCs were shown to retain their stem cell phenotype in response to culture on the SQ topography with continued expression of both stem cell markers, STRO-1 and ALCAM (note that ALCAM is a less specific skeletal stem cell marker than STRO-1 and will also label more mature, progenitor cells) and, critically, no expression of osteogenic markers were identified.

Furthermore, expression of STRO-1 and ALCAM in MSCs cultured on the SQ

topography was observed up to eight weeks post seeding, again indicating continued maintenance of the MSC phenotype over a prolonged period of time.

Quantitative assessment of marker expression over the time course study further identified a diminishing population of STRO-1+ MSCs cultured on the NSQ topography and with OGM. An increase in OPN and OCN expression were also similarly observed over time, demonstrating osteogenic differentiation. Meanwhile, STRO-1 expression in MSCs on the SQ topography remained high throughout the study, with fold change relative to the flat control increasing over time.

An additional study using qPCR to examine CD63 (another more stringent marker for MSCs) and ALCAM was used to back up the data generated using immunofluorescence. Comparative analysis with the flat control identified highly significant and very highly significant differences in ALCAM and CD63 expression respectively, providing further evidence for maintenance of the MSC phenotype in response to the SQ topography. Interestingly, commercially available skeletal and adipose derived MSCs were observed to undergo similar differentiation or phenotype maintenance in response to both nanotopographical and control substrates.

MSCs cultured on the SQ topography were also shown to undergo passaging, a common cell culture technique critical for stem cell expansion. Continuous expression of STRO-1 in MSCs cultured on the SQ topography up until passage 6 demonstrates that not only can the functionally useful lifespan of MSCs be increased, but that typical cell culture protocols can be applied to the surfaces whilst phenotypic retention is maintained.

In order to ensure that the retained cells are functional, as well as considering marker proteins, MSC multipotency must be considered as the ultimate display of stem cell phenotype (Pittenger, Mackay et al. 1999). In further studies following prolonged culture, MSCs were successfully induced to undergo differentiation using osteogenic or adipogenic differentiation media following dissociation from the SQ topography and re-culturing onto glass coverslips. Thus, MSCs cultured on the SQ topography not only expressed MSC phenotype markers, but are multipotent. Interestingly, MSCs were also treated with supplemented

media whilst still growing on the SQ nanotopography, however in this instance only modest levels of osteogenic or adipogenic differentiation were observed, indicating competitive effects between the topographical cues experienced by the cells and the soluble factors in the media.

4 Genomics

4.1 General Introduction

In this chapter, the effect of the SQ nanotopography on the activation or repression of key signalling pathways is studied using oligo- and Affymetrix microarray technology, with subsequent analysis using Ingenuity Pathway Analysis (IPA). IPA groups genes into canonical and functional pathways which have been clearly defined in literature. Canonical and functional pathways are directly linked, with changes in canonical pathway signalling feeding into, and altering the functional output or phenotype of the cell. Therefore, up- or down-regulation of gene expression in MSCs in response to the SQ nanotopography can have a direct effect on the cell phenotype.

In addition, microarrays can detect the expression of short, non-coding RNAs. These include microRNA (miRNA) and small nucleolar RNAs (snoRNA) which function as regulatory RNAs (Kiss 2001; He and Hannon 2004; Matera, Terns et al. 2007). miRNAs act as post-transcriptional regulators, binding mRNA transcripts at a complementary sequences (Ambros 2004; He and Hannon 2004). This can result in gene silencing via degradation of the mRNA or preventing subsequent translation of the mRNA (Ambros 2004; Bartel 2004). miRNAs are increasingly being implicated in regulating gene expression involved in both self-renewal and differentiation of stem cells by negatively regulating the expression of key genes involved in self-renewal or differentiation (Chen, Li et al. 2004; Viswanathan, Daley et al. 2008; Yi, Poy et al. 2008; Sartipy, Olsson et al. 2009; Melton, Judson et al. 2010).

SnoRNA's localized to the nucleolus and Cajal Bodies of eukaryotic cells, on the other hand, are known to guide the modification of ribosomal RNAs (rRNA), transfer RNAs (tRNA) and small nuclear RNAs (snRNA) by either methylation or pseudouridylation (Matera, Terns et al. 2007). SnoRNAs which modify by methylation are classed as C/D box snoRNAs, while those that are associated with pseudouridylation are classed as H/ACA box snoRNAs, however some types of snoRNAs contain both a C/D and H/ACA box and can therefore function in both types of modification (Narayanan, Lukowiak et al. 1999; Kiss 2001; Kiss,

Jady et al. 2004). The most common type of methylation is a 2'-O ribose methylation and is thought to play several roles, such as stabilizing the RNA structure, enhancing hydrophobicity and protecting the RNA from degradation (Kowalak, Dalluge et al. 1994; Bachellerie and Cavaille 1998; Clouet-d'Orval, Gaspin et al. 2005). Pseudouridylation occurs when the nucleoside uridine becomes converted to the isomer pseudouridine, and is thought to increase the number of hydrogen bonds available altering the tertiary structure of the RNA. Interestingly other types of snoRNAs have also been found to regulate alternative splicing and function as miRNAs, highlighting their functional diversity (Ender, Krek et al. 2008; Brameier, Herwig et al. 2011). Importantly, whilst the effect of these modifications on stem cell function are not fully understood, RNA editing has previously been shown to regulate embryonic and hematopoietic self-renewal and differentiation (Hartner, Walkley et al. 2009; Osenberg, Paz Yaacov et al. 2010).

4.2 Materials and Methods

4.2.1 RNA isolation

STRO-1+ cells were seeded onto nanotopographical and control substrates at a density of 1×10^4 cells per ml. Cells were maintained at 37°C with a 5% CO₂ atmosphere in α -MEM (*Invitrogen, UK*) containing 10% FBS, which was replaced twice weekly. Cells were cultured for 7, 14 (for microarray), or 28 days (for macroarrays) at which point the cells were trypsinised to maximize recovery of cells from substrates. Cells were then lysed and total RNA extracted using a Qiagen RNeasy Micro Kit as described in section 1.3.1. (*Qiagen, UK*).

4.2.2 Microarray Analysis

MSCs were cultured on PCL materials (4 material replicates) for 7 and 14 days. At these points, the cells were lysed and total RNA was extracted using a Qiagen RNeasy kit (*Qiagen, UK*) as described in section 4.2.2.. Gene expression changes were detected by hybridization of cDNA to Affymetrix HuGene 1.0 ST human arrays as per manufacturer's instructions. Initial bioinformatic analysis was based on rank product (Breitling, Armengaud et al. 2004) and a false discovery

rate of 20% was used to upload selected genes changes to the Ingenuity Pathway Analysis (IPA) server (Ingenuity® Systems, www.ingenuity.com). The functional and canonical analyses were then generated through the use of IPA.

1. Network Generation

A data set containing gene (or chemical) identifiers and corresponding expression values was uploaded into in the application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. A p-value cutoff of less than 0.05 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity.

2. Functional Analysis of an Entire Data Set

The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Molecules from the dataset that met the p-value cutoff of less than 0.05 and were associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

3. Functional Analysis of a Network

The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the molecules in the network. The network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

4. Canonical Pathway Analysis: Entire Data Set

Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Molecules from the data set that met the p-value cutoff of less than 0.05 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for

the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

4.2.3 Macroarray Analysis

Gene expression analysis was carried out using the human stem cell oligoarrays examining the expression of 96 key genes (*SABiosciences, USA*). MSCs were seeded onto PCL substrates (3 material replicas) at 1×10^4 cells per ml for 28 days. The cells were then lysed and total RNA extracted using a Qiagen RNeasy micro kit (*Qiagen, West Sussex, UK*) as described in section 3.1.3.. Total RNA was then used to synthesize the target cDNA as described in section 3.1.4.. Subsequently this cDNA was used to produce biotin - 14 CTP labeled cRNA (*Invitrogen, UK*). The cRNA was then purified and hybridized to the Oligo GEArrays overnight at 60°C. Following this the arrays were washed with a solution of 2X standard saline citrate (SSC) containing 1% SDS followed by a wash with 0.1X SSC containing 0.5% SDS. Finally, chemiluminescent detection was carried out exposing the membranes to X-ray film in accordance with manufacturer's instructions. GEArray Expression Analysis Suite on-line software was used to extract data. Detailed protocols for the isolation of total RNA, amplification and labeling of the cRNA and finally hybridization to the microarrays followed by chemiluminescent detection can be found at www.SuperArray.com.

4.3 Results

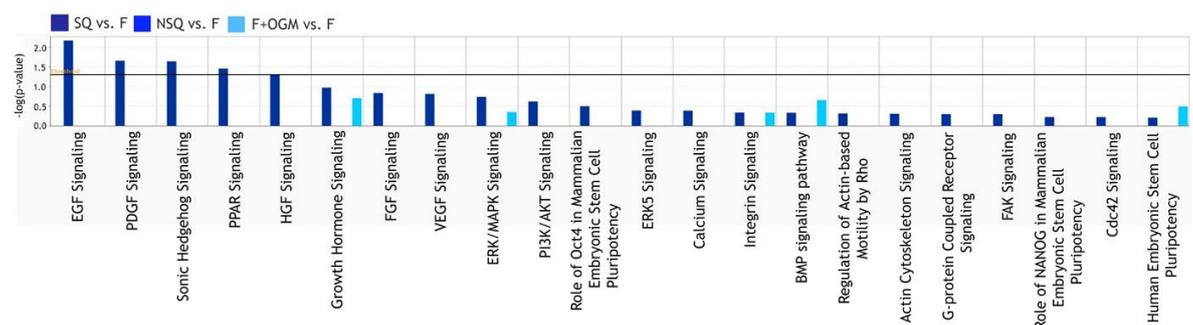
4.3.1 Canonical and Functional Analysis of MSCs in Response to Nanotopography

Analysis of microarray data at day 7, comparing MSCs cultured on the SQ, NSQ and with OGM relative to the flat control, identified mainly alterations in

canonical signalling (fig. 4-1A) on the SQ nanotopography with significant modulations in endothelial growth factor signalling (EGF), platelet-derived growth factor (PDGF) signalling, sonic hedgehog signalling, peroxisome proliferator-activated receptor (PPAR) signalling. In contrast, little change was observed in canonical signalling for cells cultured on the NSQ nanotopography and with OGM. At day 14 however, pathways associated with cellular functions were shown to undergo significant modulation in MSCs on both the NSQ and osteogenic control (mainly up-regulations) while MSCs on the SQ nanotopography showed little change in functional signalling, and with mainly down-regulations (fig. 4-1B). In particular MSCs cultured on the NSQ and with OGM were shown to undergo significant changes in functional signalling associated with tissue development, embryonic development, and cell morphology. In MSCs cultured on the SQ nanotopography significant changes were observed in cellular function and maintenance, and cell-to-cell signalling and interaction.

Further analysis of canonical signalling was then undertaken to compare MSCs cultured on the SQ, NSQ and with OGM individually, and identify whether key signalling pathways were up- or down-regulated relative to the flat control identified. Canonical signalling pathways in MSCs cultured on the SQ relative to the flat control (fig. 4-2 A) were shown to have undergone mainly down-regulations (green) at day 7. Interestingly at this time point, canonical signalling for human embryonic pluripotency was shown to be up-regulated (red), however, this was not statistically significant. At day 14, canonical signalling in MSCs on the SQ was shown to be mainly up-regulated, however only a small number of metabolic pathways were observed to be changed at this time point (fig. 4-2 B).

A



B

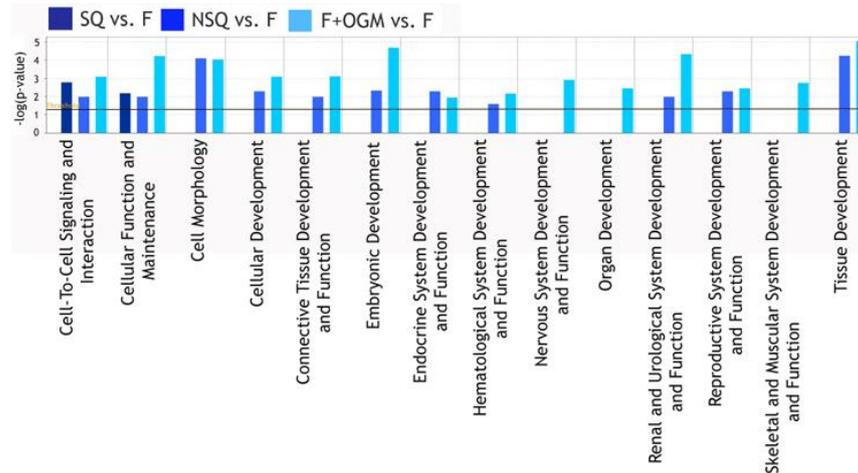
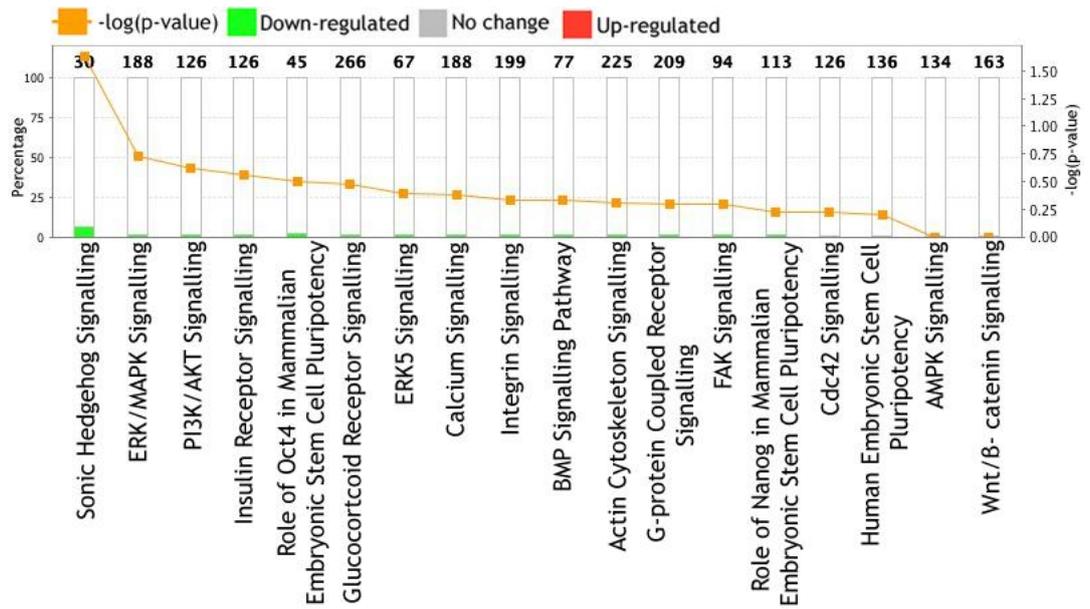


Figure 4-1 Pathway analysis of MSCs cultured on SQ, NSQ and with OGM compared with planar control. (A) At day seven, MSCs on SQ showed large-scale changes (mainly down-regulations) of major canonical pathways. Oct 4, Nanog and pluripotency pathways were not significantly affected. MSCs on the NSQ showed little change in canonical signalling compared to control; OGM showed a number of changes (mainly up-regulations) in canonical signalling. (B) At day 14 of culture, MSCs on the NSQ and with OGM showed a broad number of large functional changes (mainly up-regulations). MSCs on SQ, however, showed very few changes (mainly down-regulations). Note that the threshold is significant at $p < 0.05$ by Fischer's exact test and that the graph shows significance only as $-\log(p \text{ value})$ to show the significance as positive values over a sensible scale. $n=3$.

Examination of canonical signalling in MSCs cultured on the NSQ relative to the flat control showed that at day 7, canonical signalling was broadly down-regulated at this time point. However by day 14, MSCs on the NSQ were identified to have mainly up-regulations in canonical signalling relative to the flat control, with significant up-regulations in multiple signalling pathways (fig. 4-3 A & B), including sonic hedgehog and cell cycle related pathways. Similarly, MSCs cultured with OGM were identified to have mainly up-regulations in canonical signalling at day 7, however at this time point some down-regulations were also observed relative to the flat control (fig 4-4 A). At day 14, similar to MSCs cultured on the NSQ nanotopography, canonical signalling was shown to be broadly up-regulated. In addition, similar to MSCs cultured on the NSQ nanotopography, significant expression of genes associated with the canonical pathway sonic hedgehog, as well as several pathways associated with the cell cycle were observed.

A



B

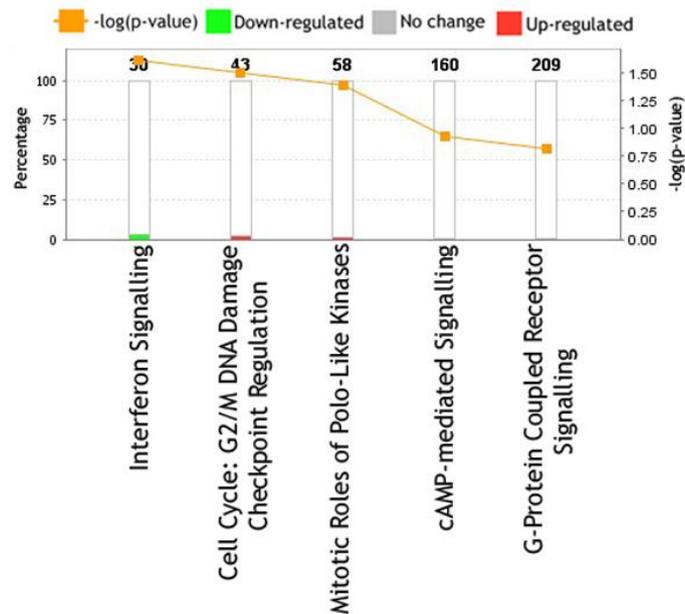
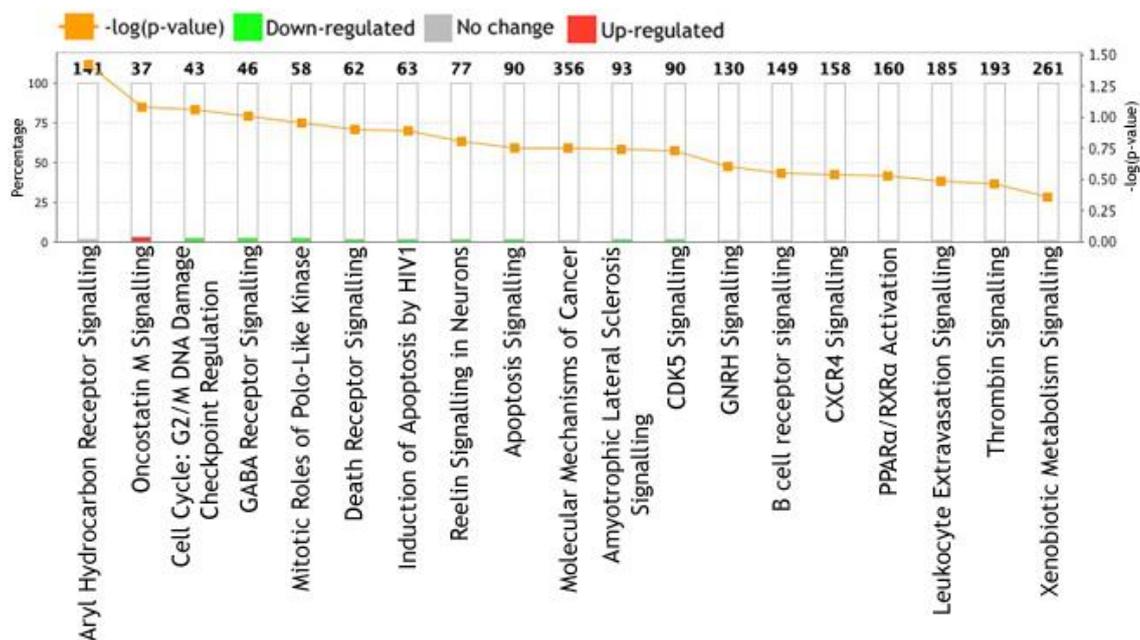


Figure 4-2 Canonical signalling in MSCs cultured on the SQ nanotopography at days 7 and 14 relative to the flat control. (A) At day 7 canonical signalling was shown to be down-regulated. (B) At day 14 metabolic signalling was shown to be mainly up-regulated, however only a small number of metabolic pathways were observed to be changed at this time point.

A



B

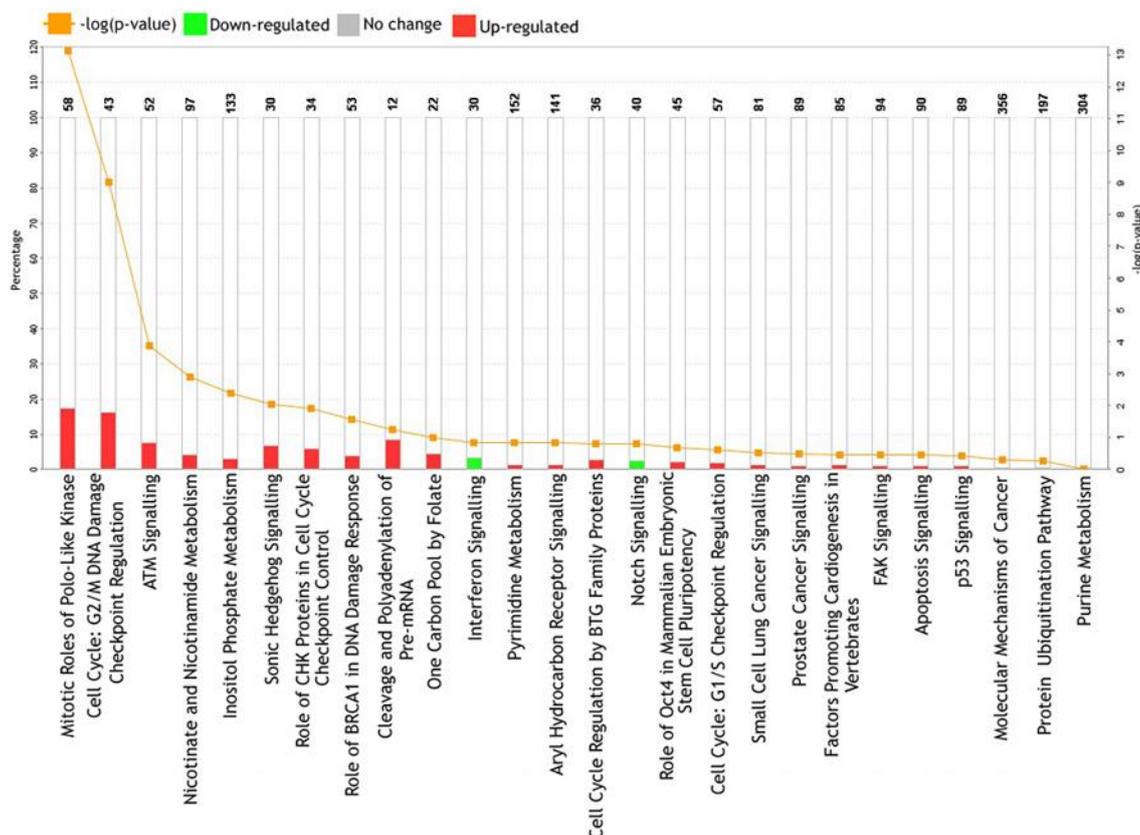
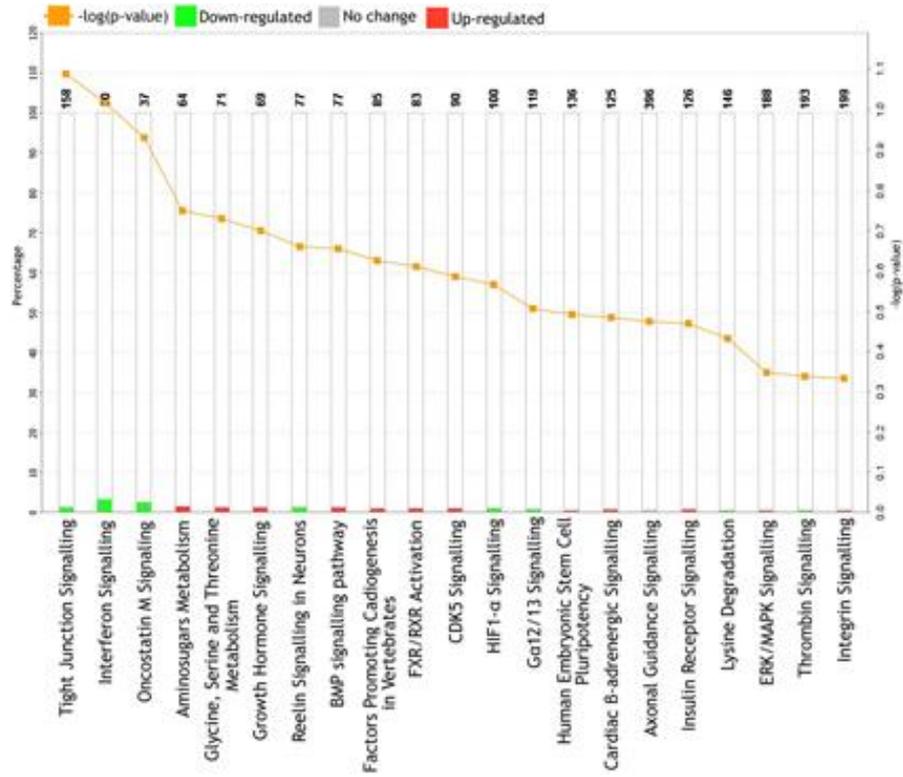


Figure 4-3 Canonical signalling in MSCs cultured on the NSQ nanotopography at days 7 (B) and 14 (B) relative to the flat control. At day 7 canonical signalling was shown to be non-significantly down-regulated, however by day 14 significant up-regulation of gene expression associated with various canonical pathways was observed.

A



B

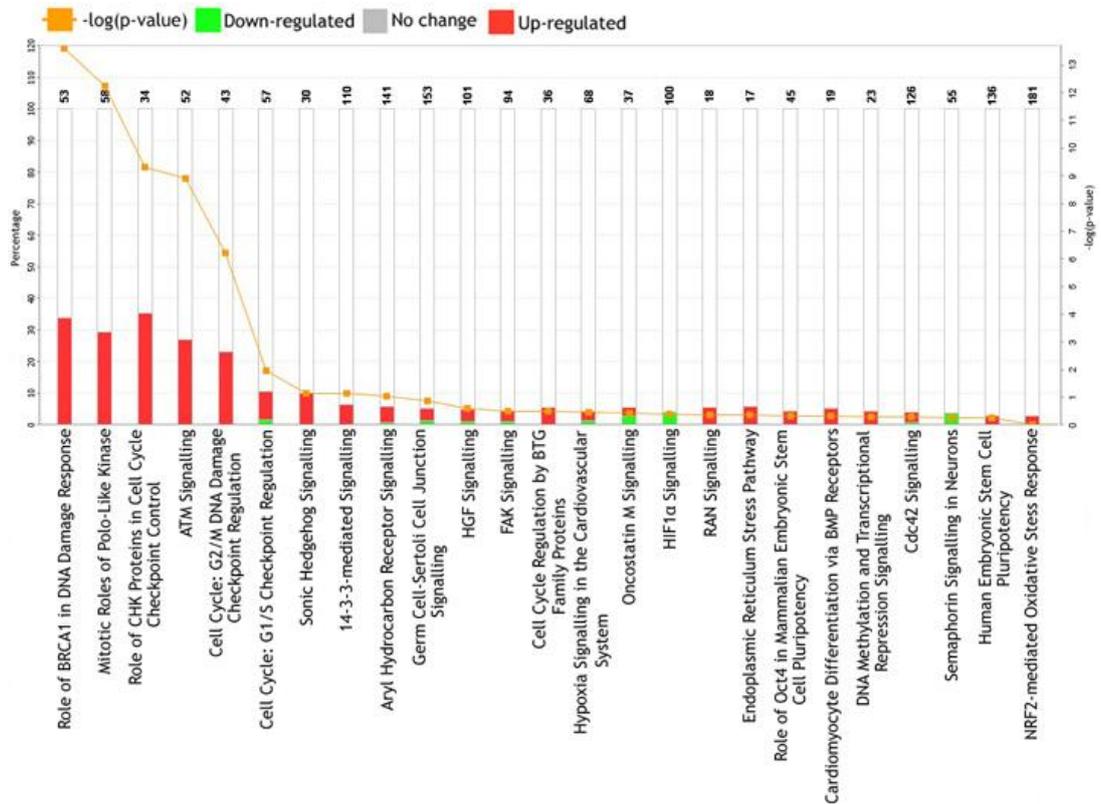


Figure 4-4 Canonical signalling in MSCs cultured with OGM at days 7 (B) and 14 (B) relative to the flat control. At day 7 canonical signalling was shown to be broadly down-regulated with up-regulation of some pathways, however by day 14 significant gene expression associated with various canonical pathways were observed similar to the NSQ.

Gene networks associated with cellular processes are classed in IPA according to the number of gene changes associated with a particular network. Networks of gene clusters were examined at days 7 and 14 in response to the SQ and NSQ nanotopographies as well as with OGM and relative to the flat control, with gene up-regulations in red and down-regulations in green. MSCs cultured on the SQ nanotopography at day 7 were found to have the most gene changes associated with gene expression, cell assembly and organisation. Additionally, most of the changes in gene expression were down-regulations at this time point (fig 4-5 A). At day 14 changes in gene networks associated with cell growth and proliferation were up-regulated. However, at this time point there were fewer changes in genes associated with the network (fig 4-5 B).

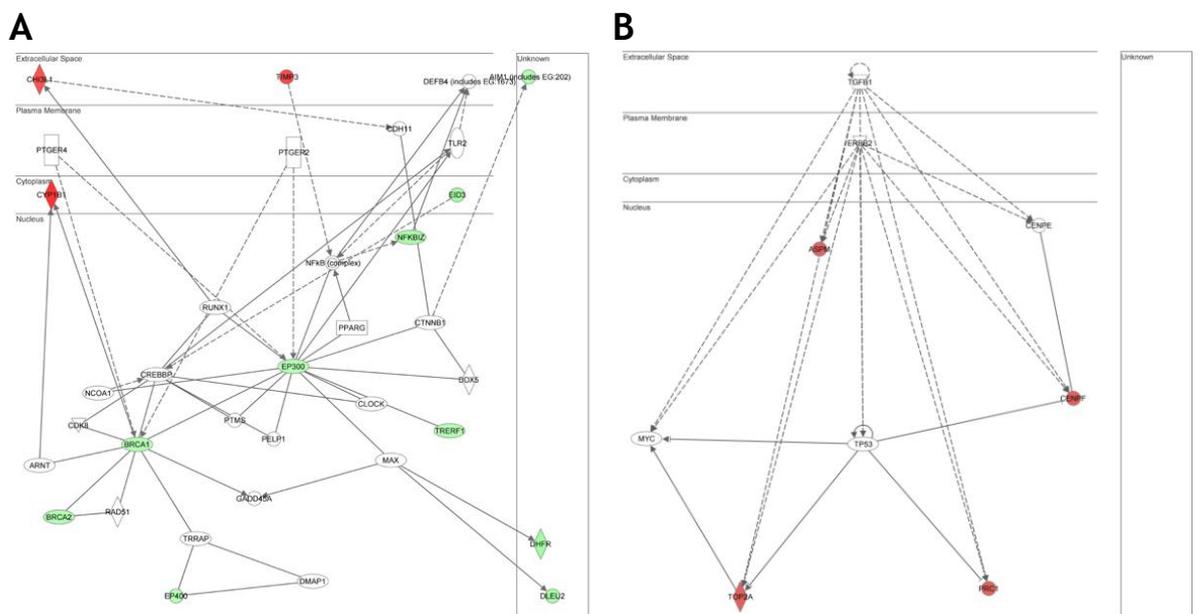


Figure 4-5 SQ vs. Flat gene networks at days 7 and 14 gene networks. (A) At day 7 mainly down-regulations were observed in genes associated with gene expression, cell assembly and organisation (B) At day 14 however up-regulations were observed in genes associated with cell growth and proliferation, however the number of gene changes observed. Red=up, green=down.

MSCs cultured on the NSQ were shown to have general down-regulations in cell morphology gene expression at day 7 however, at day 14 broad up-regulations in genes associated with the cell cycle, cell assembly and organisation, DNA reproduction and repair were observed (fig. 4-6).

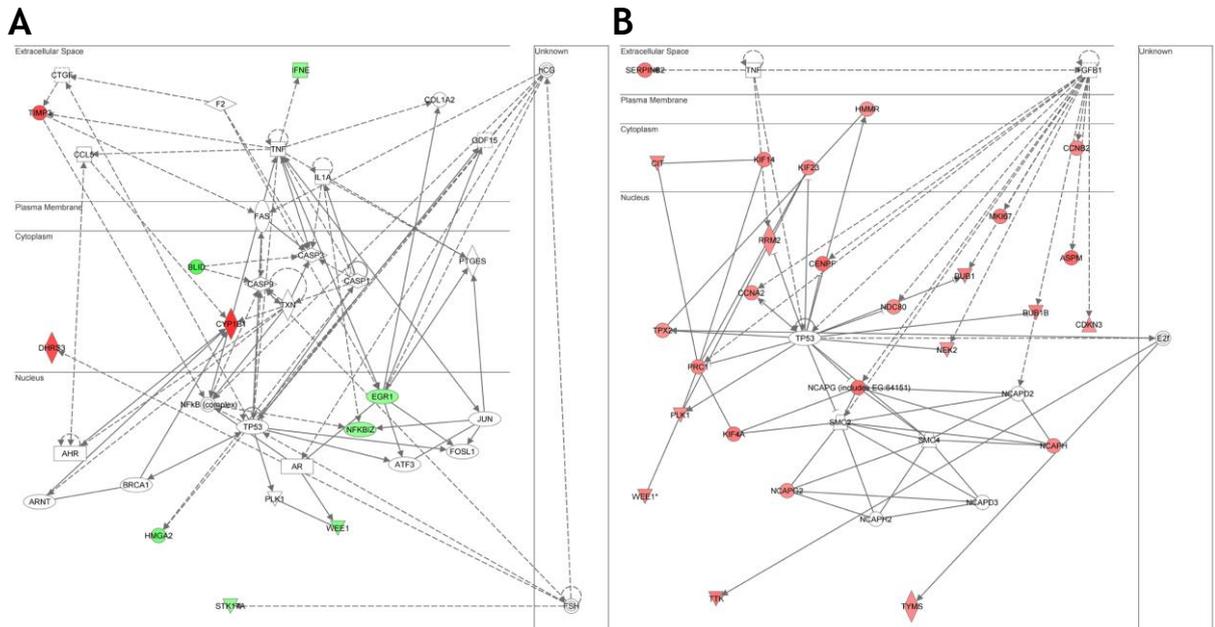


Figure 4-6 NSQ vs. Flat gene networks at days 7 and 14. (A) At day 7 general down-regulation in genes associated with the cell morphology gene network were observed (B) At day 14 up-regulation in gene expression associated with the cell cycle, cell assembly and organisation, DNA reproduction and repair gene network. Red=up, green=down.

MSCs cultured with OGM were observed to have up-regulated gene expression overall at day 7 associated with cell growth, development and tissue development, with additional overall up-regulated expression associated with signalling, movement and tissue development network at day 14 (fig. 4-7).

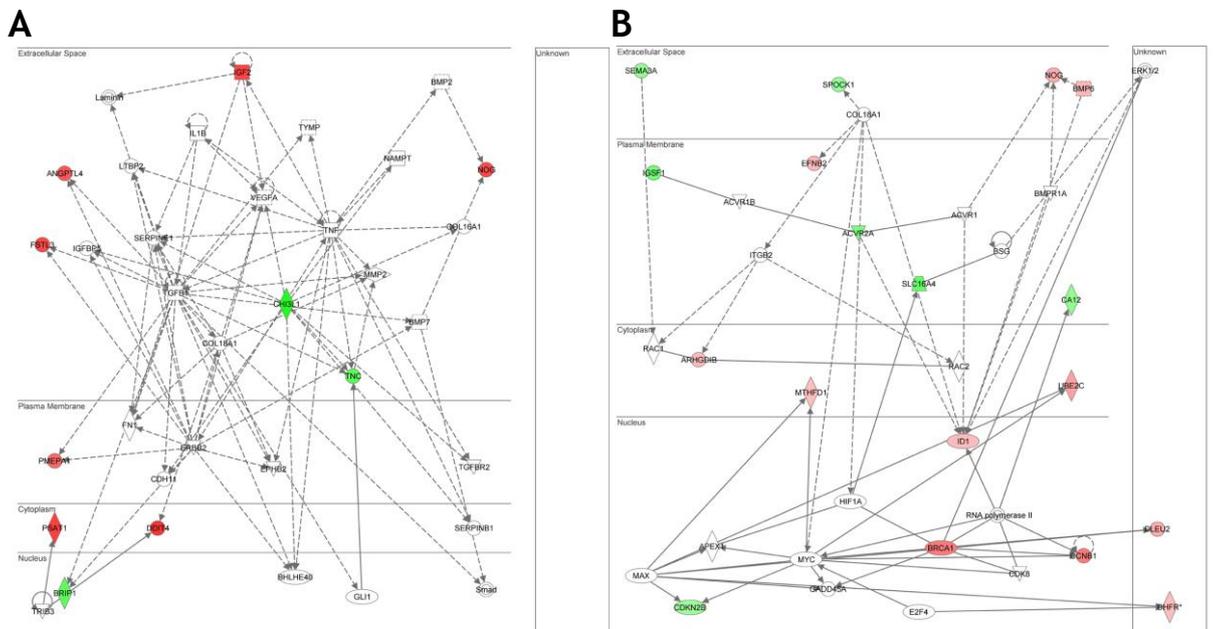


Figure 4-7 F+OGM vs. Flat gene networks at day 7 and 14. (A) At day 7 mainly up-regulations were observed in genes associated with cell growth, development and tissue development (B) At day 14 again mainly up-regulations were observed in genes associated with signalling, movement and tissue development gene network. Red=up, green=down.

4.3.2 Small RNA Expression in Response to Nanotopography

4.3.2.1 Analysis of miRNA Expression

MSCs were examined for the expression of miRNAs in response to the SQ and NSQ nanotopographies, as well as with OGM at day 7 relative to the flat control. Analysis of total miRNA expression (fig. 4-8) identified that miRNAs were mainly up-regulated in MSCs on the SQ nanotopography with only a small proportion up-regulated. In contrast MSCs on the NSQ had the most miRNAs down-regulated, with less than half of the total miRNAs being up-regulated. MSCs cultured with OGM had similar levels of miRNAs being up- and -down regulated.

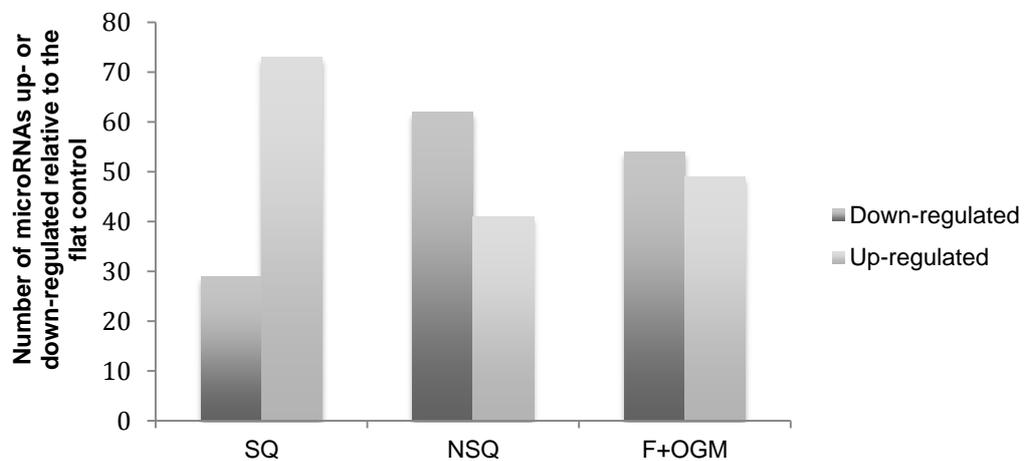


Figure 4-8 Analysis of miRNA expression at day 7. MiRNAs in MSCs cultured on the SQ nanotopography were shown to be mainly up-regulated. While data indicate that MSCs on the NSQ and with OGM, miRNAs were mainly down-regulated.

Further analysis into miRNA expression examined whether miRNAs with significant changes in gene expression relative to the flat were up-regulated or down-regulated (fig. 4-9). In MSCs cultured on the SQ nanotopography, there were approximately twice as many significantly up-regulated miRNAs than those down-regulated. Interestingly, mir-302, a miRNA normally associated with embryonic stem cell pluripotency, is significantly up-regulated only in MSCs cultured on the SQ nanotopography, while miRNAs mir-125 and mir-135, thought to suppress differentiation are also up-regulated. Additionally, miRNAs which are thought to promote differentiation are shown to be down-regulated in MSCs cultured on the SQ nanotopography. These include mir-154, mir-21, and let-7. In contrast, MSCs cultured on the NSQ were found to have significant changes in miRNAs mainly as down-regulations with only one significant up-regulation.

While MSCs cultured on the NSQ nanotopography also had significant down-regulations in miRNAs associated with differentiation, miRNAs thought to be under expressed in osteo-differentiated MSCs were also significantly down-regulated compared to the flat control. MSCs cultured with OGM were identified to have only two miRNAs significantly changed, interestingly one of these miRNAs, mir-24 was shown to be down-regulated in MSCs with OGM, and is again thought to be under expressed in osteo-differentiated MSCs.

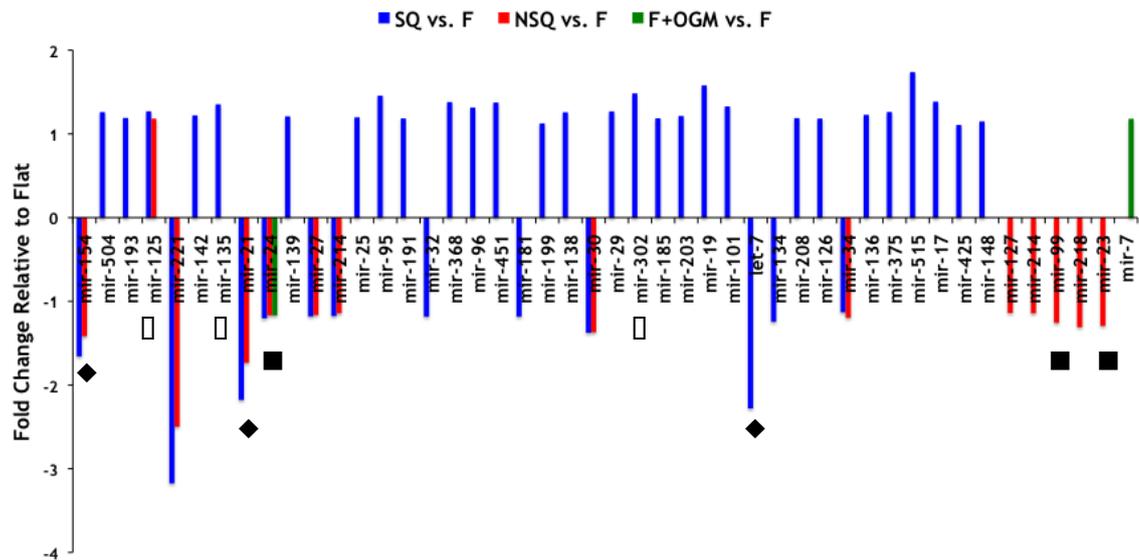


Figure 4-9 Analysis of miRNAs in MSCs cultured on the SQ, NSQ and with OGM relative to the flat control. Note that only miRNA with significant changes in expression are shown. Analysis at day 7 identified that expression of miRNAs found to be significantly altered in MSCs cultured on the SQ (blue) topography where mainly up-regulations. In contrast, significant changes in miRNA expression in MSCs cultured on the NSQ (red) where mainly down-regulations. MSCs cultured with OGM showed little significant change in miRNA expression compared to the flat at this time point. □miRNAs which have been associated with increased pluripotency ◆miRNAs which promote differentiation □ miRNAs which suppress differentiation ■ miRNAs which are found to be under expressed in osteo-differentiated cells.

When miRNA expression was examined in MSCs at day 7 cultured on the SQ relative to the NSQ nanotopography, it was found that significant up-regulations in miRNA expression on the SQ occurred in all instances except for one. Furthermore, again miR-135, a miRNA associated with repressing differentiation was found to be significantly up-regulated in MSCs cultured on the SQ nanotopography relative to the NSQ (fig. 4-10).

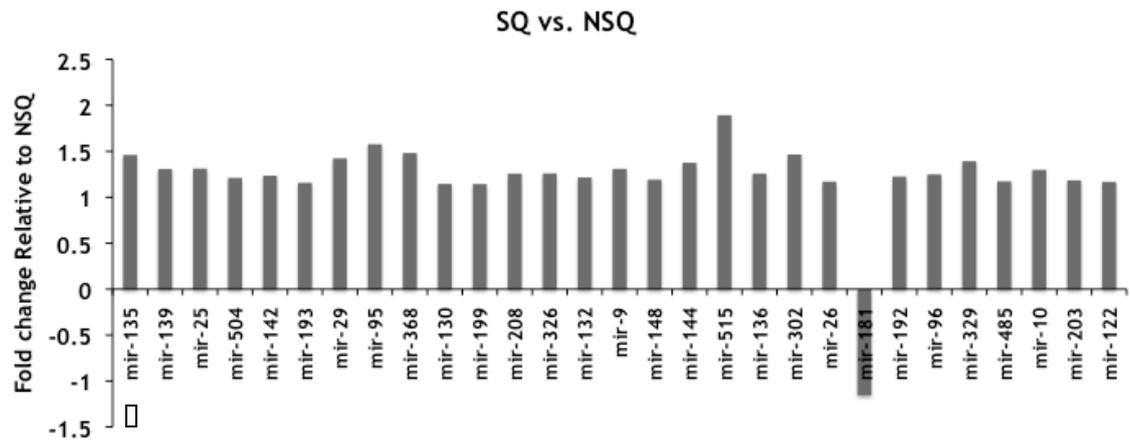


Figure 4-10 MiRNA expression analysis in MSCs cultured on the SQ relative to the NSQ, and osteogenic control, at day 7. Note that all changes are significant. Analysis shows that miRNAs in MSCs cultured on the SQ nanotopography were largely up-regulated compared to those cultured on the NSQ. □ miRNA identified to suppress osteogenic differentiation.

MiRNA expression was examined in MSCs cultured with OGM relative to those expressed on the NSQ nanotopography. In this case there were notably less significant changes in the number of RNA between the two osteogenic inducing controls. Furthermore, miRNAs associated with osteo-differentiated MSCs, miR-34 and miR-154 were up-regulated in MSCs cultured with OGM relative to the NSQ nanotopography (fig. 4-11).

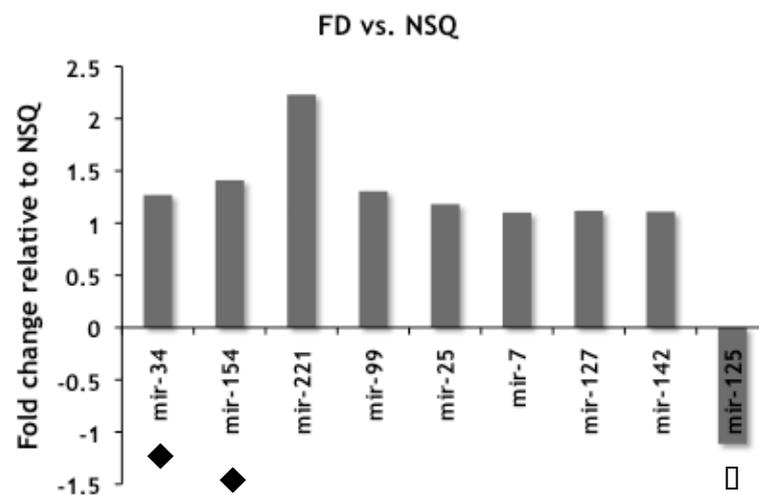


Figure 4-11 Analysis of miRNAs expressed in MSCs cultured with OGM relative to the NSQ nanotopography at day 7. In this case there were less miRNAs significantly up- or down-regulated when MSCs were cultured with OGM compared to the NSQ nanotopography. Additionally, at this time point miRNAs associated with increased osteo-differentiated were up-regulated in MSCs cultured with OGM compared to the NSQ nanotopography at this time point.

4.3.2.2 Analysis of Small Nucleolar RNA Expression

SnoRNA analysis across both topographical substrates and with OGM relative to the flat control identified broad down-regulations across all treatments, however analysis also identified significant up-regulations in MSCs cultured on the SQ and also, to a lesser extent, with OGM (fig 4-12).

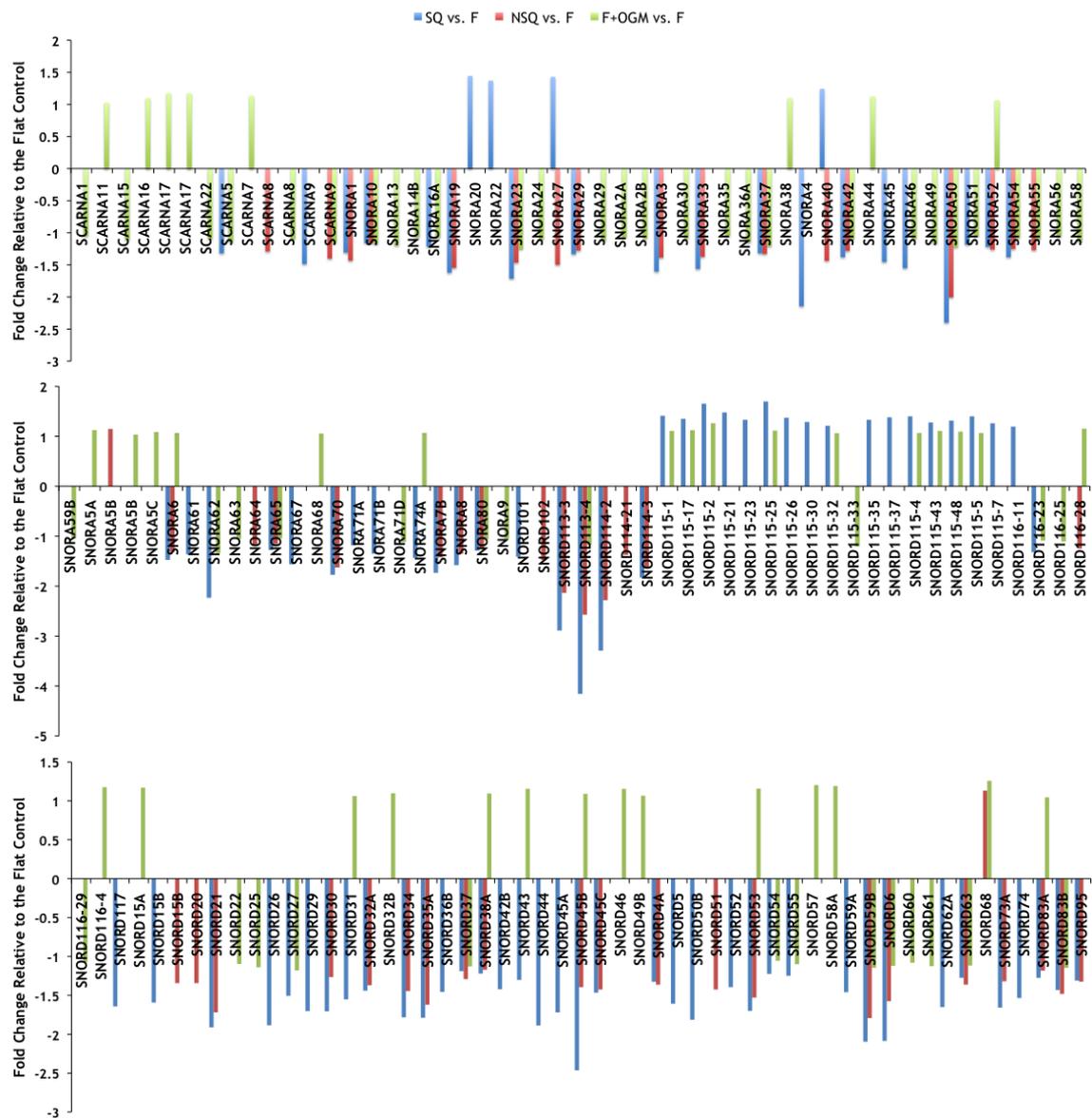


Figure 4-12 SnoRNA analysis for MSCs cultured on the SQ, NSQ and with OGM relative to the flat control at day 7. Broad down-regulations were observed for all treatments, however up-regulations were also observed in MSCs on the SQ and with OGM. All changes are significant $p < 0.05$.

SnoRNAs found to be significantly up-regulated in MSCs on the SQ nanotopography were analysed for expression on the both NSQ and with OGM. MSCs cultured on the NSQ shown no correlation in snoRNA expression compared to the SQ. Additionally, less than half of the snoRNAs up-regulated on the SQ where up-regulated in MSCs cultured with OGM (fig. 4-13).

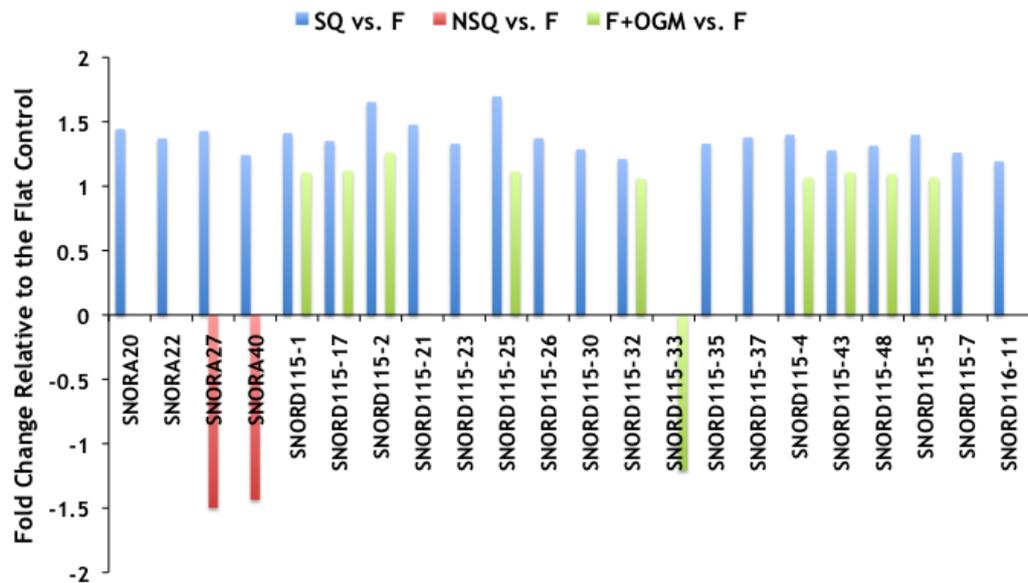


Figure 4-13. Profile of snoRNAs significantly up-regulated on the SQ nanotopography compared to the NSQ nanotopography and with OGM at day 7. Of the snoRNAs found to be significantly up-regulated on the SQ nanotopography, less than half were also found to be up-regulated in MSCs with OGM, while snoRNAs in MSCs cultured on the NSQ nanotopography were found to be down-regulated. All changes are significant with $p < 0.05$.

SnoRNA's were further examined relative to the osteogenic nanotopography, NSQ at days 7 and 14 (fig. 4-14 A & B). At this time point snoRNAs are both up- and down- regulated, however the majority of the snoRNAs are up-regulated. By day 14 however, although the number of significant changes in snoRNA expression in MSCs cultured on the SQ relative to the NSQ has decreased overall, there are over three times as many significantly up-regulated snoRNAs than those that have been down-regulated.

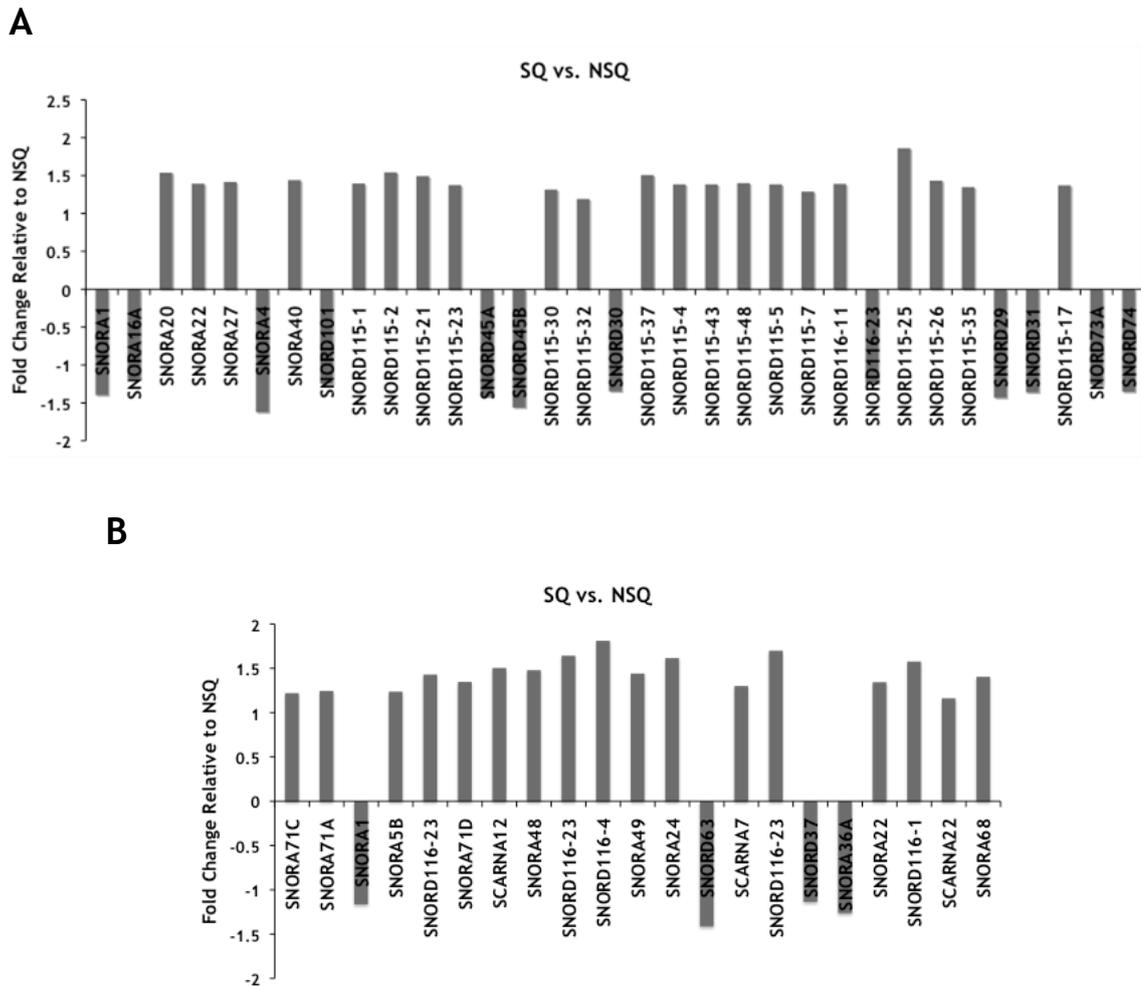


Figure 4-14 SnoRNA expression was examined in MSCs on the SQ nanotopography relative to the osteogenic nanotopography, NSQ. At day 7 significant snoRNA expression was identified, with most of the snoRNAs up-regulated relative to the NSQ nanotopography. All changes are significant $p < 0.05$.

4.3.3 Macroarray data analysis

The expression of SOX2, an embryonic stem cell marker was analysed in both Affymetrix microarray and GE Oligoarray data. The data showed that in MSCs cultured for 14 days and which underwent microarray analysis there was a slight increase in expression of SOX2 expression. Additionally, MSCs underwent 28 days of culture on the SQ nanotopography and subsequent macroarray. Similar to the microarray data analysis showed SOX2 expression was increased, although again not statistically significant relative to the flat control and also in MSCs cultured with OGM (fig. 4-15).

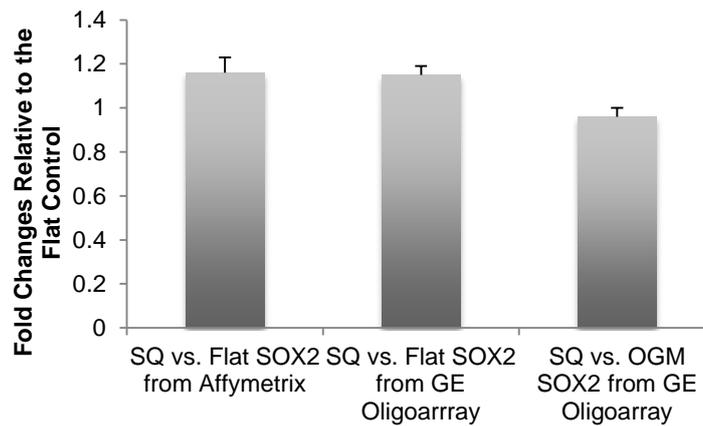


Figure 4-15 Selected microarray (Affymetrix HuGene 1.0ST array and GE stem cell oligoarray) data for SOX2 (embryonic self-renewal marker) after 28 days of culture. The graph shows non-significant fold change increase compared to (1) flat control by Affymetrix after 14 days culture (2) flat control by GE array and (3) osteogenic media (OGM) by GE array after 28 days culture.

4.4 Discussion

Microarray analysis of the MSC gene expression changes and Ingenuity® Pathway analysis of canonical and functional signalling identified widespread changes in canonical signalling in MSCs cultured on the SQ compared to the NSQ and with OGM at day 7 however, critically changes in the functional output of the cell remained unchanged at day 14 compared to widespread changes in functional pathways in MSCs cultured on the NSQ and with OGM.

Interestingly, when these changes in canonical signalling were analysed with regard to up- or down-regulations, data showed that in response to the SQ nanotopography at day 7, MSCs showed mainly down-regulations in canonical signalling. Similarly, MSCs cultured on the NSQ showed mainly down-regulation in canonical signalling at this time point, however MSCs cultured with OGM were identified to have mainly up-regulated canonical signalling at this time point indicating that the osteogenic effect of the NSQ nanotopography may have a latent effect on differentiation compared to the OGM. This is further backed up by evidence at day 14 that MSCs cultured on both the NSQ and with OGM have broad up-regulations in key canonical signalling pathways. These broad up-regulations in canonical signalling further explain the widespread changes in functional signalling at day 14 seen with both the NSQ and OGM in comparison to the little change seen in functional signalling on the SQ nanotopography at this time point.

Furthermore, additional analysis of gene networks associated with key cellular processes followed similar trends in gene expression across all topographies. These results indicate that MSCs cultured on the SQ nanotopography undergo a general down-regulation of genes associated with key canonical signalling pathways leading to little functional change, while MSCs undergoing osteogenic differentiation in response to the NSQ nanotopography and OGM undergo widespread up-regulation of key canonical signalling pathways, which ultimately results in changes in the functional signalling of the cell.

Small RNAs have been implicated in modulating canonical signalling pathways important for regulating differentiation. Here it has been demonstrated that miRNAs in MSCs cultured on the SQ nanotopography were up-regulated overall while miRNAs in MSCs cultured on the NSQ and with OGM were generally down-regulated. Furthermore, the identification of miRNA-302, a miRNA found to promote embryonic stem cell pluripotency and induced pluripotency of somatic cells via cyclin D1, an important regulator of the cell cycle (Houbaviy, Murray et al. 2003; Lin, Chang et al. 2011), may provide an additional mechanism for continued multipotency of MSCs in response to the SQ nanotopography. Interestingly, miR-302 has also been found to be regulated by the embryonic stem cell marker SOX2 (Card, Hebbbar et al. 2008). Non-significant up-regulation of SOX2, an embryonic stem cell marker, as identified by micro- and oligoarray data makes it tempting to speculate that MSCs on the SQ nanotopography may be modulating expression of embryonic-related pathways to promote maintenance of pluripotency via the cell cycle. Furthermore, down-regulation of miRNAs in MSCs on the SQ, thought to promote differentiation, again implicates a role for miRNAs in the continued maintenance of stem cells. Additionally, widespread up-regulation of miRNA expression in MSCs cultured on the SQ relative to the osteogenic NSQ, in contrast to the limited up-regulation of only a handful of miRNAs comparing MSCs cultured with OGM relative to the NSQ nanotopography provides further evidence for the importance of miRNA expression in maintaining the MSC phenotype.

Other small, un-translated RNAs such as SNORDs, although found to be mainly down-regulated across all treatments, those which were significantly up-regulated on the SQ approximately half were found to only be uniquely up-regulated on the SQ. Whilst the exact function of such snoRNAs remains unclear,

their role in RNA editing and micro-RNA like properties may provide an additional level of post-transcriptional RNA control. Furthermore, predominantly up-regulated expression of snoRNAs in MSCs cultured on the SQ nanotopography relative to the NSQ following both 7 and 14 days culture indicates their potential importance in regulating stem cell multipotency.

5 Metabolomics

5.1 General Introduction

In this chapter the metabolic profile of MSCs will be examined in response to the SQ, stem cell retaining, nanotopography. Evidence suggests that stem cells exist as relatively quiescent, metabolically inactive populations, which increase in metabolic activity as they undergo differentiation (Suda, Arai et al. 2005; Reyes, Fermanian et al. 2006; Yanes, Clark et al. 2010). *In vivo*, it is thought that embryonic cells are maintained in a metabolically inactive state by the presence of a hypoxic environment, prior to implantation and subsequent vascularisation of the embryo (Fischer and Bavister 1993). Further evidence for hypoxia as a regulator of stem cell maintenance was presented as a result of various studies which reported the presence of hypoxic conditions within various stem cell niches (Lin, Lee et al. 2006; Eliasson and Jonsson 2010; Seidel, Garvalov et al. 2010). Similarly *in vitro* culture of stem cells under hypoxic conditions was shown to promote prolonged maintenance of stem cells in their undifferentiated state (Ezashi, Das et al. 2005; Potier, Ferreira et al. 2007; Dos Santos, Andrade et al. 2010; Basciano, Nemos et al. 2011).

Hypoxia is thought to play a key role in stem cell function via an ability to maintain stem cells in a characteristically reduced redox state (Reyes, Fermanian et al. 2006). Studies have shown that hypoxia promotes maintenance of a reduced redox state via the up-regulated expression of hypoxia-induced factor-1 (HIF-1), a transcription factor known to down-regulate mitochondrial biogenesis and oxidative metabolism, mechanisms increasingly being implicated in differentiation (Cordeau-Lossouarn, Vayssiere et al. 1991; Cho, Kwon et al. 2006; Kim, Tchernyshyov et al. 2006; Papandreou, Cairns et al. 2006; Buggisch, Ateghang et al. 2007; Zhang and Gutterman 2007; Finkel 2011). Interestingly, it has been reported that induced pluripotent stem cells not only replicate the mitochondrial regulation observed in embryonic stem cells, but that hypoxic conditions can also promote the induced pluripotency of mouse embryonic fibroblasts (Yoshida, Takahashi et al. 2009; Armstrong, Tilgner et al. 2010; Prigione, Fauler et al. 2010). Overall these findings provide increasing evidence to support a role for the redox state of stem cells in the balance between self-

renewal and differentiation (Smith, Ladi et al. 2000; Reyes, Fermanian et al. 2006; Bracha, Ramanathan et al. 2010; Yanes, Clark et al. 2010). Furthermore, it has been proposed that the maintenance of a highly unsaturated or reduced metabolome *in vivo* allows stem cells to respond to oxidative processes, such as inflammation, which occur as the result of injury or disease (Yanes, Clark et al. 2010). The up-regulation of oxidative processes have been shown to increase levels of reactive oxygen species (ROS) within the cell, which have been shown to act as intracellular signalling molecules promoting stem cell differentiation (Sauer, Wartenberg et al. 2001; Su, Mitra et al. 2001; Sauer and Wartenberg 2005). In addition, increasing mechanical tension has also been shown to elevate levels of ROS (De Keulenaer, Chappell et al. 1998; Aikawa, Nagai et al. 2001; Schmelter, Ateghang et al. 2006; Sauer, Ruhe et al. 2008; Ruddy, Jones et al. 2009).

The study of metabolomics using liquid chromatography - mass spectrometry is a relatively novel technique, and as a result of more in-depth study over the last decade, it is thought that the metabolic profile of a cell can provide a particularly unique insight into the exact cellular phenotype. Metabolites are small molecules which form the intermediates and end products produced during various processes within the cell and as a result can act as chemical signatures indicative of particular cellular process. This is particularly interesting as unlike other 'omics' such as genomics or transcriptomics, which are subject to different levels of regulation and therefore can only give an indication of what may be occurring within the cell, metabolites are not. It is for this reason that the metabolic profile is thought to correlate more closely to the true phenotype of the cell (Faijes, Mars et al. 2007; Scheltema, Decuypere et al. 2010).

5.2 Materials and Methods

5.2.1 Liquid Chromatography Mass Spectrometry

Cells were seeded onto substrates (3 material replicates) at 1×10^4 cell per ml and cultured for 7 days. Following this the cells were washed in PBS before metabolites were extracted using an extraction solvent (1:3:1 chloroform:methanol:water), and placed on a rotary shaker for 1 hour at 4°C.

The solution was then centrifuged for 3 minutes at 13000g at 4°C after which samples were analysed using hydrophilic interaction liquid chromatography-mass spectrometry (Dionex UltiMate 1 with a 150 x 4.6mm ZicHilic column running at 300µl/min and Orbitrap Exactive respectively). Raw mass spectrometry data was aligned and peaks were picked using MzMine 2.0. A custom metabolite database incorporating HMDB was used to identify compounds, within a mass window of 0.5 mMU and a retention time window of 2 minutes. Means and standard errors of the mean were generated for all groups of picked peaks and the resulting data was uploaded to in-house software for pathway analysis. Data analysis was carried out using open source software on-line, Pathos: Metabolomics Web Facility (motif.gla.ac.uk/Pathos/index.html). For analysis of metabolite saturation, ratios were calculated for each metabolite at days 7 and 14, and using the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification for each metabolite and Nature Lipidomics Gateway the number of carbon-carbon double bonds for each metabolite was assessed.

5.2.2 RNA isolation

STRO-1+ cells were seeded onto nanotopographical and control substrates (4 material replicates) at a density of 1×10^4 cells per ml. Cells were maintained at 37°C with a 5% CO₂ atmosphere in α -MEM (*Invitrogen, UK*) containing 10% FBS, which was replaced twice weekly. Cells were cultured for 7, 14, or 28 days at which point the cells were trypsinised to maximize recovery of cells from substrates. Cells were then lysed and total RNA extracted using a Qiagen RNeasy Micro Kit according to manufacturer's protocols (*Qiagen, West Sussex, UK*).

Briefly, STRO-1+ MSC lysate was homogenised by vortexing and subsequent passing the lysate through a 20-gauge syringe needle. Equal volumes of 70% ethanol were added to the homogenized lysate, mixed thoroughly and added to the spin column and centrifuged at 8,000 g for 15 seconds. 350 µl of buffer RW1 containing guanidine thiocyanate was added to the spin column to denature proteins, such as RNases. The spin column was again centrifuged at 8,000 g for 15 seconds. DNA was then denatured for 15 minutes using a solution of DNase I and buffer RDD. Buffer RW1 was added again to remove the DNase I and the spin column centrifuged for 15 seconds at 8,000 g. Following washing all filtrate was

discarded. A further wash was carried out using buffer RPE followed by 80% ethanol to precipitate the RNA. The filter was then centrifuged at full speed for 2 minutes to remove any remaining ethanol. The dried filter was then transferred to a 1 ml Eppendorf tube and 14 μ l RNase-free water added. The tube and filter were subsequently centrifuged at full speed for 1 minute. Measurement of extracted RNA yields was performed with a NanoDrop® ND-1000 UV- Vis Spectrophotometer at ratios of 230/260 nm and 260/280 nm.

5.2.3 Microarray Analysis

MSCs were cultured on PCL materials (4 material replicates) for 7 and 14 days. At these points, the cells were lysed and total RNA was extracted using a Qiagen RNeasy kit (Qiagen, UK) as described in section 4.2.2.. Gene expression changes were detected by hybridization of cDNA to Affymetrix HuGene 1.0 ST human arrays as per manufacturer's instructions. Initial bioinformatic analysis was based on rank product (Breitling, Armengaud et al. 2004) and a false discovery rate of 20% was used to upload selected genes changes to the Ingenuity Pathway Analysis (IPA) server (Ingenuity® Systems, www.ingenuity.com). The functional and canonical analyses were then generated through the use of IPA.

1. Network Generation

A data set containing gene (or chemical) identifiers and corresponding expression values was uploaded into in the application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. A p-value cutoff of less than 0.05 was set to identify molecules whose expression was significantly differentially regulated. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity.

2. Functional Analysis of an Entire Data Set

The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Molecules from the dataset that met the p-value cutoff of less than 0.05 and were associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the

analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

3. Functional Analysis of a Network

The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the molecules in the network. The network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

4. Canonical Pathway Analysis: Entire Data Set

Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Molecules from the data set that met the p-value cutoff of less than 0.05 and were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

5.3 Results

5.3.1 Microarray Analysis of Metabolism

A comparative study of microarray data was undertaken to examine the metabolic profile of MSCs cultured on the SQ and NSQ nanotopographies as well as with OGM relative to the flat control. At day 7, comparative analysis of metabolic pathways showed MSCs cultured on the NSQ were identified to be undergoing widespread changes in metabolic signalling pathways, in contrast to MSCs cultured on the SQ and with OGM, with only a handful of metabolic pathways undergoing changes relative to the flat control (fig 5-1 A). At day 14

however, MSCs cultured with OGM were observed to undergo widespread changes at this time point, with MSCs cultured on the NSQ undergoing changes in only a handful of pathways. In the case of the SQ nanotopography, no changes in metabolic signalling were observed at day 14 (fig 5-1 B).



Figure 5-1 Comparative metabolic signalling in MSCs cultured on the SQ and NSQ nanotopographies as well as with OGM relative to the flat control at day 7 (A) and 14 (B). Widespread changes in metabolic signalling were observed in MSCs cultured on the NSQ at day 7. In contrast at day 14 widespread changes in metabolic signalling were observed in

MSCs cultured with OGM. MSCs cultured on the SQ nanopotography however, showed little change in metabolic signalling at either day 7 or 14.

Further analysis of microarray data was carried out to identify whether changes observed in metabolic signalling were either up- or down-regulations. MSC cultured on the SQ nanopotography where identified to have mainly down-regulations in metabolic pathways at day 7 (fig. 5-2). At day 14, however, no changes in metabolic pathways were identified, therefore no data is presented.

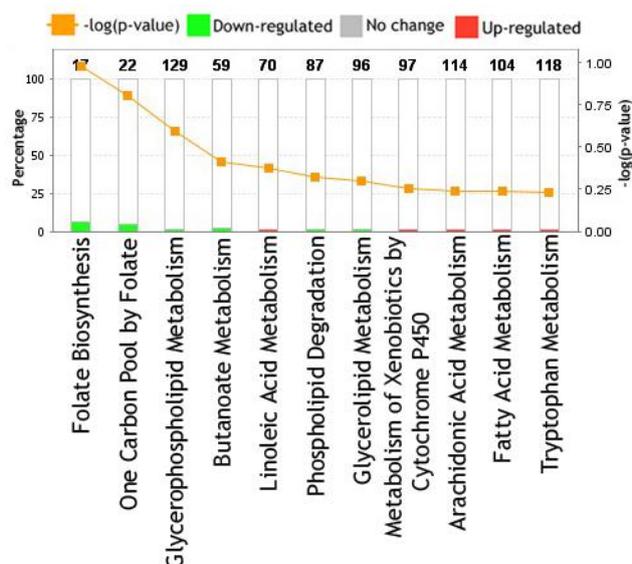


Figure 5-2 Metabolic pathway analysis for MSCs cultured on the SQ nanopotography at day 7 relative to the flat control. Metabolic pathways are shown to undergo mainly down-regulation in response to the SQ nanopotography at this time point. At day 14, no changes in metabolic signalling were observed therefore no data is shown.

In contrast, MSCs cultured on the NSQ nanopotography were identified to have mainly up-regulations in metabolic pathway signalling at day 7 (fig. 5-3 A). By day 14, MSCs on the NSQ were identified to have changes in only a handful of metabolic pathways, however these were all up-regulations (fig. 5-3 B).

At day 7 MSCs cultured with OGM were identified to have up-regulations in only a handful of metabolic pathways. However by day 14, widespread changes in metabolic signalling were observed, with these identified as mainly up-regulations (fig. 5-4 A&B).

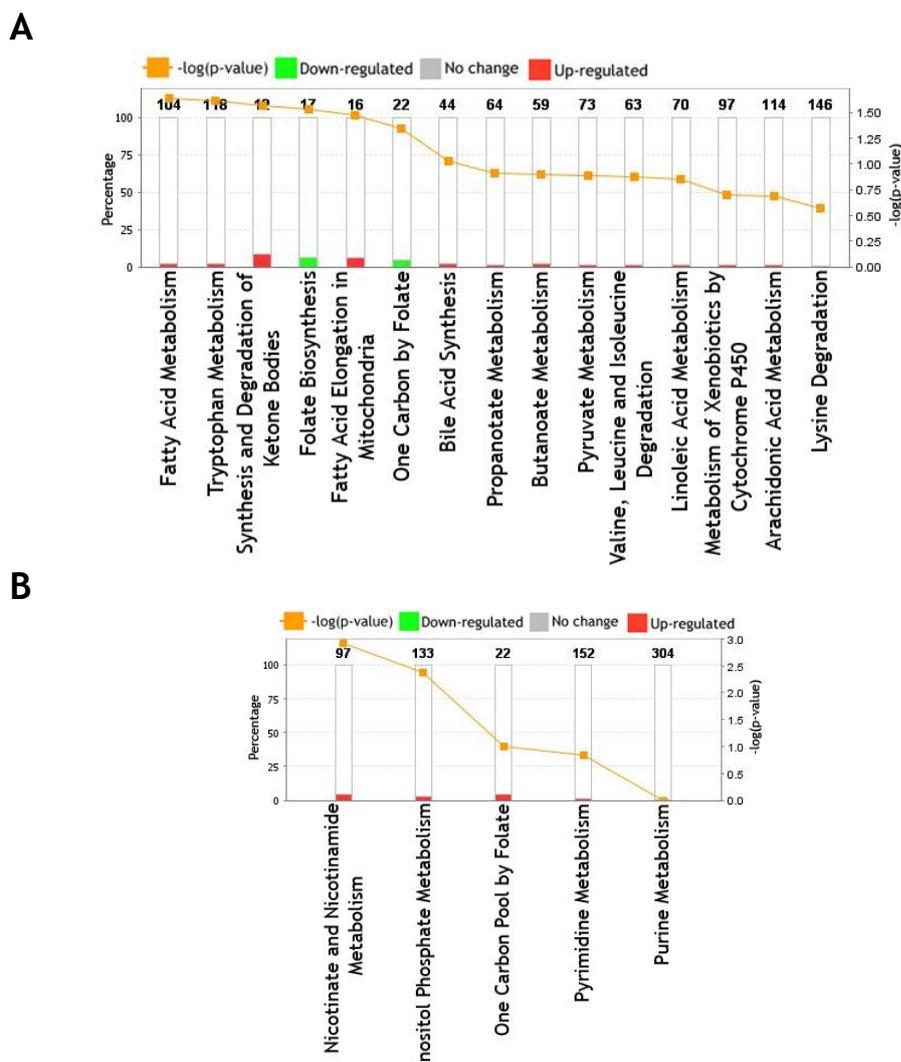


Figure 5-3 Metabolic pathway analysis for MSCs cultured on the NSQ nanotopography at day 7 (A) and day 14 (B) relative to the flat control. MSCs cultured on the NSQ nanotopography were shown to regulate several metabolic pathways at day 7, with mainly up-regulations observed.

5.3.2 Metabolite Analysis using LC-MS

Metabolites were analysed relative to the NSQ osteogenic nanotopography and with OGM at days 7 and 14. Metabolites were shown to be down-regulated in MSCs cultured on the SQ relative to both the NSQ nanotopography and with OGM. Pathways with the most metabolite changes were associated with aminoacyl-tRNA metabolism, purine metabolism and other amino acids. At day 7 a small number of metabolites associated with aminoacyl-tRNA biosynthesis were shown to be down-regulated in MSCs cultured on the SQ relative to the NSQ nanotopography (fig. 5-5 A). In addition, metabolites associated with aminoacyl-tRNA biosynthesis were shown to be broadly down-regulated in MSCs cultured on the SQ relative to with OGM at this early time point (fig. 5-5 B).

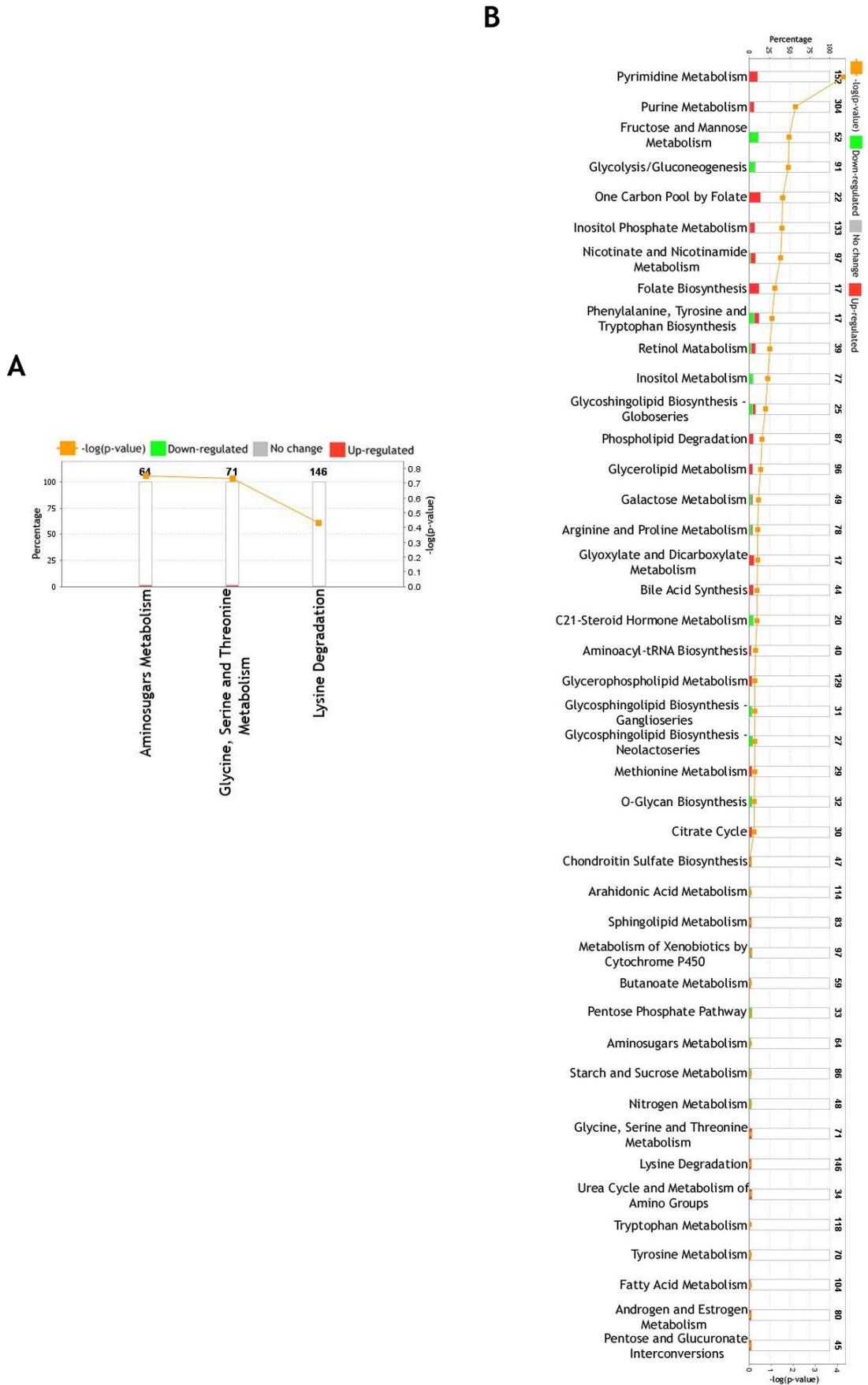
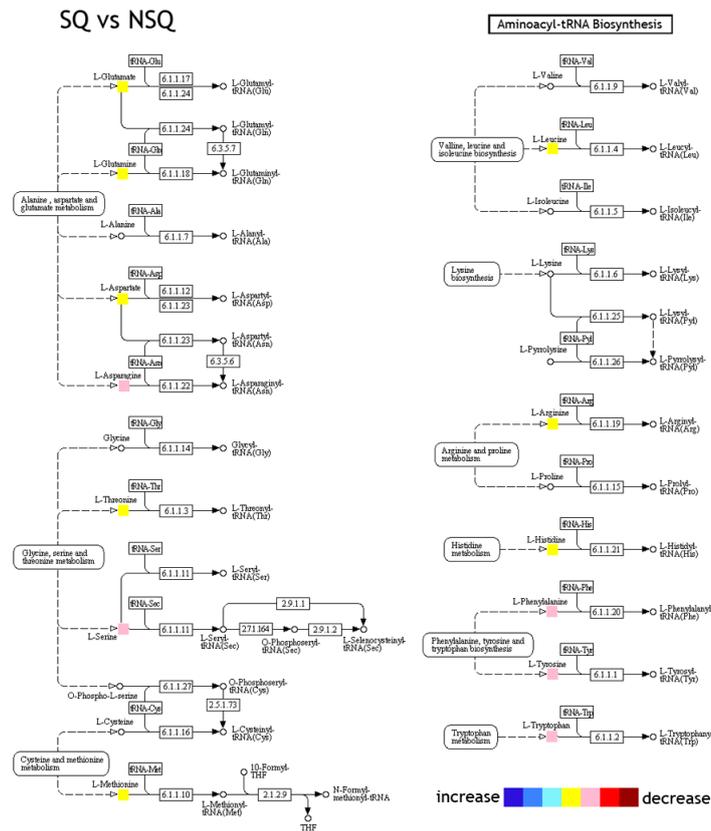


Figure 5-4 Metabolic pathway analysis for MSCs cultured with OGM at day 7 (A) and day 14 (B) relative to the flat control. MSCs cultured on the with OGM were shown to regulate several metabolic pathways at day 7, with mainly up-regulations observed.

Similarly, analysis at day 14 found metabolites associated with aminoacyl-tRNA biosynthesis were further down-regulated in MSCs cultured on the SQ nanopography relative to the NSQ (fig 5-6 A). Again at this time point, metabolites were found to be broadly down-regulated, with 18 out of 24 metabolites showing down-regulated expression in MSCs cultured on the SQ nanopography relative to those cultured with OGM (fig. 5-6 B).

Analysis of metabolite saturation at day 7 was carried out for MSCs cultured on the SQ nanopography relative to those undergoing osteogenic differentiation. Ratios were calculated for each metabolite, comparing metabolite abundance in MSCs cultured on the SQ nanopography relative to MSCs cultured in osteogenic differentiation media. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) identification for each metabolite and the Nature Lipidomics Gateway, the number of carbon-carbon double bonds was identified. At day 7, less metabolites were found to be up-regulated in MSCs on the SQ nanopography compared to those with OGM. In addition, more metabolites with higher numbers of carbon-carbon double bonds were observed to cluster towards MSCs cultured on the SQ or those which exhibit only a small increase in expression in MSCs cultured with OGM (fig. 5-7 A). At day 14, whilst more metabolites are up-regulated on the SQ compared to day 7, again metabolites with higher number of carbon-carbon double bonds are associated with MSCs cultured on the SQ nanopography than those cultured with OGM (fig. 5-7 B).

A



B

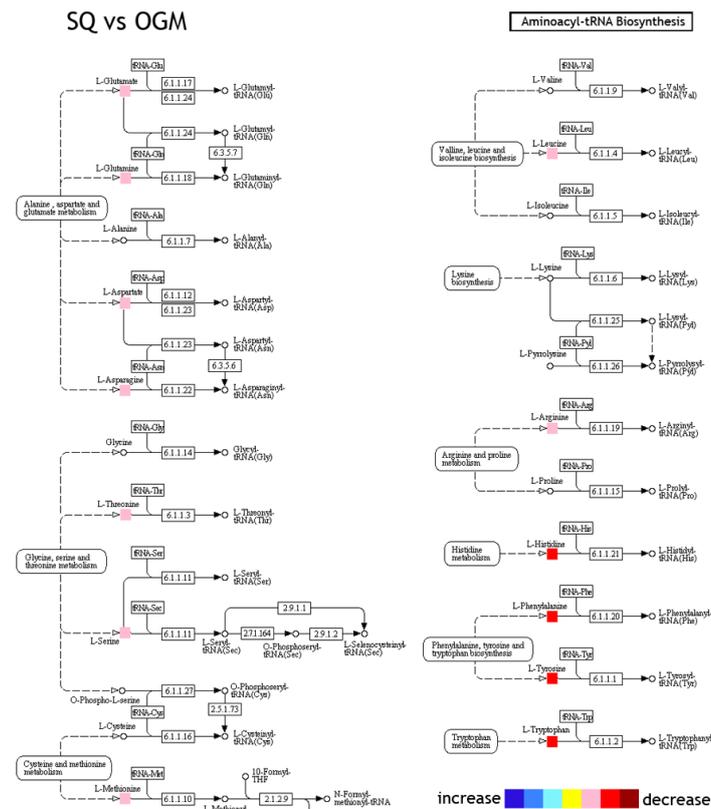
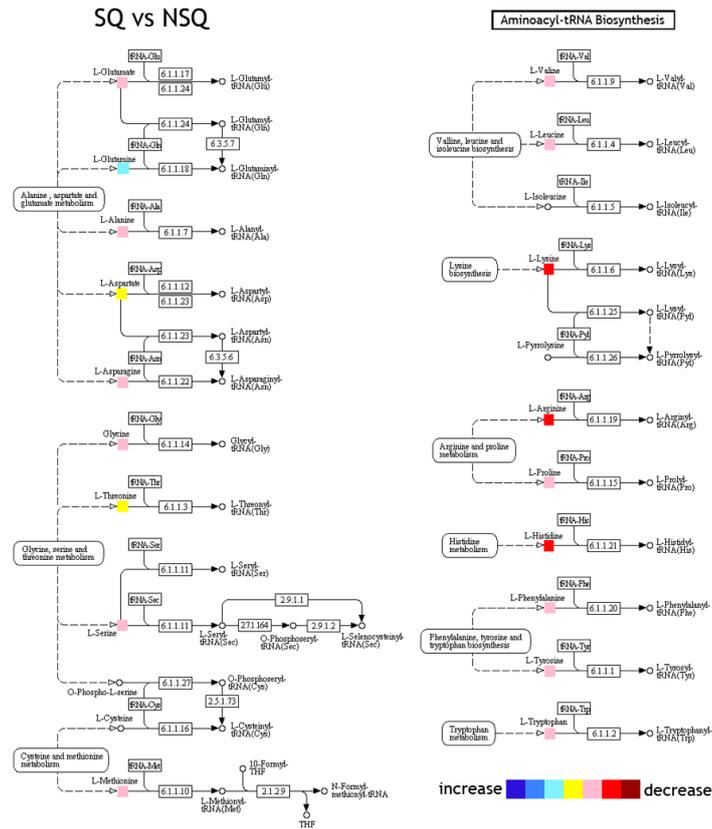


Figure 5-5 Metabolites associated with aminoacyl-tRNA biosynthesis at day 7 in MSCs cultured on the SQ relative to the NSQ nanotopography (A) and with OGM (B). A handful of metabolites were observed to be down-regulated on the SQ relative to NSQ nanotopography at day 7 whilst broad down-regulations were observed when MSCs cultured on the SQ where compared to those with OGM.

A



B

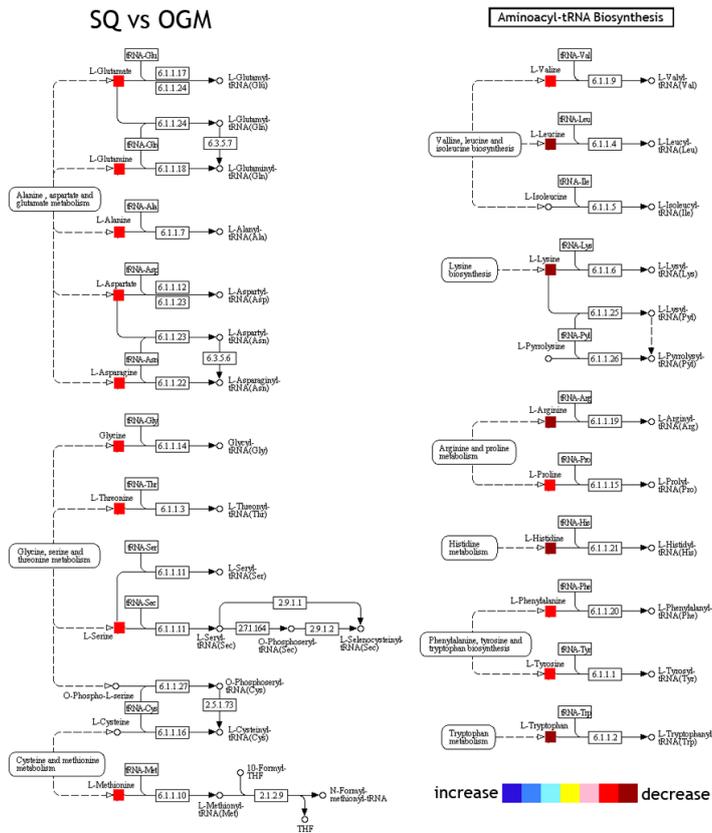


Figure 5-6 Metabolites associated with aminoacyl-tRNA biosynthesis at day 14 in MSCs cultured on the SQ relative to the NSQ nanotopography (A) and with OGM (B). At this time point a large number of metabolites were observed to be down-regulated on the SQ relative to NSQ nanotopography, whilst further broad down-regulations were observed when MSCs cultured on the SQ where compared to those with OGM.

B



Figure 5-7 Metabolic saturation in undifferentiated (SQ) and differentiating (OGM) MSCs at days 7 and 14. At day 7 (A) the number of C=C bonds observed in undifferentiated cells was higher than in the differentiating cells. At day 14 (B) again, more metabolites with higher C=C bonds were identified on the SQ relative to OGM. Note that red and green bars indicate increasing fold change.

Further analysis was carried out to examine the average number of carbon-carbon double bonds per metabolite at days 7 and 14 (fig. 5-8). Analysis identified that for the number of metabolites up-regulated at day 7, those in MSCs on the SQ nanotopography had on average double the number of carbon-carbon double bonds per metabolite than MSCs cultured with OGM. Similarly at day 14, the average number of carbon-carbon double bonds was also higher (approximately 1.7 times higher) on the SQ nanotopography than in MSCs with OGM (fig. 5-8).

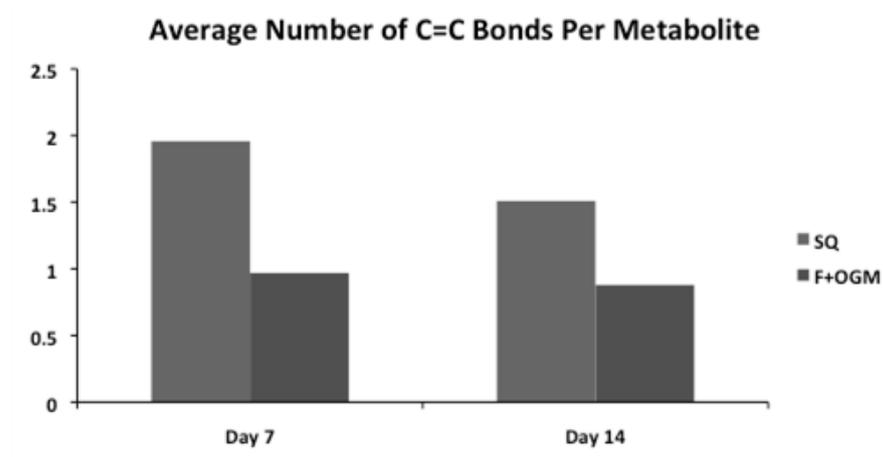


Figure 5-8 Average number of C=C bonds per metabolite. At day 7 there are approximately twice the number of carbon double bonds per metabolite in MSCs cultured on the SQ relative to with OGM. At day 14, whilst the number of metabolites with C=C have decreased on both the SQ and with OGM, the number remains higher on the SQ than with OGM.

5.4 Discussion

Analysis of microarray data for changes in gene expression associated with cellular metabolism, identified that whilst MSCs cultured on the SQ and NSQ nanotopographies underwent changes in gene expression associated with metabolic pathways, gene expression in response to the NSQ osteogenic nanotopography was mainly up-regulated. This is in contrast to MSCs cultured on the SQ nanotopography which underwent mainly down-regulations in gene expression associated with metabolic pathways at day 7. Furthermore MSCs were shown to undergo little change in metabolic signalling again at day 14 with no changes in metabolic signalling detected at this time point. Interestingly, this is in contrast to MSCs undergoing osteogenic differentiation on both the NSQ nanotopography and with OGM which were identified to have mainly up-regulated gene expression associated with several metabolic pathways at both days 7 and 14.

Further analysis using LC-MS at days 7 and 14 identified broad decreases in metabolite abundance associated with many key metabolic pathways in MSCs cultured on the SQ nanotopography relative to those cultured on the NSQ and with OGM. In-depth analysis was used to identify metabolic pathways with the most changes in metabolite abundance. Interestingly, the top pathways with the most changes (these were identified to be mainly down-regulations) in metabolite abundance comparing MSCs cultured on the SQ nanotopography relative to the osteogenic controls, included the biosynthesis of various amino acids and purines, as well as aminoacyl-tRNA biosynthesis. The down-regulation of such metabolic products in MSCs cultured on the SQ nanotopography relative to both osteogenic controls, in particular their importance in gene expression and translation correlates with previously reported observations that MSCs form a quiescent, metabolically inactive stem cell population, in contrast to actively differentiating stem cells which undergo increased metabolic activity and gene expression changes. These results further reflect those identified in chapter 4, with MSCs cultured on the SQ nanotopography exhibiting down-regulation in canonical signaling by day 14, whilst both osteogenic controls were identified to have increased gene expression associated with various signaling pathways.

As a result of previously reported work by Yanes et al., ESCs have been identified to have a characteristic unsaturated metabolite profile when compared to stem cells actively undergoing differentiation (Yanes, Clark et al. 2010). In this study, the metabolic profile of MSCs was undertaken to examine the degree of saturation for metabolites up-regulated in MSCs cultured on the SQ nanotopography and MSCs undergoing osteogenic differentiation with OGM. As a result of the study it was identified that MSCS cultured on the SQ nanotopography maintain a high degree of unsaturated metabolites compared to those cultured with OGM at both time points. Importantly, whilst the data presented here is in agreement with those by Yanes et al., the metabolite profile is less distinct for MSCs than for ESCs. However, it is proposed that this difference in metabolite saturation profile may be due to the more primitive nature of ESCs compared to MSCs.

The importance of unsaturated metabolites in stem cells were proposed to be important in maintaining 'chemical plasticity', and stem cell sensitivity to oxidative signalling processes such as inflammation. In particular, oxidative

processes such as inflammation are known increase oxidative stress as a result of increased levels of ROS, which as discussed previously are known to act as secondary messengers promoting differentiation (Sauer, Wartenberg et al. 2001; Su, Mitra et al. 2001; Sauer and Wartenberg 2005; Krutzfeldt and Stoffel 2006). MicroRNAs however, are increasingly being implicated in the regulation of metabolic homeostasis (Xu, Vernooy et al. 2003; Esau, Davis et al. 2006; Simone, Soule et al. 2009). It is tempting to speculate therefore that the increased levels of microRNA expression observed in MSCs cultured on the SQ as identified in chapter 4 may contribute to the maintenance of a reduced metabolome, however further studies would need to be conducted to support this.

Interestingly, whilst the role of nanotopography in maintaining a reduced redox state remains unsubstantiated, evidence is gathering to support the non-hypoxic up-regulation of HIF-1 in response to signalling pathways associated with cell adhesion such as PI3K/AKT/mTOR and RAF/MEK/MAPK (Hirota and Semenza 2001; Dery, Michaud et al. 2005; Diebold, Petry et al. 2010). Furthermore, research conducted during the 1970's identified a role for saturated and unsaturated lipids at the plasma membrane in the regulation of cell adhesion. Interestingly, it was found that cell adhesion increases with an increase in saturated lipids within the plasma membrane. In contrast however, cell adhesion was observed to decrease in response to an increase in unsaturated lipids at the plasma membrane (Curtis, Campbell et al. 1975; Curtis, Chandler et al. 1975; Curtis, Shaw et al. 1975). Therefore it is tempting to speculate that nanotopography may play a role in the maintenance of a metabolically reduced stem cell metabolome via non-hypoxic mechanisms, however at present further in depth research is required to support such a role.

6 Translation Across Polymers

6.1 General Introduction

The focus of this chapter is to study the effect of the SQ, stem cell retention nanotopography in different polymers to separate chemical effects from topographical effects. The ability to translate the nanotopographical effects observed on polycaprolactone (PCL) in response to the SQ nanotopography across a range of polymers is particularly important as conventional tissue culture plastics are currently produced in polystyrene (noting that polycarbonate is also used for tissue culture plastics but to a much lesser extent). In addition, the ability to transfer this technology across polymers with different surface chemistries provides the opportunity to further investigate that it is indeed the nanotopographical properties which promote maintenance of the stem cell phenotype. In this chapter therefore, MSCs cultured on nanotopographically-patterned polymers including polycarbonate (PC) and polystyrene (PS) were investigated for maintenance of the stem cell phenotype, and stem cell maintenance following standard tissue culture techniques such as passaging.

In addition, a preliminary study was carried out into the role intracellular tension plays in stem cell maintenance. Tension has been demonstrated to play a key role in the determination of stem cell fate with seminal studies previously demonstrating the importance of matrix stiffness, cell spreading and shape on cell fate determination (McBeath, Pirone et al. 2004; Engler, Sen et al. 2006; Gilbert, Havenstrite et al. 2010; Kilian, Bugarija et al. 2010). As a result, such studies have revealed the significance of the physical environment on dictating stem cell fate and self-renewal. However changes in cell fate directed by nanotopography are evolved entirely by altering the spatial arrangement of topographic cues rather than the physical properties of the substrate (Dalby, Gadegaard et al. 2007; Biggs 2008; Biggs, Richards et al. 2009; Biggs, Richards et al. 2010).

To investigate the role of topographically induced changes in cellular tension on the maintenance/differentiation of MSCs, blocking of actin/myosin contraction was carried out using the inhibitors: blebbistatin, a myosin II inhibitor and

Y27632, and an inhibitor of Rho associated protein kinases (ROCK) on all test substrates. In addition, the ERK/MAPK signalling pathway was also subject to investigation as a biochemical downstream signalling pathway of tension effectors such as FAK and Rho, and also as a key regulator of proliferation and differentiation (Giancotti and Ruoslahti 1999; Meloche and Pouyssegur 2007; Khatiwala, Kim et al. 2009; Hong, Jeon et al. 2010; Kilian, Bugarija et al. 2010).

The ERK/MAPK pathway is of particular interest due to the differential effects both the duration and magnitude of ERK/MAPK signalling ultimately have on the biological outcome. It has been shown that sustained ERK signalling, whilst in general associated with increased proliferation, can actually lead to a decrease in proliferation and induced differentiation (Sewing, Wiseman et al. 1997; Woods, Parry et al. 1997; Jaiswal, Jaiswal et al. 2000; Roovers and Assoian 2000; Kilian, Bugarija et al. 2010). In the case of sustained ERK signalling, osteogenic differentiation of MSCs is thought to be the result (Jaiswal, Jaiswal et al. 2000; Fu, Tang et al. 2008; Kilian, Bugarija et al. 2010; Jung, Kim et al. 2011). In contrast, under transient or low levels of ERK signalling, MSC proliferation is also thought to decrease, however, in this case adipogenic differentiation was observed (Jaiswal, Jaiswal et al. 2000; Kilian, Bugarija et al. 2010; Jung, Kim et al. 2011). Importantly, intracellular tension has been shown to play a key role in the induction of MAPK signalling and has thereby been implicated in playing a key role in the determination of stem cell fate (McBeath, Pirone et al. 2004; Engler, Sen et al. 2006; Khatiwala, Kim et al. 2009; Kilian, Bugarija et al. 2010). An investigation into a potential role for ERK/MAPK signalling in the maintenance of MSCs will be studied using a MAPK inhibitor, U0126.

6.2 Materials and Methods

6.2.1 Polymer Replication

An injection moulder (*Engel Victory Tech 28*) was used to generate nanotopographically-patterned and flat control surfaces as indicated in section 2.3.4.2. PC and PS samples were given a 2 and 10 second oxygen plasma treatment respectively, to allow for cell attachment (*Harrick Plasma, USA*).

6.2.2 Osteogenic/Adipogenic Differentiation

MSCs were cultured on polycarbonate replicas with SQ nanotopography and flat polycarbonate as a control. Cells were seeded at 1×10^4 cells/ml and cultured in basal media for 28 days. Following this time, MSCs were trypsinised off materials and reseeded onto glass coverslips. Basal media was replaced with either adipogenic or osteogenic induction media as previously described in section 3.1.9. MSCs were fixed following 14 days culture with induction media and immunostained for PPAR- γ and OPN, adipogenic and osteogenic markers respectively, as described in section 3.1.9.

6.2.3 Multipotency/Differentiation

MSCs were seeded at 1×4 cells/ml and cultured in basal (10%FBS/ α -MEM) or osteogenic differentiation media as previously described in section 2.2.1. and cultured for 28 days on SQ and NSQ patterned PC. Flat PC and with OGM were used as controls. Following 28 days culture MSCs were fixed and stained for OPN, OCN, ALCAM and STRO-1 as previously described in section 3.1.3. The work carried out for this experiment was conducted by Dr. Monica Tsimbouri at the University of Glasgow.

6.2.4 Cell Passaging

MSCs were cultured in basal media (10%FBS/ α -MEM) and underwent serial passaging when cultured on PS. MSCs underwent passaging up to passage 4 (as far as tested within this study) as described in section 3.1.8.

6.2.5 Runx2 expression

MSCs were cultured on PC for 3 days in basal media (10%FBS/ α -MEM) or with OGM. Following 3 days culture MSCs were fixed and stained for monoclonal mouse anti-phospho-RUNX2 (*Insight Biotechnology, UK*) as previously described in section 3.1.3.

6.2.6 Inhibition Studies

MSCs were seeded onto PC substrates at 1×10^4 cells/ml for 14 days. The MSCs were cultured in basal media (10%FBS/ α -MEM) supplemented with either a MAPK inhibitor, U0126 (10 μ m) (*Promega, UK*), blebbistatin a non-muscle myosin II inhibitor (50 μ m) (*Sigma, UK*), or Y27632 a ROCK inhibitor (10 μ m) (*Sigma, UK*). Supplemented media was changed every other day to ensure the inhibitors were active. After 14 days, cells were fixed and stained for OPN, PPAR- γ and STRO-1 as previously described in section 3.1.3.

6.3 Results

6.3.1 Multipotency/Differentiation

MSCs were cultured on PC for 28 days and assessed for expression of osteogenic markers OCN and OPN as well as ALCAM and STRO-1 using immunofluorescence. Results showed that MSCs cultured on the NSQ osteogenic nanotopography and with OGM expressed both osteogenic markers with only ALCAM expression. MSCs cultured on the flat control, however, showed only expression of ALCAM after 28 days. In contrast, MSCs cultured on the SQ nanotopography were identified to express both ALCAM and STRO-1 stem cell markers at this time point with no expression of OPN or OCN (fig. 6-1).

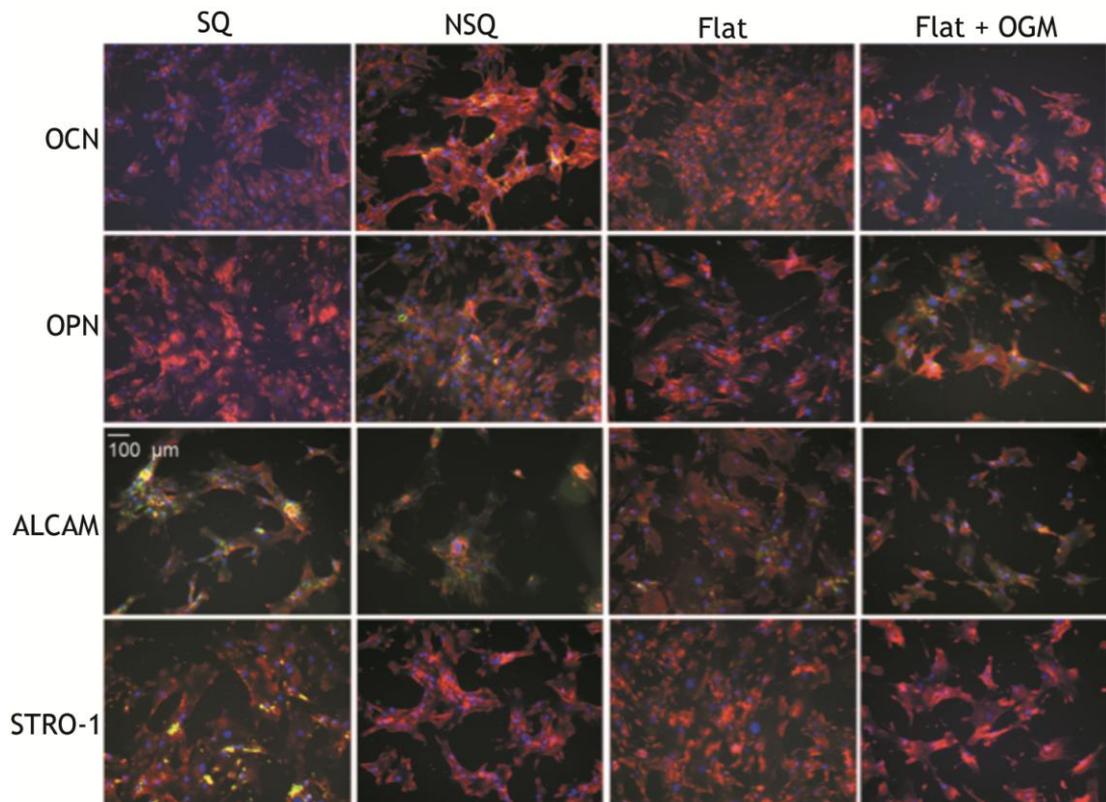


Figure 6-1 STRO-1 selected skeletal MSC growth and phenotype after culture on polycarbonate (PC). MSCs with 28 day culture on nanopatterned PC undergo similar differentiations / phenotype maintenance to MSCs cultured on nanopatterned PCL. Only MSCs cultured on the SQ surface still express STRO-1 at 28 days of culture. The MSCs cultured on NSQ and with osteogenic media expressed ALCAM to a degree but showed strong expression of the bone markers osteocalcin (OCN) and osteopontin (OPN). Red=actin, green= STRO-1, ALCAM, OCN or OPN. This work was carried out by Dr. Monica Tsimbouri at the University of Glasgow.

To demonstrate multipotency of MSCs cultured on the SQ PC nanotopography were trypsinised and reseeded onto glass coverslips. Osteogenic and adipogenic differentiation media was used to induce differentiation of MSCs. MSCs cultured on the SQ nanotopography were observed to undergo both adipogenic and osteogenic differentiation in response to appropriate differentiation media. Adipogenic differentiation was identified using PPAR- γ immunofluorescent staining, however fat droplets were also visualized. In addition, osteogenic differentiation was identified using OPN immunofluorescent staining. In contrast, MSCs originally seeded on the control PC substrates were only found to undergo low levels of marker expression in response to both osteogenic and adipogenic differentiation media (fig. 6-2)

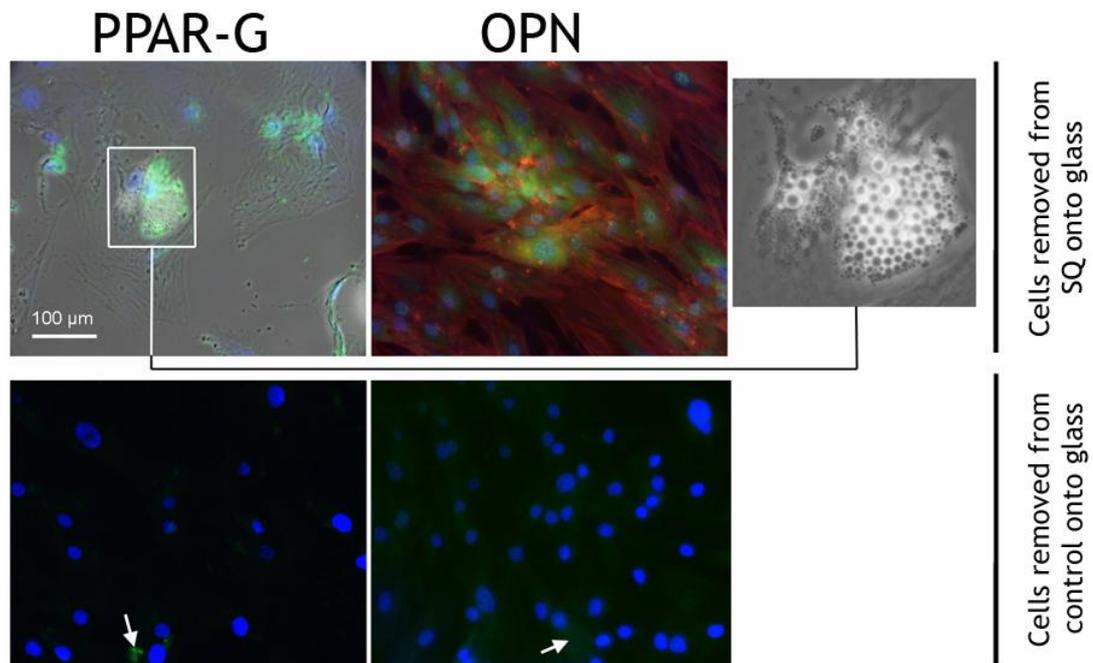


Figure 6-2 After 28 days of culture on planar control or SQ, the MSCs were trypsinised and seeded onto glass coverslips at 1×10^4 cells ml^{-1} with adipogenic or osteogenic media for 14 days before fixation and staining for the adipocyte marker PPARG or the osteoblast marker osteopontin (OPN). After culture on SQ and when treated with adipogenic media, the cells could be seen to express PPARG and had developed fat droplets (left image and magnification of the highlighted square). When treated with osteogenic media, areas of dense, positive OPN expression were noted. However, after reseeding to coverslips post-culture on flat controls, levels of differentiation induction were much lower, with only small areas of PPARG or OPN expression noted (arrows).

6.3.2 Passaging

MSCs were passaged whilst undergoing culture on SQ patterned PS. In this case, MSCs were shown to retain expression of STRO-1, the more stringent marker for MSCs for up to 4 passages (as far as tested so far). In addition, MSCs were also shown to express high levels of ALCAM (inset) (fig. 6-3). In contrast MSCs cultured on flat PS were shown to express low levels of STRO-1 in comparison to the SQ nanotopography up until passage 4.

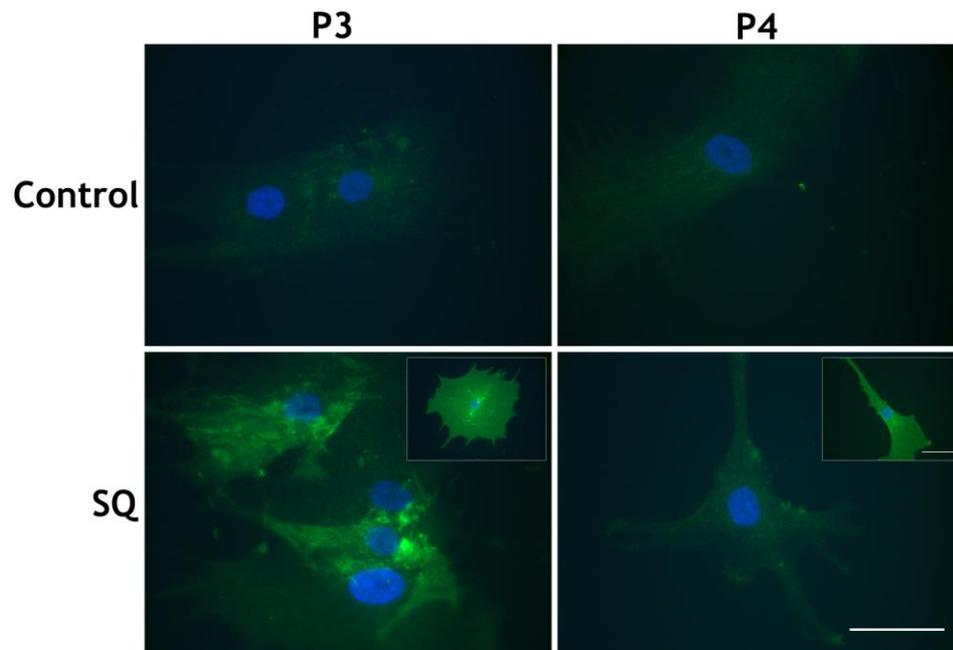


Figure 6-3. Study of STRO-1 selected MSCs after passaging. (A) (B) STRO-1 positive MSCs before passaging onto the SQ topography (after culture on tissue culture plastic for 7 days – D7) and then after subculture onto SQ for one day (P1D1), four days (P1D4) and then after serial subcultures onto SQ for two, three and four passages (images shown at 1 day after subculture – included are positive and negative controls showing antibody specificity. (B) STRO-1 stained cells cultured for up to four passages on flat and SQ patterned polystyrene. Cells on planar control were observed to have much reduced STRO-1 expression compared to MSCs on SQ (note: images of retained ALCAM are inset). Red=actin, blue = DNA, green= STRO-1 (inset = ALCAM). Scale bar = 50 μ m.

6.3.3 RUNX2 expression

Immunofluorescent analysis of phospho (active)-RUNX2 expression in response to both topographies and control substrates showed low levels of expression on both the SQ and flat control (fig 6-4 A&C), whilst a higher level of expression was noted on both the NSQ topography and osteogenic control (fig. 6-4 B&D). Comparing the osteogenic control and NSQ nanotopography to the flat control, levels of RUNX2 expression were shown to be higher in both cases.

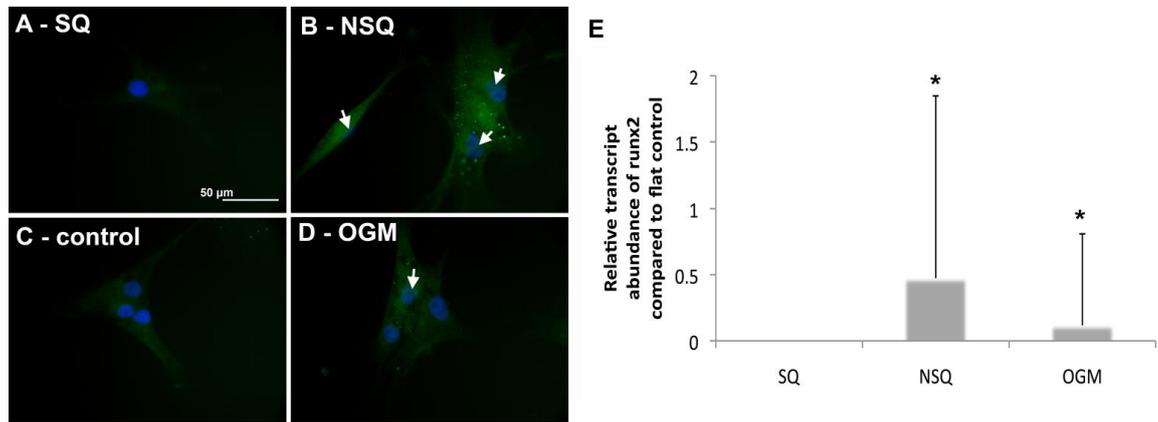


Figure 6-4 Early stage RUNX2 activation on control and test surfaces in MSCs. STRO-1 selected MSCs were cultured on polycarbonate (PC) surfaces imprinted with the SQ and NSQ nanopatterns for three days and then stained for phospho-RUNX2 (the active form). Images were taken with similar exposure and processed for brightness and contrast to the same extent. Very low levels of phospho-RUNX2 were observed on the control and retention (SQ) surfaces (A & C). However, high expression was noted in NSQ and in cells stimulated with osteogenic medium (OGM) (B & D). (E) QPCR for RUNX2 after 3 days of culture showed negligible expression on control and SQ surfaces producing incalculable Ct values (we note that only small quantities of mRNA were harvested after such a short culture time). However, RUNX2 was detected in MSCs harvested from NSQ and after culture with OGM. (n=3 material replicates, results are mean \pm SD, * = $p < 0.05$ by t-test compared to SQ and control).

6.3.4 Intracellular Tension

Disruption of intracellular tension driven by the interaction of actin and myosin was carried out using the inhibitors blebbistatin and a ROCK inhibitor. The disruption of the actin/myosin interaction resulted in a loss of STRO-1 expression in MSCs cultured on the SQ whilst adipogenic differentiation becomes apparent following expression of PPAR- γ on all substrates concomitant with a loss of osteogenic marker expression on both the NSQ and OGM controls following inhibition (fig 6-5 A & B). In addition, the inhibition of MAPK-mediated intracellular signalling was shown to result in a loss of STRO-1 expression in MSCs cultured on the SQ, as well as a loss of both osteogenic and adipogenic markers in MSCs cultured on all substrates (fig. 6-5 C).

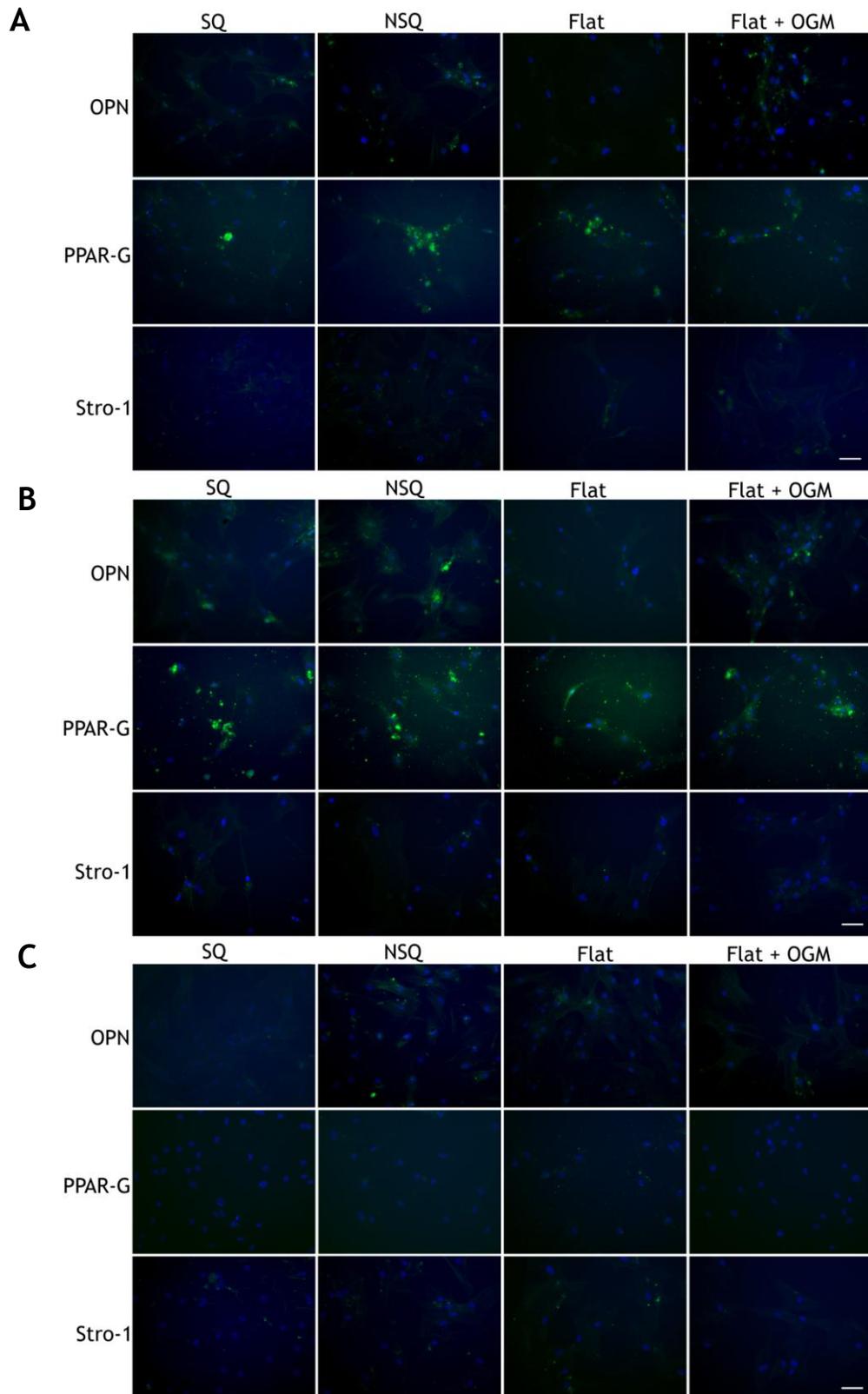


Figure 6-5 MSC phenotype retention is linked to intracellular tension and ERK signalling. Inhibition studies for actin/myosin contraction (A, RhoA kinase (ROCK) inhibition and B, blebbistatin) and extracellular-signal related kinase (ERK) (C). in MSCs cultured on planar control, test topographies (SQ retention surface and NSQ osteogenic surface) and with osteogenic media (OGM) for 14 days. When actin/myosin interaction was blocked, expression of STRO-1 and (OPN) dropped dramatically. However, PPARG expression was noted as the low-tension default (arrows) (A, B). When ERK was inhibited neither STRO-1 expression nor functional differentiation was noted, suggesting that both multipotency and differentiation are biochemically active states (C).

6.4 Discussion

In chapter three it was previously identified that MSCs cultured on PCL continued to express stem cell markers following long term culture in response to the SQ nanotopography. In this chapter MSCs were also identified to maintain expression of stem cell markers following 28 days culture of MSCs on PC. Furthermore, it was again shown that MSCs undergo osteogenic differentiation in response to the NSQ nanotopography. These results further demonstrate that it is the nanotopographical pattern which elicits such specific biological responses. In addition it was further shown that MSCs not only retain stem cell marker expression when cultured on PC but also maintain their multipotency, undergoing osteogenic and adipogenic differentiation in response to appropriate differentiation media. Continued maintenance following passaging of MSCs cultured on the SQ nanotopographically-patterned PS also indicates the ease with which common tissue culture techniques can be applied without loss of the stem cell phenotype. This is crucial if adult stem cells are to not only be maintained but also undergo expansion *in vitro*.

The importance of intra-cellular tension has previously been demonstrated for the initiation of osteogenesis (McBeath, Pirone et al. 2004; Engler, Sen et al. 2006; Kilian, Bugarija et al. 2010), but disruption of tension generated by the interaction of actin and myosin II, has now been demonstrated to result in a loss of STRO-1 expression on the SQ topography. Concomitantly, and as has been previously demonstrated in the literature, osteogenic differentiation (a high tension differentiation lineage) previously observed on the NSQ topography and with OGM becomes lost in favour of adipogenic differentiation (thought to be the default lineage in a low tension state) which indicates inhibition of intracellular tension promotes adipogenic differentiation over osteogenic differentiation (McBeath, Pirone et al. 2004; Engler, Sen et al. 2006; Kilian, Bugarija et al. 2010).

It is particularly noteworthy for this study that the loss of the stem cell marker STRO-1, following disruption of intracellular tension dictates that a degree of tension is a required for maintenance of the MSC phenotype. Additionally, loss of both differentiation and stem cell markers on all substrates, following inhibition

of ERK-mediated signalling indicates a dual role for ERK signalling as discussed in chapter 1, in both the differentiation and maintenance of the MSC phenotype. It is further proposed that ERK regulation of self-renewal may occur via control of proliferation - the percentage of stem cells undergoing proliferation on the SQ topography was observed to be similar to control at both day 7 and 14, as shown in fig. 3-12, suggesting that the cells were not becoming post-mitotic as with e.g. adipocytes and chondrocytes and that ERK negative feedback was not slowing proliferation as with osteogenesis (Stein and Lian 1993; Pumiaglia and Decker 1997; Lee, Hong et al. 2002; Boland, Perkins et al. 2004; Ebisuya, Kondoh et al. 2005).

Importantly, maintenance of the MSC phenotype over time when cultured on PC, as well as the ability to passage MSCs when cultured on PS demonstrates that this technology can be translated across a variety of polymers indicating it is indeed the nanotopography which promotes retention of the stem cell phenotype.

7 Discussion

7.1 General Introduction

The main aims of this thesis were two fold. Firstly, to investigate the response of multipotent MSCs when cultured on an ordered nanopit topography (SQ), and secondly, to further investigate the mechanism via which nanotopography mediates such cellular responses. Topography has long been known to affect a variety of cellular processes such as adhesion, proliferation and differentiation; however, the ability to maintain MSCs in their multipotent state *in vitro* has so far remained elusive (Dalby, Gadegaard et al. 2007; Lim, Dreiss et al. 2007; Nguyen, Shukla et al. 2007; Simon, Burton et al. 2007; Bettinger, Zhang et al. 2008). Therefore the main objective of this thesis was to investigate MSC self-renewal in response to nanotopography. This was undertaken by investigating key characteristics of stem cell phenotype and function.

7.2 Nanotopographical Regulation of Mesenchymal Stem Cell Function

To identify the continued maintenance of the MSCs in response to nanotopography, experiments were conducted to evaluate several key characteristics of MSCs. In general, it is proposed that stem cell characteristics *in vivo* include a slow rate of proliferation, multipotency and a low metabolic rate. As discussed in chapter 1, multipotent MSCs can be selected from the bone marrow using various antibodies, however arguably the most stringent is STRO-1 and was used throughout this study as a marker of the multipotent MSC phenotype. This study has shown that MSCs cultured on the SQ nanotopography retain expression of this key stem cell marker and ALCAM, another less stringent MSC marker, over prolonged periods of culture up to eight weeks. Furthermore it was identified that when MSCs are cultured on the SQ nanotopography their multipotency is retained over that of MSCs cultured on a flat control. Osteogenic and adipogenic induced differentiation using soluble factors was identified to be higher in MSCs reseed onto coverslips, after 28 days culture, from the SQ than those originally cultured on flat control. These results are critical as they

correlate with previous observation in the literature that when MSCs are cultured on standard flat tissue culture plastic, they undergo spontaneous differentiation losing their multipotent potential (Sherley 2002; Siddappa, Licht et al. 2007; Sarugaser, Hanoun et al. 2009).

In contrast, osteogenic differentiation was identified in MSCs cultured on the NSQ nanotopography. The NSQ nanotopography was previously identified to induce osteogenic differentiation in MSCs cultured on nanopatterned polymethylmethacrylate (PMMA) (Dalby, Gadegaard et al. 2007). Osteogenic differentiation was identified in MSCs cultured on the NSQ nanotopography by the increased expression of osteogenic markers OPN and OCN over a time course that was in agreement with previous studies of osteogenic progression (Stein, Stein et al. 1989; Stein and Lian 1993).

Water contact angle experiments were used to investigate the effect the SQ and NSQ nanotopographical patterns may have on the surface energy (Cassie and Baxter 1944). However the results indicated that there was no significant difference between the surface energy produced as a result of the two different nanotopographical patterns. These results together demonstrate that the retention or differentiation of MSCs can be tuned using only changes in the geometry of nanotopographic patterns.

The onset of osteogenic differentiation is defined by a decrease in proliferation concomitant with the up-regulation of key intracellular signalling pathways, metabolism and osteo-specific marker expression (Stein, Lian et al. 1990; Stein and Lian 1993; Reyes, Fermanian et al. 2006). The up-regulation of signalling pathways and an increase in metabolism are thought to direct the changes in cellular function required when stem cells undergo differentiation.

The up-regulation of metabolism observed in stem cells undergoing differentiation is essential to drive the increase in energy requirements associated with cellular processes and signalling (Lonergan, Brenner et al. 2006; Chen, Shih et al. 2008; Armstrong, Tilgner et al. 2010). In this thesis it was demonstrated that metabolic signalling is reduced in stem cells on the SQ, retention promoting nanotopography. This is in contrast to MSCs undergoing osteogenic differentiation on the NSQ and with OGM. Furthermore, as discussed

in chapter 5, it has been proposed in current literature that ESCs maintain a characteristic reduced metabolic profile (Yanes, Clark et al. 2010). In correlation with this study, MSCs cultured with OGM were shown to have a more oxidised metabolic profile when compared to the MSCs cultured on the SQ, stem cell retention nanotopography. Furthermore, the proposal that metabolites act like chemical signatures of cellular processes is interesting in the context of stem cells as it is also proposed that reduced metabolites undergo oxidation as a result of increased cellular processes (Yanes, Clark et al. 2010). The results presented in this thesis therefore indicate that MSCs also maintain a reduced metabolic profile which may allow the cells to respond to oxidative processes such as inflammation and undergo differentiation when required (Khodr and Khalil 2001). Therefore the more reduced metabolic profile observed in MSCs on the SQ nanotopography may indeed indicate these metabolites function as precursors for oxidative cellular processes associated with differentiation leading to increased levels of oxidative metabolites as observed in MSCs cultured with OGM.

Similarly, it was demonstrated that MSCs cultured on the SQ nanotopography modulate intracellular signalling with broad down-regulations observed. Whilst, in contrast MSCs cultured on the NSQ and with OGM were shown to broadly up-regulate intracellular signalling, with a key osteogenic signalling pathway, sonic hedgehog, shown to be up-regulated in both cases (Spinella-Jaegle, Rawadi et al. 2001; James, Leucht et al. 2010).

As mentioned in chapter 1, FAK plays an integral role in mechanotransduction, relaying signals from the extracellular environment into a biochemical signal generating downstream signalling events (Schlaepfer, Hanks et al. 1994; Katsumi, Orr et al. 2004; Leucht, Kim et al. 2007; Hong, Jeon et al. 2010; Wang, Du et al. 2011). Again, MSCs cultured on the SQ nanotopography were shown to down-regulate FAK signalling, whilst MSCs cultured on the NSQ and with OGM showed up-regulated expression. One of the downstream signalling pathways associated with FAK signalling is the ERK signalling pathway, which is critical for cell cycle progression, differentiation and, as demonstrated in this thesis, may play an important role in the retention of MSC multipotency (Schlaepfer, Hanks et al. 1994; Schlaepfer and Hunter 1996; Renshaw, Price et al. 1999; Barberis, Wary et al. 2000; Li, Wang et al. 2007; Meloche and Pouyssegur 2007). The

regulation of these different cellular processes by ERK signalling is thought to occur as a result of distinct expression patterns of ERK signalling (Ebisuya, Kondoh et al. 2005). The regulation of ERK signalling by FAK therefore makes it unsurprising that ERK signalling is increased in MSCs cultured with OGM, whilst it is decreased in MSCs cultured on the SQ.

Small RNAs including microRNAs and snoRNAs are thought to provide an additional level of regulation for gene expression (He and Hannon 2004; Brameier, Herwig et al. 2011). Their role in stem cells is only beginning to be deciphered. However, it is thought that the function of microRNAs and snoRNAs in the degradation and editing of mRNAs is a contributing factor in regulating the balance between self-renewal and differentiation (Ender, Krek et al. 2008; Hartner, Walkley et al. 2009; Sartipy, Olsson et al. 2009). Interestingly, as a role for metabolism becomes increasingly implemented in the self-renewal/differentiation balance, studies are beginning to reveal a role for microRNAs in metabolic regulation (Krutzfeldt and Stoffel 2006). Recent studies have also detected the up-regulation of microRNAs in response to adhesion and mechanical stimuli (Guan, Yang et al. 2011; Valastyan and Weinberg 2011). These microRNAs may therefore provide an additional level of control over changes in intracellular signalling and metabolism during particular stages of the cell cycle, which are sensitive to the differentiation effects of growth factors. However future studies are needed to fully investigate the role of microRNAs in response to nanotopographical control of cell adhesion and mechanotransduction, and to support their role in both the regulation of stem cell maintenance and metabolism.

In this study it was further identified that non-significant up-regulations of Sox2, an embryonic pluripotency factor, were observed in MSCs cultured on the SQ nanotopography. Whilst embryonic pluripotency factors such as Sox2 and Oct-4 are not thought to play a distinct role in the regulation of MSC self-renewal, there is evidence that sub-populations exist, called MUSE cells which have been found to express several embryonic pluripotency markers and may explain the non-significant up-regulation of Sox2 observed in MSCs cultured on the SQ (Kuroda, Kitada et al. 2010).

In addition, the function of snoRNAs in stem cell remains largely unknown. Here, snoRNAs have been demonstrated to play a role in the maintenance of stem cell self-renewal, whilst a recent study also demonstrated the role of snoRNA in increased metabolic activity, an indicator of differentiation (Hartner, Walkley et al. 2009; Michel, Holley et al. 2011). Again further investigations are required to fully elucidate their role in MSC self-renewal.

7.3 Nanotopographical Control of Intracellular Tension

Intracellular tension has previously been shown to be a key factor in determining lineage commitment during differentiation (McBeath, Pirone et al. 2004; Engler, Sen et al. 2006; Kilian, Bugarija et al. 2010) and here it is shown to be a key factor in the retention of multipotency. As discussed in chapter 1, it is proposed that the degree of nanopatterned disorder facilitates the development of intracellular tension via integrin-mediated adhesion. Huang et al have previously shown that the degree of order and disorder of RGD patterning can affect integrin-mediated binding and the formation of focal adhesions (Huang, Grater et al. 2009). Here, however, it is proposed that nanotopography may induce similar effects, leading to changes in intracellular tension and downstream signalling.

Initial focal adhesions are dynamic structures which undergo strengthening, stabilisation and enlargement as the result of increased tension (Schwartz and DeSimone 2008). As discussed in a review by Hoffman et al, a feedback loop is established whereby increased tension applied to the focal adhesions results in the generation of more mature focal adhesions called fibrillar adhesions via protein unfolding (Hoffman, Grashoff et al. 2011). It has been shown that several focal adhesion proteins such as fibronectin contain binding sites which under tension-mediated mechanical stretching become exposed creating binding sites for additional proteins associated with further downstream events (Zhong, Chrzanowska-Wodnicka et al. 1998). In this study it is proposed that changes in integrin-mediated adhesion mediated by the disruption of integrin clustering and the ability to form more mature focal adhesions, caused by the difference in nanopit geometry between the SQ and NSQ nanotopography, result in differences in intracellular tension in MSCs cultured on both these

nanotopographies. In particular it is proposed that it is this difference in intracellular tension which mediates ERK signalling to produce the different cellular responses observed on the SQ and NSQ nanotopographies.

As discussed previously, ERK signalling has been shown to induce different cellular responses as a result of both the duration and magnitude of signalling (Ebisuya, Kondoh et al. 2005). Evidence produced in this thesis has implicated a role for ERK signalling in both MSC differentiation and self-renewal. However, due to the duration and magnitude-dependent cellular response to ERK signalling, it is proposed that the formation of more mature focal adhesions, associated with MSCs cultured on the NSQ nanotopography, and subsequent higher intracellular tension may induce increased levels of ERK primarily associated with cell cycle arrest and differentiation (Lee, Hong et al. 2002; Hong, Jeon et al. 2010). In contrast, small focal adhesions and reduced intracellular tension induce only moderate levels of ERK signalling which are enough to promote cell proliferation but not differentiation (fig. 7-1) (Li, Wang et al. 2007; Meloche and Pouyssegur 2007; Wang, Gao et al. 2009).

In chapter 4, IPA data support this hypothesis with ERK signalling identified to be decreased in MSCs cultured on the SQ nanotopography, whilst MSCs cultured with OGM, and therefore undergoing osteogenic differentiation, were shown to have elevated levels of ERK signalling. However, future studies would need to be conducted to fully elucidate the mechanisms of tension mediated ERK signalling and the role that different levels of ERK signalling play in the regulation of stem cell self-renewal or differentiation.

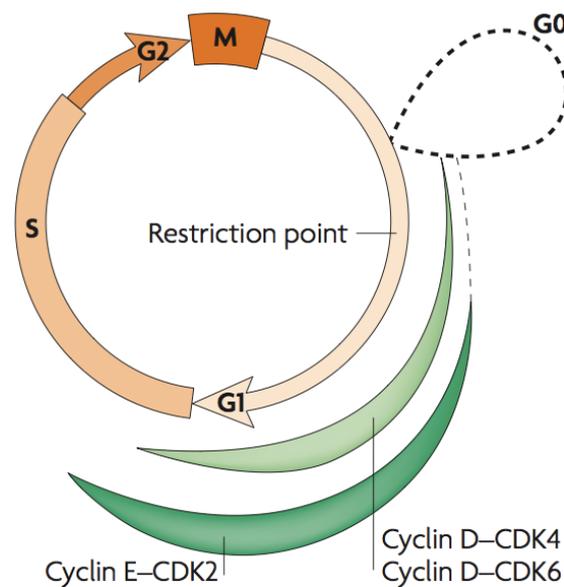


Figure 7-1 The cell cycle. The G₁ phase of the cell cycle is thought to be a critical point in maintaining the balance between self-renewal and differentiation. In particular, early G₁ phase is dependent on growth factor signalling such as ERK1/2, for cell cycle progression. The restriction point however, indicates the point where progression of the cell cycle becomes mitogen-independent. Image adapted from (Coller 2007).

Further evidence that the organisation and structure of cell cytoskeleton plays an integral role in directing stem cell differentiation was generated via a study which identified that key features of the early cytoskeletal organisation can be used to predict the ultimate fate of a stem cell (Treiser, Yang et al. 2010). This study indicates that cytoskeletal organisation and the generation of a cytoskeletal 'blue print' could be used to predict differentiation. In addition, the identification of specific cytoskeletal blueprints which are indicative of a particular stem cell fate may prove useful in further studies on cytoskeletal organisation and intracellular tension, as well as the regulation of differentiation or self-renewal.

7.4 Nanotopographical Biomaterials

Nanotopographical patterned substrates present a superior strategy over chemistry and substrate stiffness for the prolonged culture and expansion of MSCs. Nanotopography holds several advantages over other material strategies:

- Features are on the nanoscale and therefore affect cells at the protein level e.g. integrin binding.

- Nanotopography is permanent unlike other strategies such as chemical patterning which can be washed off or produce only a transient affect.
- Nanotopography promotes cellular responses in the absence of soluble chemistries. This is particularly important, as chemical supplements such as those often used for differentiating stem cells are not FDA approved and therefore cannot be used to treat cells which are to be reintroduced back into a patient. Nanotopography therefore eliminates the need for chemical supplements.
- The surfaces are relatively easy to make. Once a master substrate is produced, replicas can be produced with high-throughput using techniques such as injection moulding. Injection moulding is also a technique readily used in the fabrication of standard tissue culture plastics. Other strategies such as surface chemistry and matrices require complicated procedures to make their respective substrates. This makes them less amenable to high-throughput fabrication, slowing production time and may require specialist knowledge.
- The surfaces are relatively inexpensive to produce. Whilst the equipment required to produce injection-moulded surfaces is expensive, the high-throughput of this technology and relatively cheap cost of the polymer substrate make it amenable to providing large quantities of surfaces for distribution at relatively low cost. Moreover, they do not require specialized packaging or storage.
- Standard tissue culture techniques can be applied to the substrates. The similarity between current tissue plastic plates and nanotopographically-patterned substrate again makes them amenable to standard tissue culture techniques. This has the advantage that no additional skills are required to be learnt and the inexpensive cost makes them disposable after use.

Overall, the ability to incorporate such nanotopographical patterns with high fidelity into a range of polymers demonstrates the transferability of the technology whilst the facile nature of the materials in terms of cost and use

highlights huge potential for the generation of novel nanotopographically-patterned tissue culture plasticware.

7.5 Conclusion

Regenerative medicine is a field at the forefront of modern medicine with degenerative diseases such as osteoarthritis, Alzheimer's and Parkinson's disease, coupled with an aging population placing mounting pressure on current methods used to treat these diseases and associated illnesses. Stem cells, however, have the potential to provide an alternative solution to current treatments. By harnessing the unique properties of stem cells, both their self-renewal and differentiation capabilities, it is possible to envisage a strategy whereby a patient's own stem cells can be extracted and cultured *in vitro* to expand the stem cells *as a high-quality stem cell population*. These stem cells could later be reintroduced into the patient at the site of damage or disease, or alternatively differentiated *in vitro* prior to re-implantation.

At present, however, the limiting step of this process is caused by an inability to retain the fundamental properties of stem cells over time during *in vitro* culturing methods, caused by both the phenotypically unstable nature of stem cells and the biologically inert properties of current cell culture materials. In this thesis, therefore, it was proposed that an ordered (SQ) nanopit-patterned polymer surface might provide stem cells with the appropriate cues to promote retention of stem cell population *in vitro*.

This investigation was carried out in two ways, firstly by examining the phenotypic changes in MSCs cultured on the SQ nanotopography over a prolonged period of culture, relative to MSCs cultured on a flat and osteogenic controls, with an aim of identifying any changes in the MSC phenotype typically observed in MSCs cultured on a flat substrate and the increased expression of key proteins associated with osteogenic differentiation. Secondly, an assessment of changes in intracellular signalling and metabolic profile was undertaken to provide a more in-depth examination of the underlying gene changes associated with such changes in gene expression.

The findings presented within this thesis indicate that by reducing the overall degree of nanopattern disorder (NSQ) to as close to zero as possible (SQ) a substrate that was previously osteo-conductive can support continued maintenance of the MSC phenotype and multipotency. This is not surprising, as disorder has been shown to increase adhesion size leading to an increase in intracellular tensions, whereas ordered nanotopography has been shown to decrease adhesion size ultimately reducing the level of intracellular tension. Therefore the SQ ordered nanotopography promotes a lower intracellular tension, however not relaxed, supporting the self-renewing phenotype. Interestingly, it has been shown that collagen X, found during endochondral ossification exhibits a disordered arrangement similar in scale to the NSQ nanotopography, thus indicating a probable biomimetic cue promoting osteogenesis.

Intracellular tension has been demonstrated as a key regulator for not only differentiation but that an intermediate tension may also act as a regulator of MSC self-renewal. It is proposed that tension-dependent regulation of ERK signalling may promote self-renewal via proliferation. In addition, the identification of increased levels of small RNAs in MSCs cultured on the SQ nanotopography implicates a potential role for these small RNAs in providing an additional level of regulation, preventing changes in gene expression and cellular metabolism associated with differentiation, thereby allowing proliferation to occur without induction of differentiation. It is tempting to speculate that these small RNAs may help to down-regulate gene expression changes associated with the differentiation-inducing effects of mitogen-activated kinases particularly during progression of the cell cycle from G₁ early to late phase, a cell cycle phase which is thought to be particularly sensitive to the induction of differentiation by mitogen-activated protein kinases.

Lastly, the facile nature of the materials and scalability of the technology make the inclusion of nanotopography into polymers for tissue culture purposes an extremely potent tool for the long-term culture and maintenance of MSCs, providing a valuable resource of MSCs for both regenerative and research applications.

7.6 Future Work

The data generated throughout this thesis provides scope for various areas of investigation for future work. In particular, the application of the SQ nanotopographical surface as a tool for MSC maintenance provides not only a valuable platform for the culture of large quantities of high-quality stem cells for use in regenerative therapies, but it also provides a platform for further investigation into the mechanisms which control stem cell self-renewal. As identified throughout this thesis the mechanisms which maintain the balance in favour of stem cell self-renewal over differentiation are still not fully understood. Therefore the ability to maintain stem cells *in vitro* for research purposes is critical. Several aspects of MSC self-renewal could be investigated using the SQ nanotopography as a platform for stem cell maintenance.

7.6.1 Intracellular Tension as a Mediator of Self-renewal

The importance of intracellular tension for both stem cell retention and differentiation has been highlighted in this thesis and in the current literature. However, the exact mechanism for this action is not fully understood. In particular the degree of intracellular tension and its effects on ERK-related signalling. It is thought that an increase in intracellular tension mediates the degree of ERK signalling, resulting in different biological outcomes. The closely coupled relationship between ERK signalling, proliferation and stem cell maintenance are particularly interesting. Therefore it would be very interesting to carry out a more in-depth study on the effect intracellular tension and ERK signalling generated by nanotopography has on stem cell retention. The mechanism by which nanotopography elicits cellular responses via the manipulation of integrin binding and subsequent intracellular tension make it a particularly potent tool for researching mechanotransductive effects on stem cell self-renewal and differentiation.

7.6.2 Cell Cycle Regulation of Self-renewal

For stem cell self-renewal to occur, stem cells must also undergo proliferation. ESC and iPSCs have been identified to have shorter G₁-S phase, whilst

hematopoietic stem cells maintain their multipotent state remaining dormant in G₀ phase until required (Becker, Ghule et al. 2006; Filipczyk, Laslett et al. 2007; Wilson, Laurenti et al. 2008; Ghule, Medina et al. 2011). The role of the cell cycle in MSCs self-renewal however has not been fully investigated. Therefore, a study into the relationship between proliferation and self-renewal of MSCs cultured on the SQ nanotopography relative to MSCs undergoing differentiation could be particularly important.

Additionally, a nucleolar protein, nucleostemin has been implemented in the proliferation and differentiation of MSCs (Tsai and McKay 2002; Kafienah, Mistry et al. 2006; Ma and Pederson 2007). A loss of nucleostemin has been shown to result in cell cycle arrest at the G₁ cell cycle phase, as previously discussed, a critical point in the balance between self-renewal and differentiation, whilst the onset of differentiation has also been shown to coincide with a loss of nucleostemin expression (Tsai and McKay 2002; Ma and Pederson 2007; Kudron and Reinke 2008). Therefore, it could be worthwhile investigating the expression of nucleostemin in MSCs cultured on the SQ nanotopography and its potential role in regulating MSC self-renewal via control of proliferation. Knockdown and recovery studies could also be carried out to investigate a role for nucleostemin in self-renewal or induced pluripotency of differentiated MSCs.

7.6.3 Small RNAs

The regulation of gene expression by small RNAs creates increasingly complex regulatory mechanisms governing many cellular processes including self-renewal and differentiation (He and Hannon 2004; Ender, Krek et al. 2008; Sartipy, Olsson et al. 2009). Evidence produced as a result of this study has identified increased expression of microRNAs, which have been shown to play a role in maintaining stem cell self-renewal. Most interestingly, mir-302 was previously thought to only be expressed in embryonic stem cells. Furthermore this microRNA was not only shown to regulate embryonic stem cell pluripotency via cell cycle regulation, but was also found to promote the induced pluripotency of somatic cells (Houbaviy, Murray et al. 2003; Card, Hebbar et al. 2008; Sartipy, Olsson et al. 2009; Lin, Chang et al. 2011). The expression of this microRNA in MSCs cultured on the SQ nanotopography makes it tempting to speculate that it

may play a pivotal role in regulating MSC self-renewal, and further implicates the cell cycle as an important regulator of MSC self-renewal. In order to gain supportive evidence for this hypothesis a more in-depth investigation into the role mir-302 and other microRNAs is required to fully understand the role these small RNAs may play in regulating self-renewal and differentiation. This could be achieved using novel chemically engineering oligonucleotides called antogomirs which inhibit endogenous miRNAs, and would be particularly applicable to probe the function of miRNAs in MSC self-renewal.

SnoRNAs, which have been implicated in maintaining self-renewal of stem cells and are thought to alter gene expression via RNA editing and other microRNA-like functions (Ender, Krek et al. 2008; Hartner, Walkley et al. 2009; Brameier, Herwig et al. 2011). Various snoRNAs were also found to be up-regulated in MSCs on the SQ, however very little is known about their role in stem cells, therefore an investigation into the function of snoRNAs in self-renewal and differentiation also requires further investigation.

7.6.4 The Role of Metabolites in Self-Renewal

The metabolic profile of stem cells can act as an indicator of the stem cell state (Yanes, Clark et al. 2010). Evidence points towards increased metabolic signalling in stem cells undergoing differentiation, with undifferentiated stem cells maintaining a low metabolically active state. As a result of evidence generated in this thesis two areas of further research are required:

1. An investigation in the regulation of metabolism by microRNAs.

As discussed in section 7.6.3., microRNAs have been identified to regulate gene expression via post-transcriptional degradation. Key studies have identified microRNAs as playing a role in the regulation of metabolism. This is interesting as MSCs cultured on the SQ nanotopography were shown to have reduced metabolic activity and further research would be required to identify a definitive link between MSCs self-renewal on the SQ nanotopography and the up-regulation of many microRNAs also identified.

2. An in-depth investigation into the profile of metabolites found to be present in MSCs cultured on the SQ nanotopography.

As identified in a study by Yanes et al, the metabolic profile of ESCs was found to be made up of mainly reduced metabolites (Yanes, Clark et al. 2010). Whilst in this thesis the metabolic profile of MSCs was also identified to contain many reduced metabolites, the role these metabolites play in the continued maintenance of MSCs is still not fully understood. For instance, the use of inhibitors for oxidative pathways, the importance of these pathways in the regulation of differentiation can be evaluated when MSCs are cultured in differentiation media.

7.6.5 Other Stem Cell Types

A broad range of research has shown that MSCs and other types of stem cells are influenced by substrate nanotopography, promoting differentiation and as demonstrated in this thesis MSC retention (Fan, Cui et al. 2002; Dalby, McCloy et al. 2006; Dalby, McCloy et al. 2006; Dalby, Gadegaard et al. 2007; Yim, Pang et al. 2007; Christopherson, Song et al. 2009; Oh, Brammer et al. 2009).

Importantly, the ability to promote self-renewal of MSCs using nanotopography increases the need for further research into other types of stem cells such as ESCs, neural stem cells (NSCs) and endothelial stem cells (EDSCs) using this technology. The ability to develop defined strategies for the differentiation and retention of different types of stem cells, as well as a deeper understanding of the regulation of these processes is critical if the generation of more complex tissue is to be undertaken in the future.

Recent literature has indicated that MEK/ERK signalling may regulate the pluripotency of ESCs in a mechanism similar to MSCs (Nur, Ahmed et al. 2005; Li, Wang et al. 2007). In addition, research has shown that ESCs are responsive to nanotopography, again suggesting a potential role for tension-mediated maintenance of ESCs (Nur, Ahmed et al. 2005; Nur, Ahmed et al. 2006; Gerecht, Bettinger et al. 2007). It would therefore be interesting to investigate the potential for nanotopographically-patterned substrates in the maintenance of ESCs, and the role that mechanical tension plays in this process. This would be particularly interesting as human ESCs currently require the use of mouse embryonic fibroblast feeder layers (Martin 1981). As research strives to develop feeder-free systems for human ESC clonal expansion, nanotopography may

therefore have the potential to deliver a potent solution by providing ESCs with the necessary cues to promote their self-renewal in the absence of chemical supplements.

NSCs have the capacity to differentiate into neurons, astrocytes and oligodendrocytes and their discovery has driven research for the treatment of neurological diseases and the treatment of nerve damage as a result of injury (Reynolds and Weiss 1992; Gage 2000; Taupin and Gage 2002; Sun, Pollard et al. 2008). Neural stem cells are normally cultured as neurospheres either in suspension or as an adherent cell population (Sun, Wang et al. 2011). The self-renewal of NSCs is interesting, as it has been noted in several studies that there is a requirement for the cell adhesion molecule β_1 integrin in maintaining proliferation of these neurospheres and maintenance of self-renewal (Campos, Leone et al. 2004; Campos 2005; Leone, Relvas et al. 2005). These results are particularly interesting as β_1 integrin has been shown to regulate expression of ERK1/2 and similar to MSCs, expression of ERK1/2 has been shown to regulate proliferation (Campos, Leone et al. 2004; Campos 2005; Bettinger, Zhang et al. 2008; Lee, Jang et al. 2009; Wang, Gao et al. 2009).

Endothelial progenitor cells circulate in the blood and are multipotent cells that differentiate into cells of the blood vessels (Asahara, Murohara et al. 1997; Harraz, Jiao et al. 2001). The continuous changes in mechanical tension associated with blood vessel functioning indicate that these stem cells may be similarly mechano-responsive (Haga, Li et al. 2007; Hahn and Schwartz 2009). Indeed research has identified that changes in cytoskeletal organisation and tension act as factors that regulate the proliferation of endothelial cells (Davis and Camarillo 1995; Huang, Chen et al. 1998; Bettinger, Zhang et al. 2008). Therefore, it would be interesting to investigate the role of mechanical tension in the differentiation of endothelial progenitor cells.

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