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INFLUENCE OF TENSILE STRAIN ON RAT MESENCHYMAL STEM CELL PROLIFERATION, DIFFERENTIATION AND APOPTOSIS

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A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

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I DECLARATION

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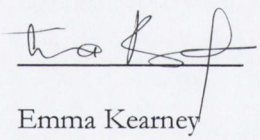

Emma Kearney

II ABSTRACT

Tissue engineering strategies that involve mesenchymal stem cells (MSCs) represent promising developments within regenerative medicine today. However, before MSCs are suitable for use in the clinic, the intricacies of how their remarkable multipotential and proliferative characteristics are controlled must be understood. One of the most potent regulators of MSC fate is biomechanical stimululation. Herein, this research examines the response of MSCs to cyclic mechanical tensile strain, and identifies the intracellular controls involved in these.
The research began by characterising both the substrate material and the cell population. Following topographical and mechanical characterisation, collagen-coated silicone that was exposed to UV was identified as the optimal surface. Adherent cells isolated from rat bone marrow stroma were characterised as a homogeneous population of cells that express the mesenchymal stem cell markers CD90 and CD105, and demonstrated an osteogenic response following incubation with osteogenic factors.
To mechanically stimulate cells, MSCs were seeded onto a collagen-coated silicone strip. Cyclic tensile mechanical strain was applied continuously at a constant frequency of 0.17Hz and varying strain magnitudes from 2.5% - 10%, for 1 – 14 days, using a custom-made bioreactor. Cell differentiation, proliferation and apoptotic responses were investigated.
Upon investigating MSC differentiation mechanical strain was found to induce an osteogenic response in MSCs in the absence of any other stimulatory mechanisms (such as osteogenic growth factors). Following cyclic tensile strain of 2.5% (at 0.17Hz) the osteogenic markers Cbfα1, collagen type I and osteocalcin were temporally expressed. Stretch-activated ion channels were identified as having a role in the detection and transduction of differentiation signals, and both the p38 mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase were involved in the strain-induced autocrine synthesis of bone morphogenic protein 2 (BMP-2).
Cyclic mechanical strain reduced the rate of MSC proliferation, thus further confirming MSC differentiation induced by mechanical stimulation. In response to 7.5% and 10% strain, cyclic tension induced mesenchymal stem cell apoptosis. L-type voltage-activated calcium channels coupled mechanical stress to activation of calpain and c-Jun NH2-terminal kinase (JNK) which lead to cell apoptosis, via caspase-3 and calpain dependent mechanisms.
The identification of the mechanical control of mesenchymal stem cell proliferation and the molecular link between mechanical stimulation and osteogenic differentiation, as well as cell death has consequences for molecular approaches based on mesenchymal stem cells, bioreactor technology, scaffold design, and computational methods in mechanobiology. Given the fundamental nature of these cell fate decisions, this research provides an insight to the mechanobiology-MSC relationship that affords its exploitation in tissue engineering strategies for the future and can contribute to regenerative medicine through a functional tissue engineering approach.

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie (1867 – 1934)

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VII LIST OF ABBREVIATIONS

2D	2 dimensional
3D	3 dimensional
^{3}H[TdR]	tritiated thymidine
α2δ1	alpha (2) delta 1
AA	ascorbic acid-2-phosphate
Ab	antibody
AFC	Ac-DEVD-7-amino-4-(trifluoromethyl) coumarin
AFM	atomic force microscopy
AFU	arbitrary fluorescent units
AIF	apoptosis-inducing factor
ALP	alkaline phosphatase
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
AP-1	activator protein-1
BCA	bicinchoninic acid
BGP	β-glycerophosphate
BK channels	Large-conductance Ca2+-activated K+ channels
BMP	bone morphogenic protein
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$[Ca^{2+}]$	calcium concentration
$[Ca2^{+}]_i$	intracellular calcium concentration
$CaCl_2$	calcium chloride
Cbfα1	core binding factor alpha 1
CC	collagen coated
Cm	centimetres
CO_2	carbon dioxide
DAPI	4',6-diamidino-2-phenylinodole
DAB	diaminobenzide
DC	dual current
Dex	dexamethasone
D-JNK1	D-JNK inhibitor 1
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothretiol
EDTA	ethylenediamine-tetraacetic acid
EGF receptor	epidermal growth factor
ERK	extracellular signal regulated kinase
EtBr	ethidium bromide
EtOH	ethanol
FAK	focal adhesion kinase
FCS	fetal calf serum

FGF	fibroblast growth factor
EC	endothelial cell
FITC	fluorescein isothiocyanate
FS	forward scatter
GAG	glycosaminoglycan
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
Gd^{3+}	gadolinium ion
$GdCl_3$	gadolinium chloride
GDF	growth and differentiation factor
GTPases	guanosine-tri-phosphatases
H_2O	water
H_2O_2	hydrogen peroxide
hr	hour
HRP	horseradish peroxidase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid]
HSC	hematopoietic stem cell
Hz	hertz
IB	islet-brain
ICE	Interlukin-1-converting enzyme
Id	inhibitor of DNA binding/differentiation helix–loop–helix proteins
Ig	immunoglobulin G
IL	interleukin
JIP-1	JNK-interacting protein
JBD	JNK-binding domain
JNK	c-Jun N-Terminal kinase
K^+	potassium ion
kDa	kilo Dalton
LIF	Leukemia inhibitory factor
LRP-5	lipoprotein receptor-related peptide 5
mA	milliamp
MACI	matrix-induced autologous chondrocyte implantation
MAP	Mitogen activated protein
MAPK	MAP kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MAU	Mean Absorbance Units
mg	milligram
MHC II	major histocompatibility complex II
$MgCl_2$	magnesium chloride
min	minute
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger RNA
MS	mechanosensitive
N	Newton
Na^+	sodium ion
ng	nanogram
nm	nanomolar
NMDA	N-methyl-D-aspartate

NO	nitrogen oxide
OF	osteogenic factors
p	phosphorylated
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCD	programmed cell death
PDGF	platelet-derived growth factor
PDMS	polydimethylsiloxane
PE	phycoerythrin
PI3kinase	Phosphatidylinosito1 3-kinase
PKA	proteinase K
PMSF	phenylmethylsulpholyl fluoride
PPAR γ	peroxisome proliferator-activated receptor γ
RGD	Arginine-Glycine-Aspartic acid
RNA	ribosomal nucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	real-time polymerase chain reaction
s	seconds
SACC	stretch activated ion channels
SAPK	stress-activated protein kinase
SDF-1	stromal cell derived factor-1
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SEM	standard error of the means
SMC	smooth muscle cells
SS	side scatter
TBS	tris-buffered saline
TBS-T	TBS-tween
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,Nn-N-tetramethylethylenediamine
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TNFR	TNF receptor
Tris-HCl	Trizma-Hydrochloride
TRP	transient receptor potential channel
TUNEL	TdT-mediated-UTP-end nick labelling
UV	ultraviolet
v/v	volume/volume
VACC	voltage activated Ca^{2+} channel
VEGF	vascular endothelial growth factor
VWA	von Willebrand factor A
w/v	weight/volume
Wnts	mammalian homologues of Drosophila wingless
μCi	microCurie
μl	microlitre
μm	micrometre
μM	micromolar
Ø	diameter

1 General Introduction

1.1 Tissue engineering

Tissue engineering is an aspect of bioengineering that has developed in recent years, partly catalysed by significant technological advancements, such as computational methods and molecular techniques. Rather than providing engineering solutions that use mechanically durable, bioinert materials to replace diseased or damaged tissues or defects in the body, tissue engineering approaches the restoration of function from a regenerative perspective, consequently providing treatments where primary pharmacological effects come from both living cellular components and incorporated bioactive elements. A definition by Langer and Vacanti states:

> *"tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ."*
>
> *(Langer and Vacanti 1993)*

The essence of the tissue engineering paradigm is to extra-corporeally generate a construct that upon maturation can be implanted into the diseased or damaged host site, where it will integrate with the local environment, and form a support, then a substitute, whereby the tissue or organ regenerates to function as before the pathology presented. Many proposals have based the treatment principles on what has been learned from developmental biology i.e. applying or recreating events during early organ development, to pathologies in the diseased adult. The most recent tissue engineering approaches have proposed for treatments to include stimulatory mechanisms for the activation of native regeneration capabilities demonstrated in early foetal development. Stem cells will contribute considerably to such regenerative approaches. However, there is a consensus in the scientific community that to achieve regeneration through such an approach, most progress will be made upon gaining an understanding of intrinsic resident stem cell behaviour and the environmental cues, both physical and chemical, needed to activate these cells (Barrilleaux et al. 2006; Chamberlain et al. 2007; Giordano et al. 2007; Gurtner et al. 2007; McGonagle et al. 2007; Satija et al. 2007; Watt and Hogan 2000).

Tissue engineering is therefore a novel, clinically relevant area that aims to provide a complimentary or alternative approach to traditional medicine. Most major

organ systems can benefit from tissue engineering solutions including cardiovascular, e.g. for the treatment of myocardial infarction (L'Heureux et al. 2007; Xiang et al. 2006), musculoskeletal injury such as osteoarthritis (Adachi et al. 2006; Martin et al. 2007), urological conditions such as sphincter incontinence (Becker and Jakse 2007; Feki et al. 2007), in the treatment of neurological conditions such as spinal cord injury (Chalfoun et al. 2006; Sykova et al. 2006), skin for damage due to burns for example (Metcalfe and Ferguson 2007) and many others, emphasising the scope for tissue engineering solutions in healthcare today. Although regenerative solutions for many of the systems described are in the early stages, treatments for injury to the skeletal system have received considerable interest, partly due to the accessibility of osteoprogenitor cells, and tissue engineering for skeletal injury has made promising developments in recent years.

1.1.1 Skeletal tissue engineering

Pathologies of the skeletal system are of high incidence, stemming principally from sports related injury (Giza and Micheli 2005), impact injuries through accidents (Kwong et al. 2006), congenital conditions (Bolder et al. 2001), disease such as Paget's disease (Hosking 2006), and a growing number from age related degenerative conditions such as osteoporosis (Johnell and Kanis 2006). The tissue types that are affected are bone, cartilage and meniscal tissue, in particular at the articulating interfaces, and the supporting connective tissues including ligaments and tendon. Reflective of the progressively aging population trend occurring in the developed world, disease and injury of the skeletal system are increasing. For a number of years now, bone grafts are second only to blood transfusions on the list of transplanted material. Every year, up to four million bone-replacement procedures are performed worldwide, which require the use of a bone graft or bone graft substitute. Therefore, it is a major medical area that justifies the allocation of resources.

Damage to certain skeletal tissue, particularly cartilage, is irreparable by the healing mechanisms within the body. While bone tissue has a strong regenerative potential (Marsh and Li 1999; Martin 2007), a critical size of defect exists where endogenous regenerative mechanisms do not suffice, and healthy remaining bone will be mechanically overloaded with a high risk for additional trauma due to fractures, for example, those associated with the failure of hip implants, segmental bone defects and tumour surgery. Current intervention is essentially limited to

prosthetic replacement (Zimmerma et al. 2002) or transplantation of homologous tissue (Somers et al. 2002). While prostheses have been largely successful for large progressed joint defects (e.g. hip joint replacement), they do not integrate with the surrounding tissue, are time-limited by the device durability and are less likely as an option for younger patients and small articulating surface defects where the prescription is often compromised healing and treatment through physical therapies. Often, original tissue form, and therefore function, is often never restored. One option is autologous osteochondral repair or mosaicplasty (van der Kooy and Weiss 2000), but donor tissue availability and donor site morbidity limits this technique. For bone trauma, human cortical bone has been used as the gold standard to fill segmental defects more than 5cm for over 50 years (Megas 2005). However, they have limited bone forming and remodelling capabilities and as with cartilage tissue, the volume of bone that can be safely harvested from the donor site is limited and can result in donor site pain and morbidity (Goulet et al. 1997). Transplantation of allograft skeletal tissue carries the associated immune reactions and risk of virus and prion transfer representing major challenges for the orthopaedic surgeon. Modern allografting, using material stored within regulated bone banks, overcomes these complications; however, structure and healing can be unpredictable (Togawa et al. 2004); there are concerns regarding disease transfer (Barriga et al. 2004; McCann et al. 2004) and demand outstrips supply. In light of these difficulties, a new functional tissue alternative free from the limitations of supply, inconsistency, and disease that encourages bone formation and increases bone density is a major clinical need. To address these challenges, tissue engineering has been explored with the hope of redefining the use of medical device implants and tissue grafts to the use of biodegradable scaffolds combined with cells or bio-molecules to repair and/or regenerate structurally compatible extracellular matrix (ECM) of the skeletal system.

A requirement common to all tissue engineering approaches is the organised delivery of cells to the area of interest, and the activation of these cells. To achieve this, tissue engineering solutions require a suitable cell source, combined with an extracellular matrix upon which the cells can be supported and appropriate cues to direct the cellular behaviour (Figure 1.1). For bone tissue engineering, these can be more specifically defined as cells with an osteogenic potential, easily obtained and handled *ex vivo*, a 3-dimensional (3D) substrate that will be osteoinductive and will provide mechanical stability and facilitate vascularisation, and an osteoinductive signal that will promote and maintain the osteogenic phenotype so

that the implant can functionally contribute in the injured area. These 3D environments, referred to as 'scaffolds', are designed specific to the requirements of the target environment, and are often modelled upon that natural extracellular matrix; however a number of general design criteria apply. Scaffolds should facilitate the localisation and delivery of cells to specific sites in the body, define and maintain a three-dimensional space for the formation of a new tissue with appropriate structure and guide the development of new tissues with their appropriate functions to facilitate the attachment, survival, migration, proliferation and differentiation of stem cells and progenitors (Bouhadir and Mooney 1998; Freed et al. 1994). The most critical scaffold properties are biocompatibility, vascularisation and chemotaxis.

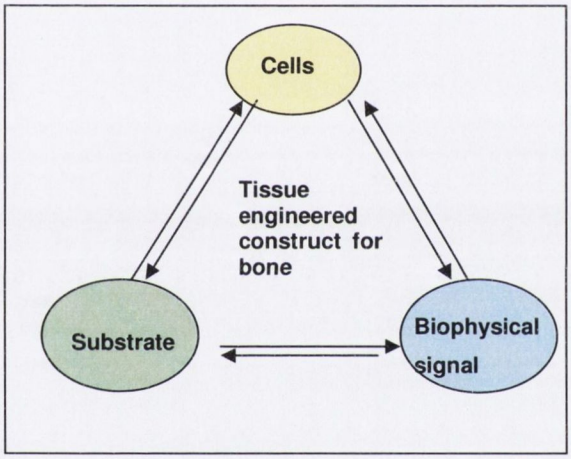


Figure 1.1 The tissue engineering paradigm

A synergistic interaction between the cells, substrate and extracellular influences is required to achieve the organised delivery of cells for tissue engineering approaches.

1.2 Cell substrate

The extracellular environment has a profound effect on cell behaviour. For example, in cell death studies, it has been revealed that in the absence of signals from the extracellular environment, a cell will undergo programmed cell death (apoptosis) (Raff 1992). In tissue engineering, the extracellular environment can act as a platform for systematic control of cell behaviour through accurately defined ECM compositions (Harley et al. 2007). All ECM is comprised physically of the collagen family, elastic fibres, glycosaminoglycans (GAG) and proteoglycans, and adhesive glycoproteins; and chemically of soluble growth and differentiation factors. It is through different combinations, immobilization, and

spatial organization of these substances that different types of scaffolds are formed that characterise the different body tissues and organs. Physically, the ECM influences cell behaviour through its mechanical properties, composition, and pore structure. In terms of the influence on stem cell differentiation, Even-Ram et al. (2006) have discovered a substrate-stiffness sensitivity in the bifurcation of lineage commitment: soft matrices that mimic brain are neurogenic, stiffer matrices that mimic muscle are myogenic, and comparatively rigid matrices that mimic collagenous bone prove osteogenic (Even-Ram et al. 2006).

The skeleton plays an important role as the load-bearing tissue of the body, providing the mechanical structural support; hence one of the major design challenges for skeletal tissue engineering scaffolds is site-specific matching of the structural mechanical properties. As well as being biocompatible with the host tissue, it is necessary for the implant to withstand physiologic loading until sufficient tissue regeneration occurs. The implant architecture should be porous to initially allow cell seeding *ex vivo*, then *in vivo* for effective transport of nutrients and waste products and provide a volume void whereby vascularisation, new tissue formation, and remodelling can occur. Because mechanical signals are important mediators of the differentiation of connective tissue progenitors, the cell scaffold must create an appropriate stress environment throughout the site where new tissue is desired.

Recent advances in scaffold materials have made a transition from nonporous biologically inert materials to more porous, osteoconductive biomaterials and cell-matrix composites. Some of the material properties that are considered are physicochemical properties such as surface area porosity, local acidification, material chemistry, dimensional architecture, mechanical integrity, degradation characteristics, natural v synthetic, potential for drug delivery; and biological factors such as the ability to support cellular attachment, proliferation, differentiation, matrix deposition, angiogenesis, and to prevent dedifferentiation.

Bone is made up of a cellular component and an extra-cellular matrix. The cells of bone are osteoblasts that are responsible for bone formation, osteoclasts that resorb bone as part of the controlled remodelling process, and osteocytes which are aged osteoblasts that have become trapped in the stiff extracellular matrix that they produce. The extracellular matrix has an organic component and inorganic component. Collagen forms the major organic component, providing elasticity, and hydroxyapatite, an analogue of calcium phosphate, forms the major inorganic

component. In addition, bone cells produce growth factors such as bone morphogenic proteins (BMPs) and transforming growth factor beta (TGF-β). Tissue engineering strategies for bone that aim to closely match the structural ECM of the native material will form the most appropriate scaffold materials. Matching the growth factor environment would considerably add to their performance.

Successful materials that act as carriers for cell delivery in orthopaedic applications can be both naturally occurring materials (Malafaya et al. 2007) such as collagen (Farrell et al. 2006) and chitosan (PP et al. 2005), or biodegradable synthetic polymers such as polyglycolic acid (Freed et al. 1994). In recent years however, the role of the scaffold in bone tissue engineering has widened to serving as a delivery device for extrinsic cues, including biochemical and mechanical signals that direct the fate of the cells seeded within. For example, scaffold materials can be designed to be multifunctional for example simultaneously provide osteoconductive surfaces to promote bone regeneration, while releasing angiogenic factors to drive neo-vascularisation in the regenerating tissue (Wolf-Brandstetter et al. 2006). This extends to exploiting the endogenous tissue engineering capabilities through applying advances in the understanding of developmental biology to implant a treatment that will stimulate the *in vivo* niche to initiate a healing regenerative process, through the incorporation of bioactive molecules and cells in scaffolds. Eliciting a signalling profile from the native environment capable of activating resident stem cells is emerging as an important therapeutic component (Gurtner et al. 2007), for example, using the model of the hypoxic environment during foetal development, in which wound regeneration occurs in the absence of scars.

Other developments in the compatibility of scaffold biomaterials, such as the incorporation of adhesion peptides for example RGD (Arg-Gly-Asp) and nanoscale surface manipulation, have enhanced cell adhesion and migration (Gurtner et al. 2007). Scaffolds can also function to facilitate the retention and distribution of cells about the region where new tissue is desired. Through pre-clinical examinations, it is emerging that the biggest challenge in scaffold design is to match the native environment in terms of chemical and mechanical cues that are generated in native bone, as well as having the appropriate physical presence. For example, native bone tissue is a major storage site for growth factors such as BMPs (Gautschi et al. 2007) and TGF-β. The mechanical environment will also regulate the response of cells within the scaffold and the surrounding tissue.

Therefore, matching the release of growth factors and a controlled mechanical environment will bring the success of bone tissue engineering one step closer to reality for patients through subsequent tissue formation and regeneration of adult bone.

1.3 Mesenchymal stem cells

The cell source for skeletal tissue engineering applications can be somatic cells, e.g. chondrocytes for cartilage defects using the matrix-induced autologous chondrocyte implantation (MACI) technique (Marlovits et al. 2005), or stem cells. Stem cells are defined as cells that can both self renew and give rise to clonal progeny (Gurtner et al. 2007; Weissman 2000). Development begins with the totipotent zygote which is capable of giving rise to any tissue in the body. The inner cell mass of the blastocyst, which forms after 7-8 cell divisions, contains pluripotent cells capable of generating all tissues in the developing foetus. These are embryonic stem cells (ESCs). Further differentiation forms multipotent lineage specific stem cells, for example, hematopoietic stem cells (HSCs) that form blood cells, or mesenchymal stem cells (MSCs) (Gurtner et al. 2007). Due to their uncommitted nature and responsiveness to various chemical and physical inductive cues towards a variety of specialised lineages, MSCs are a suitable cells source for tissue engineering applications. Progress in bone tissue engineering has been assisted by the accessibility and availability mesenchymal stem cells, since they are an osteoprogenitor cell source.

MSCs are unspecialised cells present in many adult tissues including the bone marrow, that act as a progenitor reservoir for the regeneration of multiple tissue types including bone, cartilage, adipose, tendon and ligament ((Caplan and Dennis 2006; Pittenger et al. 1999) Figure 1.2) in response to trauma or as part of the natural cycle of cell turnover. Their most distinctive properties are their ability to proliferate in culture, their multilineage differentiation potential and their isolation through plastic adherence (Tuan et al. 2003). In addition, there is an emerging body of data on their immunomodulatory properties (Bocelli-Tyndall et al. 2007; Nauta and Fibbe 2007) and their capibilities for migrating and homing to injury sites (Barbash et al. 2003; Metheny-Barlow et al. 2004), both *in vitro* and *in vivo* following systemic infusion of *ex-vivo* expanded MSCs.

MSCs were originally isolated by Friedensteins' lab in the 1960's from bone marrow and stroma of spleen and thymus (Friedenstein et al. 1970; Friedenstein et

al. 1966). Using the adherence technique for plating these cells, which is the most common method for their isolation today, Freidenstein and colleagues observed adherent colonies of cells that after an initial latent period began to multiply rapidly. Following passaging, a fibroblastic morphology was reported. Subsequent investigations on this population provided evidence for multipotential differentiation (Bab et al. 1986; Mardon et al. 1987). Because of their ability to differentiate into cells of mesenchymal origin, they are currently referred to as either mesenchmal stem cells or marrow stromal cells by the acronym MSCs as they appear to arise from the complex array of supporting structures found in the marrow (Caplan 1991; Horwitz et al. 2005; Prockop 1997).

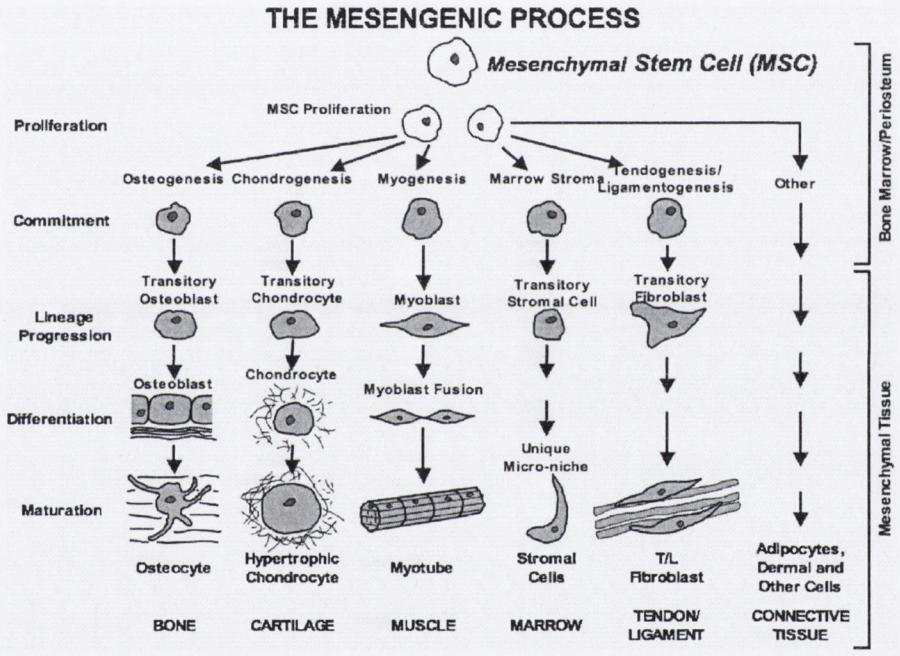


Figure 1.2 Mesengenesis

Multi-potent stem cells found in the bone marrow, and in other tissues, are capable of proliferating and differentiating along distinctive mesenchymal lineages to form highly specialised phenotypes capable of forming many connective tissues (Adapted from (Caplan and Dennis 2006)).

The most accessible and enriched tissue source for the isolation MSCs is the bone marrow; however MSC-like cells have been isolated from other sites including adipose tissue, amniotic fluid, periosteum and muscle, and show functional heterogeneity (In 't Anker et al. 2003; Lee et al. 2000a; Nakahara et al. 1990; Zuk et al. 2002). While the frequency of MSCs within the bone marrow is low, thought to be between 0.0001 – 0.01% (Baksh et al. 2004; Pittenger et al. 1999) the lack of specific markers for MSCs makes this estimation difficult. MSCs have successfully

been used to reform tissues when encased in tissue-specific scaffolds and implanted into different tissue sites. For example, in rodents, dogs and humans, MSCs have been delivered to long-bone repair sites in calcium phosphate porous ceramics to produce morphologically and biomechanically superior bone (Bruder et al. 1998; Kon et al. 2000). The transplantation of *ex vivo*-expanded allogeneic MSCs has shown little immunogenic responses *in vivo* (Koc and Lazarus 2001), thought to be due to the absence of expression of the major histocompatibility complex II (MHC II) antigens that are responsible for immune rejection. Recent reports indicate that MSCs do not acquire MHC II cell surface antigens upon differentiation along adipogenic, chondrogenic and osteogenic lineages. In addition, MSCs can modulate immune responses by suppressing both B and T cell functions (Corcione et al. 2006; Rasmusson et al. 2003), further supporting MSCs as a cell source for tissue engineering.

1.3.1 MSC isolation

There is currently no unique marker or combination of markers for MSCs; however, several non-specific markers have been identified that aid their isolation from hematopoetic cells. For human MSCs these include endoglin (CD105) (Barry et al. 1999; Haynesworth et al. 1992), CD73 (Barry et al. 2001) and Stro-1 (Simmons and Torok-Storb 1991). MSCs do not express the hematopoetic markers CD45 and CD34 or the endothelial cell marker CD31 (Baddoo et al. 2003). In light of the absence of specific markers, MSCs can defined by a combination of physical, morphological and phenotypical properties, or retrospectively using their multiple tissue differentiation properties *in vitro* and *in vivo* as a functional criterion (Dennis et al. 1992). The selective adherence of MSCs to plastic surfaces and the lack of adherence of hematopoetic cells (Friedenstein et al. 1970; Luria et al. 1971) is an effective method of producing a homogeneous population, and the most widely adopted method for the isolation of MSCs from bone marrow. With each passage and medium change, more non-adhering contaminating cells are removed and MSCs become further enriched. This adherent population of cells is heterogeneous, containing osteoblasts, adipocytes, endothelial cells and vascular pericytes, all of which originate from MSCs (Tropel et al. 2004), therefore, enrichment of the MSC population is desirable to optimise tissue engineering applications that are dependent on the multipotential differentiation capabilities. A number of approaches are used to enrich a more homogeneous population based on a panel of both positive and negative markers.

Percoll centrifugation (Lennon and Caplan 2006), based on the density of the fraction, separates nucleated cells from red blood cells of the marrow aspirate. While cells isolated by these methods express appropriate markers in response to *in vitro* osteogenic, chondrogenic and adipogenic differentiation cocktails, the isolation is non-specific. Using the expression profile of antigens, cytokine receptors, and adhesion molecules characteristic of MSCs that has been described, fluorescent-activated cell sorting (FACS) has recently been used to positively select cells expressing a panel of surface markers, or for immunodepletion of cells expressing hematopoietic and/or other lineage antigens (Baddoo et al. 2003; Jones et al. 2002). A series of monoclonal antibodies raised towards surface MSC antigens has been used for the immunophenotypical characterisation of this cell population. These antibodies target a range of cell surface receptors that include SH2 which recognises CD105 (Barry et al. 1999), SH3 and SH4 that recognise distinct epitopes on CD73 (Barry et al. 2001), and a group of other adhesion molecules and growth factor/cytokine receptors (Alhadlaq and Mao 2003). Many isolation protocols are based on the process of negative selection of cells lacking the expression of hematopoietic and endothelial cell markers CD45, CD34, CD14 and CD11b which are prominently expressed on monocytes and macrophages, the most likely hematopoietic cells to be found in an MSC culture; CD79α and CD19 which are markers of B cells that may also adhere to MSC in culture; and HLA-DR molecules are not expressed on MSC unless stimulated, e.g. by IFN-γ (Dominici et al. 2006; Pittenger et al. 1999), as well as markers of endothelial cells such as CD31 (Alhadlaq and Mao 2003; Baddoo et al. 2003; Chamberlain et al. 2007). The antibodies Stro-1, anti-Sca-1 and HOP-26 have also been used to enrich osteoprogenitor cells in bone marrow culture (Alhadlaq and Mao 2003; Joyner et al. 1997; Simmons and Torok-Storb 1991; Van Vlasselaer et al. 1994).

1.3.2 MSC fate

At each passage in culture, human MSCs exhibit a lag phase of growth, followed by a log phase and finally a growth plateau state; the rate of growth in log phase and the final number of cells after a fixed period in culture diminishes as a function of continued passaging. The average number of population doublings for marrow-derived adult human MSCs is 38±4 (Bruder et al. 1997). As human MSCs reach their population doubling capacity, they become very broad and flattened before degenerating (Caplan et al. 1998). The capacity of MSCs to differentiate into the mesenchymal lineages is independent of the age of the donors although

MSC titers decrease with age (Caplan 2007), and the osteogenic potential of MSCs does not decline with the passage or cryopreservation (Caplan et al. 1998; Leskela et al. 2003). Adult progenitor cells in their niche environment remain undifferentiated until stimulated (Tuan et al. 2003). The developmental potential of a particular progenitor cell can be pre-programmed at the time of asymmetric division dependent on the segregation of cell fate determinants or because of differential influences from the surroundings (Rabbany et al. 2003; Watt and Hogan 2000), thus emphasising that intrinsic regulation and environmental regulation contribute to cell fate. Quiescent adult progenitor cells become mobilised during repair and remodelling through regulation by external chemical and physical signals that control their activation, proliferation, migration differentiation and survival i.e. their fate. The extrinsic control influenced by exogenous factors including secreted factors, cell-cell interactions through membrane proteins, integrins and their relationship with the extracellular matrix (Ball et al. 2004; Caplan et al. 1998) are important from a tissue engineering perspective as they provide an opportunity to influence stem cell fate decisions for specific outcomes. Exogenous factors influence MSC responses by activating networks of molecular regulators including signalling molecules and transcription factors that ultimately determine stem cell fate (Viswanathan et al. 2003). *In vivo*, the microenvironment of these cells interfaces with systemic physiology and the extra-cellular matrix, which are capable of transmitting biochemical and biophysical signals to influence local development events (Caplan et al. 1998).

1.4 Cell Proliferation

Proliferation is a fundamental requirement for developmental establishment and renewal of tissues (Stein 1998). Insight into the control of proliferation will facilitate the design of protocols for expansion of stem cells, regulation of the proliferative process and cell cycle control. Cell cycle studies in culture revealed that while a small fraction of MSCs are actively engaged in proliferation, the vast majority of cells are standing at the Go/G1 phase of the cell cycle (Conget and Minguell 1999). Although check points and length of each phase of the cell cycle have not been determined, the high percentage of Go/G1 cells suggests a high competence of MSCs to differentiate (Tamir et al. 2000). Moreover, the Go/G1 population of MSCs includes a minor and variable subset of resting quiescent cells, as evidenced by RNA and DNA content (Conget and Minguell 1999) or by FACS analysis of size and granularity (Colter et al. 2000).

With the onset of *in vitro* culture, MSCs exhibit a large but highly variable expansive potential. While some preparations of MSCs can be expanded through over 15 cell doublings, others cease replicating after about 4 cell doublings (Bruder et al. 1997; Digirolamo et al. 1999). Sixty population doublings have also been reported for mouse MSCs (Tropel et al. 2004). This can be due to several factors, including the procedure used to harvest the marrow e.g. cells have a steeper growth curve in log phase when fed daily as compared to twice weekly (Bruder et al. 1997; Digirolamo et al. 1999), and the age or condition of the donor from which progenitor cells were prepared e.g. colony forming unit-fibroblasts (CFU-f) frequency reduced with age of donor and bone marrow transplant patient CFU-f frequencies are reduced and do not recover (Digirolamo et al. 1999; Galotto et al. 1999). Despite the high *ex vivo* expansive potential, MSCs do not loose their normal karyotype and telomerase activity (Pittenger et al. 1999). However, extensive subcultivation impairs cell function by the onset of evident signs of senescence (Digirolamo et al. 1999) and/or apoptosis (Conget and Minguell, 1999).

1.5 Cell differentiation

Cell differentiation proceeds from unspecialised cells to tissue specific cells through selective environmentally-induced protein expression. Almost all organs and tissues have a pool of progenitor cells that can respond to normal cell turnover demands, or during injury or damage response. To efficiently function in the organ or tissue where progenitor cell differentiation is required, differentiation must result in a phenotypically matched cell. Therefore through targeting specific genes, many signalling possibilities exist that can differentially control cell phenotype. Several *in vitro* studies have been conducted to assess the differentiation potential of MSCs, as well as to set up culture conditions, differentiation stimuli, and methods for the identification of each differentiated phenotype. These are supported by *in vivo* studies demonstrating that bone marrow-derived MSCs develop into terminally differentiated phenotypes, like those forming bone (Goshima et al. 1991; Kadiyala et al. 1997; Kuznetsov et al. 1997; Pereira et al. 1995; Richards et al. 1999), cartilage (Kadiyala et al. 1997; Kataoka and Urist 1993; Pereira et al. 1995), tendon (Butler et al. 2007; Young et al. 1998), muscle (Ferrari et al. 1998), neural (Parr et al. 2007), and adipose tissues (Mauney et al. 2007).

Differentiation involves the expression of a specific set of genes empowering the cell to carry out specialised functions necessary for survival of the target tissue type. Several instructive signalling molecules have been identified that induce differentiation and the expression of lineage specific genes, including various forms of TGF- β, BMP, growth and differentiation factor (GDF), tumor necrosis factor-α (TNF-α) and Wnt ligands. Many differentiation factor combinations for MSCs in culture mimic the *in vivo* signalling molecule expression seen during embryogenesis, e.g. BMPs for osteogenic differentiation.

1.5.1 MSC Osteogenesis

MSC osteogenesis follows the upregulation of gene activity stimulated by osteogenic transcription factors. The expression of osteogenic proteins contributes to the formation of a functional mineralised extracellular matrix. Each of these features can be used to detect stages of osteogenic differentiation. Additionally, cell morphology can be monitored as a marker of the development stage of osteoblastic differentiation (Jaiswal et al. 1997). Under induction by osteogenic factors, the morphology of MSCs changes from a fibroblastic to a polygonal appearance (Liu et al. 1999). Dexamethasone stimulates alkaline phosphatase activity as one of the initial responses during osteogenic differentiation. Thus, assays for the detection of alkaline phosphatase are commonly carried out to detect early differentiation. Other methods for the detection of osteogenic differentiation are reactivity with anti-osteogenic monoclonal antibodies, for proteins including Cbfα1, collagen type I, osteocalcin and BMP2 (Lee et al. 2000a).

Cbfα1, a member of the developmental-associated runt homology domain family of transcription factors, is required for osteoblastic differentiation and endochondral and intramembranous bone formation (Ducy et al. 1997; Komori et al. 1997). Cbfα1 is expressed just before osteoblast differentiation and only in mesenchymal progenitor cells committed to become either chondrocytes or osteoblasts. Once terminal commitment has occurred, expression is limited to osteoblasts (Ducy et al. 2000). The molecular mechanisms by which Cbfα1 can function as a master regulatory gene for activating the program of osteoblastogenesis has provided novel insights for transcriptional regulation of tissue specific genes. The unique properties of Cbfα1 mediate several key functions necessary for regulating skeletogenesis, controlling osteoblast growth and differentiation, and integrating the complex pathways required for bone

formation and turnover (Lian et al. 1998). Cbfα1 is required for the activation of genes encoding osteopontin (Hijiya et al. 1994), osteocalcin (Towler et al. 1994), and collagen type I (Rossert et al. 1996) and is involved in the regulation of their expression (Ducy et al. 2000). Binding sites for Cbfα1 exist in the promoter regions of most genes that are required for the synthesis of extracellular matrix (Ducy et al. 1997). Sustained expression of Cbfα1 in osteogenic and pluripotent cell lines up-regulates osteoblast-specific gene expression and induces *in vitro* mineralization in a cell type-dependent manner (Byers et al. 2002). The role for Cbfα1 in bone formation has been demonstrated *in vivo* by the complete absence of osteoblasts and bone formation in Cbfα1 null mice, however in these studies, mature cartilage was formed (Otto et al. 1997).

Type I collagen is the major structural protein in bone comprising 95% of the extracellular non-mineral bone matrix, and is essential for the formation and maturation of mineralised matrix (Dunitz 2001). It is formed by the combination of two α1 and one α2 collagen polypeptides containing hydroxylated proline and lysine residues. This structure is known as procollagen and as it is secreted from the osteoblast, terminal regions are cleaved. The rest of the molecule joins the growing collagen fibril. Stabilisation of the helical structure occurs by cross-linking and it is the pattern of this linking that makes this collagen specific to bone. Studies on rat osteoblast precursor cell differentiation have shown collagen type I to peak at day 3 (Quarles et al. 1992) making it a protein marker of early MSC osteogenesis.

Several non-collagenous proteins are prominent constituents of, and essential for, the formation and maturation of mineralized tissues. The most notable among these are osteocalcin, osteopontin, and osteonectin, also secreted by osteoblasts to form the osteoid or organic substance in which mineralization occurs (Quarles et al. 1992). Osteopontin and alkaline phosphatase are both associated with the bone matrix formation and are maximally expressed during matrix maturation. Osteocalcin is regulated by one of only two specific osteoblast transcription factors (Ducy et al. 2000). This protein is up-regulated in the late stages of osteoblast differentiation, and maximum elevation of osteocalcin marks the transition form matrix maturation to mineralisation (Ducy et al. 1996; Owen et al. 1990) and thus, plays an important part in the mineralisation of the matrix (Owen et al. 1990). It is thought that the role of osteocalcin in matrix formation is to serve as a site for hydroxyapatite crystals (Watts 1999). It has been observed by a

number of studies that osteocalcin is a later marker in the ECM, peaking from 7 days after stimulation of osteoblasts with osteogenic differentiation medium. (Ebeling 2001; Quarles et al. 1992).

BMPs are bioactive bone growth factors that provide important and specific signals that are essential for full osteogenic differentiation and in the regeneration of bone. They become up-regulated in response to differentiation signals in allograft bone fracture healing, bone regeneration and heterotopic bone formation, and the upregulation of BMP2 in response to osteogenic factors (Frank et al. 2002; Oreffo et al. 1999) is linked to osteogenic differentiation in mesenchymal progenitor cells (Lee et al. 2000b; Yamaguchi et al. 2000). Additionally, they are the only family of proteins known to individually induce ectopic bone formation (Wang et al. 1990). Upregulation of BMP2 has been used as a marker of the osteogenic differentiation potential of MSCs (Sumanasinghe et al. 2006).

The maturation of osteoblasts is marked by their ability to mineralise the osteoid matrix in their extracellular environment. Matrix mineralisation involves calcification of the osteoid to hydroxyapatite by other secreted substances such as alkaline phosphatase. The formation of a mineralised matrix is the only morphological distinguishing feature of osteoblast cells in culture (Ducy et al. 2000). The detection of calcium (in the form of hydroxyapitite) in extracellular deposits forms a marker for mature osteoblasts in culture (Coelho and Fernandes 2000; Jaiswal et al. 1997). Two methods for the detection of mineralised matrix deposition are von Kossa and alizarin red staining. The von Kossa method stains the phosphate ion of calcium phosphate mineral black (Bonewald et al. 2003), and the alizarin red dye binds selectively to calcium salts binding approximately 2 mol of Ca^{2+} per mole of dye (Gregory et al. 2004; Lievremont et al. 1982; Puchtler et al. 1969) and both used widely for calcium mineral histochemistry (Jaiswal et al. 1997; Ogura et al. 2004; Stanford et al. 1995a).

1.6 Cell apoptosis

Apoptosis is a highly regulated mechanism of cell death, whereby a programmed sequence of events lead to the highly organised destruction, degradation and phagocytotic deletion of the cell (Arends and Wyllie 1991; Kerr et al. 1972), significantly, in the absence of a local inflammatory response (Raffray and Cohen 1997). Apoptosis is active in development, contributing to the sculpturing of

many organs and tissues (Meier et al. 2000; Raffray and Cohen 1997), and in the regulation and maintenance of cell populations in tissues during physiological and pathological conditions (Raff 1998). Multiple inducers of apoptosis have been identified including ionising radiation, oxidative stress, sustained increases in $[Ca^{2+}]i$, or lack of survival signals (Gerschenson and Rotello 1992; Raff 1998; Raff 1992). Apoptosis is distinguished by unique morphological features including chromatin condensation and nuclear fragmentation, cell shrinkage, membrane blebbing, the formation of apoptotic bodies containing cellular organelles and nuclear fragments and the externalisation phosphatidylserine identifying the cell as a phagocytic target (Figure 1.3, adapted from (Walker et al. 1988)). A major hallmark of apoptosis is the internucleosomal fragmentation of double-stranded DNA into fragments of 180- to 200-bp length (Ziegler and Groscurth 2004).

Apoptosis is an active process, and the molecular characterisation has recently been examined. Cell death through apoptosis follows complex biochemical events including cell to cell and intracellular signal transduction, ordered enzyme cascades, and gene transcription (Hengartner 2000; Yuan et al. 1993). Despite the diversity in signal transduction, all apoptotic pathways are believed to converge ultimately with the activation of the caspases (Blatt and Glick 2001), leading to the characteristic features of apoptotic cell death.

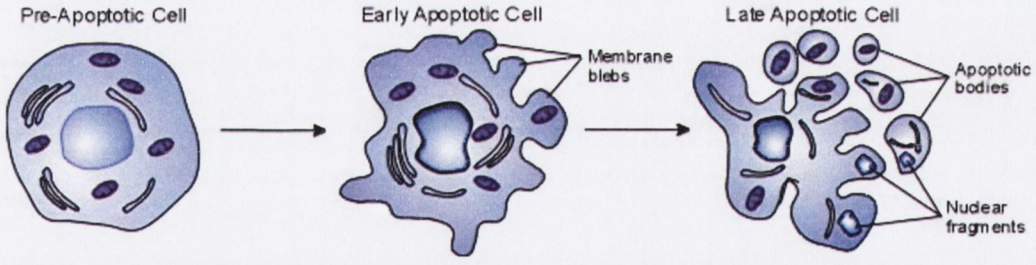


Figure 1.3 Morphological features of apoptotic progression

Apoptosis involves condensation of the nuclear chromatin and cytoplasm, fragmentation of the nucleus and the formation of membrane-bound bodies (Adapted from (Walker et al. 1988)).

Apoptosis is commonly identified using biochemical methods that detect key events in the apoptotic process (Otsuki et al. 2003; Sgonc and Gruber 1998). One method, the TUNEL technique, detects DNA strand breaks through labelling of free 3'-OH DNA ends generated by DNA fragmentation in apoptosis (Bursch et al. 1990). The TUNEL system provides an accurate quantitative assessment of cell

death by providing a colourimetric DAB based system to distinguish cells containing fragmented DNA (Gavrieli et al. 1992).

Other methods for detecting apoptosis involve probing for the biochemical activity of the caspases, Calpain and by examining the JNK signalling pathway, all hallmark biochemical signalling cascades that become active during apoptosis.

1.6.1 Caspases

The complex molecular signature of apoptosis is distinct, and is genetically encoded to all cells to provide an instant response to a death signal. Interlukin-1-converting enzyme (ICE) is the mammalian homologue of the protein first discovered in C. elegans (ced-3) to be required for apoptosis. The ICE family are intracellular protein-cleaving enzymes, known as caspases (cysteine-dependent aspartate-specific proteases), that mediate proteolytic cleavage events through an amplifying proteolytic cascade, cleaving one another in sequence. Procaspases are made up of a large and a small subunit. Proteolytic processing between these subunits forms an active caspase. Once activated, some of the caspases cleave other specific proteins that support the nuclear membrane, activate a DNA-degrading enzyme that cleaves the DNA in the cells nucleus; and cytoskeletal proteins involved in cell attachment and tensegrity, thereby promoting detachment, making apoptotic bodies easy to ingest by phagocytic cells.

Caspases are activated by extrinsic and intrinsic signals (Figure 1.4). Following death receptor assembly on the cell surface, specific adaptor molecules such as perforin facilitate the protease granzyme B through transmembrane channels. This protease cleaves and activates certain procaspases to begin the proteolytic death cascade (Atkinson and Bleackley 1995). Extrinsic mechanisms can also operate through the Fas ligand protein. This binds to Fas receptors on the surface, causing aggregation of the receptors (Nagata and Golstein 1995). Procaspase-8 molecules aggregate, then cleave and activate each other to begin the apoptotic sequence (Ashkenazi and Dixit 1998). Intrinsic controls activate upon release of cytochrome c from the mitochondria. Cytochrome c normally functions in the electron-transport processes in mitochondria that generate most of the cells adenosine-tri-phosphate (ATP). Upon intracellular damage however, it accumulates in the cytoplasm (Reed 1997) usually in association with alterations of mitochondrial membrane proteins, such as the Bcl-2 family of proteins (Antonsson 2001). The Bcl-2 family consist of 2 opposing groups that regulate

apoptosis: pro-apoptotic members induce cytochrome c release from the mitochondria and anti-apoptotic members prevent its' translocation from the mitochondria (Korsmeyer 1999). In the cytosol, cytochrome c creates the apoptosome, a cytosolic death signalling protein complex. Procaspase-9 activation is downstream of this complex which proteolytically activates caspase-3, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death (Earnshaw et al. 1999).

Examination of MSC apoptosis has revealed that they follow caspase-dependent apoptosis and exhibit many of the hallmark features (Raz et al. 2006). Several studies have shown that embryonic and perinatal progenitor cells undergo apoptosis via the caspase pathway and/or involvement of the Fas receptor (D'Sa-Eipper and Roth 2000). Apoptosis through caspase-3 activity has been observed in MSCs that were prevented from adhering to their extracellular matrix (anoikis) (Feng et al. 2007). MSCs undergo apoptosis in response to hypoxia and serum deprivation (Zhu et al. 2006).

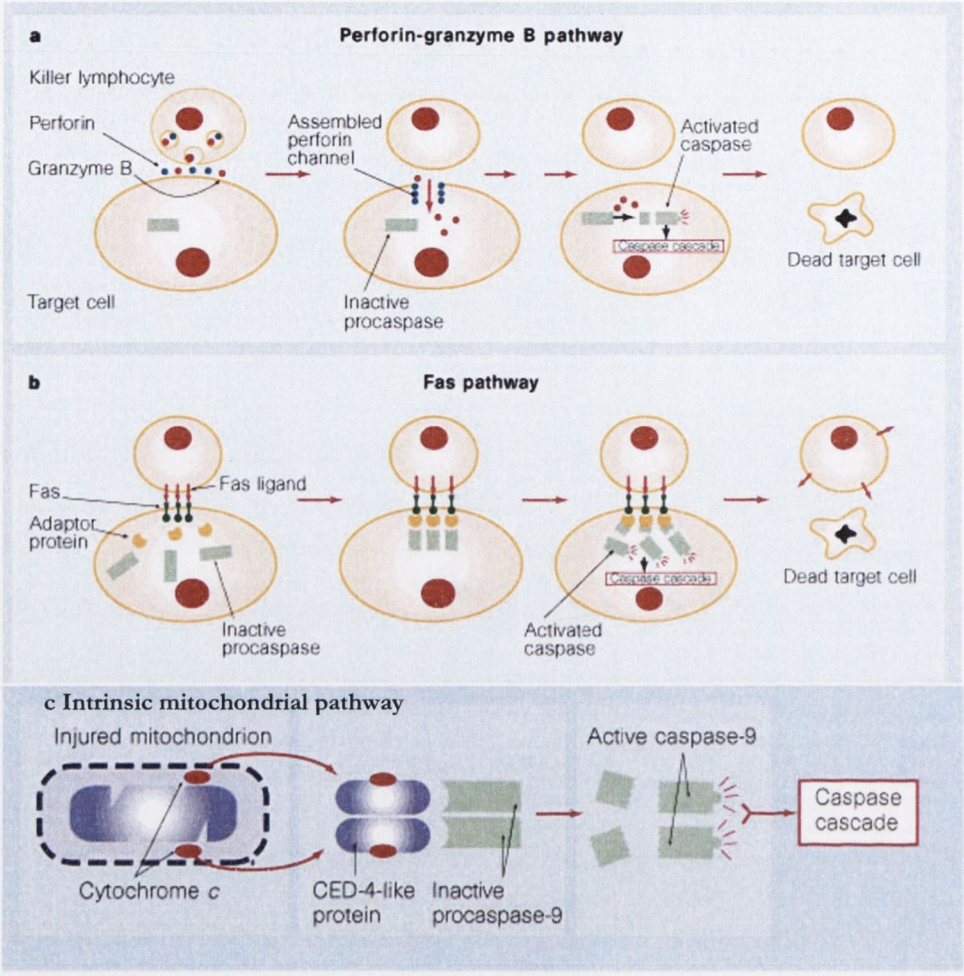


Figure 1.4 Caspase activation during apoptosis

In extrinsically triggered apoptosis, a) lymphocytes e.g. in response to infection, secrete proteins onto the target cell surface: the perforin protein assembles into channels that enable the granzyme B protein to enter the target cell and activate procaspases. b) Fas ligand on the lymphocyte surface binds to the Fas protein on the target cell, causing the Fas molecules to form clusters. An adaptor protein then binds to the cluster and recruits inactive procaspase-8 molecules, which then activate one another to initiate the caspase cascade. c) Following cell stress or damage, intrinsically activated apoptosis can occur. The released cytochrome c molecules bind to CED-4-like adaptor proteins. The adaptor proteins then aggregate and bind procaspase-9 molecules. The clustered procaspase-9 molecules activate one another, and the activated caspase-9 molecules then activate other procaspases, leading to apoptosis (adapted from Raff (1998)).

1.6.2 Calpain

Calpains are a family of calcium–activated intracellular cysteine proteases that carry out proteolytic cleavage on a diverse range of substrates. Calpain is responsive to stimuli such as radiation and ischemia (Kohli et al. 1999; Waterhouse et al. 1998). There is evidence that calpain is tightly regulated post-transcriptionally by many mechanisms including its endogenous inhibitor calpastatin, calcium requirements, and autoproteolytic cleavage (Molinari and Carafoli 1997). Mechanisms of calpain regulation include an elevation of calcium concentration (Guroff 1964; Suzuki et al. 1981), phospholipid binding (Suzuki et al. 1992), the endogenous calpain inhibitor calpastatin (Wendt et al. 2004) and phosphorylation by the extracellular-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) (Glading et al. 2002). Among more than 100 proteins identified as calpain substrates are transcription factors, transmembrane receptors, signalling enzymes and cytoskeletal proteins including numerous adhesion complex components and migration-related proteins (Goll et al. 2003) such as actin, alpha-actinin, talin, filamin, fodrin, gelsolin, focal adhesion kinase (FAK), integrinβ3, c-fos, c-jun, p53, and procaspase-3 and -9. (Cooray et al. 1996; Hirai et al. 1991; Kubbutat and Vousden 1997; Meredith et al. 1998; Vanags et al. 1996; Wolf et al. 1999; Yuan et al. 1997). Several calpain substrate proteins are cytoskeletal proteins or proteins that can associate with cell membranes. This has led to speculation that calpains may be particularly important in destruction of cellular architecture during apoptosis. Ca^{2+} stimulated calpains induce cell death by activating pro-apoptotic members of the Bcl-2 family (Cao et al. 2003; Chen et al. 2001), by facilitating the release of apoptosis-inducing factor (AIF) from mitochondria through proteolytic cleavage of a membrane anchor that retains AIF on the inner mitochondrial membrane (Polster et al. 2005), by cleaving essential components of the cytoskeleton (Bano et al. 2005), and by inducing the release of lysosomal cathepsins (Artal-Sanz and Tavernarakis 2005).

Cell stimulation by mechanical shear stress elicits a Ca^{2+} influx sensitive activation of calpain that is facilitated primarily through Phosphatidylinositol 3-kinase (PI3kinase) (Miyazaki et al. 2007). Elevated mechanical stress induces apoptosis in smooth muscle cells via p53 protein expression and transcriptional upregulation of p53 responsive genes (Sedding et al. 2007; Wernig et al. 2003). This has recently been linked to increased calpain activity but as an anti-apoptotic mechanism, as inhibition of calpain further induced p53 protein expression and

transcriptional activity (Sedding et al. 2007). The pro-apoptotic effect of calpain in response to mechanical stress has been demonstrated in neurones, whereby Ca^{2+} influxes associated with permeability changes following tensile forces have been linked to cytoskeletal degredation via spectrin proteolysis through calpain activity (Buki and Povlishock 2006). Calpain activation has been assessed using several different assays including *in vitro* whole cell assays to detect cleavage of the fluorogenic calpain substrate Suc-Leu-Tyr amidofluorocourmarin (AFC) (Boland and Campbell 2003).

1.6.3 c-Jun amino-terminal kinase (JNK)

The c-Jun amino-terminal kinase, also a stress activated protein kinase (SAPK), is stimulated primarily by a plethora of stress conditions, such as UV irradiation, DNA damage, heat shock and oxidants, genotoxic stress, as well as by inflammatory cytokines (Davis 2000). The JNK protein kinases are activated by phosphorylation on tyrosine and threonine residues, separated by a proline, by MKK4 and MKK7. The JNKs bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity (Pulverer et al. 1991). A major target of the JNK signalling pathway is the activation of the AP-1 (Activator protein-1) transcription factor that is mediated, in part, by the phosphorylation of c-Jun and related molecules. JNK appears to be essential for AP-1 activation caused by stress and some cytokines, but is not required for AP-1 activation in response to other stimuli (Yang et al. 1997). AP-1 transcription complex is an important regulator of gene expression contributing to the control of many cytokine genes and is activated in response to environmental stress, radiation, and growth factors - all stimuli that activate JNKs. Regulation of the JNK pathway is extremely complex and is influenced by many MKKKs. Activation of the JNK pathway results in the phosphorylation and activation of several transcription factors in addition to c-Jun, such as ATF-2, Elk-1, p53, and c-Myc, as well as factors such as Bcl-2, Bcl-xL, paxillin, and MAP2 (Figure 1.5) (Davis 2000; Nishina et al. 2004; Wada and Penninger 2004).

JNKs are important in controlling apoptosis. It has been proposed that JNK activation triggers apoptosis in response to stimulation via a mitochondria-dependent mechanism (Tournier et al. 2000). It appears that the various roles of the JNK pathway in apoptosis depend on the cell types and conditions observed (Wada and Penninger 2004) Although JNK activation is predominantly associated with promotion of cell death, the SAPK/JNK stress pathway, under certain

conditions, participates in many different intracellular signalling pathways that control a spectrum of cellular processes, including cell proliferation, differentiation, transformation, apoptosis, migration, and cytoskeletal integrity (Johnson and Lapadat 2002; Katz et al. 2007).

The treatment of human MSCs with osteogenic factors also stimulates JNK activation late in the matrix synthesis phase of osteogenic differentiation process (Jaiswal et al. 2000). Through inhibiting JNK activity during osteogenic and adipogenic factor treatment, osteogenic differentiation is suppressed while adipogenic differentiation is promoted. (Tominaga et al. 2005). JNK signalling therefore is central to the bifurcation of lineage commitment as well as in late stage matrix formation. The involvement of the JNK cascade in proliferation has also been demonstrated in human adipose-derived MSCs, where inhibition resulted in the prevention of platelet-derived growth factor (PDGF) induced proliferation (Kang et al. 2005).

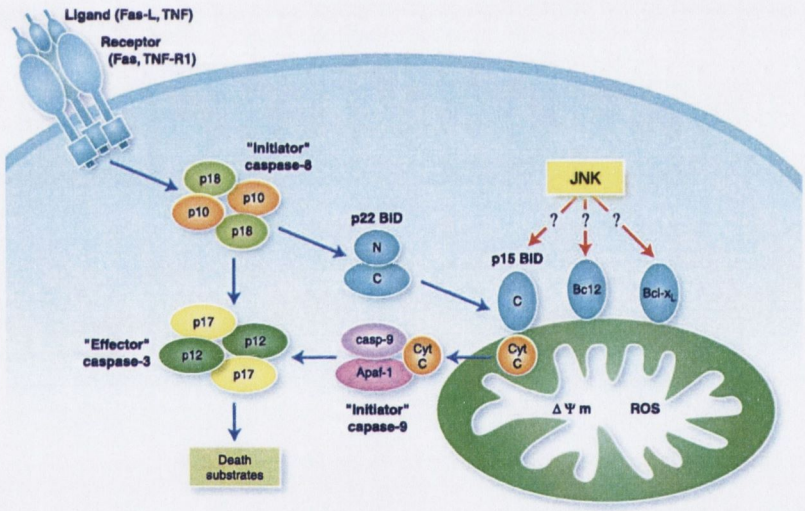


Figure 1.5 JNK signalling in apoptosis.

JNK is not required for death receptor signalling, but is required for caspase-9 activation by the mitochondrial pathway. Potential targets of JNK include members of the Bcl2 group of apoptotic regulatory proteins. Adapted from (Davis 2000).

JNK activation has been reported in response to a number of biomechanical stimuli, including, stretch. In response to mechanical stretch, renal epithelial cells undergo apoptosis through the specific activation of JNK/SAPK and p38 SAPK-2 pathways and this is dependent on the activation of caspase-3 and -9 (Nguyen et

al. 2006). JNK mediated apoptosis is also seen as a strain response in vascular smooth muscle cells and human patellar tendon fibroblasts (Skutek et al. 2003; Sotoudeh et al. 2002). Transient JNK activation is often seen in response to biomechanical stimuli, however apoptosis is not induced (Richards et al. 1999). The apoptotic effects and mechanisms of MSC response to biomechanical stimuli have yet to be fully defined. JNK is also involved in the mechanotransduction of osteogenic lineage commitment signalling (Matsuda et al. 1998), therefore, is a potent SAPK and can regulate a number of cell fates in response to extracellular stimulation.

The intrinsic regulators of multipotent progenitor cell proliferation, differentiation and apoptosis are responsive to influences of the microenvironment, or niche, where the cells normally reside e.g. apoptotic cell signalling cascades can be initiated following extracellular biomechanical or biochemical stimulation. For MSCs to follow a specific differentiation pathway, biochemical cascades initiate within the cell in response to extracellular cues, that result gene transcription leading to the expression of lineage specific proteins (Luginbuehl et al. 2004). Similarly, progenitor cells *in vivo* are quiescent and will only divide in response to a microenvironmental stimulus (Moore and Lemischka 2006). This can be extrinsic signalling from surrounding stromal cells or influence from outside the bone marrow which can direct stem cell activation and subsequent proliferation or differentiation and matrix synthesis (Gurtner et al. 2007). This extrinsic signalling takes the form of biochemical or biomechanical stimulation and has been shown to be required for and enhance *in vivo* tissue regeneration responses (Chen and Mooney 2003).

1.7 Biochemical factors in MSC osteogenesis

Bone formation *in vivo* follows high alkaline phosphatase activity in osteoblasts that produces a mineralised collagenous bone matrix. The induction of MSC osteogenesis is a highly programmed process, and can be chemically directed *in vitro*. Following the first reports of the colony-forming unit assembly from bone-marrow, Freidenstein and colleagues demonstrated induction of osteogenesis from mesenchymal stem cells (Friedenstein et al. 1970; Friedenstein et al. 1966). Subsequent investigations of this population provided evidence for osteogenic differentiation (Bab et al. 1986; Mardon et al. 1987) and *in vivo* bone formation from MSCs (Krebsbach et al. 1997; Mankani et al. 2001).

Endogenous systemic glucocorticoids are involved in bone formation and bone remodelling, and the synthetic glucocorticoid dexamethasone is a commonly used analog for *in vitro* osteogenic studies. Dexamethasone stimulates MSC proliferation and has been shown to regulate alkaline phosphatase gene expression (Bellows et al. 1990; Both et al. 2007; Cheng et al. 1996; Leboy et al. 1991; Milne et al. 1998) as well as many other genes involved in osteogenesis including collagen type I, osteocalcin, osteopontin, bone sialoprotein, parathyroid hormone receptor, insulin-like growth factor, and osteopotegrin (Beresford et al. 1994; Cheng et al. 1996; Leboy et al. 1991). Alkaline phosphatase has a role in the mineralisation of newly formed bone and its activity is a biochemical marker of osteoblastic differentiation (Watts 1999).

Ascorbic acid functions as a cofactor in the post-translational modification of proline in collagen to hydroxyproline (Pinnel et al. 1987; Schwarz et al. 1981) and also stimulates the synthesis of collagen I and III (Nusgens et al. 2001), as well as increasing the synthesis of non-collagenous bone matrix proteins. Ascorbic acid is not stable in solution at 37°C, therefore the stable analogue L-ascorbic acid-2-phosphate which has similar activity in tissue culture (Jaiswal et al. 1997) is used for the maturation of collagen in osteogenic cultures.

Organic phosphates, such as β-glycerol phosphate also support osteogenesis by playing a role in the mineralisation and modulation of osteoblast activities (Chung et al. 1992). β-glycerophosphate is an artificial alkaline phosphatase substrate, and once hydrolyzed is a source of phosphate ions for the formation of a mineralised extracellular matrix through calcium phosphate deposition *in vitro* (Bellows et al. 1991; Chung et al. 1992; Maniatopoulos et al. 1988; Pittenger et al. 1999; Tenenbaum and Heersche 1982). Free phosphates can induce mRNA and protein expression of osteogenic markers such as osteopontin and these phosphates have known effects on the production and nuclear transport of the key osteogenesis regulator gene Cbfα1 (Ducy et al. 1997; Fujita et al. 2001).

While dexamethasone is capable of stimulating alkaline phosphatase activity and other genes related to osteogenesis, calcium accumulation and subsequent matrix mineralisation are only observed when MSCs are cultured with all three osteogenic supplements, i.e dexamethasone, L-ascorbic acid-2-phosphate and β-glycerophosphate (Bellows et al. 1991; Chang et al. 2006; Nusgens et al. 2001; Tenenbaum and Heersche 1982). There now exists a standardised protocol for MSC osteogenic differentiation (Jaiswal et al. 1997). From that study, through a

systematic approach, an optimal osteogenic factor cocktail was determined that directs MSCs towards the osteogenic lineage, and cumulates in mineralised matrix production. The development of the osteoblast phenotype from induction with these osteogenic factors, follows a temporal sequence of differentiation involving active cell proliferation, expression of osteoblastic markers, synthesis, deposition and maturation of a collagenous extracellular matrix and, matrix mineralisation (Lian et al. 1997; Marie et al. 1989; Owen et al. 1990; Robey and Termine 1985), that is not dissimilar to that seen during development and fracture repair (Caetano-Lopes et al. 2007).

1.8 Biomechanical factors in MSC osteogenesis

Co-existing with the biochemical influences, another biophysical influence in the extracellular microenvironment is physical stimuli including mechanical force. Mechanical forces exist in tissue microenvironments as a result of muscle contraction, differing growth rates of two connected tissues and through transmission of external loading. During normal tissue growth or repair, mechanical events at the tissue level can be thought of as hydrostatic pressure imposed on cells and tensile deformation of cells and local extracellular matrix (Prendergast et al. 1997). Imposed stresses originating outside the cell come from externally applied loads and the tension and pressure gradients generated from differences in growth rates of neighbouring tissues (Henderson and Carter 2002). Mechanical forces are important in development, growth, and the maintenance and function of tissues such as the production of bone, the contraction of muscle, the vibrations of the eardrum and the remodelling of cardiovascular tissues and can have potent influence over progenitor cell fate control. Wolff, in 1892, discovered the relationship between the structural adaptation of bone and physical requirements, stating that

> *'Every change in the form and function of bone or of its function alone is followed by certain definite changes in the bone internal architecture, and equally definite alteration in its external conformation, in accordance with mathematical laws.'*
>
> *Wolffs Law (Wolff 1892).*

Mechanobiology has developed to provide detailed studies on the regulation of skeletal formation by signals to cells generated by mechanical loading. In 1941, Pauwels proposed that the physical factors of dilatational and distortional strain (change in volume and change in shape) determine the skeletal mesenchymal

mesengenic pathway (Figure 1.6;(Pauwels 1980)). Endochondral bone formation would require a stable mechanical environment and therefore would be stimulated in areas of low distortional and high dilatational strain. Carter and colleagues developed this theory to include cyclic mechanical loading that delivered hydrostatic and deviatoric stresses, and found that cyclical hydrostatic stress inhibits cartilage ossification while octahedral shear stress encourages ossification (Carter et al. 1998). This relationship can be applied more generally to tissue differentiation (Figure 1.7). Prendergast et al. (1997) have considered the microenvironment of the cells further, and included relative fluid/solid velocity in their mechano-regulation model as a biophysical stimulus involved in regulating tissue differentiation *in vivo*. Fluid velocity is created in skeletal tissue during movement of the fluid phase upon mechanical loading. Fluid flow will act to increase the deformation of the cells further therefore at high velocities there will be a decreased potential for differentiation (Figure 1.8).

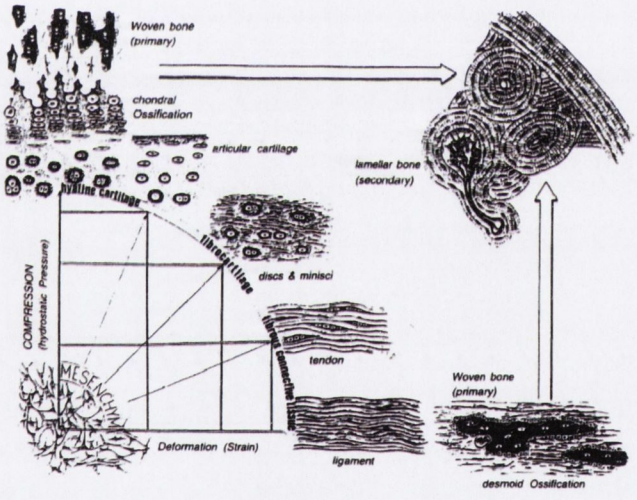


Figure 1.6 Pauwels' concept of tissue differentiation.

Tissue-differentiation processes starting from mesenchymal cell condensations to fibrous tissue, fibrocartilage or bone, are governed by mechanical stimuli. Depending on the response of the mechanical environment to the presence of these tissues, osteoblast proliferation and ossification can occur (Adapted from Pauwels (1980))

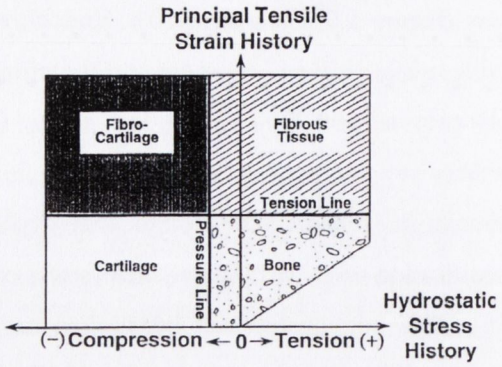


Figure 1.7 Carters representation of the relationship between mechanical stimuli and tissue differentiation.

This tissue differentiation theory relates mechanical loading history, defined in terms of solid matrix tensile strains and fluid pressures, to tissue phenotype. 'Tension' on the x-axis represents negative fluid pressure. For example, it proposes that cartilage forms under excessive hydrostatic compressive stress, fibrous tissue forms with excessive tensile strain, and fibrocartilage forms with combined hydrostatic pressure in the presence of tensile strain (Adapted from (Carter et al. 1998)).

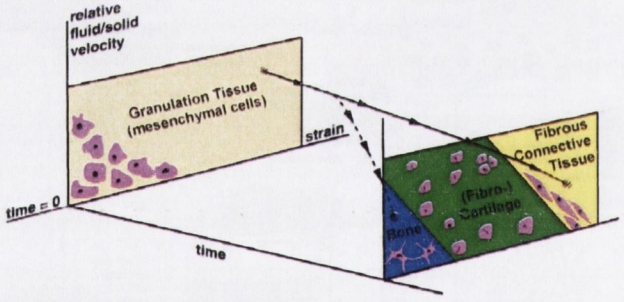


Figure 1.8 A mechanoregulation model by Prendergast et al. (1997) for the hypothesised interaction between biophysical stimuli and tissue phenotype.

Over time, cells that are capable of differentiating, synthesise collagenous matrices. The combination of the mechanical properties in the tissue determine the pattern of ossification, for example, if the collagen synthesis reduces motion, then the shear strains and relative velocities will reduce and ossification will occur-but intermediate tissue types may be required (Adapted from (Prendergast et al. 1997)).

1.8.1 MSC Mechanotransduction

Recent evidence shows that mechanical factors such as fluid shear stress, tensile forces, compressive loads, hydrostatic pressure and the rigidity if the extracellular matrix can regulate gene induction, protein synthesis, cell growth, death, and differentiation (Sato et al. 2007). For an imposed tension or pressure to act as an inductive signal cells must have the ability to detect mechanical stimulation, there must exist a mechanism of mechanotransduction through which the cell translates the mechanical signal into a particular biological response. Although cell response to mechanical stimulus is recognised as a fundamental biological phenomenon, the cellular mechanisms underlying mechanotransduction are still under examination and recent research has identified a number of mechanoreceptors involved in the transduction of force in to an electrochemical cascade. Major mechanosensors and cell components involved in the mechanotransduction mechanisms include the cytoskeleton, integrins and focal adhesions, guanine nucleotide-binding proteins (G-proteins), and ion channels (Figure 1.9; Wang and Thampatty (2006)).

Ion channels form pores that span the plasma membrane and are therefore ideally situated to respond to external forces applied to the cell. The activation of mechanosensitive ion channels has also been proposed as a transduction mechanism (Hamill and Martinac 2001). Amongst the mechanosensitive channels are the stretch-activated cation channels (SACCs) (Kalapesi et al. 2005) and transient receptor potential channels (TRPs) (Lin and Corey 2005). However, mechanosensitive channels also include other ion channels such as voltage-dependent Ca^{2+} channels (el Haj et al. 1999), K+ channels (Dopico et al. 1994; Olesen et al. 1988) the NMDA receptor (Casado and Ascher 1998) and Ca^{2+}-dependent BK channels (Kawakubo et al. 1999). In the latter, mechanosensitivity may be secondary to their normal physiological functions. In particular, the roles of the mechanosensitive ion channels - SACCs and L-type VACC, in mechanotransduction form the focus for investigations in this research.

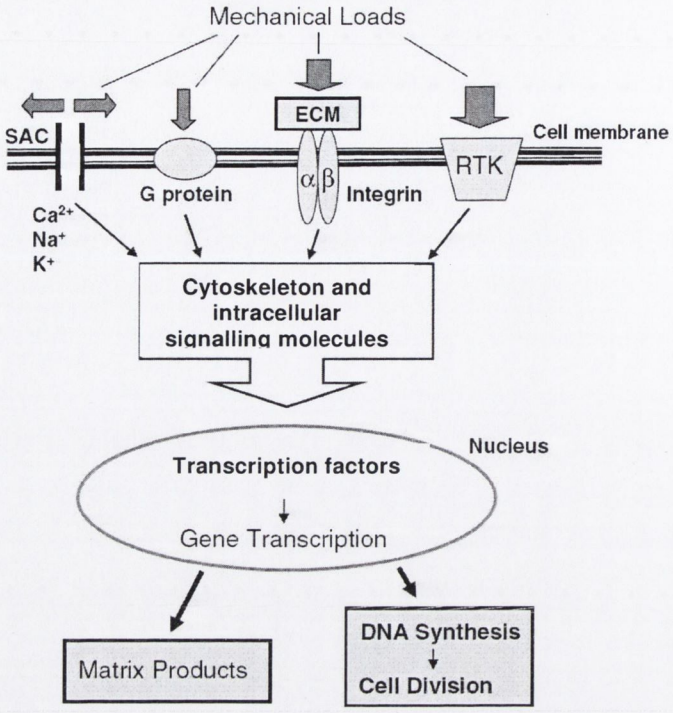


Figure 1.9 Mechanotransduction mechanisms

Integrins, G proteins, receptor tyrosine kinases (RTKs), and stretch-activated ion channels (SAC) are involved in the mechanotransduction of physical stimuli. Intracellularly, mitogen-activated protein kinases (MAPKs) and the cytoskeleton are down stream of these and link to the activation of transcription factors that control matrix formation and DNA synthesis. (Adapted (Wang and Thampatty 2006)).

1.8.1.1 Stretch-activated cation channels

The precise cellular mechanisms underlying mechanosensation and the conversion of mechanical signals to cellular responses such as new bone formation remain to be fully resolved; however, stretch-activated cation channels (SACC) in the plasma membrane have been the focus as mechanoreceptors in a number of studies of mechanoregulation through tensile strain (Guharay and Sachs 1984; Rawlinson et al. 1996). These channels serve as mechano-electrical switches converting a mechanical force into a change in the cellular electrochemical gradient (Ingber 2006). It has been suggested that mechanosensitive (MS) channels may be gated directly by changes in the lipid bilayer tension, or in the 'tethered model' of gating, by displacement of the MS channel with respect to components of the cytoskeleton or extracellular matrix (Hamill and Martinac 2001). Their response to membrane tension leads to an influx of cations including Ca^{2+}, and extracellular

Ca^{2+} influxes are believed to be necessary for downstream anabolic effects of mechanotransduction in osteocytes (Mikuni-Takagaki 1999). Rises in intracellular calcium leads to further $[Ca^{2+}]_i$ increases through the mobilisation of intracellular stores, and many intracellular signal transduction pathways are sensitive to elevated calcium. Changes in intracellular Ca^{2+} levels regulate a wide range of cellular processes, including cell growth, cell motility, contraction, apoptosis, and differentiation to direct the appropriate cell response to mechanical stimulation (Lammerding et al. 2004). Mechanical stimulation elevates Ca^{2+} in many types of cells, including smooth muscle cells (SMC), fibroblasts, osteoblasts, and vascular endothelial cells (Kirber et al. 2000; Pommerenke et al. 1996; Shen et al. 1992; Sigurdson et al. 1992).

1.8.1.2 Voltage activated calcium channels

L-type voltage activated Ca^{2+} channels (VACC) have previously been shown to have mechanosensitive properties (Lyford et al. 2002). A recent study on the use of mechano-active scaffolds has confirmed the activation of these channels in response to *in vitro* mechanical conditioning, and an enhancement of osteogenic markers in response to calcium influxes from prolonged opening of the channels (Wood et al. 2006). In that study, mechanical conditioning increased levels of the VACC subunit alpha (2) delta 1 ($\alpha 2\delta 1$). Mechanisms of mechanosensation in VACC have yet to be fully defined, however, research has recently identified the von Willebrand factor A (VWA) type domain on the extracellular sequence of the $\alpha 2\delta 1$ subunit of the VACC (Olesen et al. 1988). Since this domain mediates binding to proteins such as collagen, found in the extracellular matrix, it follows that the $\alpha 2\delta 1$ subunit may be involved in the detection of extracellular mechanical events and link subsequent activation of the channels.

A direct coupling may exist between mechanosensitive channels and the cytoskeleton since agents that disrupt the organization of actin filaments, such as colchicine and cytochalasin-D, affect mechano-sensitive channel activity. Depolymerisation of actin with cytochalasin-D in cos-7 cells, transfected with mechanosensitive channels, enhances channel activity (Wan et al. 1999), and in chick muscle cells cytochalasin-D increases the stretch sensitivity of mechanosensitive channels (Guharay and Sachs 1984); these studies suggest that the cytoskeleton exerts a tonic inhibition on mechanosensitive channel activity and support the contention that mechanosensitive ion channels are functionally linked with the dynamics of the intracellular cytoskeleton.

Mechanical strain is prevalent in numerous tissue types. Tensile strain may be cyclic in nature, as in the rhythmic distension of a blood vessel, or it may be fairly static, as in maintaining the extension of a bundle of skeletal muscle. Additionally, the applied load may occur in any number of directions within the 3D architecture of a given tissue, ranging from predominantly linear to more intricate multi-axial strains. The *in vitro* response to mechanical strain often depends on several factors including the type, magnitude, frequency and duration of the strain, the matrix molecules used in culture, and the cell type being investigated. This array of factors has repeatedly led to seemingly conflicting reports on cell responses in the scientific literature. A number of cell culture systems have been developed in which the response of bone-derived cells to physical forces has been observed. The loading techniques for *in vitro* stimulation include hydrostatic pressure (Stanford et al. 1995), stretching (Neidlinger-Wilke et al. 1994), bending (Kaspar et al. 2002) and fluid shear stresses (Billotte and Hofmann 1999), and all have induced responses in bone cells including proliferation, differentiation and cell orientation. In one study, cyclic stretching has increased osteoblast-like cell proliferation, collagen type I production and reduced alkaline phosphatase activity and osteocalcin expression (Kaspar et al. 2002), suggesting that cyclic strain stimulates matrix production but inhibits matrix mineralisation.

1.9 Intracellular Signalling

Following biochemical or biomechanical stimulation of mesenchymal stem cells from their microenvironment, mechanotransduction through second messenger systems translates the signal detected by the mechanosensors by mainly chemical (or electrical) soluble signalling cascades, to the nucleus, where gene expression is controlled. Signalling pathways in the transduction of extracellular cues into intracellular responses include the mitogen-activated protein kinase (MAPK) cascade (Figure 1.10) and the phospholipase enzyme PI3-kinase (Cano and Mahadevan 1995; Marshall 1995). The MAPK pathway is a highly conserved three layer signalling cascade, in which the MAPK elements (the most downstream tier) are activated upon tyrosine and threonine phosphorylation. This causes dimerisation of the receptor and an intermolecular cross-phosphorylation of the two receptor molecules. The phosphorylated receptors then interact with adaptor molecules that trigger downstream events in the cascade. The MAPK then phosphorylates a variety of substrates that control transcription, the cell cycle, or rearrangements of the cytoskeleton. Activation of these kinases can have potent

effects leading to cell fates such as differentiation, proliferation or apoptosis and responses such as migration and growth.

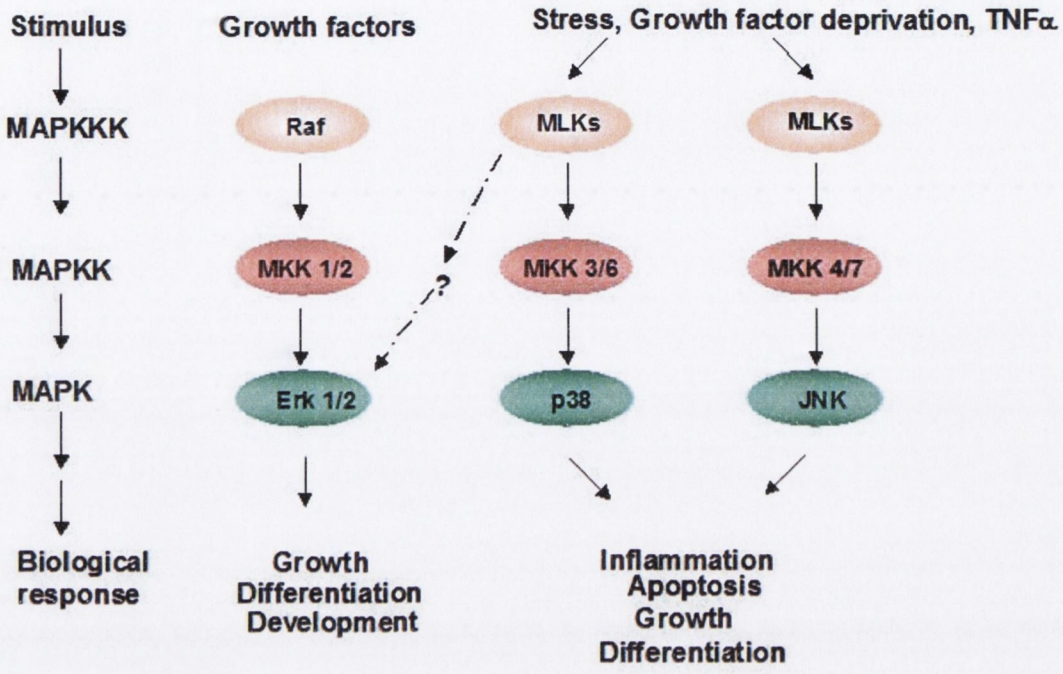


Figure 1.10 MAPK signalling

Extracellular stimuli such as growth factors of physical disruption initiate a cascade of sequential kinase stimulation that amplifies and specifies signals over at least 3 tiers to eventually activate several regulatory molecules in the cytoplasm and in the nucleus that initiate cellular processes such as proliferation, differentiation, and development.

The generic MAPK signalling pathway is shared by four distinct cascades: the extra-cellular-signal-regulated kinases ERK1 and ERK2, the c-Jun NH2-terminal kinases 1 and 2 (JNK 1 and JNK2), p38-MAPK and Erk5 (Katz et al. 2007). p38–MAPK and JNKs are activated by environmental stresses and inflammatory cytokines and thus are known as stress activated protein kinases (SAPKs). These MAPK cascades are regulated by both growth factor signalling and physical stimulation. Growth factors are considered to be the major regulators of the Erk1/2 cascade, whereas Erk5 is regulated by both growth factors and stress, and out of the two major stress-induced cascades, the JNK pathway is partially regulated by growth factors, and only a few studies have documented activation of the p38-MAPK cascade by growth factors (Katz et al. 2007). MAPKs phosphorylate specific serines and threonines of target protein substrates which

include other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins. MAPK-catalysed phosphorylation of substrate proteins functions as a switch to turn on or off the activity of the substrate protein (Johnson and Lapadat 2002). By and large, activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are linked to induction of apoptosis (Xia et al. 1995).

Several lines of evidence suggest that signalling through MAPKs is essential for the early stages of osteoblast differentiation (Franceschi and Xiao 2003; Gallea et al. 2001; Hipskind and Bilbe 1998). Moreover, it has been shown that ERK MAPK signalling is involved in the stimulation of osteoblast-related gene expression by extracellular matrix-integrin receptor interaction as well as mechanostressing (Rubin et al. 2002; Suzawa et al. 2003).

1.9.1 Extracellular regulated kinase (ERK)

The ERK cascade is a central signalling pathway responsive to a large number of extracellular stimuli including growth factors, hormones, G protein-coupled receptors and other mechano-transducers. ERKs are activated by phosphorylation on threonine and tyrosine residues by an up-stream dual specifity kinase (Blumer and Johnson 1994). Active ERKs have a wide range of substrates including multiple cytoplasmic and cytoskeletal proteins, protein kinases and phosphatises, transcription factors and regulators of apoptosis (Yoon and Seger 2006). The ERK cascade bridges the gap between the receptors and their intracellular targets thus are involved in a wide range of cellular responses such as proliferation, migration and differentiation, (Katz et al. 2007).

In response to biochemical stimuli such as osteogenic factor supplements, ERK has been shown to differentially mediate the commitment of human MSCs towards the osteogenic or adipogenic lineages, favouring osteogenesis when activation is sustained (Jaiswal et al. 2000). Transient ERK activation following receptor tyrosine kinase signalling has been associated with proliferation (Marshall 1995). ERK phosphorylation has also been associated with EGF-induced MSC expansion *in vitro* (Tamama et al. 2006). TGFβ2 an important osteogenic mediator, stimulates ERK activation (Huwiler and Pfeilschifter 1994). The exposure of osteogenic treated human MSCs to cyclic mechanical strain promotes the production of mineralised matrix, and this response is largely mediated through ERK1/2 signalling (Simmons et al. 2003). ERK activation in MSCs has also been

observed during *in vivo* distraction osteogenesis suggestive of a mechanoregulatory mechanism associated with ERK mediated bone generation (Rhee et al. 2006). Subsequent *in vitro* simulations however, have revealed opposing responses in ERK phosphorylation following mechanical stimulation. For example, an inhibition of ERK1/2 activation occurred in the preosteoblast cell line MC3T3-E1 following long-term loading (up to 25 days). This correlated with persistent matrix deposition but inhibited mineralisation (Jackson et al. 2006). Contrastingly, ERK activation has been reported in response to mechanical stimulation of human MSCs in the absence of osteogenic conditioned culture medium. While this was measured as an early response (< 120 mins), it appeared to be a potent regulator of lineage commitment towards osteogenic differentiation as inhibition during strain was associated with increases in non-osteogenic lineage marker expression levels (Ward et al. 2007). ERKs therefore, have demonstrated independent biochemical and biomechanical sensitivity, and form part of the early lineage commitment signalling in MSC differentiation.

1.9.2 p38

The p38 MAPKs are strongly activated *in vivo* by environmental stresses and inflammatory cytokines, and less by serum and growth factors. Environmental cellular stresses include alterations in the concentrations of nutrients, growth factors, cytokines and cell damaging agents as well as physical stimulation. Activation of p38 MAPK is mediated by dual phosphorylation on a conserved motif that includes a glycine residue between the canonical threonine and tyrosine (Kyriakis and Avruch 2001). Targets of p38 MAPK are both cytoplasmic and nuclear, indicating that multiple cellular functions are under their control. Physiological substrates include transcription factors, other protein kinases which in turn phosphorylate transcription factors, cytoskeletal proteins, and translational machinery components, and other proteins such as metabolic enzyme, glycogen synthase or cytosolic phospholipase (Kyriakis and Avruch 2001). The p38 MAPK pathway has been identified as having a key role in differentiation and migration. Such studies have been facilitated greatly due to the sensitivity of p38 to chemical inhibitors including SB203580 (Cuenda and Rousseau 2007).

In human osteoblasts, p38 MAPK activation is essential in the BMP-2 up-regulation of type I collagen, osteocalcin, and alkaline phosphatase, and p38 MAPK and ERK activation is essential in the up-regulation of fibronectin and osteopontin (Lai and Cheng 2002). During biochemical induction of osteogenesis

by osteogenic factors, the phosphorylation of p38 has been observed (Jaiswal et al. 2000). p38 activation followed a temporally distinct pattern to ERK activation. Coupled with the distinct activation stimuli and substrate specificity, p38 signalling is likely to be involved in other cellular processes during osteogenic exposure such as maturation and mineralisation. In serum-deprived MSCs, p38 is activated in response to TGF-β and is implicated in protection from apoptosis through an autocrine activation of the pro-survival PI3kinase/Akt pathway (Horowitz et al. 2004). The TGF-β/p38 cascade has also been associated with the progression of apoptosis (Yu et al. 2002). Recent studies have shown that ectopic BMP-2 induces the osteoblast phenotype via p38-MAPK-dependent mechanism in both the MC3T3-E1 cell line and calvaria-derived osteoblastic cells (Guicheux et al. 2003). In response to mechanical strain, activation of p38 has also been observed in human MSCs (Simmons et al. 2003). As with growth factor activation, this follows a temporally distinct pattern to ERK1/2 activation, however its expression is more likely to be associated with inhibition of late osteogenic markers such as matrix mineralisation. Recently, Yu et al. (2002) have identified a role for compressive force activation of p38 in the expression osteogenic and chondrogenic genes in the mesenchymal stem cell line C2C12 (Yu et al. 2002). The p-38 MAPK therefore, has a parallel to ERK role as a second messenger during biochemical and biomechanical signal transduction, with the additional role in stress induced apoptotic events.

1.9.3 Phosphatidylinositol 3-kinase (PI3-kinase)

PI3-kinase is a heterodimeric enzyme that phosphorylates phospholipids at the plasma membrane (Berridge 1987) and is responsive to external stimuli. PI3-kinase phosphorylates the D3 hydroxyl of phosphoinsitides and produces Phosphatidyl-Inositol -3-phosphates, which are involved in the regulation of diverse cellular processes, including proliferation, survival, cytoskeletal organization, vesicle trafficking, glucose transport, and platelet function (Fruman et al. 1998). PI3-kinase is activated by several receptors and non-receptor protein tyrosine kinases (Karnitz et al. 1994) by binding of its p85 regulatory subunit to an activated receptor. The downstream target of PI3-kinase is the serine-threonine kinase Akt, which transmits survival signals from growth factors (Duronio et al. 1998; Kandel and Hay 1999). PI3-kinase is associated with numerous cellular functions including cell growth, survival and proliferation (Duronio et al. 1998).

Studies have demonstrated that activation of the PI3-kinase/Akt pathway is essential for BMP-2 induced osteoblast differentiation (Ghosh-Choudhury et al. 2002; Osyczka and Leboy 2005). Inhibition of this kinase has led to the absence of the alkaline phosphatase, therefore indicating the requirement for PI3-kinase for osteoblast differentiation. Involvement of PI3-kinase has recently shown to regulate some biological properties of TGF-β such as epithelial-mesenchymal transition and matrix protein expression (Bakin et al, 2000). Recently PI3-kinase has been implicated in myogenic and neuronal (Jori et al. 2005) differentiation (Jiang et al. 1998; Kaliman et al. 1996), as a mediator of cell survival in fibroblasts and neurones following apoptotic insults (Kulik et al. 1997; Yao and Cooper 1995) and in cell migration during chemokine induced responses (Li et al. 2007). In addition, inhibitor studies have revealed a basic role for PI3-kinase in cytokine induced proliferation of progenitor cells (Gong et al. 2006). Mehanical stretch activates PI3-kinase in osteoblastic cells and cardiomyocytes and retinal pericytes in response to stretch (Danciu et al. 2003; Petroff et al. 2001; Suzuma et al. 2000).

Since PI3-kinases interact with membrane proteins, it is possible that they have an involvement in signalling of extracellular mechanical stimulation. In response to shear stress, PI3-kinase mediates Akt phosphorylation, which is then associated with an anti-apoptotic response (Dimmeler et al. 1998). Cyclic strain also activates PI3-kinase via p21ras, and this activation has been demonstrated to be upstream of strain induced ERK activity (Ikeda et al. 1999). p21ras is a small GTP binding protein which has been shown to play a pivotal role in the signal transduction pathways of cellular responses to extracellular mitogenic stimulation. PI3kinase has been shown to modulate many mechanically induced events in a number of cell types including vascular endothelial, lung, muscle and bone (Danciu et al. 2003; Dogra et al. 2006; Miyahara et al. 2007; Wang et al. 2007).

1.10 Aims and objectives of the research

Tissue engineering is driven by the integration of cells, substrate and biophysical stimuli in an appropriate combination that once implanted, leads to functional tissue regeneration. Mechanical forces can act as MSC stimulatory controls in a manner that is reflective of the physiological microenvironment (Satija et al. 2007). To develop an understanding of biomechanical microenvironmental influences on MSCs, the aim of the research was to investigate how physical stimuli, in the form of tensile mechanical strain, can act as a regulator of MSC fate, at the molecular level.

The first objective was to provide a characterisation of the parameters of the study. Firstly, the author aimed to characterise the cell substrate biomaterial (silicone), and to determine the optimal cell-seeding conditions for this material. Secondly, the author aimed to phenotypically characterise the cell population, and to examine the proclivity of osteogenic factors to induce MSC differentiation on a silicone-substrate. Additionally, the contribution of coating the substrate with collagen on this response was examined. Finally, the author aimed to characterise the role of ERK, p38 and pI3kinase signalling pathways in the control of osteogenic factor-induced MSC differentiation.

The second objective was to use a custom-made uniaxial strain device to examine the effect of cyclic tensile mechanical strain on MSC osteogenic differentiation by probing for expression of Cbfα1, collagen type I, osteocalcin and BMP2. Furthermore, the author aimed to determine the molecular mechanisms involved in this response, beginning by examining the involvement of SACCs in the cell membrane, then by investigating the role of the intracellular signalling pathways ERK, p38 and pI3-kinase in the mechanotransduction of strain-induced MSC osteogenic differentiation.

The final objective of this research was to investigate the effect of tensile mechanical strain on MSC proliferation and apoptosis. The author aimed to determine how MSC proliferation changes during strain by measuring DNA synthesis, and to examine the limits of strain magnitude that can be applied before the mechanical stimulation becomes an extrinsic apoptotic signal, by examining DNA fragmentation. Furthermore, the author wished to delineate the involvement of the mechanosensors – L-type VACCs and SACCs in strain-

induced apoptosis and probe for activation of the intracellular signalling molecules JNK, caspase-3 and calpain during a strain-induced apoptotic response.

2 Materials and Methods

2.1 Preparation and Procedures for Cell Culture

2.1.1 Material Sterility

To avoid bacterial, viral or fungal contamination that can alter normal cell function, all materials that were used during cell culture were sterile and all methods during cell culture were carried out aseptically.

2.1.2 Sterilisation Procedure – Equipment

Re-usable equipment such as glassware (Schott, Germany) and non-sterile equipment such as pipette tips (Sarstedt, Leicester, England) were wrapped in aluminium foil and autoclave tape and autoclaved at 121°C for 20 min (Priorclave Ltd., Model #EH150, London, England). Dissection instruments were baked at 200°C overnight in a hotbox oven (Sanyo-Gallenkamp Hotbox Oven, Model #OHG050, Loughborough, England). All equipment was sprayed liberally with 70% alcohol before placing inside a laminar flow hood (Astec-Microflow laminar flow workstation, Florida, U.S.A.). Sterile consumables e.g. falcon tubes and petri-dishes (Sarstedt, Leicester, England) were opened only inside the laminar flow hood.

2.1.3 Sterilisation Procedure – Reagents

Dulbecco's Modified Eagles Medium (DMEM; Sigma-Aldrich, England) was purchased sterile and all solutions that it was supplemented with were sterile filtered using a 0.2 μm cellulose acetate membrane syringe filter (Pall Gellman, Michigan, U.S.A.). Water was autoclaved and all other reagents were stored in autoclaved glass bottles, aliquoted under sterile conditions and were sterile filtered before use.

2.1.4 Culture Environment

All culture work was carried out in the laminar flow hood (Astec-Microflow laminar flow workstation, Florida, U.S.A.). Disposable latex gloves (Arista Latindo Ind Ltd., Indonesia), also liberally sprayed with 70% alcohol, were worn during all culture procedures. Gloves were changed regularly and the hood was routinely wiped down with alcohol. The ultra-violet (UV) light in the hood was switched on, normally overnight, to further assist sterilisation.

2.1.5 Waste Disposal

All hazardous material (lab plastic, sharps, gloves and carcasses) were separated into appropriate UN approved primary packaging and then sent to the hazardous material facility (Trinity College, Dublin) where the waste was sent for disposal in accordance with Irish and EU legislative requirements.

2.2 Isolation and Culture of Mesenchymal Stem Cells

2.2.1 Animals

Young adult Wistar rats (3 months; approx.200 – 250g), bred at the BioResources unit of Trinity College Dublin, were used for this research. Animals were maintained in a 12 hr light dark cycle at an ambient temperature of 21 – 22°C. Food and water were available *ad libitum*. Animals were sacrificed by CO_2 asphyxiation. Folowing 5 mins in a CO_2 chamber, animals were retrieved for dissection.

2.2.2 Dissection

The area around the hind limbs was sprayed liberally with 70% alcohol and the skin from this area was removed. The hip joint was dislocated and the leg was cut free from the body. The femur was dislocated from the tibia at the knee joint and was pulled free from any muscle. Any remaining muscle was carefully dissected from the femur. The femur was placed in pre-warmed DMEM supplemented with 2% penicillin/streptomycin (Gibco BRL, Dublin), 10% Foetal Bovine Serum (FBS; Gibco BRL, Dublin), 0.5% l-Glutamine (Gibco BRL, Dublin), 0.5 % Glutamax (Gibco BRL, Dublin), and 1% non-essential amino acids (Gibco BRL, Dublin). To obtain the tibia, the Achilles tendon and several other tendons on the near side of the foot were severed. The foot was levered away from the experimenter and pushed towards the knee joint. The remaining muscles were pulled free from their insertions by their tendons towards the kneecap. The kneecap was then removed and with it all adherent muscle tissue. Once all bones were removed and freed of muscle tissue they were transferred to a laminar flow hood to maintain sterile conditions.

2.2.3 Mesenchymal Stem Cell Isolation and Culture

Bones were cut at both epiphyses and the marrow was flushed into a 50ml falcon tube using 5 ml of pre-warmed (37°C) supplemented DMEM, a 25 gauge needle

(Beckton Dickson Labware Europe, France) and syringe (Beckton Dickson Labware Europe, France). The suspension was centrifuged at 2000g (Sigma Aldrich, Model #2K15C, St Louis, U.S.A) for 5 minutes at 22°C. The supernatant was discarded and the pellet re-suspended in 10 ml of complete medium. This suspension was serially passed through 16, 18 and 20 gauge needles, 3 times each, to obtain a single cell suspension. The suspension was passed through a 40 μm sterile nylon mesh filter to remove any cell clumps. The suspension was plated onto a petri dish for half an hour, after which time the supernatant was removed and the adherent cells discarded. The cell suspension was counted using a haemocytometer (Sigma-Aldrich, England) and diluted accordingly with supplemented DMEM, to give a cell suspension of approximately $5x10^7$ nucleated cells per 75 cm^3 tissue culture flask. Cells were plated onto T75 culture flasks (Sarstedt, Leicester, England). After 24 hours the flask culture medium was replaced to remove non-adherent cells. The flask was rinsed once with supplemented DMEM and 10 ml of supplemented DMEM was added to the flask. Culture medium was replaced every 3 to 4 days. Cells were maintained at 37°C in a humidified incubator (Nuaire, Plymouth, UK) with 5% CO_2 and 95% air.

2.2.4 Cell Passaging

Upon reaching 80 – 90 % confluency (Fig 2.1), as assessed by visual inspection under an inverted microscope, (Nikon Labophot, Nikon Instech Co. Ltd. Kanagawa, Japan) cells were passaged at lower densities onto new culture flasks. Flasks were rinsed twice with pre-warmed sterile phosphate buffered saline (PBS; 100mM NaCl, 80mM Na_2HPO_4, 20mM $Na_2H_2PO_4$; Sigma-Aldrich, England). 5 ml of Trypsin EDTA (Sigma-Aldrich, England) was added to each flask to detach the cells from the surface and flasks were incubated at 37°C for 5mins. The cultures were examined under an inverted light microscope for detachment and the flask was tapped gently to release any remaining cells. The suspension was added to a 50 ml falcon and the flask rinsed twice with 5 ml of supplemented DMEM, also added to the falcon. The cell suspension was centrifuged at 2000g for 5 min at 22°C. The supernatant was discarded and the pellet re-suspended in 10 ml of supplemented DMEM. The suspension was passed through a 20 gauge needle 3 times to obtain a single cell suspension and the cells were re-plated onto T75 culture flasks at half their density before re-plating. Cultures were maintained in an incubator at 37°C in 95% air and 5% CO_2. Supplemented DMEM was changed every 3 days.

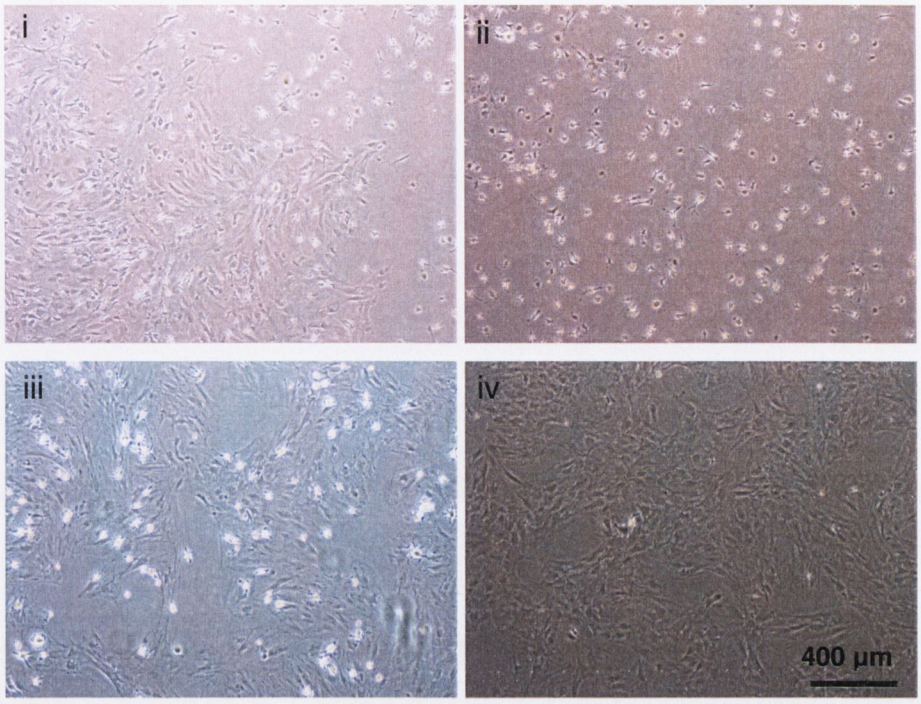


Figure 2.1 Light contrast microscopy of cells in culture flasks

3 – 4 days after isolation from the bone m arrow cells begin to form colonies on the surface of the tissue culture flasks (i). Other areas of the flask have cells attached however they do not form colonies (ii). Upon confluence cells are passaged. These cells form a more evenly distributed pattern of colonies (iii). At passage 4 (iv) cells are used for experimentation.

2.2.5 Fluorescence-activated cell sorting (FACS) - Immunophenotyping by flow cytometry

Flow cytometry is a useful technique due to the fact that the cells can be monitored, providing sensitive and specific information about each single cell. In relation to the optics of flow cytometry, when the light source hits a cell, the amount of light scattered to the side is detected in the side scatter (SS). The information provided from side scatter describes the size and shape of the cell. Flow cytometers use lasers as their source to excite cells. The excitation from the lasers must be equivalent to the absorption wavelengths of the fluorochromes used (Robinson, 2004). The argon laser is the most commonly used since it produces several lines in the UV, and can excite fluorescin, which is a common fluorochrome. The other parameter detected is forward scatter (FS) and it provides information about the surface properties, complexity of the cell and can determine how granulated the cells are. Various populations of cells can be distinguished from the information provided by side and forward scatter following acquisition of samples. In addition, antibodies have fluorescent labels attached enabling the surface expression of specific cell markers.

2.2.5.1 Preparation and staining of MSCs

Passage 4 cells were analysed for the positive MSC marker CD90 and the negative MSC marker CD45 by flow cytometry with a CyanADP flow cytometer (DakoCytomation). A single cell suspension of MSCs was obtained at a concentration of $3x10^6$cells/ml as previously described (2.2.4). Samples were centrifuged at 250 *g* for 5 mins at 4°C. The supernatant was discarded and pellets re-suspended in 3ml of blocking buffer (50% FCS in PBS) on ice for 20 mins. Samples were centrifuged again and washed 2 times with fluorescence-activated cell sorting buffer (FACS buffer; PBS containing 2% Heat inactivated fetal calf serum (v/v) 0.09% sodium azide (w/v), pH 7.4). Cells were then re-suspended at $3x10^6$ cells/ml in FACS buffer and incubated on ice for 20 mins. MSCs were stained with phycoerythrin (PE)-conjugated mouse anti-rat CD90 (Thy-1) monoclonal antibody (BD Biosciences, Oxford, UK) to identify mesenchymal stem cells and fluorescein isothiocyanate (FITC) -conjugated mouse anti-rat CD45 monoclonal antibody (BD Biosciences, Oxford, UK) to identify hematopoetic stem cells. For isotype controls, PE- and FITC-conjugated mouse IgG1,κ monoclonal immunoglobulins were used.

Relevant antibodies (2µl), isotype controls (2µl) and 100µl of cell suspension were added to wells of a 96-well-plate (Table 2.1). As controls, cells were added to wells without antibody (100µl). Plates were vortexed gently and incubated for 45 min at 4°C in the dark. Plates were centrifuged and cells washed 2 times with FACS buffer (200µl) before finally re-suspending samples in 200µl of FACS buffer. This solution was made up to 500µl in 5ml FACS tubes. Tubes were covered with tin-foil and stored on ice at all times to prevent decay of fluorochromes and cell clumping, respectively.

2.2.5.2 Acquisition

Samples were analysed using flow cytometry to acquire 10,000 events (cells) on a Dako Cytomation CyAn™ ADP, and Summit software was used for analysis. The fluorescence was measured on a logarithmic scale with band pass filter 520 for FITC and 576 for PE (Table 2.2).

Cell marker	Primary Monoclonal antibodies	Fluorescent conjugate	Supplier
Mesenchymal stem cells	Mouse anti-rat CD90 (Thy-1)	PE	BD Biosciences, Oxford, UK
Hematopoetic stem cells	Mouse anti-rat CD45	FITC	BD Biosciences, Oxford, UK

Table 2.1 Primary antibodies and fluorescent conjugates used in immunopheotyping

Fluorochrome	Excitation Peak	Emission Peak	Laser Wavelength
FITC	495	520	488
PE	565	576	488,514,568

Table 2.2 Fluorochromes used for immunophenotyping

2.3 Substrate preparation and cell seeding

2.3.1 Preparation of the collagen coated silicone strips

Silicone elastomer strips (10×60 mm; Goodfellow, Huntington, England and Speciality Manufacturing, Saginaw, USA) were sterilised by spraying with alcohol before placing them in a tissue culture petri-dish. Strips were rinsed with sterile water twice and allowed to dry. A 1% collagen solution was prepared aseptically by autoclaving rat-tail tendon collagen type I (Sigma-Aldrich, England) in ultra-pure water. The solution was added to the dish for two hours and incubated at 37°C. The collagen solution was poured off, and strips were washed two times with sterile water and allowed to dry. Strips were then exposed to UV light in the laminar flow hoods overnight.

2.3.2 Cover-slip preparation

Silicone cover slips were prepared by cutting 13-mm diameter discs from silicone sheeting using a custom-made circular punch (Workshop, Mechanical Engineering Department, Trinity College Dublin). Discs were sterilised by spraying with alcohol before placing them in a tissue culture Petri-dish. Coverslips were rinsed twice with sterile water and allowed to dry. Half of the discs were coated with a 1 % collagen solution as described above (2.3.1), and all exposed to UV overnight.

Plastic coverslips (Sarstedt, Wexford, Ireland) were purchased sterile. Packages containing 50 coverslips were sprayed with 70% EtOH before opening them in the laminar flow hood. Silicone discs and plastic coverslips were placed inside 24-well plates (Sarstedt, Wexford, Ireland) for seeding.

2.3.3 Cell seeding

Upon reaching 80-90% confluency after 21 days in culture, as determined by visual inspection using light microscopy (Figure 2.1), cells were seeded onto collagen-coated silicone substrates (strips and 13mm discs) and 13mm sterile coverslips (Sarstedt, Wexford, Ireland). Supplemented DMEM was poured from the flasks, followed by two rinses with pre-warmed PBS (37°C). 5 ml of trypsin EDTA was added to each flask and flasks were incubated for 5 minutes at 37°C. Flasks were examined under an inverted microscope for detachment and the flask was tapped gently to release any

remaining fixed cells. The suspension was added to a 50 ml falcon and the flask rinsed twice with 5 ml of supplemented DMEM, also added to the falcon. The cell suspension was centrifuged at 2000g for 5 min at 22°C. The supernatant was discarded and the pellet re-suspended in 1 - 2 ml of supplemented DMEM. The suspension was passed through a 20 gauge needle 3 times to obtain a single cell suspension. Cell numbers were determined using a haemocytometer and suspensions were diluted to give approximately 250,000 cells/ml. Substrates were seeded with volumes of cells in suspension that estimated to 20,833 cells/cm^2 (0.5ml for strips and 0.11ml for coverslips). These were then incubated at 37°C in 95% air and 5% CO_2. After 30 minutes to allow attachment of the cells, supplemented DMEM was added to petri-dishes (Sarstedt, Wexford, Ireland) (9.5ml) and 24-well plates (0.89ml). Cells were incubated for 2 days. After 2 days treatments began. Half of the medium in each well was then replaced every 3 days in order to make use of any soluble cell bioactive factors that may be released by the cells.

2.3.4 Cell density assay

To investigate the optimal seeding density for cells on 60×10 mm silicone membranes, attachment of cells was examined after seeding 0.5 ml of cells in solution at 3 different densities – 125,000, 250,000 and 500,000 cells/ml. Attachment was examined intermittently up to 48 hrs after initial seeding by washing unattached cells from the surface of the strip using ice-cold PBS and scraping the remaining attached cells into 100µl of PBS. Cells were transferred to a 0.5ml microtube and centrifuged at 650 *g* for 5 min. Supernatant was removed and samples were stored at -85°C or further analysis. Cell number was analysed using the Hoechst method described below (2.7.1.4).

2.3.5 Surface treatment assay

To investigate the effect of silicone substrate surface treatment on cell attachment, 0.5 ml of cells in solution (density 250,000 cells/ml) were seeded on 4 different substrate surfaces. All silicones were sterilised by spraying with 70% alcohol. Strips were then washed twice using sterile water and allowed to dry. Surface treatments were as described in Table 2.3. Surface 1 received no further treatment. Surface 2 was coated with collagen as described in section 2.3.1. Surface 3 was collagen-coated then exposed overnight to UV light (100 microwatts/sec) in the laminar flow hood.

Surface 4 had no collagen, and was exposed to UV light. Cell attachment was determined over 48 h using the Hoechst method (Section 2.7.1.4).

Surface	Collagen Coat	UV light
1	-	-
2	+	-
3	+	+
4	-	+

Table 2.3 Silicone surface conditions for assessing cell attachment

2.4 Two Dimensional Mechanical Loading of Mesenchymal Stem Cells.

A mechanical stimulus in the form of cyclic tensile strain was applied to the mesenchymal stem cells using a mechanical rig to stretch a substrate that the cells were attached to.

2.4.1 The Rig

The rig is a custom made mechanical device (Mechanical Engineering, Trinity College, Dublin) that is capable of delivering cyclic uniaxial stretch (Figure 2.2). The stainless steel equipment employs a 12V dc geared motor to drive a cam that delivers the cyclic stretching to a linear slide assembly. The slide assembly has a 26mm stroke and uses an eccentric circular cam to deliver displacement. The motor is connected to a transducer (Farnell Instruments Ltd., England) that is connected to the mains supply.

2.4.2 Application of Mechanical Load

Tensile strain was applied to the cells using the rig. The rig was prepared by checking the cam was set to the minimum position, removing the screws and clamps from their position and placing an uncovered petri dish under the linear slide assembly. One seeded collagen-coated strip was carefully removed from the petri dish. It was

aligned perpendicularly across the clamps and fixed into position one side at a time so that it was firm between the clamps with zero strain applied. The petri dish was filled with enough supplemented DMEM to just cover the strip. The apparatus was incubated at 37°C in 95% air and 5% CO_2. Power connections were established. Fresh supplemented DMEM was added to the control strip and this was also moved to the incubator. The rate of stretch was set to a frequency 0.17 Hz by adjusting the voltage from the transducer that was connected to the power supply accordingly so that the strip would complete one stretch-relax cycle every 6 seconds, calculated using a timer. The cells were stretched for 1 - 14 days. Strain magnitudes were adjusted from 2.5% to 5% to 7.5% to 10% by changing the cam on the rig.

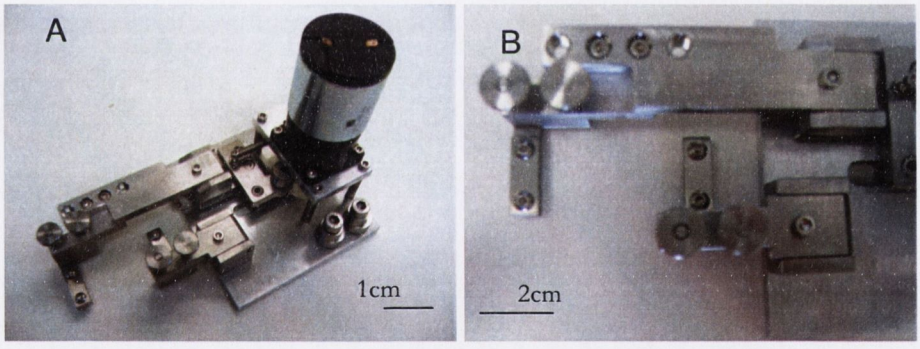


Figure 2.2 Uniaxial strain device

A) Uniaxial strain device (Moretti et al. 2004). B) Clamps between which substrate is attached. One side is fixed; the other is displaced by an eccentric cam and linear slide assembly.

2.5 Cell Treatments

2.5.1 Treatment with osteogenic factors

For static experiments, 2 days after seeding, cells were treated with osteogenic factors (OF; 0.68 nM dexamethasone (Sigma-Aldrich, England) 10 mM β-glycerophosphate (Sigma-Aldrich, England) and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, England)). Medium was removed from each well and replaced with 1 ml of medium containing the osteogenic supplements. For strips in petri-dishes, medium was removed and replaced with 10ml of supplemented medium. Medium in control wells/dishes was replaced with fresh DMEM. Medium was changed every 3- 4 days by removing half of the medium and replacing it with the same amount of fresh supplemented medium to make use of any soluble bioactive factors that may be released by the cells.

2.5.2 ERK inhibitor U 0126

The ERK inhibitor 1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio]butadiene (U0126; Calbiochem, Nottingham, UK; (Favata et al. 1998)) was stored as a 1mM stock solution in dimethylsulfoxide (DMSO; LGC Promochem, Middlesex, UK) at -20°C, and diluted to a final concentration of 2μM in warm culture media for addition to cultures. Medium containing U0126 was replaced every 3 days during the treatment. U0126 is a selective inhibitor of MEK1 and MEK2, upstream kinases in the ERK1/2 pathway (Favata et al. 1998).

2.5.3 p38 inhibitor SB 203580

The p38 inhibitor 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580; Calbiochem, Nottingham, UK; (Cuenda et al. 1995) was stored as a 1mM stock solution in dimethylsulfoxide at -20°C, and used at a final concentration of 10μM. As SB203580 is light sensitive, the cells were treated in the dark for the duration of the study. SB 203580 is a specific inhibitor of the p38 MAPK (Cuenda et al. 1995).

2.5.4 Phosphatidylinositol 3-kinase (PI3-Kinase) inhibitor LY294002

The PI3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; Cell Signalling Technology, Danvers, USA, Vlahos et al.(Vlahos et al.

1994)) was stored as a 10mM stock solution in dimethylsulfoxide at -20°C, and used at a final concentration of 2μM. As LY294002 is light sensitive, the cells were treated in the dark for the duration of the study. Medium containing the LY294002 was replaced every 3 days during the treatment. LY294002 is a specific inhibitor of PI3-kinase.

2.5.5 L-type calcium channel blocker Nicardipine

The L-type voltage-sensitive calcium channel blocker 1,4-Dihydro-2,6-dimethyl-4-[3-nitrophenyl]methyl-2-[methyl- (phenyl-methyl)amino]-3,5- pyridinedicarboxylic acid ethyl ester (nicardipine hydrochloride; Sigma-Aldrich, Wexford, Ireland; (Iwanami et al. 1979)) was stored as a 1mM stock solution in dimethylsulfoxide at -20°C, and used at a final concentration of 0.5μM. Nicardipine belongs to a group of calcium-channel blockers called dihydropyridines. L-type calcium channels have a unique pharmacological sensitivity to dihydropyridine agonists and antagonists.

2.5.6 Calpain inhibitor MDL 28170

The cell-permeable peptidyl-aldehyde calpain inhibitor MDL 28170 (carbobenzoxy-valinyl-phenylalaninal also known as calpain inhibitor III Z-Val-Phe-CHO; Calbiochem, Nottingham, UK; (Mehdi et al. 1988)) was stored as a 20mM stock solution in dimethylsulfoxide at -20°C, and used at a final concentration of 10μM. MDL 28170 is a potent cell permeable synthetic calpain inhibitor (Donkor 2000).

2.5.7 c-Jun NH2-terminal kinase (JNK) inhibitor D-JNKI1

The JNK inhibitor, D-JNK-1, (JNK$_i$; Alexis Biochemicals, Lausanne, Switzerland) was obtained as a 1mM stock dissolved in sterile PBS and was stored at -20°C. The stock was diluted in pre-warmed culture medium to a final concentration of 1μM. JNK$_i$ is a protease resistant synthetic peptide based on the cytoplasmic inhibitor JNK-binding domain (JBD) of the (Dickens et al. 1997). JNK$_i$ peptide inhibits the interaction between JNK and its substrates, blocking activation of the transcription factor c-Jun by JNK (Bonny et al. 2001).

2.5.8 Stretch activated cation channel (SACC) inhibitor gadolinium III chloride

The stretch-activated cation channel inhibitor gadolinium III chloride (Sigma-Aldrich, Wexford, Ireland; (Yang and Sachs 1989)) was stored as a 2mM stock solution in dH_2O at -20°C, and used at a final concentration of 10μM. SACC are nonselective membrane channels (Yang and Sachs 1990) sensitive to membrane tension (Saitou et al. 2000; Sokabe et al. 1991). The non-selective channels are reversibly blocked by the trivalent lanthanide gadolinium, which is the most potent blocker for mechanoreceptive transducers (Yang and Sachs 1989).

2.6 Substrate Analytical techniques

2.6.1 Atomic Force Microscopy (AFM) analysis of Substrate surface

Uncoated silicone strips, collagen-coated silicone and collagen-coated silicone that had been exposed to UV light were labelled, placed on glass coverslips and imaged blindly. The coverslips were placed on the AFM scanner (Asylum Research, Santa Barba, USA) directly above the objective lens of the inverted microscope. When focused in this position, the AFM cantilever tip could be visualised and placed over a specific location on the silicone strip. The AFM cantilevers used for indentation were rectangular NSC36 cantilevers, with a stiffness range from 0.5 to 2.5 N/m (Mikromasch, Germany). The stiffness and sensitivity of the cantilever were calibrated in air using the Sader method (Sader, 1998). The AFM cantilever was brought to the substrate by engaging in contact mode. This allowed any cantilever deflection during advancement towards the silicon surface to be controlled in a feedback loop that maintains deflection at a predetermined 'setpoint' value via appropriate movement of the piezo driver. This ensured a 'soft landing' and minimises risk of tip damage and accidental damage to the silicon membrane. An image of the silicone substrate was then acquired. Using Asylum Research software images were analysed and a 3D reconstruction was created. Surface roughness was calculated using Asylum Research software.

2.6.2 Scanning Electron Microscopy analysis of Substrate surface

Dry samples of silicone and collagen-coated silicone strips were mounted on 10mm aluminium stubs using conductive carbon cement, Leit C. When dry, a gold film of approximately 10 - 30nm was grown by means of a Polaron sc500 sputter coater at 25mA for approximately 3 minutes. Micrographs were obtained with a Hitachi S-4300 Field Emission Scanning Electron Microscope operating at 5kV acceleration voltage with a tungsten filament and equipped with an energy-dispersive X-ray microanalysis system (EDAX 9100). The SEM microscope covers the practical magnification range from 20× to 100000×. The resolution is 2.5nm at 5Kv. Acceleration voltage is 0.2 – 30Kv in 100V steps.

2.6.3 Tensile tests

Sample grips were mounted on a Zwick Z005 materials testing system with a 5N load cell attached. Four samples from each of the 4 treatments listed above (Table 2.3) were tested. Each test section was cut to a width of 3.5mm using a custom-made cutter comprising of 2 scalpel blades 3.5mm apart, and loaded in the longitudinal direction. A preload of 0.005N was applied to each sample after which, each specimen was strained to 100% at a rate of 3.33%/s. The stress was calculated as the load divided by the original cross sectional area and the strain was calculated as the change in length divided by the original length. The Young's modulus was determined from the linear slope of the corresponding stress-strain curve from 0 to 10%.

2.7 Cellular analytical techniques

2.7.1 Examination of Proliferation

Proliferation was examined by assessing the synthesis of DNA through ^{3}H-thymidine uptake (van Susante, 2001). During the last 24 hours of the experiment, strained and control unstrained cells were pulsed with 2.0 μCi/ml methyl tritiated-thymidine (Amersham, UK). At the end of the 24 hour period, strips were removed and washed with 5 ml of pre-warmed PBS and placed separately in new petri-dishes. Using a cell scraper (Sarstedt, Wexford, Ireland) cells were removed from the substrate into 250 μl of pre-warmed medium. The cells suspension was added to a 0.5ml microtube

(Sarstedt, Wexford, Ireland) and centrifuged at 2000g for 5 min to obtain a cell pellet. Supernatant was removed using a pipette and pellets were stored at -20°C for analysis.

2.7.1.1 Cell Digestion.

Batches of frozen samples were defrosted at room temperature. Cells were digested by adding 150µl of 0.1 % papain in buffer (PBS, 5mM EDTA pH8, 5mM cysteine-HCl (Sigma-Aldrich, Dublin, Ireland)). After a gentle vortex samples were incubated at 60°C overnight.

2.7.1.2 Proliferation analysis

120µl of cell digest was transferred to a clear 96-well plate (Sarstedt, Dublin). Cells were harvested onto a filtermat (PerkinElmer, Dublin, Ireland) using a TomTech cell harvester. The Filtermat was allowed to dry then placed inside a plastic bag (PerkinElmer, Dublin, Ireland), to which 5ml of scintillation cocktail (PerkinElmer, Dublin, Ireland) was added. The bag was rolled to ensure even distribution of scintillation cocktail throughout the filtermat, then heat sealed and accurately aligned inside a scintillation reader cassette. The cassette was placed in a beta-plate scintillation counter and a 'stop' plate added above. A tritiated thymidine protocol was selected on the computer and radioactive counts were automatically detected. Counts per sample were normalised to the total DNA in each sample (see 2.7.1.4).

2.7.1.3 DNA quantification using Hoechst 33258 - Standard Curve

Standard curves were generated by quantifying the signal from known concentrations of DNA from papain digested cells. MSCs were counted using a hemocytometer. A sample containing $2x10^6$ cells was centrifuged at 2000*g* for 5 min to obtain a cell pellet. This was digested as described above (2.7.1.1) in 2 ml of 0.1 % papain solution to give 1 x 10^6 cells/ml. Microtubes (1.5ml) with volumes corresponding to $5x10^4$ cells, $3x10^4$ cells, $2x10^4$ cells, $1.5x10^4$ cells and $1x10^4$ cells, were taken from the digested sample and dilutions were made (in papain solution) to give $5x10^3$ cells, $2.5x10^3$ cells, $1.25x10^3$ cells, and 625 cells. Samples for each cell number were repeated in triplicate. 200µl of 0.01% Hoechst 33258 in buffer (10 x 10mM Tris, 1mM Na_2EDTA, 1M NaCl, pH 7.4) was added to each microtube, ensuring protection from light. After gentle vortexing the contents of each microtube were transferred to a black 96-well plate (Sarstedt, Wexford, Ireland) Plates were analysed on a Labsystems Multiscan

fluorescent plate reader (Fluroskan Ascent, Germany). The excitation wavelength was 365nm and the emission wavelength was 458 nm.

2.7.1.4 DNA Assay

Cell digests were gently vortexed and 3×10µl samples from each cell digest were added to empty 0.5ml microtubes. 200µl of 0.01 % Hoechst was added to each microtube, ensuring protection from light. After light vortexing the samples were transferred to a black 96-well plate which was read on a fluorescent plate reader at an excitation of 365nm and emission of 458nm.

2.7.2 Histological methods

Cells were examined for viability and the expression of the bone related proteins collagen type I and osteocalcin by fluorescence immunocytochemistry, and markers of mineralisation by examining calcium deposits using Alizarin Red and Von Kossa stains. All histological methods on mechanically loaded samples proceeded by removing the outer 5 mm from the strip that was clamped in the rig, dividing the remaining part of the strip into equal sections and placing each section onto a separate slide. For static studies cover-slips were stained in 24-well plates or removed to separate glass slides. A wax border was created around the substrate using a PAP pen (Agar Scientific, England), wherein all solutions were contained to a maximum volume of 40µl.

2.7.2.1 Fixation of Cells

Before histological examination, samples were fixed by adding excess paraformaldehyde (4% paraformaldehyde in dH_20; pH 7.4; Sigma-Aldrich, England) to each sample for 30 min at room temperature. The paraformaldehyde was removed and samples were stored in Tris-buffered saline (TBS; 20mM TrisHCl, 150mM NaCl; pH 7.4; Sigma Aldrich, England) or H_2O (for inspection of mineralisation), at 4°C until required for histological examination.

2.7.2.2 Fluorescence immunocytochemistry staining for Collagen I, osteocalcin, CBFα1, p38, pI3Kinase, endoglin and Thy1.

Cells were permeabilised by covering the silicone strip in 0.1% Triton-X100 in TBS for 10 minutes (Sigma, Aldrich, England) and re-fixed in paraformaldehyde for 30 min at room temperature. Cells were washed three times in TBS (5 min each). Non-reactive sites were blocked for 2 hours at room temperature using blocking buffer (2% BSA in TBS with 0.1% Triton-X100 and 20% heat-inactivated horse serum, (Gibco BRL, Dublin). The slides were placed in a humidified chamber and a primary antibody (see Table 2.4) was added overnight.

Cells were washed three times (5 min each) with TBS and incubated with an appropriate secondary anti-goat IgG (1:50 in blocking buffer; Vector, California, USA; see Table 2.4) for 1 hr at room temperature. Cells were washed again three times with TBS. Extravadin FITC (1:50 TBS; Sigma Aldrich, England) was added for 1 hour at room temperature in a darkened room. Cells were washed 6 times in distilled water. In some cases nuclei were counter-stained with 4',6-diamidino-2-phenylinodole (DAPI) stain (Sigma-Aldrich, Dublin, Ireland). The silicone strips were transferred to a new slide and mounted cell side up in Vectashield® (Vector, California, USA). A coverslip was placed in top and the edges were sealed with nail varnish. Slides were observed at 40× magnification with a fluorescence microscope (Leitz Orthoplan Microscope, Leica Microsystems AG, Wetzlar, Germany) using Improvision software (Improvision, Coventry, UK), or a confocal microscope (Zeiss, LSM-510-META). Cells were observed under excitation 490 nm; emission, 520 nm for FITC labelled antibodies. Images were taken with an AxioCam digital microscope camera (Zeiss, Karl Zeiss, UK).

2.7.2.3 Fluorescence quantification

Fluorescence intensity was quantified using a pixel-intensity method modified from that described in Lehr et al (Lehr et al. 1997). Digital images were opened with Adobe® PhotoShop® Creative Suite 2 Version 9.0 (Adobe Systems Incorporated, San Jose, CA, USA). Using the magic wand tool from the select menu, the cursor was placed in a dark area of the image containing no cells. The tolerance level of the magic wand tool was adjusted so that all contacting extra-cellular dark areas were

selected. Using the 'similar' command in the select menu, all dark areas in the image containing no cellular areas were selected by adjusting the tool threshold appropriately. Subsequently the background was deselected and the foreground containing the stained cells was selected by choosing the Inverse tool in the Select menu. By selecting Expanded View and Show Statistics in the histogram window, the mean staining intensity could be recorded. This was repeated for 4 images of each treatment, with approximately 20 cells in each image.

Protein Target	**Antibody Source**	**2°Antibody**	**Antibody dilution in blocking buffer**	**Supplier**
Osteocalcin	Goat Polyclonal	Biotinylated horse anti-goat IgG	1° 1:1000 2° 1:50	Diagnostic Systems Labs: DSL-7600
Collagen type I	Goat Polyclonal	Biotinylated horse anti-goat IgG	1° 1:500 2° 1:50	Santa Cruz Biot, Cal. USA: sc-8784
Cbfα1*	Rabbit Polyclonal	Biotinylated goat anti-rabbit IgG	1° 1:200 2° 1:50	Alpha Diagnostic International: CBFA11-A
Phospho-p38	Mouse Monoclonal	Biotinylated horse anti-mouse IgG	1° 1:250 2° 1:50	Abcam: ab32557
PI3 Kinase	Mouse Polyclonal	Biotinylated horse anti-mouse IgG	1° 1:250 2° 1:50	Santa Cruz Biot, Cal. USA sc-56464
Thy-1 (CD90)	Mouse Monoclonal	Biotinylated horse anti-mouse IgG	1° 1:250 2° 1:50	AbCam, Cambridge, UK : ab225
Endoglin (CD105)	Rabbit Polyclonal	Biotinylated goat anti-rabbit IgG	1° 1:250 2° 1:50	Santa Cruz Biot, Cal. USA sc-20632
Active Caspase-3 **	Rabbit polyclonal	Biotinylated goat anti-rabbit IgG	1° 1:150 2° 1:50	Promega Corporation, Wisconsin, USA: G748
BMP2	Rabbit polyclonal	Biotinylated goat anti-rabbit IgG	1° 1:250 2° 1:50	AbCam, Cambridge, UK ab14993
Phospho-ERK	Rabbit polyclonal	Biotinylated goat anti-rabbit IgG	1° 1:250 2° 1:50	Santa Cruz Biot, Cal. USA: sc-23759
Phosphorylated JNK	Mouse monoclonal	Biotinylated horse anti-mouse IgG	1° 1:200 2° 1:50	Santa Cruz Biot, Cal. USA sc-6254

Table 2.4 Antibodies used for immunocytochemistry

* Cells were incubated with proteinase-K (0.02%; dH_2O) prior to incubation with primary antibody to permeabilise the nucleus.

** Specifically recognises a neo-epitope only present in the active cleaved form of caspase-3.

2.7.2.4 Cell Death (Apoptosis) Detection

Apoptotic cell death was identified using the DeadEnd™ Colorimetric TUNEL system (Promega Corporation,Madison, USA) in accordance with the manufacturers instructions. Fixed sections of the silicone strip were washed twice with TBS. Cells and nuclei were permeabilised with 0.2% Proteinase-K and 0.2% triton for 5 minutes then washed 3 times with TBS. This was followed by incubation with equilibration buffer for 10 minutes. Biotinylated nucleotide was incorporated at the 3'-OH DNA ends by adding the reaction mix of 1% biotinylated nucleotide mix and 1% terminal deoxynucleotidyl Transferase (TdT) Enzyme in equilibration buffer at 37° for 1 hour. 20X SSC was diluted 1:10 with distilled water and added for 15 minutes to terminate the reaction. Unincorporated nucleotides were removed by washing in TBS three times, 5 minutes each. Endogenous peroxidases were blocked by adding hydrogen peroxide (1:100 distilled water) for 3 – 5 mins, and strips were washed twice, 5 minutes each. A solution of horseradish-peroxidase-labeled Streptavidin (1:500 TBS) was incubated at room temperature on the strips for 40 minutes to bind to the biotinylated nucleotides. After washing, the peroxidase was detected using the peroxidase substrate hydrogen peroxide, the stable chromagen diaminobenzadine (DAB) and nickel ammonium sulphate. Upon a colour change (approx. 3 – 10 mins), the strips were washed several times in distilled water, dehydrated, mounted in DPX and left overnight to dry. Observation was by x 40 magnification light microscopy. The nuclei of TUNEL positive cells stained darker than the negative stained nuclei. Positive cells were counted and expressed as a percentage of the total number of cells in the view or up to 400 cells in total.

2.7.2.5 Alizarin Red Staining for Extracellular Calcium Deposition

Alizarin Red S had been used extensively in histological methods for the detection of calcium deposits (Puchtler et al. 1969). Slides were placed in 250 ml of Alizarin Red (2% in dH_2O, Sigma-Aldrich, England) for 30 sec before washing in distilled water and dehydrating through 70% alcohol, spirit, absolute alcohol and xylene for 2 mins each. Silicone samples were imaged and photographed instantenously as DPX could not be used with the silicone material. For plastic coverslips, a drop of DPX was

placed on each section, and allowed to dry. The slides were examined at 10×, 20× and 40× using phase contrast microscopy (Olympus CKX41, Japan). Images were acquired by a ColorView camera (Soft Imaging System, Germany) and analysed using Analysis software.

2.7.2.6 Alizarin Red S dye extraction and assay

ARS staining is particularly versatile in that the dye can be extracted from the stained monolayer and readily assayed. The following method, based on the procedure described in Gregory et.al.(2004), was used to extract the Alizarin Red dye from the stained monolayer and semi-quantify the calcium present in the samples. Fixed monolayers on coverslips in 24 well plates were washed twice with excess dH_2O prior to addition of 1ml of 40mM ARS (pH 4.1). Plates were incubated at room temperature (RT) for 20 min on a rocker roller platform. Unincorporated dye was aspirated off using a transfer pipette and wells were washed 4 times with excess dH_2O (5 mins; on shaker). Plates were tilted for 2 mins to facilitate removal of excess water and reaspirated. For quantification of staining, 500µl of 10% (v/v) acetic acid was added for 30min at RT while shaking. The monolayer was then scraped from the coverslip with a cell scraper (Sarstedt, Wexford, Ireland) and transferred with 10% acetic acid to a 1.5ml microtube (Sarstedt, Wexford, Ireland) using a transfer pipette (Sarstedt, Wexford, Ireland). After vortexing for 30s the slurry was overlaid with 312.5µl mineral oil (Sigma-Aldrich, Dublin) heated to 85°C for 10 min and transferred to ice for 5 min. Care was taken to avoid opening the tubes until fully cooled. The slurry was then centrifuged at 20,000*g* for 15 min and 500µl of the supernatant was removed to a new 1.5ml microtube. 100µl of 10% (w/v) sodium hydroxide was added to neutralise the acid. Aliquots (150µl) of the supernatant were transferred in triplicate to an opaque-walled, transparent-bottomed 96-well plate and read at 405nm.

2.7.2.7 Von Kossa Staining for Extracellular Calcium Phosphate Deposition

Cells were incubated in 5% silver nitrate (500 µl per slide in dH_2O; Sigma-Aldrich, England) under an electric lamp for 1 hour at room temperature. Cells were washed five times in distilled water (3 min each) and placed in 5% sodium thiosulphate (in dH_2O, Sigma-Aldrich, England) for 2 min. Cells were washed again three times in distilled water and placed in Toludine Blue for 10 secs. Cells were washed three times before dehydrating through 70% alcohol, spirit, absolute alcohol and xylene for 2

minutes each. Cells were images instantaneously (for silicone substrates) or a drop of DPX was placed over each section, and a glass coverslip placed on top. The slides were examined at 10×, 20× and 40× using phase contrast microscopy (Olympus CKX41, Japan).

2.8 Protein quantification

Protein concentration in cultured cell samples was assessed using the bicinchoninic acid (BCA) Protein Assay (Pierce, Lieden, Netherlands) in accordance with manufacturers' instructions. Standards were prepared from stock ampules of 2mg/ml bovine serum albumin (BSA) provided in the kit. Dilutions were made with dH_2O to prepare a range of standards from 1500μg/ml to 0μg/ml. Samples (10μl) and standards (10μl) were added to a 96-well plate (Sarstedt, Wexford, Ireland) in duplicate and working reagent concentrate (1:50 reagent B in reagent A, as per manufacturers instructions (Pierce, Lieden, Netherlands; 200μl) was added to both. Plates were covered and incubated at 37°C for 30min. Plates were allowed to cool, then absorbance was assessed at 562nm using a 96-well plate reader (EIA Multiwell reader, Sigma-Aldrich, Dorset, UK). The concentration of protein in samples was calculated from the regression line plotted (Excel, Microsoft Office 2003) from the absorbance of the BSA standards.

2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.9.1 Preparation of total cell culture protein.

To analyse expression of protein in MSC cultures, cells were washed in ice-cold PBS (Sigma-Aldrich, Dublin) before harvesting by scraping cells from silicone substrates into lysis buffer (120μl/strip; 25mM HEPES, 5mM $MgCl_2$, 5mM EDTA, 5mM dithioethreitol (DTT), 2mM PMSF, 10μg/ml leupeptin, 6.25μg/ml pepstatin, 6.25μg/ml apoprotein, 10μl/ml sodium orthovanodate, pH 7.4) on ice using a cell scraper. Lysates were collected and homogenised (×20 strokes) in lysis buffer on ice using a 1ml glass homogeniser (Jencons, Bedfordshire, UK), then transferred to 0.5ml microtubes. The total protein concentration of each sample was determined using the BCA method (2.8). Samples were diluted by a factor of 2 with 4X SDS sample buffer (150 mM Tris-HCl pH 6.8, 10% glycerol (v/v), 4% SDS (w/v), 5% β-

mercaptoethanol (v/v), 0.002% bromophenol blue (w/v), and samples were boiled for 5 mins using a heat block (Stuart SBH1300, Carl Stuart, Dublin, Ireland) Samples were stored at -85°C for analysis.

2.9.2 Preparation of polyacrylamide gels

Polyacrylamide separating gels (1mm thick) with a monomer concentration of 12% overlaid with stacking gel were cast by setting them between two 10cm wide glass plates (Sigma-Aldrich, Dorset, UK) which were mounted on a gel electrophoresis unit (Sigma Techware, Dorset, UK) using spring clamps. The upper and lower reservoirs of the unit were flooded with electrode running buffer (25mM Tris-base, 200mM glycine, 0.1% SDS (w/v)). Samples (10µl) were loaded into the wells and proteins were separated by the application of a 32mA current to the gel apparatus. Pre-stained molecular standards (5µl) were used to confirm the molecular weight of protein bands. The migration of the bromophenol blue was monitored and the current switched off when the dye reached to bottom of the gel (approx. 30 min).

2.9.3 Semi-dry electrophoretic blotting of proteins

The gel was removed from the apparatus and placed on top of a sheet of nitrocellulose blotting paper (0.45µm pore size; Sigma-Aldrich, Dorset, UK), which had been cut to the approximate size of the gel and soaked in ice-cold transfer buffer (25mM Tris-base pH 8.3, 192mM glycine, 20% methanol (v/v), 0.05% SDS (w/v)). A sandwich was made by placing one piece of filter paper (Standard Grade N0. 3, Whatman, Kent, UK) on top of the gel and one piece beneath the nitrocellulose paper. This sandwich was soaked in transfer buffer and placed on the platinum-coated titanium electrode (anode) of a semi-dry blotter (Sigma-Aldrich, Dorset, UK). The lid of the blotter (stainless steel cathode) was shielded with a mylar cut-out (Sigma-Aldrich, Dorset, UK), ensuring that all the current passed through the sandwich. A constant current of 225mA was applied for 90 min.

2.10 Western immunoblotting

After transfer of proteins to nitrocellulose membrane, non-specific binding was blocked by incubating the nitrocellulose in TBS containing 5% BSA (w/v) for 2 hours at RT with gentle agitation. The membrane was washed with TBS-T (3x15

min). The nitrocellulose blotting paper was then probed with the following primary antibodies (Table 2.5):

- a rabbit polyclonal anti-phospho-ERK1/2 recognising ERK 1 phosphorylated at Thr-202 and Tyr-204 and ERK 2 phosphorylated at Thr-185 and Tyr-187 (1:500 dilution TBS-T containing 0.2% BSA; Santa Cruz Biotechnology Inc, California, USA).
- a rabbit polyclonal anti-phospho-p38 recognising p38 phosphorylated at thr-180 and tyr-182 only (1:500 dilution TBS-T containing 0.2% BSA; Santa Cruz Biotechnology Inc, California, USA)
- a rabbit polyclonal antibody raised against the synthetic peptide corresponding to amino acids 45-60 of BMP2 protein (1:500 dilution in TBS-T containing 0.2% BSA (w/v); Santa Cruz Biotechnology Inc. California, USA)
- a mouse monoclonal antibody raised against the epitope maping the gamma-carboxylated residue at position 17 of the full length osteocalcin protein of cow origin (1:500 dilution in TBS-T containing 0.2% BSA (w/v); Santa Cruz Biotechnology Inc. California, USA)

This was incubated overnight at 4°C with gentle agitation. The membrane was then washed with TBS containing 0.05% Tween (TBS-T, v/v, 3x15mins) and incubated with an appropriate secondary antibody (Table 2.5) that was horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich Dorset, UK) for 2 hrs at RT, followed by 3 washes of 20 mins in TBS-T). A chemiluminescent detection chemical (SuperSignal Ultra; Pierce, Lieden, Netherlands) was added and the blotting paper exposed to 5 x 7in photographic film (Hyperfilm®, Amersham, Buckinghamshire, UK) and developed using a Agfa film processor (Agfa-Gervart Group, Dublin, Ireland).

Following western immunoblotting for pERK (1/2), p-38, BMP2 and osteocalcin, blots were stripped with an antibody stripping solution (1:10 dilution in dH_2O; Reblot Plus Strong antibody stripping solution; Chemicon, California, USA) and reprobed for the following primary antibodies respectively (Table 2.5):

- a rabbit polyclonal antibody raised against a peptide mapping the C-terminus of ERK2 of rat origin (1:500 dilution in TBS-T containing 0.2% BSA (w/v); Santa Cruz Biotechnology Inc. California, USA)

- a rabbit polyclonal antibody raised against the recombinant full length p38 protein of human origin (1:500 dilution in TBS-T containing 0.2% BSA (w/v); Santa Cruz Biotechnology Inc. California, USA)
- a mouse monoclonal antibody raised against the full length native protein of human erythrocytes (1:500 dilution in TBS-T containing 0.2% BSA (w/v); Santa Cruz Biotechnology Inc. California, USA) (for osteocalcin and BMP2).

Protein Target	**Antibody source**	**Secondary antibody**	**Antibody dilution % BSA in TBS-T**	**Protein band Size**	**Supplier**
Total ERK	Rabbit polyclonal	Goat anti-rabbit IgG	1o 1/500 2% BSA 2o 1/1000 2%	42 kDa	Santa Cruz Biotech. sc-154
Phosphorylated ERK1/2 (Thr-185 and Tyr-187)	Goatpolyclonal	Rabbit anti-goat IgG	1o 1/500 2% BSA 2o 1/1000 2%	42/4 kDa	Santa Cruz Biotech. sc-16982
Total p38	Rabbit polyclonal	Goat anti-rabbit IgG	1o 1/500 2% BSA 2o 1/1000 2%	38 kDa	Abcam: ab7952
Phosphorylated p38 (thr-180 and tyr-182)	Rabbit monoclonal	Goat anti-rabbit IgG	1o 1/500 2% BSA 2o 1/1000 2%	42 kDa	Abcam: ab32557
Osteocalcin	Mouse monoclonal	Goat anti-mouse IgG	1o 1/500 2% BSA 2o 1/1000 2%	12 kDa	Abcam ab13418
BMP2	Rabbit polyclonal	Goat anti-rabbit IgG	1o 1/500 2% BSA 2o 1/1000 2%	45 kDa	Abcam ab14933
GAPDH	Mouse monoclonal	Goat anti-mouse IgG	1o 1/500 2% BSA 2o 1/1000 2%	36-40 kDa	Abcam ab9484

Table 2.5 Antibodies used for western blotting.

2.10.1 Densitometry

In all cases quantification of protein bands exposed on photographic film was achieved by densitometric analysis using the GelDoc-It imaging system and LabWorks image acquisition and analysis software (UVP Bioimaging Systems, Cambridge, UK). In all cases ratios of phosphorylated target protein/target protein or target protein/total GAPDH were quoted using arbitrary units.

2.11 Enzyme activity analysis

2.11.1 Measurement of calpain activity

Clevage of the fluorogenic calpain substrate (Suc-Leu-Tyr conjugated to aminofluorocoumarin (AFC); Calbiochem, Nottingham, UK) to its fluorescent product was used as a measure of calpain activity. Following treatment (10% strain, 0.17Hz, 2 days) cells were scraped from silicone substrated using a cell scraper and homogenised in lysis buffer (120μl;, 5mM $MgCl_2$, 5mM EDTA, 5mM dithioethreitol, 2mM PMSF, 10μg/ml leupeptin, 10μg/ml pepstatin, 10μg/ml apoprotein, pH 7.4) on ice. Samples (25μl) were incubated with the Suc-Leu-Tyr peptide (10μM; 2μl) and incubation buffer (23μl; 50mM HEPES, 10mM dithiothreitol, 20% glycerol (v/v), pH 7.4) or as an internal control incubation buffer (25μl) for 1hr at 37°C. Fluorescence was assessed (excitation, 400nm; emission, 505nm) using a spectrofluorimeter (Fluroscan Ascent FL platereader; Labsystems, Vantaa, Finland).

2.12 Image Processing

Images taken under phase-contrast microscopy were captured using a digital camera (Soft Imaging System, Germany) and acquired using Analysis software (Soft Imaging System, Germany). Enhancements to the images captured of the fluorescent staining were carried out using Photoshop software (Adobe Inc.).

2.13 Statistical Analysis

Data are expressed as means of the combined result from each of the repeated tests ± standard error of the means (SEM). Statistical analysis to compare treatments was carried out by a paired Student's t-test to determine whether significant differences existed between the populations for the apoptosis study. Unless otherwise specified

one-way analysis of variance (ANOVA) was used for all other statistical analysis when comparing more than 2 groups with Tukeys or Newman-Keuls post hoc tests. Test for normal distribution were carried out using the Kolmogorov-Smirnov test. For non-normally distributed data, the Wilcoxon-signed ranks test was used to test for significance. In all cases the alpha level was set to 0.05. All statistical analysis was carried out using Graphpad Instat software.

3 Substrate characterisation

3.1 Introduction

One method of delivering tensile mechanical stimulation *in vitro* is by seeding cells onto a flexible membrane which is capable of withstanding tensile strains, and applying a defined load using a bioreactor (Butler et al. 2000; Chen and Hu 2006). The most common substrate material for the application of tensile strain to cells in a 2D environment is silicone (polydimethylsiloxane, PDMS (Cavalcanti-Adam et al. 2002; Deutsch et al. 2000; Lateef et al. 2002; Simpson et al. 1996; Wang et al. 2005)), due to its chemical inertness (Sia and Whitesides 2003), biocompatibility (Gorman and Woolfson 2002; Musolf 1990), thermostability, transparency (Masuda et al. 2007) and elastic properties that allow the delivery of physiological and supra-physiological strains (Brown et al. 2005). Additionally, silicone membranes can withstand sterilisation by alcohol washing. However, silicone polymers are hydrophobic, thus do not promote cell adhesion. Surface treatments including plasma, UV-ozone or laser (Hillborg et al. 2004; Khorasani and Mirzadeh 2004; Morra et al. 1990) induce hydrophilic modifications, but can deteriorate over time (Williams et al. 2004). As an alternative, silicone substrates are often coated with cell adhesive proteins including collagen type I. Adhesion promoting proteins contain the Arginine-Glycine-Aspartic (Arg-Gly-Asp; RGD) amino acid sequence which is the site for cell attachment by the integrin cell surface receptors (Pierschbacher and Ruoslahti 1984; Ruoslahti 1996). This practice was introduced in seminal studies on the cell-substrate-flexibility relationship (Pelham and Wang 1997) since it provides a mechanism for cell attachment to hydrophilic materials; therefore, in this study, silicone membranes coated with collagen type I are used as a substrate material for the application of tensile strain to MSCs.

In conjunction with the alcohol immersion sterilisation technique, collagen-coated silicone was further sterilised by exposure to UV light. In addition to the anti-microbial action imparted by UV exposure, it causes topographical modification of the silicone surface. Exposure creates a hydrophilic surface and can modify surface roughness (Ouyang 2000), dependent on the homogenicity of the silicone, through the formation of an oxidised silica-like layer which also has improved mechanical properties that are sustained with increasing time after exposure (Lew et al. 2007; Oláh 2005; Sia and Whitesides 2003; Ye et al. 2006). UV is also used in scaffold fabrication for tissue engineering applications as a controllable, non-toxic

protein cross-linking agent (Fujita et al. 2005). UV irradiation produces free radicals on tyrosine and phenylalanine residues that cross-link collagen fibres (Weadock et al. 1995). When UV exposure for 20mins is used on a poly(glycolic acid)-collagen scaffold, improved cell attachment and proliferation occurs (Fujita et al. 2005). It is thought that this is as a result of the effects of UV suppressing collagen degradation and improving the retention of its adsorption onto the scaffold (Fujita et al. 2005). However, it is known that UV light also causes the break-down of the collagen type I helix (Miles et al. 2000) as well as cross-linking and the relative proportions depend on the presence of oxygen, pH of solution, type of collagen, wavelength of the UV (Fujimori 1988) and time of exposure (Suh et al. 1999).

Through precise control of specific properties of substrate materials, it is possible to direct cell behaviours in a specific way. The surface roughness of cellular substrates plays an essential role in cell attachment. Generally, the greater the level of topography of the substratum, the greater the degree of cytoskeletal involvement and time required to negotiate these surface features (Curtis and Wilkinson 1997). Increased surface roughness improves cell adhesion (Price et al. 2004) and gene expression responses (Hatano et al. 1999; Kim et al. 2006). There are a number of conflicting reports on the effects of UV on surface roughness, however all studies report a modifying effect. In most reports UV has a smoothing effect on the surface, thus reducing the surface roughness dramatically during 2 hrs of exposure, with a slight increase after 12 hrs (Ouyang 2000), and this is due to the formation of a continuous silicone oxide surface layer. A study on UV sterilisation of an electrospun scaffold found short-term UV exposure (20 seconds) to reduce surface roughness although this was also associated with lower cell coverage (Andrews et al. 2007). Opposing effects of UV exposure of silicone surfaces are reported when differences in the homogeneity of the silicone substrates is present. In heterogeneous substrates, UV exposure increases surface roughness, through what is thought to be the collapse of the oxide layer around the underlying aggregates (Oláh 2005).

Cells have the ability to detect and actively respond to the flexibility of their substrate (Pelham and Wang 1997). Force-sensing can activate mechanotransduction and result in intracellular signalling that affects cell fate (Ingber 2006). Changes in the stiffness of the extracellular matrix can have a

profound influence on the fate decisions made by stem cells (Deroanne et al. 2001; Engler et al. 2004). For example, MSCs grown on a soft substrate, which replicates the elasticity of brain tissue, adopt a neuronal phenotype whereas cells grown on a stiff substrate, which mimics bone tissue, assume an osteoblast phenotype (Engler et al. 2006; Leucht et al. 2007). Thus, for tissue engineering applications, scaffold materials with mechanical properties that mimic natural matrices provides another way of directing stem cell differentiation (Lutolf and Hubbell 2005). The intrinsic resistance of a solid material to a stress, i.e. its stiffness, is measured by the solids elastic modulus (Young's modulus) E, which is obtained by measuring the resistance offered by a material to the development of a uniaxial elastic strain (ε) in response to the applied stress (σ). It is a common citation of the stiffness of a material. Young's modulus is expressed as:

$$E = \sigma / \varepsilon \qquad (1)$$

In a detailed study on the chemical and mechanical effects of modifying substrate stiffness on cell behaviour, Brown et al (Brown et al. 2005) conclude that surface properties dominate the initial cell attachment and spreading i.e. adhesion, whereas the mechanical properties influence the long-term cell growth.

In this research, a deformable silicone membrane was used as the cellular substrate. To address the hydrophobic nature of silicone, it was coated with a thin collagen layer, followed by exposure to UV (see Methods section 2.3.1). The topography, chemical composition and hydrophobicity of *in vitro* cellular substrates can have an influence on cell interaction and behaviour; these can have an effect on cellular behaviours such as adhesion, migration, proliferation, and differentiation. Therefore, since surface topography can be affected by the preparation methods and mechanical stiffness can affect cell attachment and proliferation behaviour, it was important to characterise the silicone membrane, particularly to optimise the cell-substrate relationship. To examine the properties of the substrate to gain a better understanding of cellular responses and to optimise cell attachment, firstly the presence of collagen type I on the surface was examined using fluorescence immunological detection, scanning electron microscopy (SEM; see Methods section 2.6.2) and atomic force microscopy (AFM see Methods section 2.6.1). SEM focuses a beam of electrons on the sample. Information from the resultant low energy secondary and high energy back scatter electrons can be gathered to create an image. Silicone and collagen-coated silicone

were prepared for SEM by coating with a thin layer of gold, to improve conductivity and to attract the electron beam. AFM acquires images through translating changes in deflection of a cantilever at the end of which is a sharp tip, which is used to sense the forces between the tip and the sample. Changes in the deflection of the cantilever, as it is scanned across the sample, are measured through an optical system consisting of a laser beam reflected off the upper surface of the cantilever and onto a position-sensitive photodiode, which can measure changes in the position of the incident laser as small as 1nm. From AFM, surface roughness before and following collagen and/or UV exposure will be quantified. By performing a tensile test on silicone with and without treatments, the mechanical properties were determined (see Methods section 2.6.3). Cell seeding density and tissue culture substrate is known to affect the developmental potential of cultured cells (Jaiswal et al. 1997). Thus, the effect of membrane treatments on seeding density was examined using a DNA assay (see Methods section 2.7.1.4) to determine an optimal cell seeding density.

3.2 Results

3.2.1 Surface examination of silicone elastomer.

To detect adsorption of collagen onto the silicone surface, membranes were examined by fluorescence immunochemistry using an antibody for collagen type I (Figure 3.1A). On silicone that had received no collagen-coating (Figure 3.1Ai), only background non-specific staining was evident, and no collagen type I immunoreactivity was observed. Following incubation with a collagen type-1 solution (Figure 3.1Aii), silicone membranes displayed an irregular punctate pattern of collagen type I immunoreactivity on their surface. This finding demonstrates the presence collagen type I on the silicone membrane surface.

To obtain topographical information on the silicone membrane before and after collagen-coating, SEM was used to image membranes at 400× to 500,000× magnification and a representative magnification of 1800× in Figure 3.1B demonstrates that when membranes were imaged using SEM, there were no distinguishable topographical features between silicone (Figure 3.1Bi) and collagen-coated silicone (Figure 3.1Bii).

For AFM of silicone elastomer, samples were examined in intermittent mode and a 90μm area was raster scanned. A third group (collagen-coated + UV) was included in the surface analysis. Figure 3.1C of 3D surface reconstructions demonstrates that a variance exists in the surface roughness between (i) silicone, (ii) collagen-coated silicone, and (iii) collagen-coated silicone that has been exposed to UV light.

The average roughness can be computed from the height of each feature detected on the surface, relative to the area scanned (Figure 3.1D). To aid with accuracy when calculating surface roughness data, sections of the acquired image can be masked when artefacts are evident in order to avoid skewness created by a feature that is not native to the surface. Figure 3.1D demonstrates the variation in the ratio of roughness/surface area between samples exposed to different treatments; In masked images surface roughness increases from 0.0044nm/μm^2 for silicone to 1.1426 nm/μm^2 for collagen-coated silicone but decreases to 5.0386×10^{-4} nm/μm^2 for collagen coated silicone that has been exposed to UV; and that unmasked and masked surface roughness calculations follow the same trend between samples.

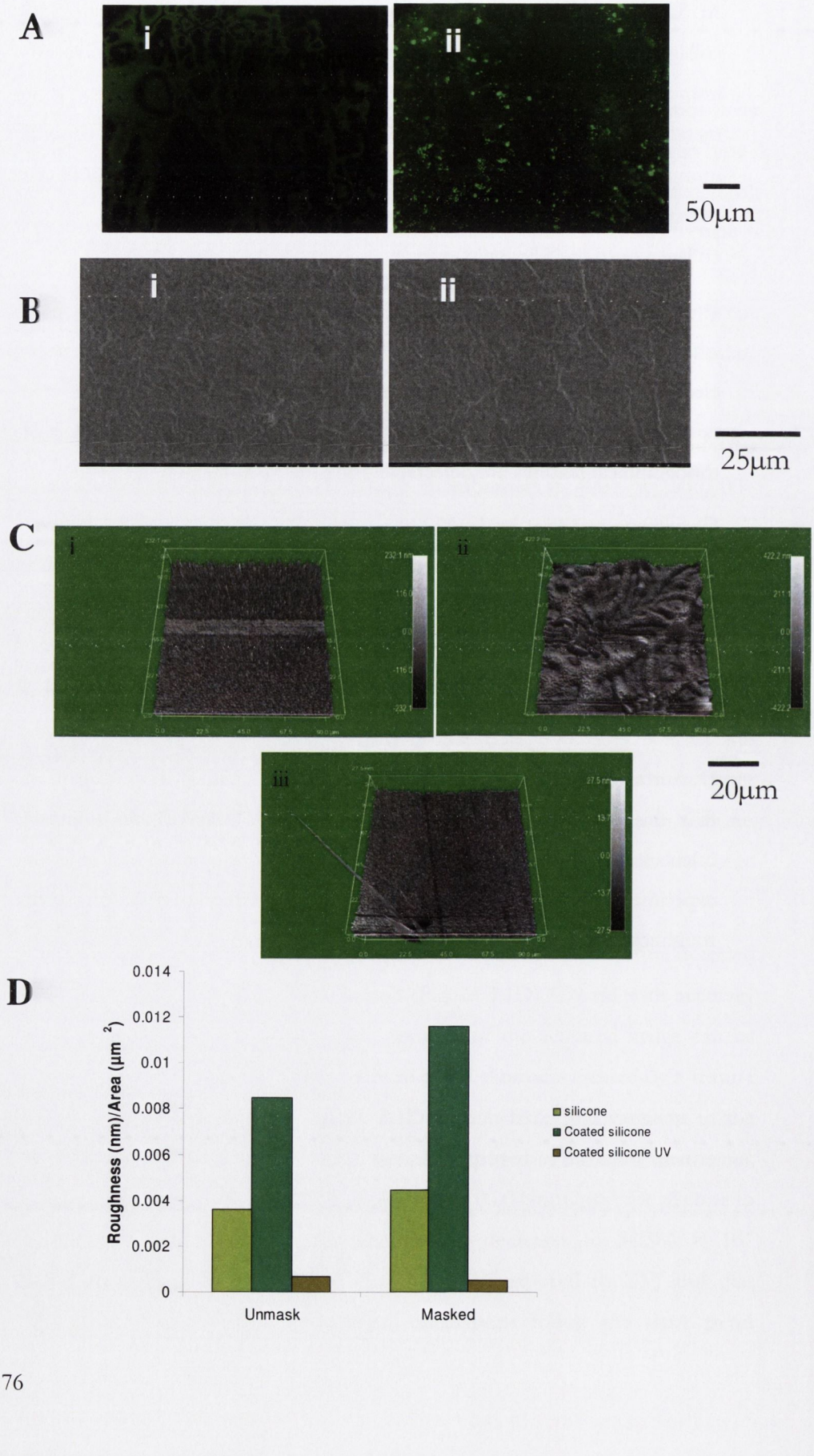
Figure 3.1 Surface analysis of silicone substrate.

A: Silicone membrane was prepared untreated (i), or coated with a 1% collagen solution (ii). Membranes were then examined using fluorescence immunochemistry for the presence of collagen type I on the surface of the material. Untrerated silicone (i) displays background non-specific immunofluorescence, while silicone coated with collagen type I (ii) shows punctate features of positive immunoreactivity, indicating the adsorption of collagen onto the silicone membrane.

B: Silicone membrane was prepared untreated (i), or coated with a 1% collagen solution (ii). Both surfaces were then imaged using scanning electron microscopy. Representative images of (i) silicone and (ii) collagen-coated silicone at 1800× magnification demonstrate that no distinguishable topographical features are detectable using the SEM technique.

C: Silicone was prepared without surface treatment (i), coated with a 1% collagen solution (ii) or coated with collagen and exposed to UV light for 12hrs (iii). 3D reconstructions of AFM 90μm^2 scans of each surface were created. Silicone (i) displays a granular appearance, fibrils can be seen on collagen-coated silicone (ii) and UV exposure has a smoothing effect on the collagen-coated silicone (iii).

D: Surface roughness increases when silicone is coated with collagen, and that this decreases following exposure to UV. Masked and unmasked calculations follow the same trend. Results are representative of 1 individual experiment, thus represents a trend for the effect of UV on surface roughness.


A
i
ii
50µm
B
i
ii
25µm
C
i
ii
iii
20µm
D
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Roughness (nm)/Area (µm 2)
Unmask
Masked
silicone
Coated silicone
Coated silicone UV

3.2.2 Silicone membrane mechanical properties.

Another surface characteristic that affects cell-substrate attachment is the stiffness of the substrate. A number of links have been identified that associate more rigorous cell attachment, spreading and then proliferation, with stiffer substrates (Brown et al. 2005; Pelham and Wang 1997). To examine the stiffness of the silicone substrates, a tensile test was carried out that can measure the Young's modulus of the test material. To determine the Young's modulus, silicone, collagen-coated silicone, collagen coated silicone that was subsequently exposed to UV light for 12 hrs, and silicone without collagen that was exposed to UV light for 12 hrs were tested in tension using a materials testing machine. The Young's modulus was determined from the first 10% of the corresponding stress-strain curve for each sample.

Figure 3.2 shows that silicone has a Young's modulus of 1.72 ± 0.03 MPa. (mean ± SEM). When the membrane was coated with collagen, this significantly increased to 1.81 ± 0.03 MPa ($p<0.05$, One way ANOVA; n=5). Exposing the collagen coated silicone to UV for 12 hrs caused the Young's modulus to significantly decrease to 1.65 ± 0.02 MPa, compared to collagen-coated silicone ($p<0.01$, One way ANOVA; n=5). Following exposure to UV alone, the Young's modulus significantly decreased to 1.56 ± 0.01 MPa compared to untreated silicone and collagen coated silicone ($p<0.01$, One way ANOVA; n=4). Finally, the Young's modulus was significantly lower for UV irradiated silicone compared to collagen-coated silicone that was exposed to UV ($p<0.01$, One way ANOVA; n=4).

While the range of stiffness measured for these 4 material treatments is narrow (1.56 ± 0.01 to 1.81± 0.03 MPa), it is possible to extract trends that follow the topographical changes that were observed through surface roughness measurements. That is, an increase in stiffness corresponds with an increase in surface roughness, and the decrease in surface roughness that is observed with exposure of the collagen coated substrate to UV light, corresponds with the decrease in stiffness measured in this test. This finding demonstrates that by adding the collagen-coating to the silicone membrane, the substrate stiffness is increasing, and that UV exposure, before or after coating with collagen, causes the stiffness of the silicone to decrease.

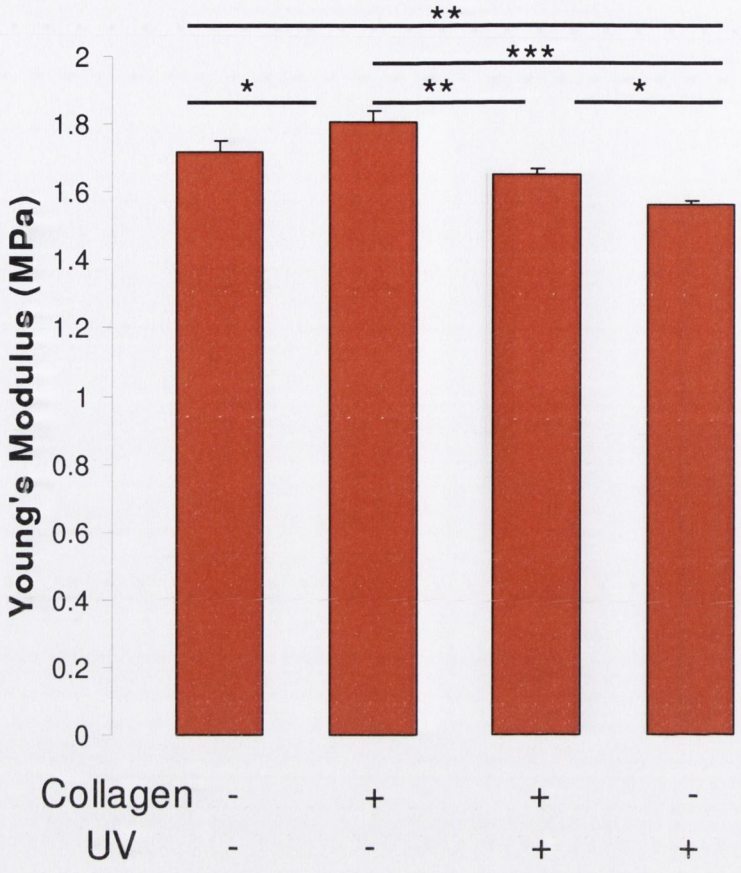


Figure 3.2 Effect of surface treatment on the Young's modulus of silicone substrate tensile mechanical modulus

The stiffness of silicone, collagen-coated silicone, collagen-coated silicone that was exposed to UV for 12 hours and silicone without collagen, but exposed to UV light for 12 hours were examined by measuring the Young's modulus of the material. Silicone that was collagen-coated was significantly stiffer than untreated silicone. Exposure of silicone and collagen-coated silicone to UV light significantly decreased the stiffness compared to uncoated and collagen-coated respectively. Results are expressed as a mean ± SEM for 4/5 independent observations One way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.2.3 Optimal MSC seeding density.

Cells were seeded onto collagen-coated silicone membranes 48hrs prior to the application of mechanical strain. In order for MSCs to populate their substrate, there is a minimum density at which they are seeded so as they are at least 50% confluent following initial attachment. However, for optimal transfer of mechanical strain from the substrate to the cells, a sub-confluent monolayer (~80%) following 2 days in culture is desirable. This is to ensure that cell dynamics are not influenced or restricted by limited space availability, in order that histology can be carried out on fixed cells and to rule out any possibility of spontaneous differentiation that can occur in MSCs that have reached confluency. To determine the optimal seeding density, 0.5ml of passage 4 cells were seeded at 3 densities – 125,000, 250,000, and 500,000 cells/ml; i.e. 62,500, 125,000 and 250,000 cells per silicone strip. Attachment was monitored 7.5, 12, 24 and 48 hrs post-seeding and cell number was measured using the Hoechst assay for quantifying DNA. Figure 3.3A shows that at the lowest seeding density of 62,500 cells, 73,347 ± 44113 cells (mean ± SEM) were attached 7.5 hrs post-seeding however following an initial increase, this declined steadily to 37,256 ± 16164 cells 48hrs post-seeding. Following seeding at 125,000 cells, 83,389 ± 32224 cells were attached 7.5 hrs post-seeding and this increased to 127,257 ± 41552 cells at 48 hrs. At 7.5 hrs post-seeding with 250,000 cells, 194,540 ± 45855 cells were attached. This increased to a maximum of 223,184 ± 54 cells after 12hrs, then declined steadily to 193,526 ± 48543 cells 48hrs post-seeding. Since cell attachment is increasing steadily at 48hrs following seeding with 125,000 cells, this is the optimal seeding density for MSCs on this substrate.

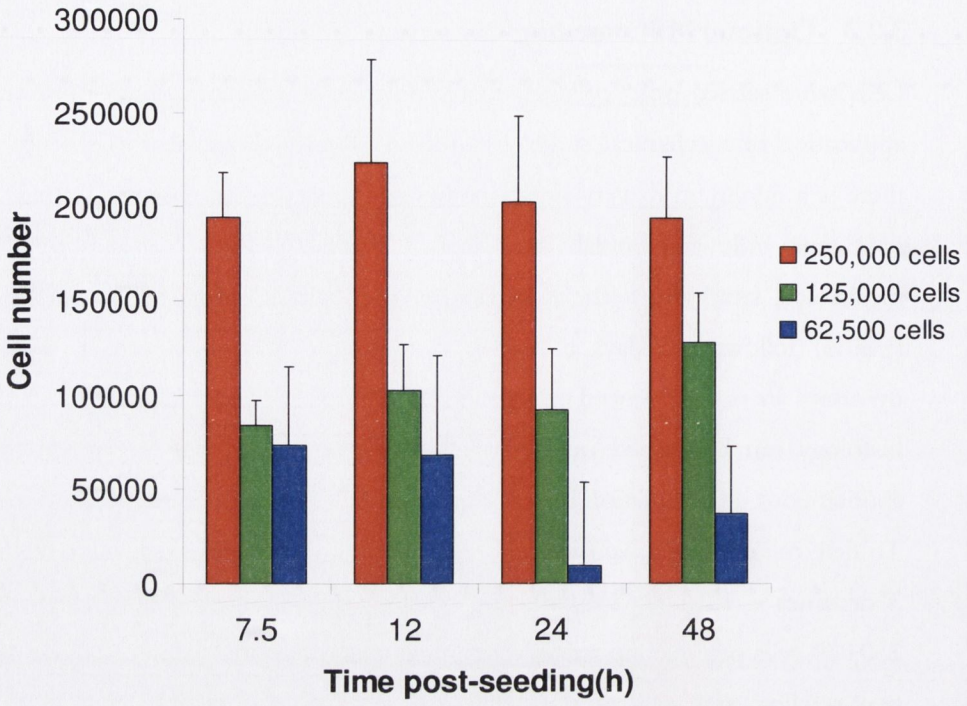


Figure 3.3 Optimal MSC seeding density for 60mm^2 silicone strips

MSCs were seeded onto collagen-coated silicone that had been exposed to UV light for 12hrs, with 62,500 cells, 125,000 cells and 250,000 cells. To determine the optimal cell-seeding density, cell attachment was monitored at intermittent time-points over a 48hr period by harvesting attached cells from strips and quantifying total cell number using the Hoechst assay.

Following seeding with the 62,500 cells, cell numbers decreased steadily over the course of the assessment. By seeding with 125,000 cell numbers increased gradually up to the end of the assessment. Seeding with 250,000 cells displayed a consistent increase in cell numbers until 12 hrs post-seeding, when cell number decreased. Results are expressed as a mean ± SEM for 4 independent observations.

3.2.4 Cell attachment on silicone with different surface treatments.

The properties of a substrate surface will determine the degree of cell attachment. It is known that improved cell attachment can be achieved on rougher stiffer surfaces. To this end, I coated the silicone membrane with collagen. Adsorption of collagen to the surface has been demonstrated and an increase in the surface roughness as a result of collagen-coating has been determined (Figure 3.1). To examine then, the effect of enhanced surface characteristics on cell attachment, cell number was quantified on 4 different silicone surfaces (uncoated, collagen-coated, collagen coated + UV exposure, UV exposure only) intermittently, post-seeding, over a 48hr period. Figure 3.4 demonstrates that between 0.5 and 6hrs, only small changes in cell attachment are detectable between surface treatments; however silicone membranes that had been treated offered improved attachment compared to untreated silicone at both time-points. There was a trend for the best cell attachment best on surfaces that were collagen-coated, and exposed to UV. Results are expressed as a mean ± SEM for 3/4 independent observations.

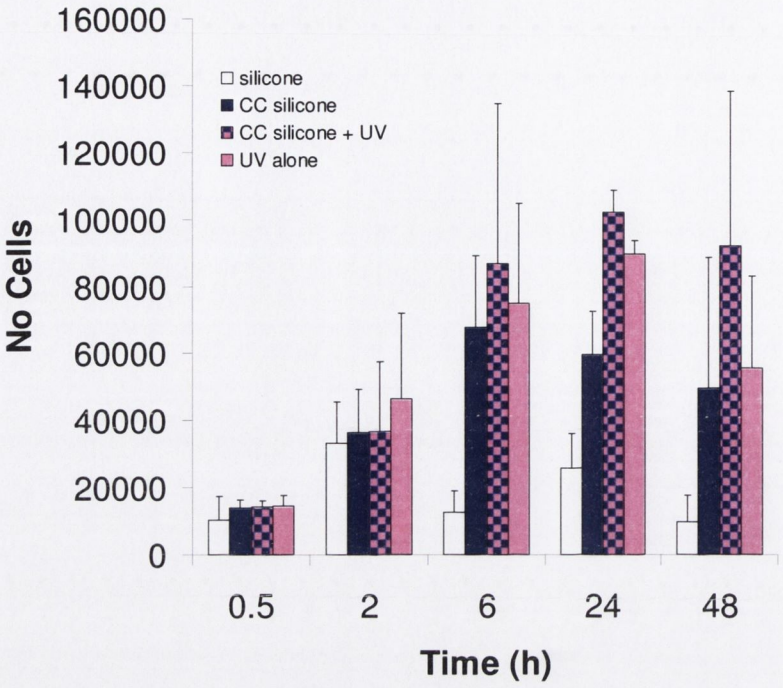


Figure 3.4 Optimal surface treatment for improved cell confluency

MSCs (125,000 cells) were seeded onto silicone, collagen-coated (CC) silicone, collagen-coated silicone that had been exposed to UV light for 12 hours (CC silicone + UV) and silicone (untreated) that had been exposed to UV light for 12 hrs (UV alone). Cell attachment was monitored at 0.5, 2, 6, 24 and 48hrs post-seeding, using the Hoechst assay.

While cell attachment at 0.5 – 2 h was similar on all substrates, by 6 hrs there was a trend for better cell attachment collagen-coated ± UV-treated silicone. This trend continued 24 and 48 hrs post-seeding, however changes were not significant.

Results are expressed as a mean ± SEM for 3/4 independent observations.

3.3 Discussion

The relationship between cells and the substratum to which they attach is a critical determinant of cell behaviour (Brown et al. 2005; Engler et al. 2006; Leucht et al. 2007). To mechanically stimulate cells, this research seeds MSCs onto a collagen-coated silicone substrate. To understand this cell-substratum relationship and whether it has an influence on cell responses such as attachment, it was pertinent to examine the properties of the substrate for this study. This allowed both a topographical and mechanical characterisation of the substrate, and subsequently, optimisation of substrate treatment and cell seeding density was determined. Using immunochemistry, the presence of collagen type I was identified on the silicone surface following coating with collagen in solution. Scanning electron microscopy examined the collagen-coating topographically, however scanning at a range of magnifications failed to reveal distinguishable features between silicone and collagen-coated silicone. By using atomic force microscopy, the presence of collagen could be identified on the collagen-coated membrane, and silicone and collagen-coated silicone were topographically distinguishable. Collagen-coated silicone that had been exposed to UV for 12 hrs was also examined by AFM and surface reconstructions demonstrate a topographically distinct pattern from both silicone and collagen-coated silicone. Corresponding surface roughness measurements from the AFM images indicated that collagen-coating increased surface roughness while subsequent exposure of collagen-coated silicone to UV markedly reduced the surface roughness, when compared to uncoated, untreated silicone however this was a trend observed in 1 experiment. Mechanical testing revealed that exposure to UV significantly decreased the Young's modulus of collagen-coated silicone. The optimal seeding density was determined as 125,000 cells/600mm^2, and collagen-coated silicone that had been exposed to UV, which was not the most mechanically stiff material and furthermore had the least topography, was determined as the best substrate for cell attachment during a 48 hr period.

The use of silicone for studies on mechanobiology, particularly for tensile stimulation, is an ideal material due to its elastic, transparent and biocompatible properties (Masuda et al. 2007). However, for cell culture, modifications to the surface are necessary in order to improve the intrinsically inert and hydrophobic properties. Collagen contains the RGD peptide sequence, and is widely used as an

adhesion promoter, and to mask the hostility of non-biological materials in tissue engineering applications (Galvin et al. 2002; Le Bellego et al. 2006; Mazzocca et al. 2007; Pelham and Wang 1997). This research uses collagen to improve the silicone surface properties for MSC attachment. Adsorption is achieved by incubating the silicone membranes with collagen in solution at 37°C for 2 hrs, which is a method that has previously been used for protein adsorption (Gurdak et al. 2006; Pamula et al. 2004). The adsorbed layer could then be detected by immunochemistry and AFM. From the AFM image, the large fibrillar appearance may be due to the fact that when the collagen adsorbs, the adsorbed amount is equivalent to 8–10 monolayers of close-packed molecules (Pamula et al. 2004), or may be a more globular structure, as seen with adsorbed denatured collagen (Gurdak et al. 2006), thus giving an inflated collagen molecule appearance. The fibril formation on the surface can be largely dependent on physicochemical properties of the substrate (Pamula et al. 2004) .

When silicone and collagen-coated silicone were examined using SEM, it was not possible to visualise any topographical variations between samples. However, SEM specimens are sputter-coated with a few nanometres of gold, while atomic force microscopy does not need metal coating. For SEM, this is necessary for the sample to interact with the electron beam, but may render the collagen-coat undetectable, particularly if the collagen adsorption interaction was weak. In this study, collagen is prepared aseptically by solution in water and autoclaving. Collagen can become denatured to gelatin upon heating and this effect is a irreversible kinetic process (Miles et al. 1995; Wright and Humphrey 2002) at greater than 41.5 °C for human collagen type I (Persikov and Brodsky 2002), therefore exposure of collagen to autoclaving at 121°C will almost certainly denature the molecule. It is still possible for denatured collagen to adsorb onto the silicone membrane, however the ability of collagen to assemble fibrils is lost (Gurdak et al. 2006). Thus the adsorbed layer that is seen by immunochemistry and AFM in this study is not the intact collagen helix, but the heat transformation of collagen to gelatin whereby disintegration of the collagen triple helical structure into random coils had occurred. Denaturation causes collagen to shrink (Wright and Humphrey 2002) and adsorbed denatured collagen forms aggregate 'islands' on a monolayer of flattened coils, which can become easily displaced upon drying (Gurdak et al. 2006). Furthermore, when a protein adsorbs, it usually undergoes a change in conformation, which may include denaturing or unfolding (Muschler et

al. 2004). Since this can lead to the formation of 'holes' in the adsorbed layer in which the substrate is exposed, it is possible that the SEM scan was on an area where the adsorbed layer had been displaced. Immunochemical detection was possible since the collagen type I antibody binding site was unaffected, as the recognition sequence is a specific short polypeptide chain that is not disrupted by unravelling of the collagen triple helix structure that occurs through breaking of the hydrogen bonds between the chains.

Imaging by AFM of the collagen-coated surface that had been exposed to UV revealed a much smoother appearance than both silicone and collagen-coated silicone. Subsequent surface roughness measurements confirmed this trend. The measurement of roughness also identified that the collagen-coating technique creates a rougher surface than that of silicone on the micrometre scale. It is known that UV exposure decreases the surface roughness of a homogeneous substrate, through the formation of an oxidised silica-like layer (Oláh 2005; Ye et al. 2006). Also, when collagen is exposed to UV it causes crosslinking. Limited exposure of collagen to UV results in an intermediate state consisting of molecules that are still in a native triple helix, but with the possibility of multiple chain scissions along the helix, however prolonged exposure (from 1 to 8 hrs) leads to denaturation (Miles et al. 2000; Suh et al. 1999) and the formation of random coils that rearrange into a dense flattened pattern (Suh et al. 1999). Therefore, the combination of heat exposure to the collagen in solution and UV exposure to the adsorbed layer, contribute to the decreased topography of collagen-coated silicone exposed to UV that is observed with the AFM surface roughness measurements.

UV irradiation of scaffolds for tissue engineering can positively regulate cell adhesion and a number of studies report increased hydrophilicity, surface roughness, cell attachment and subsequent cell spreading of osteoblast-like cells on a silicone substrate that was exposed to UV before adsorption of a fibronectin to the surface (Fujita et al. 2005; George et al. 2006; Toworfe et al. 2004). This study has identified a trend for that the best initial cell attachment on silicone that had been exposed to UV, however spreading and proliferation with time was preferential on collagen-coated silicone that had been exposed to UV, a trend which is in agreement with the study by Fujita on a poly(glycolic acid)-collagen scaffold (Fujita et al. 2005) where they report improved proliferation on scaffolds

that were also exposed to UV. Therefore, while collagen becomes degraded following UV exposure, the collagen-coated silicone exposed to UV it retains the ability to achieve the best cell attachment and proliferation during the 48 hr pre-culture period prior to the application of mechanical stimulation. It is possible that this is due to the persistence of the RGD amino-acid sequence cell attachment domain following heat and UV exposure; however this requires further experimentation to be confirmed.

From the mechanical tests carried out on the substrates, it is possible to link the improved cell attachment with the significant decrease in stiffness of collagen-coated silicone following exposure to UV. The mechanical properties (i.e. Young's modulus) of a substrate can be increased following short (15 mins) exposure to UV (Cornwell et al. 2007; Oláh 2005) as a result of protein cross-linking. In this study the opposite trend occurred, possibly through saturation of cross-linking and subsequent protein degradation (Miles et al. 2000; Suh et al. 1999; Weadock et al. 1995). The efficiency of crosslinking and degradation depends mainly on the sample preparation and irradiation dose. The exposure time in the current study was prolonged (12 hrs), therefore protein degradation may be reflected the decrease in Young's modulus following exposure to UV. Previous studies on mechanical properties on collagen type I have shown following exposure to UV irradiaton for 15 mins improves the mechanical properties but that increasing durations of UV irradiation lead to a decrease in the Young's modulus of collagen (Sionkowska and Wess 2004; Weadock et al. 1995).

In the study by Brown et al (2005), they report that mechanically stiffer substrates provide the best conditions for initial cell attachment, while more flexible substrates promote cell proliferation, and thus associate surface properties to dominate attachment and mechanical properties to influence long term growth. In that study, the range of substrate stiffness' were 0.05, 0.26 and 1.79 MPa, however in the current study there is a much narrower range from 1.65 – 1.72 MPa, therefore direct comparisons on initial attachment behaviours is difficult. In this study, there were no differences in the proclivity for initial cell attachment on each of the substrates examined and it is possible that this is linked to the very narrow range of Young's modulus. This consistency in cell attachment on equally stiff substrates would then agree with the study by Brown (Brown et al. 2005) since he found cell attachment to vary as mechanical stiffness varies. While the range of

mechanical stiffness was very narrow for each of the substrates, they had varying surface chemical and roughness characteristic from the treatments they received. The effects of varying substrate treatments are reflected in the cell attachment relationship, which is most marked at 48 hrs after initial cell seeding demonstrating that the collagen-coated silicone that had been exposed to UV was the best substrate for promoting proliferation of the cells. From the AFM data on the roughness of the substrate surfaces, collagen-coating exposed to UV creates the lowest surface roughness and therefore topography. It is known that cells interact best when there is a greater level of topography in their substratum (Curtis and Wilkinson 1997), and increasing surface roughness promotes the proliferation of calvarial osteoblastic cells (Hatano et al. 1999; Kunzler et al. 2007) fibroblasts (Kunzler et al. 2007) and human bone marrow cells (Deligianni et al. 2001), which is in contrast to the result found in this study. Therefore, the increased number of cells on the collagen-coated UV silicone substrate must depend on the surface chemistry more than topography or mechanical factors. The collagen-coated silicone that was exposed to UV irradiation had the lowest mechanical properties and the best proliferation which is in agreement with the study by Brown et al (Brown et al. 2005) but since the variability in the mechanical properties between substrates was within a very narrow range (1.65 – 1.72 MPa) the mechanical properties may not have the strongest influence on cell proliferation.

To conclude, this study has demonstrated the presence of an adsorbed protein layer on silicone, following incubation with autoclaved collagen, which is most likely to be a degraded product of the collagen helix. This structure could be visualised by AFM but not SEM. I postulate that this may be due to the local displacement of the adsorbed layer that can occur when the adsorbed collagen is denatured (Gurdak et al. 2006). Exposure of the collagen-coated substrate to UV radiation caused a reduction in the surface roughness, however this surface, while mechanically relatively dissimilar from the other surfaces, provided the best properties for cell proliferation over 48 hours. Therefore, it must be a feature of the surface chemistry, which depends on the properties of the material, resulting from collagen adsorption and subsequent UV irradiation that creates the best environment for cells, although this remains to be investigated. It should be stated that data from SEM and AFM are limited to 1-2 individual tests, and analysis could benefit from further testing, however this was due to limited access to the

equipment. In a paper on the osteogenic differemtiation of MSCs, by Jaiswal et al (1997), they discuss the practice of achieving subconfluency in cultured cells avoiding the formation of multilayers, since differentiation is known to be triggered at high densities (Caplan et al. 1983). Therefore it was determined that the optimal seeding density was 125,000 cells/6000mm^2 since at this density cells were confluent but not forming multilayers, and still continuing to proliferate. From this study, it is clear that 1) cells are very sensitive to the features of their extracellular environment and 2) surface features are very sensitive to the treatments they are exposed to. This is encouraging for the use of silicone in the study of mechanobiology, since further optimisation of the surface properties (Lew et al. 2007) can be carried out to emhance the silicone membrane biomaterial.

4 A Study on the osteogenic factor induction of mesenchymal stem cell differentiation

4.1 Introduction

The multipotential differentiation properties of MSCs make them an interesting cell source for tissue engineering applications. This is of particular interest for applied mechanobiology in the tissue engineering of skeletal tissues, since the skeletal system is known to be particularly mechanoreceptive. Since there is no specific marker or combination of markers that definitively identify MSCs thus these cells are currently defined by a combination of physical, morphologic, phenotypic, and functional properties. A series of monoclonal antibodies raised towards surface MSC antigens including CD105 (Barry et al. 1999) CD73 (Barry et al. 2001), and a group of other adhesion molecules and growth factor/cytokine receptors (Alhadlaq and Mao 2003) Many isolation protocols are based on the process of negative selection of cells lacking the expression of hematopoietic and endothelial cell markers e.g. CD45 and CD34.

Isolation through plastic adherence of rat bone-marrow derived MSCs is a widely recognised method and both *in vitro* and *in vivo* evidence supports the multipotential properties of this population. Surface antigen expression allows for a rapid identification of a cell population, and has been used extensively in immunology and haematology (Dominici et al. 2006). Recently, upon phenotypic analysis of a CD45 low population of rat MSCs, Jones et al (2002) found that they were uniformly positive for CD105, CD90 and a panel of other receptor-antigens, and were capable of retrospective functional characterisation using differentiation assays. $CD105^{+}/CD90^{+}/CD45^{-}$ markers have formed part of the characterisation and/or isolation strategies for a number of *in vitro* and *in vivo* studies involving MSCs (Djouad et al. 2005; Jager et al. 2005; Lu et al. 2005; Pasquinelli et al. 2007; Pittenger et al. 1999; Prat-Vidal et al. 2007; Rochefort et al. 2005; Vogel et al. 2003; Weiss et al. 2006), and form a specific representative panel of markers sufficient for this study, however, enrichment can be improved by increasing both positive and negative marker criteria.

The presence of dexamethasone (Dex), β -glycerophosphate (βGP), and L-ascorbic acid-2-phosphate (AA), are known to favour the expression of the osteoblastic phenotype (Aronow et al. 1990; Maniatopoulos et al. 1988). The optimal osteogenic supplement profile was determined as 100nM dexamethasone, 10 mM β -glycerophosphate, and 0.05 mM and L-ascorbic acid-2-phosphate, however a range of concentrations for each supplement was shown to have osteogenic effects (Jaiswal et al. 1997). Dexamethasone is involved in the

regulation of alkaline phosphatase gene expression (Cheng et al. 1996; Leboy et al. 1991; Milne et al. 1998) as well as many other genes involved in osteogenesis including collagen type I and osteocalcin (Beresford et al. 1994; Cheng et al. 1996; Leboy et al. 1991). L-ascorbic acid-2-phosphate is used for the maturation of collagen in osteogenic cultures. β-glycerophosphate is a source of phosphate ions, for the formation of a mineralised extracellular matrix through calcium phosphate deposition *in vitro* (Bellows et al. 1991; Chung et al. 1992; Maniatopoulos et al. 1988; Pittenger et al. 1999; Tenenbaum and Heersche 1982). Osteogenic differentiation can be detected by probing for the expression of proteins including Cbfα1, collagen type I and osteocalcin, and through the detection of calcium deposits in the extracellular matrix (Jaiswal et al. 1997; Ogura et al. 2004; Stanford et al. 1995a).

The induction of *in vitro* osteogenic differentiation of MSCs in response to osteogenic factors has been extensively examined and optimised conditions have been determined for induction of cells seeded on standard tissue culture substrates (Aronow et al. 1990; Chang et al. 2006; Coelho and Fernandes 2000; Jaiswal et al. 1997; Maniatopoulos et al. 1988; Tropel et al. 2004). However, given the development of substrate-driven differentiation, and since MSCs are highly responsive to their extracellular environment, the consideration of the influential nature of the substrate on cell behaviour is pertinent (Cancedda et al. 2003) and so the osteogenic treatment of MSCs on a collagen-coated silicone substrate is examined in this study. A number of material factors in the immediate environment of the cells such as surface chemistry are likely to play a role via cell-matrix-osteoinductive factor cell interactions, in the control of stem cell behaviour, initial protein and cell adhesion, morphology and ultimately differentiation (Badylak 2007). Some substrates affect attachment and spreading alone, without influencing the osteogenic process (Deligianni et al. 2001; Ogura et al. 2004), while others can directly influence lineage specific differentiation (Engler et al. 2006). Also of interest when examining differentiation on other substrates, is the activation of cell signalling during that process.

The MAPK pathway plays a crucial role in the transmission of extracellular mitogenic signals from membrane to nucleus, and extracellular stimulation with osteogenic factors has been shown to stimulate the activation of this pathway (Jaiswal et al. 2000; Lee et al. 2002). It has been shown that ERK MAPK signalling is involved in the stimulation of osteoblast-related gene expression by

extracellular matrix-integrin receptor interaction, as well as mechanostressing (Rubin et al. 2002; Suzawa et al. 2003), and activation of the p38 MAPK promotes osteogenesis by inducing the expression of Cbfα1 (Runx2) (Satija et al. 2007). Several lines of evidence suggest that signalling through MAPKs is essential for the early stages of osteoblast differentiation (Franceschi and Xiao 2003; Gallea et al. 2001; Hipskind and Bilbe 1998). Osteogenic differentiation of MSCs by biochemical induction has been shown to be differentially regulated by ERK1/2 and p38 (Jaiswal et al. 2000). The phosphatidylinositol (PI) 3-kinase signal transduction pathway is also responsive to osteogenic factor induced differentiation (Ghosh-Choudhury et al. 2002).

The experiments in this study began by phenotypically characterising the adherent cell population following 21 days of expansion and 3/4 passages in culture. This was done by monitoring the homogeneity of the population by quantitative detection of positive and negative MSC markers CD90 and CD45 respectively, using flow cytometry (see Methods 2.2.5). Endoglin (CD105) and CD90 expression were also examined using imunocytochemistry (see Methods 2.7.2). Since it has been established that the cell-substrate is a key regulator of many cell fates, the propensity of MSCs to differentiate on a collagen-coated silicone substrate in response to the osteogenic factors of dexamethasone, β-glycerophosphate, and L-ascorbic acid-2-phosphate (see Methods 2.5) was examined by probing for differentiation markers during a 28 day study, using imunocytochemistry and other histological techniques (see Methods 2.7.2). The effects of collagen in the extracellular environment have also been noted as having an influential effect on MSC osteogenesis. Thus the effect of a collagen-coated substrate on the osteogenic factor induction of osteogenesis was examined by probing for osteogenic markers in cells cultured on 2 distinct substrates: silicone and collagen-coated silicone, during a 28 day study. Protein and matrix markers were detected using histological techniques (see Methods 2.7.2) Furthermore, to examine the signalling mechanisms activated during osteogenic factor induced MSC osteogenesis on collagen-coated silicone, the involvement of ERK, p38 and PI3-kinase was determined by culturing cells in osteogenic factors and in the absence or presence of pharmacological inhibitors of the signalling pathways (see Methods 2.5), then probing for osteogenic markers over 21 days, using histological techniques (see Methods 2.7.2).

4.2 Results

4.2.1 Cultured marrow stromal cells express the mesenchymal stem cell surface marker endoglin (CD105)

To isolate adult mesenchymal stem cells from a preparation of bone marrow, the established method based on adherence of marrow-derived fibroblast-like cells to plastic tissue culture plates, and the concomitant lack of adherence of other marrow derived cells was used (Alhadlaq and Mao 2003). By the third or fourth passage, it is thought that a large percentage of osteoblasts, fibroblasts, fat and other stromal cells, will be removed from the culture, however, isolation by this method does produce a heterogeneous population (Ahmed et al. 2006; Phinney et al. 1999). Specific MSC cell surface markers present on adherent MSCs include endoglin (CD105) and CD90, and these are used in MSC isolation criteria (Dominici et al. 2006; Rochefort et al. 2005) and to enrich mesenchymal precursors (Majumdar et al. 2000). To examine qualitatively the expression of endoglin and CD90 on 21 day old cultures, cells were fixed and probed for the endoglin and CD90 expression using immunofluorescence.

Following 3-4 passages and 21 days in culture, the marrow stromal cell population express the MSC surface marker endoglin and CD90 (Figure 4.1). Figure 4.1 (i) demonstrates background fluorescence in negative controls i.e. cells that were stained without the addition of primary antibody. In contrast, in cells that were stained with endoglin antibody Figure 4.1 (ii), strong punctate immunoreactivity is evident in all cells. Similarly, Figure 4.1 (iii) demonstrates background fluorescence in negative controls; in cells that were stained with the CD90 antibody, strong immunoreactivity is evident in all cells (Figure 4.1 (iv)). This data indicates that the adherent population of marrow stromal cells following 21 days in culture express the endoglin and CD90, reported to be present on MSCs (Dominici et al. 2006).

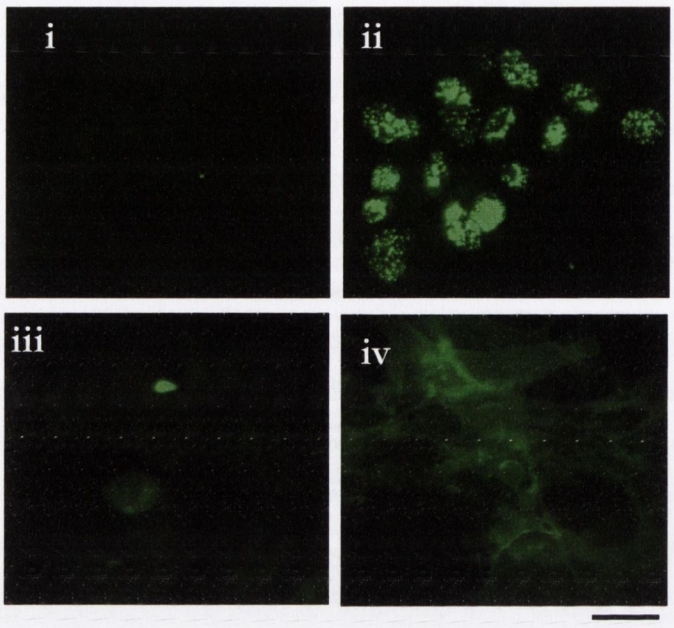


Figure 4.1 Cultured marrow stromal cells express the cell surface markers endoglin (CD105) and CD90

To examine the phenotype of the adherent marrow stromal population that had been cultured for 21 days and reached passage 3 or 4, cells were probed for the expression of the mesenchymal stem cell surface markers, endoglin and CD90, using immunofluorescence on fixed cells.

Fluorescent images of cultured cells that have been stained (i) without primary antibody and (ii) with a primary antibody that recognises an epitope corresponding to amino acids 27 – 326 mapping near the N-terminus of endoglin, demonstrate that strong endoglin immunoreactivity is present in a punctate pattern in cultured marrow stromal cells.

Similarly fluorescent images of cultured cells that have been stained (i) without primary antibody and (ii) with a primary antibody that recognises the CD90 antigenic determinant, demonstrate that strong CD90 immunoreactivity is present in cultured marrow stromal cells.

Scale bar is 50μm.

4.2.2 MSC phenotying using flow cytometry.

Expression of the positive mesenchymal stem cell markers endoglin and CD90 has been demonstrated (Figure 4.1). It has been noted that the markers used for positive selection of MSCs have broad cell reactivity. Therefore, isolation by negative selection has been utilised in procedures for MSC purification and enrichment (Baddoo et al. 2003; Hachisuka et al. 2007; Jones et al. 2002; Majumdar et al. 1998). It is known that MSCs do not express the hematopoietic cell marker CD45 (Chamberlain et al. 2007; Dahlke et al. 2004). Cell phenotyping by flow cytometry is a powerful analytical technique that quantifies cells presenting specific labelled antigens and due to the range of MSC phenotype markers, has been a useful tool in examining the marrow stromal cell population (Gronthos and Simmons 1995; Zangi et al. 2006). To examine the homogeneity of the endoglin+/CD90+ marrow stromal cell culture, fluorescent-activated cell sorting (FACS) was utilised. Passage 4 cells were analysed for the positive MSC marker CD90 and the negative MSC marker CD45. The overlay graph in Figure 4.2 demonstrates high expression of the positive MSC marker CD90 and low expression of the negative CD45 marker. Quantification revealed that 96.89 ± 0.41% of cells (mean ± SEM) were positive for CD90 whereas 2.14 ± 0.81% of cells were positive for CD45 (Table 4.1). These data indicate that the adherent population of marrow stromal cells following 21 days in culture is a homogeneous $CD90^{+}/CD45^{-}$ population and thus herein will be referred to as a mesenchymal stem cell (MSC) population. Results are representative of 7 independent observations.

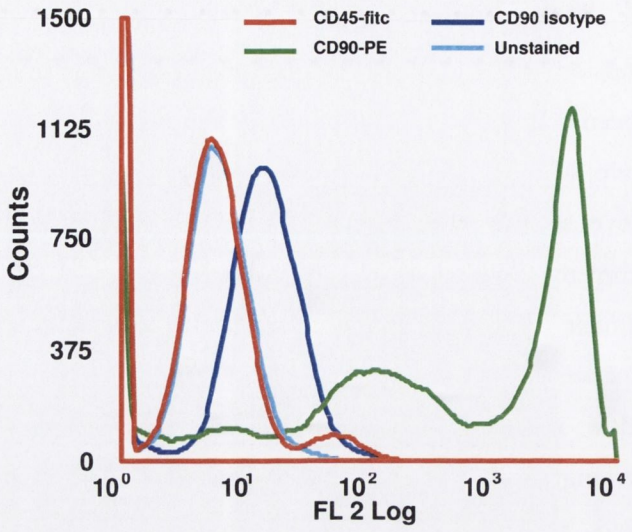


Figure 4.2 MSCs after 3 weeks in culture are a homogenic population

To examine the homogeneity of the adherent cells in culture for 21 days and demonstrate CD45/CD90 expression, flow cytometry was used to quantify $CD90^{+}$ and $CD45^{-}$ cells.

Overlay representations of fluorescent labels indicated that phycoerythrin (PE), which was conjugated to CD90, was detected at high levels as indicated by the majority of events falling at the upper end of the log fluorescence scale on the x-axis. Fluorescein isothiocyanate (FITC), which was conjugated to CD45, was not present on the majority of cells as indicated by the large number of detections at the low level of fluorescence on the log fluorescence (x-axis). Results are representative of 7 independent observations.

MSCs	CD90+	CD45+
Mean	96.89	2.14
S.E.M	0.41	0.81

Table 4.1 Quantification of FACS positive events (see Appendix I).

4.2.3 Silicone elastomer supports MSC differentiation induced by soluble factors.

The capacity of MSCs to differentiate has been well documented (Chamberlain et al. 2007; Pittenger et al. 1999) and typically proceeds by incubating MSCs with an appropriate combination of soluble factors for 2-3 weeks. For osteogenesis, these are ascorbic acid, β-glycerophosphate and dexamethasone. To investigate the differentiation capacity of MSCs seeded on a 2D silicone elastomer coverslip (13mmø), culture medium was supplemented with the osteogenic cocktail of soluble factors (dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM)). Following 7, 14, 21, and 28 days in culture, cells were fixed and monitored for markers of osteogenesis (Figure 4.3). The protein markers collagen type I and osteocalcin (Aubin et al. 1995) were examined using fluorescence immunocytochemistry; and extra-cellular matrix was examined using histology for extracellular calcium via the alizarin red method (Puchtler et al. 1969); and using the von Kossa method of detection for extracellular calcium phosphate deposits. In treated cells, collagen type I imunoreactivity was evident after 7, 21 and 28 days (Figure 4..3A) and osteocalcin immunoreactivity could be detected after 28 days (Figure 4..3B). After 21 and 28 days, extracellular matrix mineralisation could be detected with both von Kossa (Figure 4.3C) and alizarin red (Figure 4.3D) methods of detection. These observations confirm that silicone elastomer supports MSC osteogenesis, and therefore is a suitable substrate for further investigations in MSC mechanobiology. Results are representative of 4 independent observations.

Figure 4.3 Silicone supports MSC osteogenesis induced by soluble factors

MSCs were seeded onto silicone elastomer coverslips and cultured in osteogenic medium for 7–28 days. Collagen type I and osteocalcin expression was examined using fluorescence immunocytochemistry; matrix mineralisation was examine using the alizarin red and von Kossa histological stains.

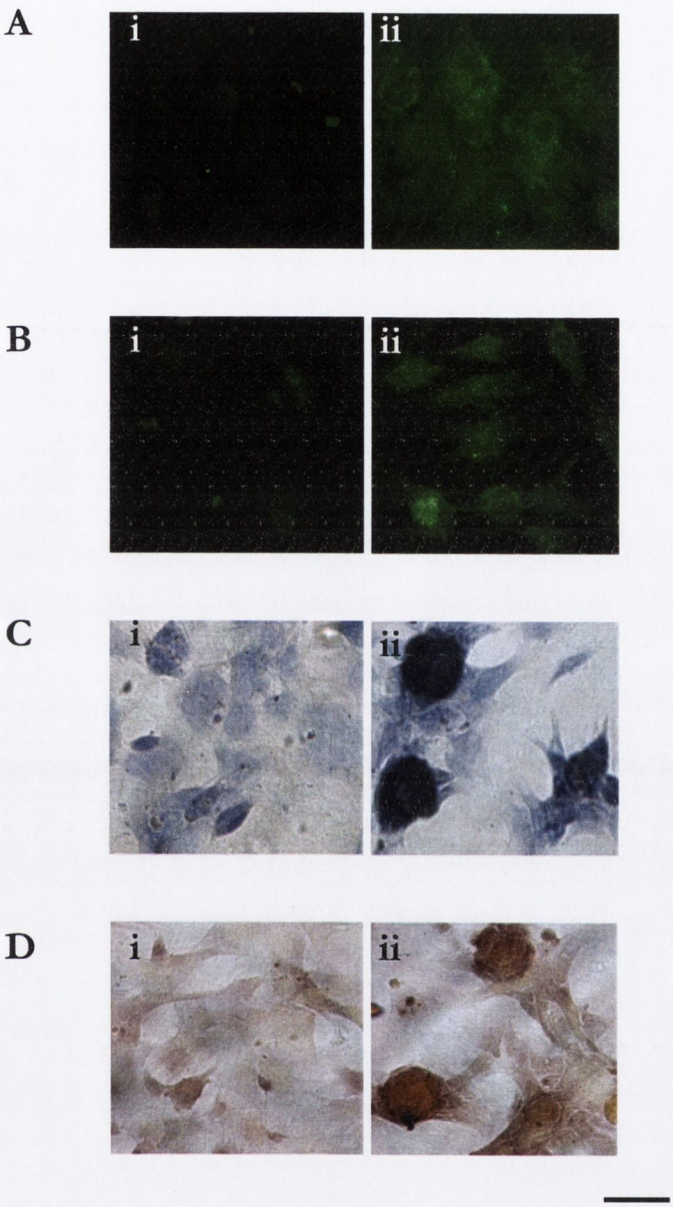
A: Collagen type I immunoreactivity is not evident in control untreated cells (i), however following treatment with osteogenic factors (ii), intense collagen immunoreactivity is observed after 7, 21 and 28 days. Images are from cells cultured for 7 days and are representative of cells at the 21 and 28 day time points.

B: Similarly, osteocalcin immunoreactivity is not observed in control cells (i) however after 28 days of treatment with osteogenic factors (ii), intense osteocalcin immunoreactivity is evident.

C: In control cells (i) examined for the presence of calcium in the extra-cellular matrix using the von Kossa method, no positive staining is evident. However in 21 and 28 day treated cells (ii), areas of positive black staining can be observed, particularly in areas of nodule aggregation, a feature of cultured MSCs following osteogenic treatment. Images are from cells cultured for 28 days and are representative of cells at the 21 day time points.

D: Likewise, untreated cells (i) demonstrate no positive staining for alizarin red, however following exposure to osteogenic factors (ii) for 21 and 28 days, positive alizarin red staining is observed, again, particularly in the nodular aggregations. Images are from cells cultured for 28 days and are representative of cells at the 21 day time points.

Results are representative of 6 independent observations. Scale bar is 50μm (A,B)/ 100μm (C,D) (Approx. 90% cells demonstrate positive immunoreactivity).


A
i
ii
B
i
ii
C
i
ii
D
i
ii

4.2.4 The induction of MSC differentiation by osteogenic factors on silicone membranes is unaffected by a collagen-coating on the substrate.

To improve the biocompatibility of the silicone elastomer substrate, it is coated with collagen. It has been noted that MSCs respond to collagen type I in their environment (Xiao et al. 1998) by expressing osteoblast markers such as osteocalcin. If the regulation of MSC osteogenic marker expression in response to a stimulus is to be monitored, it is important that the impact of the extracellular composition is known. Thus, cells seeded on silicone induced to differentiate towards the osteogenic lineage using soluble factors, were examined for the influence of a collagen-coated substrate, on their propensity to differentiate.

To investigate the influence of collagen-coating of the silicone substrates, MSCs were seeded on 2D silicone elastomer coverslips and coverslips that had been coated with collagen (1%). Cells were grown in the absence and presence of osteogenic factors (dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM)). Following 7, 14, 21, and 28 days in culture, cells were fixed; collagen type I and osteocalcin expression was monitored using fluorescence immunocytochemistry, and extra-cellular matrix mineralisation was examined using alizarin red and von Kossa histological stains.

Figure 4.4A demonstrates that in treated cells seeded on silicone (ii, vi, x) and collagen-coated silicone (iv, viii, xii), collagen type I imunoreactivity was evident after 7, 21 and 28 days. Figure 4.4B demonstrates that osteocalcin immunoreactivity could be detected in cells on silicone and cells on collagen-coated silicone after 28 days. After 21 and 28 days, in both cells on silicone and cells on collagen-coated silicone, extracellular matrix mineralization could be detected with both alizarin red (Figure 4.4C) and von kossa (Figure 4.4D) detection.

These observations indicate that collagen-coating on the silicone elastomer substrate supports, but does not influence, MSC osteogenesis, and therefore is a suitable substrate for further investigations into MSC mechanobiology. Results are representative of 4 independent observations.

Figure 4.4 Coating of silicone substrate with collagen does not influence osteogenic factor-mediated osteogenesis.

The influence of collagen type I in the extracellular environment on the osteogenesis of MSCs was examined. Cells were seeded onto silicone coverslips, and silicone coverslips coated with a 1% collagen solution, and cultured in osteogenic medium (dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM)) for 28 days. Collagen type I and osteocalcin expression was examined using fluorescence immunocytochemistry and matrix mineralization was examined using the alizarin red and von Kossa histological stains after 7, 14, 21 and 28 days of treatment.

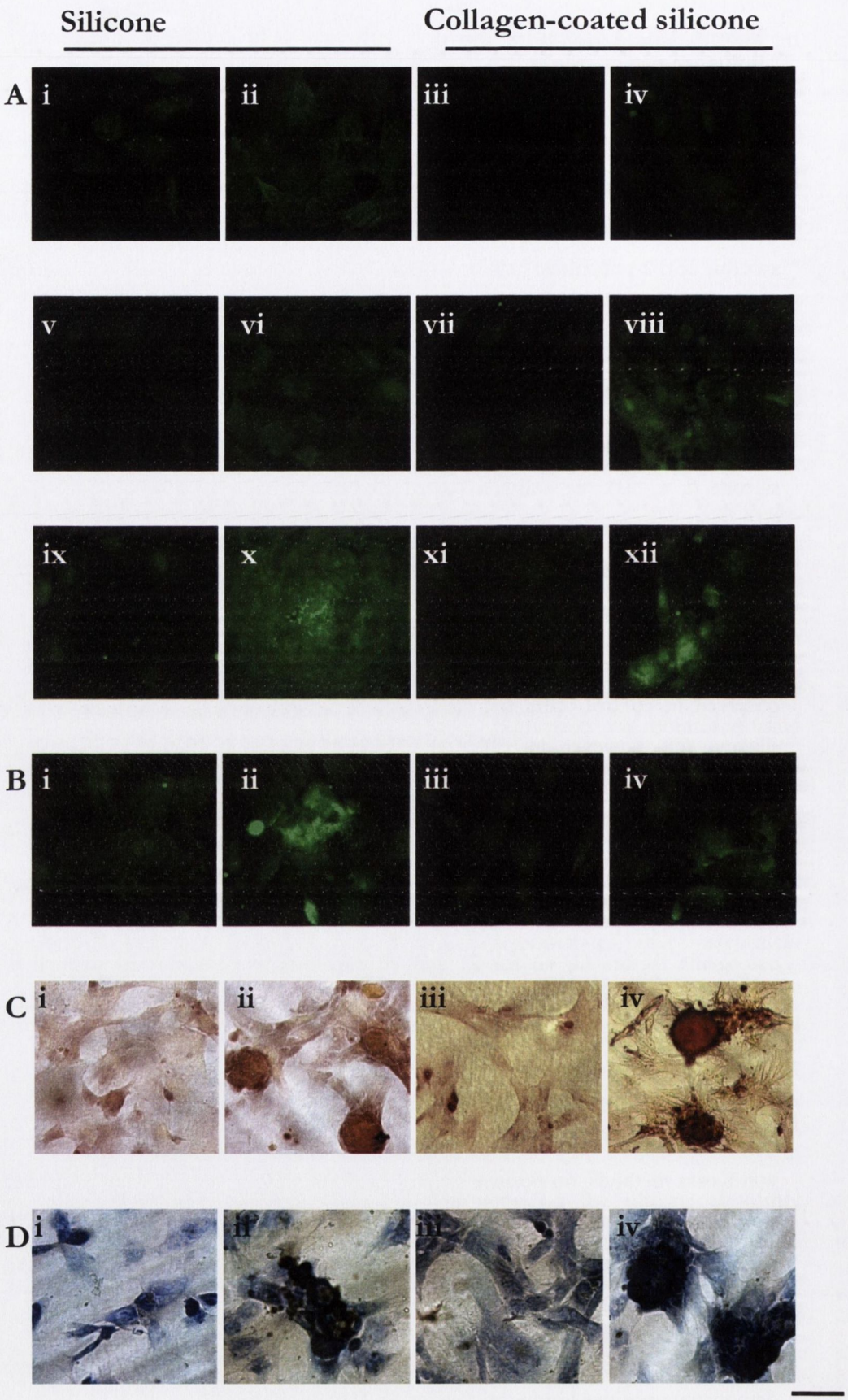
A: Collagen type I immunoreactivity is not evident in control untreated cells on silicone (i, v, ix) or collagen coated silicone (iii, viii, xi), however following treatment with osteogenic factors for 7 (ii, iv), 21 (vi, viii) and 28 days (x, xii), cells on silicone (ii, vi, x) and collagen coated silicone (vi, viii, xii) display collagen type I immunoreactivity.

B: Similarly, in cells cultured for 28 days, osteocalcin immunoreactivity is not observed in control untreated cells cultured on silicone (i) or collagen-coated silicone (iii) however in cells treated with osteogenic factors, osteocalcin immunoreactivity is evident when cells are cultured on both silicone and collagen-coated silicone.

C: Control untreated cells cultured on silicone (i) and collagen-coated silicone demonstrate no positive staining for alizarin red, however following exposure to osteogenic factors (ii, vi) for 21 and 28 days, positive alizarin red staining is observed in both substrates. Images are from 28 day cultures and are representative of 21 and 28 day cultures.

D: In control cells cultured for 28 days on silicone (i) and collagen coated silicone (ii) and examined for the presence of calcium in the extra-cellular matrix using the von Kossa methods, no positive staining is evident. However following treatment with osteogenic factors, (ii, iv), areas of positive black staining can be observed in cell grown on both substrates. Images are from 28 day cultures and are representative of 21 and 28 day cultures.

Results are representative of 4 independent observations, approx. 90% cells demonstrate positive immunoreactivity. Scale bar is 50μm.


Silicone
Collagen-coated silicone
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4.2.5 p38 and pI3-kinase signalling in osteogenic factor-induced osteocalcin expression in MSCs.

In investigating the underlying molecular mechanisms of MSC differentiation through the influence of osteogenic supplements, Jaiswal et al. (2000) identified the MAP kinase family members extracellular signal-regulated kinase (ERK) the stress-activated protein kinases (SAPK) c-Jun N-terminal kinase (JNK), and p38 to be temporally activated during osteogenic factor treatment of MSCs. A further MAP kinase - phosphatidylinositol 3-kinase (PI3-kinase), which is responsive to differentiation factors (Shepherd et al. 1998) and is involved in cell growth and apoptosis (Toker and Cantley 1997), has been identified as having an involvement in osteoblast differentiation (Ghosh-Choudhury et al. 2002).

To examine the molecular control of osteocalcin expression, the role of p38 and pI3-kinase during MSC osteogenesis induced by osteogenic factors was investigated. Thus, MSCs were cultured in the absence or presence of dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM). To investigate the roles of p38 and pI3-kinase, medium was further supplemented with the p38 inhibitor SB 203580 (10μM) and the pI3-kinase inhibitor LY 294002 (2μM) respectively. After 7, 14, and 21 days in culture, cells were fixed and examined for osteocalcin expression using fluorescence immunocytochemistry.

Immunoreactivity was quantified by measuring the fluorescence intensity (Table 4.2). Figure 4.5A demonstrates that following 21 days of osteogenic treatment, this significantly increased from 11.2 ± 0.65 AFU in untreated controls to 17.94 ± 0.67 AFU following 21 days of osteogenic treatment, however, in the presence of SB 203580 (10μM), immunoreactivity intensity significantly decreased to 12.66 ± 0.59 AFU. In the presence of LY 294002 (2μM), immunoreactivity intensity was reduced (14.57 ± 0.64 AFU), however not significantly so. Results are representative of 160 cells (ANOVA, Newman-Keuls post hoc).

Figure 4.5B demonstrates that osteocalcin expression is upregulated in MSCs exposted to osteogenic factors (ii), compared to untreated control (i). In MSCs exposed to osteogenic factors and the p38 inhibitor SB 203580 (10μM; iii), osteocalcin expression was comparable to expression in control untreated cells. Similarly, in MSCs that were exposed to osteogenic factors and the pI3-kinase

inhibitor, LY 294002 (2μM; iv), osteocalcin expression was comparable to expression in control untreated cells.

This finding demonstrates the expression of the osteogenic marker osteocalcin following 21 days of osteogenic treatments, and suggests the involvement of p38 and a trend for pI3-kinase involvement in osteogenic factor-induced MSC osteocalcin.

Time point (days)	CONTROL	+ Osteogenic factors	+ Osteogenic Factors + SB 203580	+ Osteogenic Factors + LY 294002
7	7.05 ± 0.57	8.54 ± 0.73	4.66 ± 0.55	4.13 ± 0.23
14	25.28 ± 1.11	31.18 ± 1.56	19.43 ± 1.22	27.19 ± 1.40
21	11.2 ± 0.65	17.94 ± 0.67*	12.66 ± 0.59**	14.57 ± 0.64
Arbitrary Fluorescent Units AFU (mean ± SEM); * = significant increase from control; ** = significant decrease from + osteogenic factors; n = 160 cells, ANOVA, Newman-Keuls post hoc				

Table 4.2 Quantification of osteocalcin fluorescence following treatment with osteogenic factors and p38/pI3-kinase inhibitors.

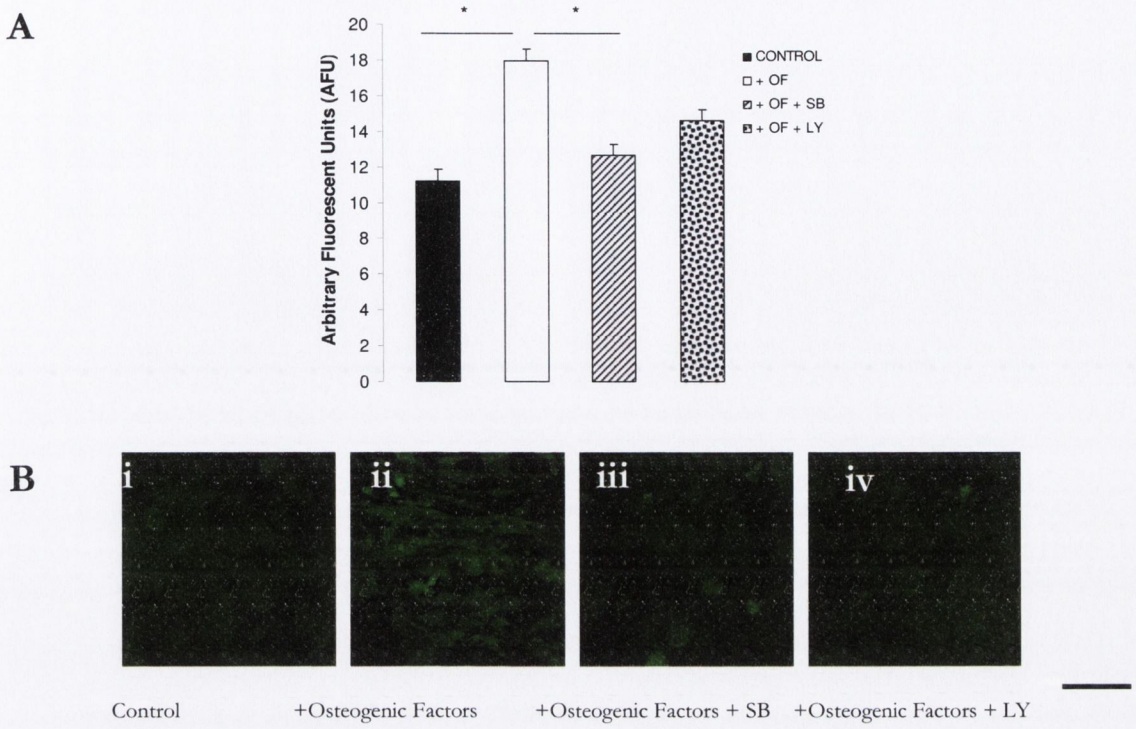


Figure 4.5 Examination of the role of p38 and PI3-kinase in osteogenic factor induced osteocalcin expression in MSCs after 21 days

MSCs were grown in control culture medium, medium supplemented with osteogenic factors (dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM ; OF), medium supplemented with osteogenic factors and the p38 inhibitor SB 203580 (10 μM; OF + SB) or medium supplemented with osteogenic factors and the pI3-kinase inhibitor LY 294002 (2 μM; OF + LY) for 21 days. Cells were fixed and the expression of osteocalcin was assessed by immunofluorescence.

A: Expression of osteocalcin was quantified by measuring the grey level intensity of the fluorescent images. A significant increase in osteocalcin fluorescence intensity was measured in cells exposed to osteogenic factors compared to untreated controls. In the presence of SB 203580 (10μM) this was significantly decreased. In the presence of LY 294002 (2μM) this was decreased but not significantly. Results are expressed as mean ± SEM for 160 cells.

B: MSCs exposed to osteogenic factors for 21 days (ii) displayed more intense osteocalcin immunoreactivity compared to untreated control cells (i;). In the presence of SB 203580 (10μM; iii) and LY 294002 (2μM; iv), osteocalcin immunoreactivity in MSCs exposed to osteogenic factors was comparable to control levels. Scale bar is 50μm.

4.2.6 The role of p38 and pI3-kinase in osteogenic factor-induced MSC matrix mineralisation.

Matrix mineralisation is a marker of mature osteogenic differentiation. To examine the roles of signalling pathways involved in osteogenic factor-induced matrix formation, MSC osteogenesis was induced by supplementing culture medium with the osteogenic factors dexamethasone (0.68nM) β-glycerophosphate (10mM) and ascorbic acid-2-phosphate (0.05 mM). To investigate the roles of p38 and pI3-kinase, medium was further supplemented with the p38 inhibitor SB 203580 (10μM) and the pI3-kinase inhibitor LY 294002 (2μM) respectively. After 7, 14, and 21 days in culture, cells were fixed and examined for matrix mineralisation using the histological stain, alizarin red. Using the method described by Gregory et al. (Gregory et al. 2004), bound dye was extracted and semi-quantified using colorimetric detection at 405nm (Table 4.3). Figure 4.6 demonstrates a trend towards reduced matrix mineralisation following 14 and 21 days of exposure to osteogenic factors when p38 is inhibited, and at 21 days when pI3-kinase is inhibited. This identifies a trend for the involvement of p38 and pI3-kinase in MSC osteogenesis. Results are representative of 6 independent observations.

Time point (days)	CONTROL	+ Osteogenic factors	+ Osteogenic Factors + SB 203580	+ Osteogenic Factors + LY 294002
14	0.03 ± 0.01	0.08 ± 0.04	0.02 ± 0.01	0.06 ± 0.03
21	0.08 ± 0.03	0.17 ± 0.05	0.09 ± 0.04	0.09 ± 0.03
mM (mean ± SEM); n = 6 individual experiments				

Table 4.3 Quantification of matrix mineralisation following treatment with osteogenic factors and p38/pI3-kinase inhibitors.

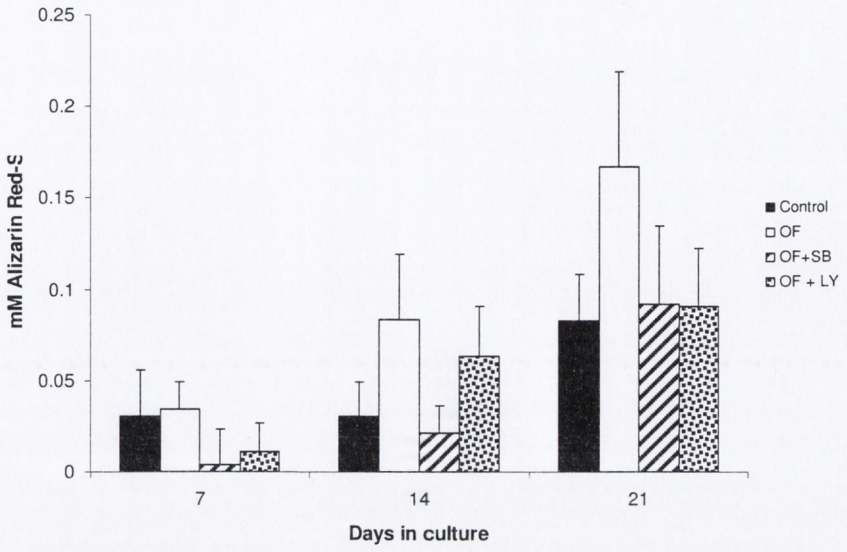


Figure 4.6 Matrix mineralisation, as assessed by alizarin red semi-quantification

Osteogenesis was induced in MSCs via osteogenic factor (OF) supplementation for 21 days. The role of p38 and pI3-kinase in matrix mineralisation were examined by blocking the p38 pathway using the inhibitor SB 203580 (10μM), and the pI3-kinase pathway using the inhibitor LY 294002 (10μM) respectively .Cells were examined after 7, 14, and 21 days in culture for matrix mineralisation using the alizarin red histological stain for calcium deposition, followed by extraction and colorimetric detection. An increase in calcium deposition following 14 and 21 days of osteogenic treatment is reflected by increased concentrations of alizarin red measured in treated samples, compared to controls. When cells are exposed to osteogenic treatments in the presence of the p38 inhibitor SB 203580 however, this increase is no longer observed. Osteogenic treatments in the presence of the pI3-kinase inhibitor LY 294002 reduced matrix mineralisation after 21 days of exposure. It can be noted that this is a trend detected and that there are no statistically significant changes between groups. Results are representative of 6 independent observations.

4.3 Discussion

The aim of this chapter was to phenotypically verify the adherent cell fraction of a marrow stromal cell isolate as MSCs, and to examine their osteogenic differentiation potential on silicone and collagen-coated silicone. In addition, this study aimed to examine the intracellular signalling mechanisms of osteogenic factor-induced osteogenic differentiation. Following 21 days in culture, adherent cells from a marrow stromal preparation at passage 4 expressed the positive MSC markers CD105 (endoglin) and CD90. Furthermore, quantitative flow cytometry revealed that 97% of adherent cells expressed CD90, whereas 2% of cells expressed the haematopoietic cell marker CD45. Therefore, preparations of adherent cells are homogeneous for the MSC marker CD90 and contain only a small fraction of cells of the hematopoietic phenotype. Following treatment with the osteogenic factors dexamethasone, β-glycerophosphate, and L-ascorbic acid-2-phosphate cells seeded on silicone and collagen-coated silicone differentiated toward the osteogenic phenotype with equal proclivity. Thus MSCs on a silicone substrate are responsive to osteogenic factors, and this substrate is capable of supporting osteogenesis as measured by collagen type I and osteocalcin expression and the formation if a mineralised matrix. Coating the silicone with collagen has no affect osteogenic differentiation when MSCs are stimulated by osteogenic factors. Collagen type I was detected following 7, 21 and 28 days of osteogenic treatment. Osteocalcin expression was detected following 28 days but not after 21 days of osteogenic treatment. Matrix mineralisation was detected following 21 and 28 days of osteogenic treatments. It was demonstrated that p38 is involved in osteocalcin expression, and following osteogenic factor treatments, by blocking p38 and pI3-kinase signalling pathways, mineralisation at 21 days was reduced, however not significantly.

The isolation of MSCs by plastic adherence is a widely practiced technique (Colter et al. 2000), however it is accepted that while containing a large percentage of cells with the classical features of MSCs (i.e. ability to proliferate in culture and differentiate), they are not a homogeneous population. To study mechanoregulation of MSCs, it is important that the findings are an accurate reflection of the cells' behaviour; thus definition of the cell population is of importance. Of equal importance is the homogeneity of the sample, so that effects cannot be attributed to the reactions of other cell types present in the culture. To do this, 2 positive MSC markers (CD90 and CD105 (endoglin)) and 1 negative

MSC marker (CD45) were chosen for qualitative histological and quantitative FACS analysis. These are a sub-selection of the known positive and negative markers however, were considered sufficient for the purposes of this research. These markers were chosen based on the literature, and match, in part, the minimal criteria for defining multipotent mesenchymal stromal cells, set out by the International Society for Cellular Therapy for human MSC (Dominici et al. 2006). In that criteria, greater than or equal to 95% expression of the positive surface antigen expression is the minimum acceptable level of purity, and less than or equal to 2% expression of hematopoietic antigens is the maximum acceptable level of purity.

Immunohistochemical analysis confirmed the expression of CD90 and CD105 on the adherent cell population, and FACS analysis demonstrated a 97% homogeneous population for CD90, while only 2% of cells expressed CD45. Following analysis, since such a large percentage of the population was positive for the MSC marker CD90, and the negative marker fraction was within the limits of a homogeneous population criteria as defined by The International Society for Cellular Therapy (Dominici et al. 2006), further isolation techniques to remove the 2% of cells that were positive for the hematopoietic cell marker CD45 were not carried out. Furthermore, MSCs with this expression profile have been used without further isolation on a number of *in vitro* and *in vivo* studies (Rochefort et al. 2005). Therefore, the marrow stromal fraction of cells that is used in this study are adherent to plastic, express 2 MSC positive surface antigens, and contain a negligible percentage of $CD45^+$ cells, are highly proliferative in culture and show potent differentiation potential. In light of the lack of universally accepted criteria, this sufficiently addresses the requirements for the minimal criteria to define mesenchymal stem cells.

The induction of MSC osteogenic differentiation by osteogenic factors now follows an standardised established protocol (Jaiswal et al. 1997), however, since MSCs are sensitive to their extracellular matrix, it was deemed important in this study to examine whether this induction was possible on collagen-coated silicone, and whether the collagen adsorbed on the membrane had an effect in the osteogenic response. It was found that the silicone membrane used for this research was capable of supporting osteogenic differentiation of MSCs and that the collagen substrate did not affect the progress of differentiation. When seeded onto both silicon and collagen-coated silicone, MSCs expressed collagen type I at

7, 21 and 28 days, osteocalcin at 28 days, and calcified mineral was detected in the extracellular matrix in both environments after 21 and 28 days in culture. This temporal expression is reflective of the distinct stages described for the formation of bone that include the proliferation phase during which collagen type I is expressed; the down regulation of proliferation and the onset of matrix maturation; and finally matrix mineralisation which coincides with osteocalcin and other matrix related proteins. One unexpected observation was that osteocalcin could not be detected at the 21 day time-point; however, matrix mineralisation was proceeding. It is possible that this is connected to the lower concentration of dexamethasone that was used in this study. In addition to the osteogenic markers analysed here, other analyses are commonly carried out, and their application can help to more accurately define the stages and transitions in osteogenic differentiation. These include enzymatic activity analysis of alkaline phosphatase, and X-ray diffraction to analyse mineral deposition (Jaiswal et al. 1997). The latter is often used in conjunction with von Kossa staining, as the von Kossa technique detects phosphate ions which can be found both in the intracellular cytosolic compartment, and from the culture medium used with cells.

In the study by Jaiswal et al. (Jaiswal et al. 1997) on the characterisation of a reproducible system for the induction of osteogenic differentiation from purified, culture expanded MSCs *in vitro*, the optimal dose of dexamethasone was determined by monitoring alkaline phosphatase activity and extracellular mineral deposition. Following a dose dependency study, it was found that all concentrations of dexamethasone examined (1 – 1000 nM) significantly increased alkaline phosphatase activity. At the lowest concentration (1nM), osteogenic morphological and mineralisation features were not detected following 16 days of treatment; however alkaline phosphatase activity demonstrated a 3-fold increase. Thus, based on high alkaline phosphatase activity and cell attachment characteristics, that study chose 10 nM as the optimal concentration (Jaiswal et al. 1997). In this study, dexamethasone is used at a concentration of 0.68nM, and this may explain the delayed onset of mineralisation markers at 21 days. Jaiswal et al (1997), report mineralisation at later time-points when using low concentrations of dexamethasone. The concentration of dexamethasone used in this study is considerably lower than that which was found to be optimal by Jaiswal et al. (1997). Since emphasis has been placed upon its role as the promoter of alkaline phosphatase and other enzymatic activity, this low concentration may not have induced optimal temporal osteogenic marker expression. However, osteoblastic

differentiation has been reported in the absence of glucocorticoid in fetal murine marrow stromal cells (Coelho and Fernandes 2000), and in some studies, more emphasis is placed upon the presence of ascorbate and a phosphate ion source (Chang et al. 2006) .

Since MSCs differentiated with similar temporal expression patterns on both silicone and collagen-coated silicone substrates, this indicates that in this model, the osteogenic stimulus is dominant. In other studies where collagen is present on the extracellular substrate, it has not been seen to be influential over osteo-inductive factors (Farrell et al. 2006; Fujita et al. 2005). This is supported by the absence of osteogenic markers on both substrates in control culture conditions. In contrast, in a study where collagen type I was presented in the presence of osteoinductive factors as a further solubilised supplement in the culture medium, exogenous collagen enhanced both early and late osteogenic differentiation, demonstrating the role it can have in the differentiation process (Kihara et al. 2006). A collagen component in the cell substrate has been demonstrated to promote cell proliferation and attachment (Fujita et al. 2005; Simmons et al. 2003) and from Chapter 3, cell attachment was favoured by a collagen-coated substrate. The absence of an osteoinductive effect may be due to the degradation of the collagen helix as a result of the preparation method. Therefore, differentiation of MSCs in this study is unaffected by the collagen-coated substrate.

Upon investigating signalling pathways, this study found a trend towards the MAPK p38 and pI3-kinase to have an involvement on the expression of osteogenic proteins and in matrix mineralisation. Following 14 and 21 days in osteogenic conditions, in the absence or presence of p38 and pI3-kinase inhibitors, there was a trend towards an increase in calcium in the matrix following osteogenic treatments, which could be blocked in the presence of inhibitors. This trend was also observed following fluorescent immunohistochemical detection of osteocalcin after 21 days of treatments. While these data collectively infer trends for mechanisms, further studies are necessary, that might use more refined techniques for quantification of alizarin red staining, to clarify this trend. Several studies have suggested that p38 MAPK is activated and involved in the expression of numerous osteogenic markers following osteogenic factor induction of osteogenesis (Suzuki et al. 2002). PI3-kinase is also responsive to extracellular stimuli, and has been shown to be involved in the osteogenic response upon growth factor treatment in osteoblast pre-cursor cells

(Osyczka and Leboy 2005). While the responses in this study were consistent within samples, to statistically confirm putative roles for these signalling pathways, further investigations should be carried out. The discrepancies between immunohistochemical staining and statistical analysis upon quantification, may be due to large standard errors of the mean caused variation in these particular samples. This can benefit from increasing the number of experimental repetitions. Furthermore, use of the semi-quantitative technique, western blots, is an approach that may address the inconsistencies and variations in the data from the signalling experiments. In addition, the real time polymerase chain reaction (RT-PCR) could provide more qualitative data for earlier cellular responses through detection of mRNA levels.

To summarise, this study has characterised the cell population used in this research as mesenchymal stem cells, and has identified the cell population to be homogeneous. These cells were responsive to osteogenic factor treatments when seeded on collagen-coated membranes, and collagen did not affect the differentiation of the cells. Investigations on the signalling in these cells identified trends for roles for p38 and pI3-kinase in osteogenic protein expression.

5 The effect of cyclic strain on mesenchymal stem cell differentiation

5.1 Introduction

Physical stimulation is an important regulator of tissue homeostasis and the effects on bone remodelling are well known (1978). While the application of physiological mechanical stimulation occurs at the tissue or organ level, this resonates down to the cellular unit where the mechanical stimulus is transduced to a biochemical signal. A cellular response is initiated, and this can in turn have effect at the whole tissue level. Bone formation during embryological skeletogenesis and development during fracture repair involves the recruitment and differentiation of progenitor cells (Bruder et al. 1994; Caplan 1987). In combination with biochemical influences, the mechanical microenvironment regulates the differentiation of these osteoprogenitors to form skeletal tissue suitable for the structural properties of the local area. Theoretical models of skeletal mechanobiology have been of interest for some time; however it is just recently that these have been applied to osteochondral progenitor models (Prendergast et al. 1997), and experimental validation remains incomplete. Additionally, a precise molecular signature that couples strain to osteogenic differentiation remains to be fully defined. Therefore, in this study the effect of tensile mechanical strain on the osteogenic protein expression of MSCs was examined, with an investigation into the accompanying mechanotransduction mechanisms.

Physiological osteoblast development during both embryological skeletogenesis by intramembranous ossification, and fracture repair, follows a defined sequence of events that begins with the proliferation of progenitor cells (Karsenty 2003; Mackie 2003). The differentiation that follows is particularly responsive to the mechanical composition of the microenvironment. For example, embryonic motility represents an important epigenetic component of development (Muller 2003) and biophysical stimulation promotes bone fracture repair through the stimulation of cell responses that follow the bone remodelling principle regulated by the hypothesis proposed by Wolff (Chao and Inoue 2003; Wolff 1892). Osteoblast formation is regulated by the key transcription factor, Cbfα1 (Harada and Rodan 2003). Mechanical stress influences the differentiation of precursor cells by increasing the expression of the Cbfα1 gene. Following mechanical loading of the periodontal ligament, there is an up-regulation of Cbfα1 expression corresponding to areas experiencing tensile strain (Kawarizadeh et al. 2005). This has also been observed following the application of tensile strain *in vitro* (Ziros et

al. 2002), and in both environments, a concomitant up-regulation of phosphorylated ERK1/2 is observed (Kawarizadeh et al. 2005; Ziros et al. 2002). These findings implicate an ERK-mediated Cbfα1 regulation of osteoblast differentiation, in response to a strain stimulus. Since MAPKs are known to be responsive to mechanical strain among other extracellular signals (Franceschi and Xiao 2003), it follows that the mechanotransduction of tensile strain may act through these molecules. Developmental differentiation of osteoblasts continues with the expression of a characteristic pattern of genes (Caetano-Lopes et al. 2007) that is mirrored *in vitro* (Lian et al. 1997; Marie et al. 1989; Robey and Termine 1985). These include collagen type I osteopontin, alkaline phosphatase BMP2 and osteocalcin.

Stretch-activated cation channels (SACCs) in the plasma membrane have been the focus of mechanoreceptors in a number of studies of mechanoregulation. They have been reported in a variety of cell types including primary osteoblast cells (Charras et al. 2004; Sachs and Morris 1998) and studies report whole cell cytosolic calcuim responses following their manipulation, supporting a putative role of SACCs as the first step in the transduction of extracelluar stimuli into a cell fate response (Kirber et al. 2000; Zou et al. 2002). Gadolinium, which inhibits SACCs (Yang and Sachs 1989), significantly attenuates the increase in intracellular calcium concentration in mechanically stimulated osteoblasts (Danciu et al. 2003).

The MAPK pathway plays a crucial role in cell proliferation and differentiation by transmitting extracellular signals from the membrane to the nucleus. Since tensile stimulation is an extracellular stimulus, the MAPK pathway has formed a central focus for investigations of mechanotransduction. Osteogenic differentiation of MSCs by biochemical induction has been shown to be differentially regulated by ERK1/2 and p38 (Jaiswal et al. 2000). Mechanical stimulation of MSCs also initiate MAPK responses (Simmons et al. 2003) and it is thought that these act through BMP-2 to regulate bone associated protein responses (Lai and Cheng 2002). Through the relationship of PI3-kinase with cell membrane phospholipids, its associated signalling has also received attention in mechanotransduction studies (Danciu et al. 2003; Petroff et al. 2001; Suzuma et al. 2000). Mechsanosensitive integrins of the G-protein family such as Ras (Takai et al. 2001) are involved in the activation of the PI3-kinase pathway (Vanhaesebroeck et al. 2001).

Although the biochemical conditions have been identified that stimulate MSC differentiation, it is likely that biomechanical forces, such as those associated with skeletal loading, are also involved in this process. Despite the knowledge of

osteoblast responses to mechanical stimulation, studies on biomechanical stimulation alone (i.e. in the absence of osteogenic growth factors) in MSCs have yet to be completed and stand to contribute to tissue engineering. The aim of this study was to assess the effect of cyclic tensile mechanical stimulation on the osteogenic protein expression of MSCs and to identify the mechanotransduction behind strain-mediated MSC osteogenic differentiation. A custom-designed uniaxial bioreactor delivered a strain stimulus to MSCs seeded on a collagen-coated silicone membrane for up to 14 days, duing which time, protein expression was examined. Tensile strain affects the cell membrane by physically deforming it. SACCs respond to tensile strain by opening and allowing an inward current of non-specific cations. It is not clear whether these membrane channels are involved in transmitting mechanical signals in MSCs to regulate osteogenic differentiation. Thus, using the SACC blocker, gadolinium chloride, during the delivery of tensile strain, the role of SACCs as mechanosensors involved in strain-induced protein expression was examined. To investigate the intracellular signalling pathways in the mechanotransduction of strain-induced protein regulation, MAPK signalling was investigated using inhibitors for ERK and p38 during the delivery of tensile strain. Phospholipids in the cell membrane are also involved in the transmission of extracellular events (Duronio et al. 1998; Kandel and Hay 1999) through enzymatic breakdown in response to receptor stimulation, to products with second messenger activity. Since PI3-kinase forms part of this biochemical pathway, a role for this enzyme in mechanotransduction was examined using an inhibitor of PI3-kinase. This study investigated the hypothesis that mesenchymal cell fate, in particular osteogenic tissue differentiation, could be regulated, in the absence of biochemical stimulation, by mechanical strain via SACCs and intracellular signalling associated with the MAPK members ERK and p38, and the PI3-kinase enzyme.

5.2 Results

5.2.1 Collagen type I expression is unchanged between clamped, flow and static control conditions

In experiments designed to examine the effect of strain on MSC differentiation, the control unstrained cells ware cultured on collagen-coated silicone that is placed in a tissue culture dish. This was validated against cells that ware cultured on collagen-coated silicone and attached in the clamps of the mechanical straining device statically (i.e. clamped control). Also, during tensile cyclic loading of the silicone membrane, the movement of the clamp through the culture medium causes a disturbance of the culture medium surrounding the cells. This can exert a fluid shear stress over the surface of the cells. Since fluid shear stresses have shown to have potent effects on cellular fate (Li et al. 2004; Vezeridis et al. 2006), it was appropriate to examine the effect of this fluid movement in this experimental setup. To replicate the fluid shear stimulus alone, in the absence of the tensile strain stimulus, one end of the cell-seeded silicone strip was attached to the fixed clamp in the rig, while the other end remained unclamped. The rig was then turned on so that the shear flow conditions would be created through the movement of the clamp in the culture medium.

Cells were (i) cultured statically in a tissue culture dish, (ii) clamped in the mechanical device, (iii) clamped at one end or (iv) strained (2.5%;Figure 5.1A). After 3 days, collagen type I expression was assessed by fluorescence immunocytochemistry (Figure 5.1B). MSCs in (i) static control (ii) static clamped conditions and (iii) flow conditions express background levels of collagen type I immunoreactivity. However, MSCs exposed to cyclic strain (iv) display strong collagen type I immunoreactivity. Collagen type I fluorescence intensity was quantified by examining the average grey level intensity for at least 80 cells in each group (Figure 5.1C). Fluorescence intensity was greater than negative controls only in strained conditions.

These data suggest that both clamping and the fluid shear force created by flow of the culture medium has no effect on protein expression in cultured MSCs and indicates that any changes in protein expression through the application of strain can primarily be attributed to the tensile forces applied to the silicone, and not to the fluid shear force created concomitantly through the application of tension. This validates the method of culturing MSCs in a tissue culture dish without the

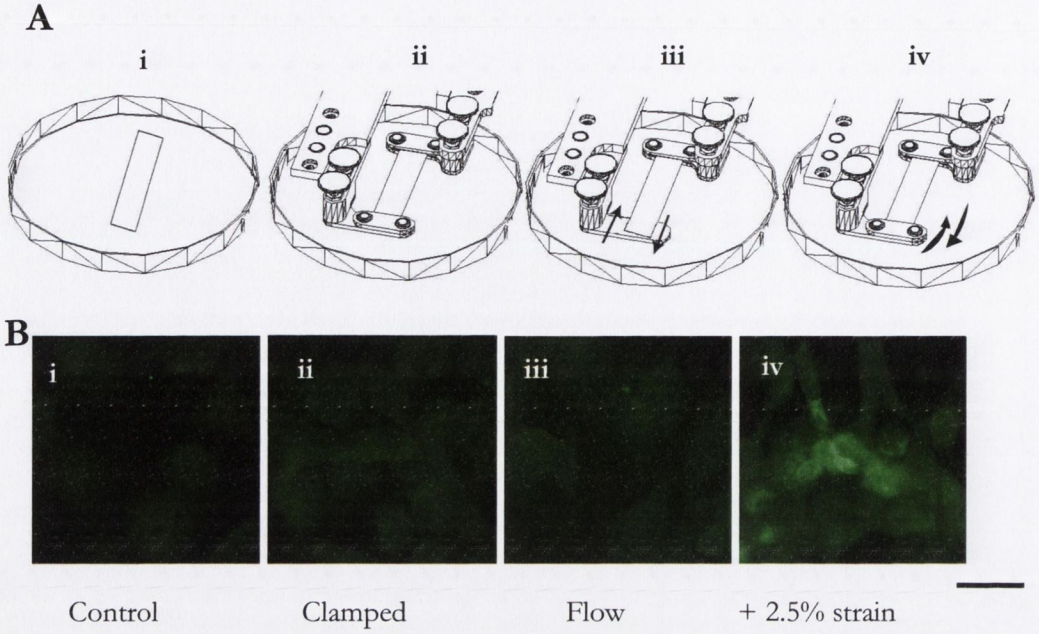
application of clamps on the silicone as appropriate control conditions. Results are representative of 4 independent experiments.

Figure 5.1 Clamping and concomitant fluid flow have no effect on MSC collagen type I expression

A: MSCs on collagen-coated silicone were cultured statically in a tissue culture dish (i), statically but clamped in the mechanical stretching device (ii), clamped at 1 end (iii), or clamped in the mechanical stretching device and 2.5% strain applied to the substrate (iv). After 3 days, cells were fixed and the expression of Collagen type I was assessed by immunofluorescence.

B: In static controls in the tissue culture dish (i), the clamped control (ii) and the strip clamped at 1 end (iii), only background immunostaining is evident. Immunofluorescent staining for collagen I is markedly increased in cells exposed to mechanical loading (iii; approx. 90% of cells).

C: Expression of collagen type I was quantified by measuring the grey level intensity of the fluorescent images. Fluorescence intensity in each of the 4 conditions is compared to negative controls. Only in cells that were stimulated by 2.5% strain did fluorescence intensity increase to above negative control. Results are expressed as mean ± SEM for 100 cells in 4 independent experiments, *$p<0.05$. Scale bar is 25μm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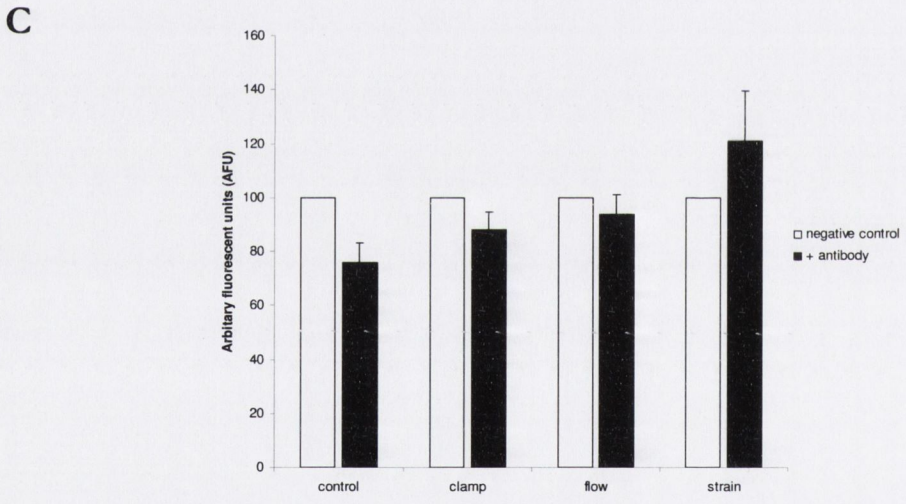
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5.2.2 Strain of 2.5% induces Cbfα1 expression after 3 and 6 days.

Strain-mediated differentiation towards the osteogenic lineage can be assessed through examining changes in cellular protein expression. One of the early events in osteogenesis is the activation of the transcription factor Cbfα1 that is known to induce the expression of osteoblast specific genes (Komori et al. 1997).

To assess the influence of strain on Cbfα1 expression, cyclic tensile strain was applied to MSC seeded collagen-coated silicone membranes for 3 and 6 days. The effect of mechanical stimulation on MSC differentiation was assessed using immunocytochemistry in conjunction with fluorescence microscopy. Immunoreactivity was quantified by measuring the average grey level intensity.

Cells were fixed and probed for the expression of Cbfα1. Tensile mechanical strain of 2.5% at 0.17 Hz for 3 days induced the expression of Cbfα1. Figure 5.2A demonstrates that (i) MSCs in static culture display background immunoreactivity and (ii) this immunoreactivity was increased in all mechanically stimulated cells. Quantification of the grey level intensity in control and strained cells (iii) demonstrates that immunoreactivity significantly increased from 13.66 ± 1.15 AFU (mean ± SEM) in control cells to 27.07 ± 1.84 AFU in cells exposed to mechanical strain ($p<0.05$, Students paired t-test; n=200 cells).

Tensile mechanical strain of 2.5% at 0.17 Hz for 6 days induced the expression of Cbfα1. Figure 5.2B demonstrates that (i) MSCs in static culture display background immunoreactivity and (ii) this immunoreactivity was increased in all mechanically stimulated cells. Quantification of the grey level intensity in control and strained cells (Figure 5.2B) demonstrates that immunoreactivity significantly increased from 15.91 ± 1.04 AFU (mean ± SEM) in control cells to 20.11 ± 1.04 AFU in cells exposed to mechanical strain ($p<0.05$, Students paired t-test; n=4), This result indicates an induction of Cbfα1 in MSCs in response to a 3 and 6 day 2.5% mechanical strain.

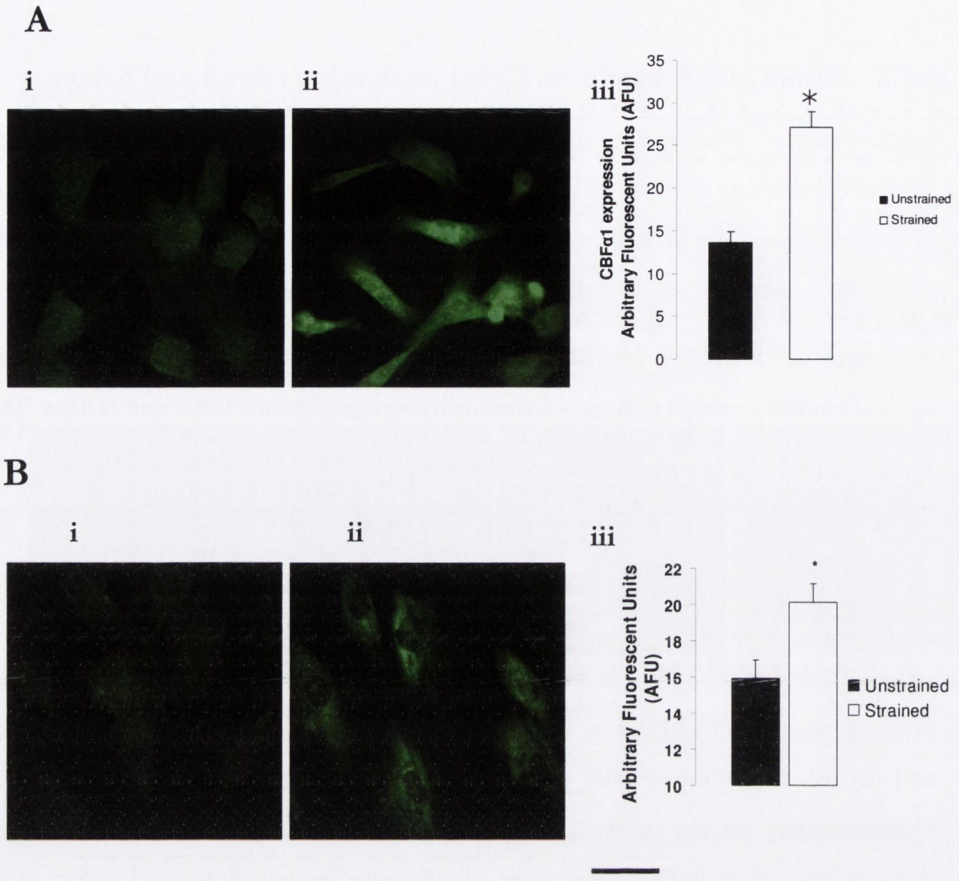


Figure 5.2 Strain of 2.5% induces Cbfα1 expression after 3 and 6 days.

MSCs seeded onto collagen-coated silicone were (i) grown statically or (ii) exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz. Cells were fixed and the expression of Cbfα1 was assessed by immunofluorescence.

Following 3 days (A) and 6 days (B), MSCs in static culture (i) display only background staining for Cbfα1; however MSCs exposed to mechanical strain (ii) display intense Cbfα1 immunoreactivity. Quantification of grey level values (iii) demonstrates a significant increase in fluorescence intensity in cells exposed to mechanical strain compared to unstrained controls. Results are expressed as mean ± SEM for 200 cells, *$p<0.05$. Scale bar is 25μm.

5.2.3 Strain of 2.5% induces collagen type I expression after 3, 6 and 9 days, but not 12 days.

It has previously been demonstrated by this lab that tensile mechanical strains of 2.5% 5% and 10% promote the expression of collagen type I in MSCs compared to unstrained controls (Kearney 2004). To repeat this, to investigate whether this expression is sustained with continued mechanical strain, and whether strain regulates any later markers of osteogenic differentiation, cyclic tensile strain was applied for 3, 6, 9 and 12 days. MSC differentiation was assessed using immunocytochemistry in conjunction with fluorescence microscopy, and immunoreactivity was quantified by measuring the average grey level intensity.

Following a 3 day strain, cells were fixed and probed for the expression of collagen type I. Tensile mechanical strain of 2.5 % at 0.17 Hz for 3 days promoted the expression of collagen type I in MSCs. Figure 5.3A demonstrates that (i) MSCs in static culture display background immunoreactivity however (ii) immunoreactivity was increased in all mechanically stimulated cells. This result is in agreement with the previous findings of this laboratory, that mechanical strain induces collagen type I expression in MSCs.

Tensile mechanical strain of 2.5% at 0.17 Hz for 6 days (B) and 9 days (C) also induced the expression of collagen type I in MSCs. In static culture (Figure 5.3Bi, Ci), MSCs display background collagen type I immunoreactivity which was increased in all mechanically stimulated cells (Figure 5.3Bii, Cii). Quantification of the grey level intensity demonstrates that collagen type I immunoreactivity significantly increased from 12.53 ± 1.29 AFU (mean ± SEM) in control cells to 21.53 ± 1.44 AFU in cells exposed to mechanical strain for 6 days ($p<0.05$, Students paired t-test; n=4, Figure 5.3Biii) and from 10.02 ± 0.97 AFU in control cells to 20.75 ± 2.12 AFU in cells exposed to mechanical strain for 9 days ($p<0.05$, Students paired t-test; n=4, Figure 5.3Ciii)

Tensile mechanical strain of 2.5% at 0.17 Hz for 12 days did not affect the expression of collagen type I in MSCs (Figure 5.3D). Figure 5.3D demonstrates that in both (i) static and (ii) strained conditions, MSCs do not display collagen type I immunoreactivity. Quantification of the grey level intensity in control and strained cells (Figure 5.3 Diii) reveals that in static conditions, collagen type I immunoreactivity was 9.4 ± 1.08 AFU and this was not altered following a 12 day 2.5% mechanical stimulus (11.9 ± 2.05). These data suggest that 2.5% mechanical strain for 3, 6, and days induces expression of collagen type I in MSCs which is

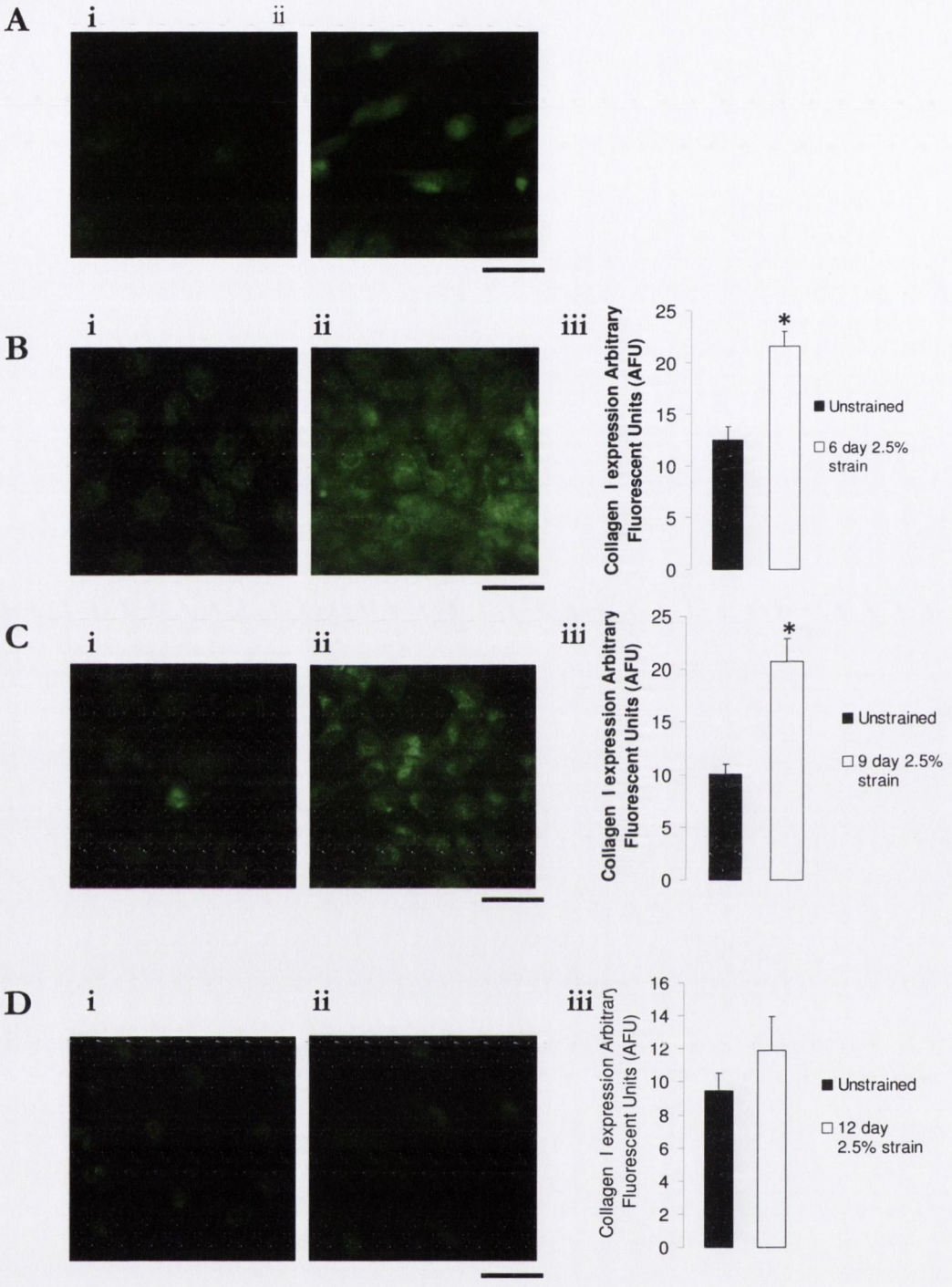
not seen following 12 days, thus collagen type I expression is dependent on the duration of strain stimulus.

Figure 5.3 Strain of 2.5% induces collagen type I expression after 3, 6, 9 but not 12 days

MSCs seeded onto collagen-coated silicone for 3 (A), 6 (B), 9 (C) and 12 (D) days, were grown (i) statically or (ii) exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz. Cells were fixed and the expression of collagen type was assessed by immunofluorescence. Following 3, 6, and 9 days, MSCs in static culture (i) display background staining for collagen type I; however, MSCs exposed to mechanical strain (ii) display intense collagen type I immunoreactivity. Following 12 days in culture (D), MSCs in both static (i) and under strained conditions (ii) display background immunoreactivity for collagen type I.

Expression of collagen type I was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in fluorescence intensity was observed in cells exposed to mechanical strain for 6 and 9 days compared to unstrained controls. A modest change in fluorescence intensity was observed in cells exposed to mechanical strain for 12 days compared to unstrained controls, however this was not statistically significant. Results are expressed as mean ± SEM for 100 cells in 4 independent observations, Students paired t-test *$p<0.05$

Scale bar is 50μm.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5.2.4 Strain of 2.5% induces osteocalcin expression after 6, 9 and 12 days.

Osteocalcin is a protein associated with the osteogenic phonotype and is reported to become up-regulated at later stages of osteogenic differentiation. Previous investigations in this laboratory have reported no change in osteocalcin expression in response to a 2.5% 5% and 10% 3 day tensile mechanical stimulation of 2.5% (0.17Hz)(Kearney 2004). Since osteocalcin expression occurs later in osteogenic differentiation, it was decided to probe for expression of this protein following strains of 6, 9 and 12 days.

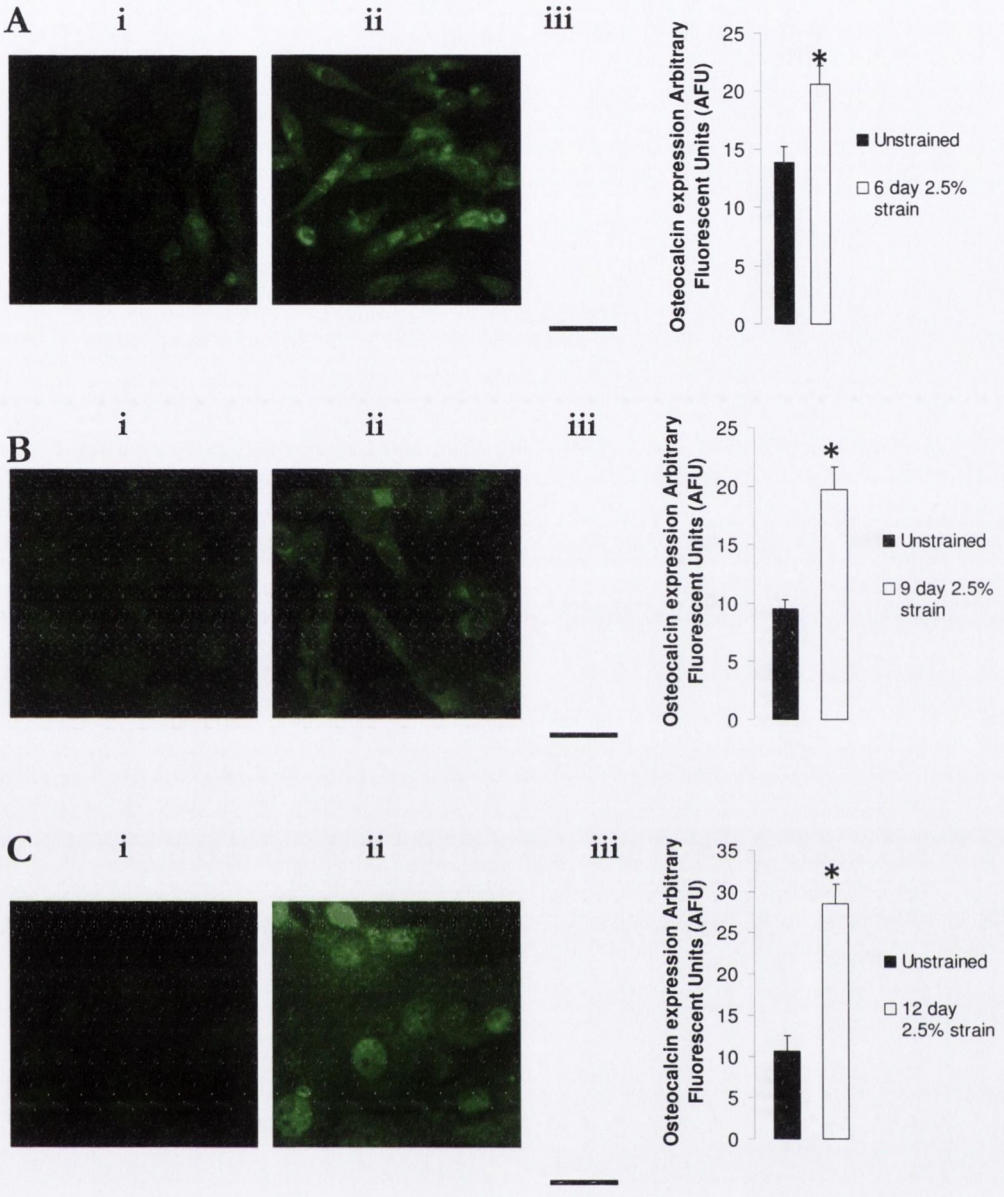
Cells were fixed and probed for the expression of osteocalcin using immunofluorescence. Tensile mechanical strain of 2.5% at 0.17 Hz for 6, 9 and 12 days promoted the expression of osteocalcin in MSCs. Figure 5.4 demonstrates that MSCs in static culture for 6 (A), 9 (B) and 12 (C) days display background osteocalcin immunoreactivity (i) and immunoreactivity was increased in all mechanically stimulated cells (ii). Quantification of the grey level intensity in control and strained cells (iii) demonstrates that osteocalcin immunoreactivity significantly increased from 13.83 ± 1.36 AFU (mean ± SEM) in control cells to 20.6 ± 1.6 AFU in cells exposed to a 6 day mechanical strain ($p<0.05$, Students paired t-test; $n=4$), from 9.52 ± 0.76 AFU in control cells to 19.73 ± 1.94 in cells exposed to a 9 day mechanical strain ($p<0.05$, Students paired t-test; $n=4$) and from 10.65 ± 1.86 AFU in control cells to 28.47 ± 2.42 in cells exposed to a 12 mechanical strain ($p<0.05$, Students paired t-test; $n=4$) These data indicate that osteocalcin expression is induced in response to 2.5% cyclic mechanical strain for 6, 9 and 12 days.

Figure 5.4 Strain of 2.5% induces osteocalcin expression after 6, 9 and 12 days

MSCs seeded onto collagen-coated silicone were grown (i) statically or (ii) exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz for 6 (A), 9 (B) or 12 (C) days. Cells were fixed and the expression of osteocalcin was assessed by immunofluorescence. MSCs in static culture (i) display background staining for osteocalcin; however, (ii) MSCs exposed to mechanical strain display intense osteocalcin immunoreactivity.

Expression of osteocalcin was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in fluorescence intensity was observed in cells exposed to mechanical strain of 6 (A), 9 (B) and 12 (C) days compared to unstrained controls. Results are expressed as mean ± SEM for 100 cells in 4 independent observations, Students paired t-test *$p<0.05$

Scale bar is 50μm.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5.2.5 Osteocalcin expression in response to a 14 day 2.5% strain

To examine the effect of a 14 day strain on osteocalcin expression, cells were cultured in control or strained (2.5%) conditions for 14 days. Expression levels of the osteocalcin protein were measured by western immunoblot using a monoclonal antibody raised against the epitope mapping the gamma-carboxylated residue at position 17 of the full length osteocalcin protein, in conjunction with densitometry (Figure 5.5). Expression levels of the housekeeping protein GAPDH were also measured by western immunoblot, and osteocalcin expression in control and strained conditions was normalised to GAPDH levels as a representation of the total number of cells in each sample.

Figure 5.5A demonstrates that exposure to a 2.5% strain stimulus for 14 days increases osteocalcin in MSCs but not significantly so. Thus osteocalcin expression was 0.74 ±0.08 arbitrary units (mean ± SEM) in control and 1.07 ± 0.2 arbitrary units in strained cells ($p=0.3$, Wilcoxon signed rank test, $n=7$ observations) This finding indicates that 2.5% tensile strain for 14 days does not affect osteocalcin expression in MSCs. A sample immunoblot demonstrating osteocalcin expression in response to strain relative to GAPDH levels is shown in Figure 5.5B.

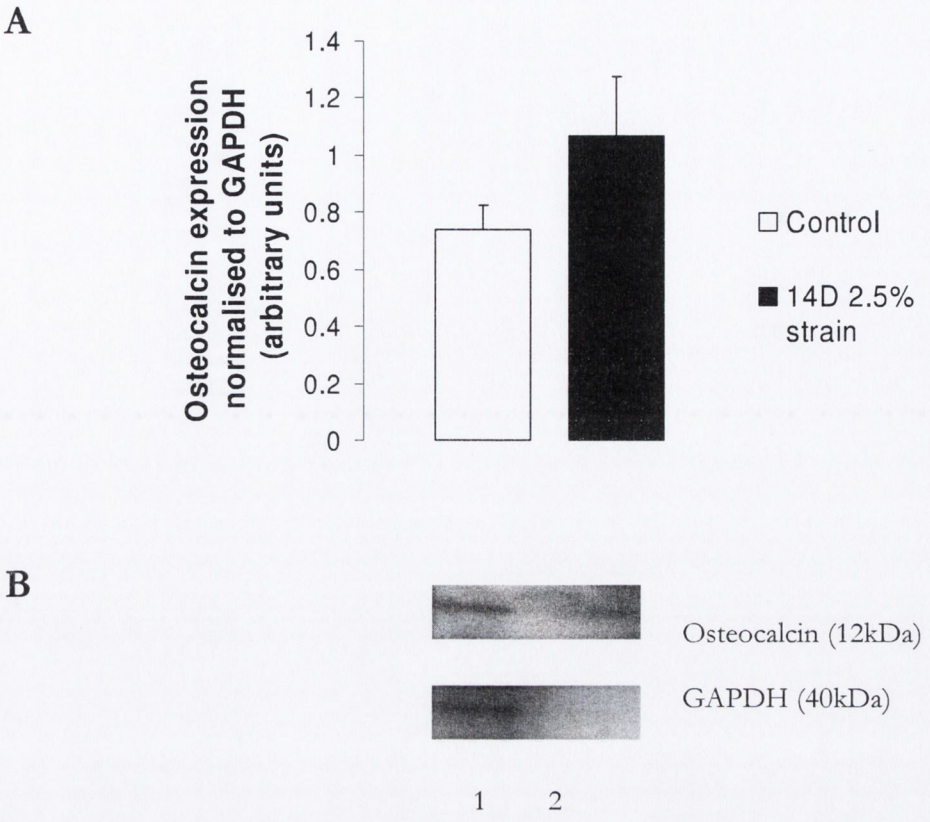


Figure 5.5 Strain mediated expression of osteocalcin.

The effect of a 14 day 2.5% strain on osteocalcin expression in MSCs was examined. Following 14 days in static culture or exposed to 2.5% tensile mechanical strain MSCs were harvested and analysed for expression of osteocalcin using western immunoblotting. Osteocalcin expression was normalised to the housekeeping protein GAPDH expression.

A: There was a marked increase in osteocalcin in response to a 14 day 2.5% strain; however, this failed to reach statistical significance. Results are expressed as mean ± SEM for 7 independent observations.

B: A sample western immunoblot demonstrating osteocalcin protein expression at 14 days in control (lane 1) and strained MSCs (lane 2). GAPDH immunoblots display the variability in protein loading of samples.

5.2.6 Strain of 2.5% induces BMP2 expression after 14 days.

Following a 14 day strain, cells were fixed and probed for the expression of BMP2 using immunofluorescence. Tensile mechanical strain of 2.5 % at 0.17 Hz for 3 days promoted the expression of BMP2 in MSCs (Figure 5.6). Figure 5.6A demonstrates that MSCs in (i) static culture display background immunoreactivity and (ii) this immunoreactivity was increased in approx 90 % of mechanically stimulated cells (n = 100 cells).

Expression levels of BMP2 protein were also measured by western immunoblot using a polyclonal antibody raised against the synthetic peptide corresponding to amino acids 45-60 of BMP2 protein, and band widths were quantified using densitometry (Figure 5.6B). Expression levels of the housekeeping protein GAPDH were also measured by western immunoblot, and BMP2 expression in control and strained conditions was normalised to GAPDH levels as a representation of the total number of cells in each sample. Following a 14 day 2.5% strain stimulus, BMP2 expression was significantly increased from 0.79 $\pm$ 0.15 arbitrary units (mean $\pm$ SEM) in unstrained control cells to 4.34 $\pm$ 1.88 arbitrary units ($p<0.05$, Wilcoxon signed rank test, $n=10$ observations; Figure 5.6B). This finding indicates that 2.5% tensile strain for 14 days induces BMP2 expression in MSCs.

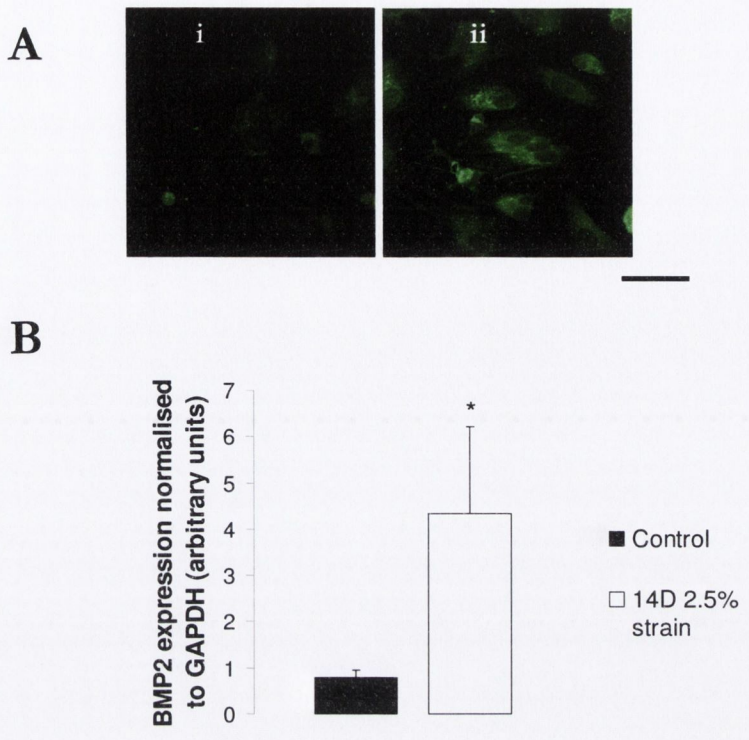


Figure 5.6 Effect of 2.5% strain on BMP2 expression after 14 days.

MSCs seeded onto collagen-coated silicone were grown statically or exposed to cyclic mechanical tensile loading if 2.5% at 0.17 Hz for 14 days.

A: Cells were fixed and the expression of BMP2 was assessed by immunofluorescence. MSCs in (i) static culture display only background staining for BMP2; however (ii) MSCs exposed to mechanical strain display intense BMP2 immunoreactivity. Scale bar is 50μm. n = 100 cells.

B: Following 14 days in static culture or exposed to 2.5% tensile mechanical strain MSCs were harvested and analysed for expression of BMP2 using western immunoblotting. BMP2 expression was normalised to the housekeeping protein GAPDH expression. Strain of 2.5% significantly increased BMP2 expression following a 14 day stimulation. Results are expressed as mean ± SEM for 10 independent observations, Wilcoxon signed rank test, *$p<0.05$.

5.2.7 Summary of strain-induced protein expression

Protein expression in response to a continuous 2.5% cyclic tensile strain for 3 – 14 indicates that following 3 days Cbfα1 expression is increased compared to unstrained controls and this continues to 6 days, beyond which it was not examined. Collagen type I expression is increased following 3, 6, and 9 days, but not 12 days of strain, the maximum time examined. Increased expression in osteocalcin was observed after 6 days of strain; this trend continued at 9 and 12 days, but not following 14 days of strain. BMP2 expression was examined only following a 14 day stimulation and expression was up-regulated when compared to unstrained controls. This summary is represented schematically in Figure 5.7.

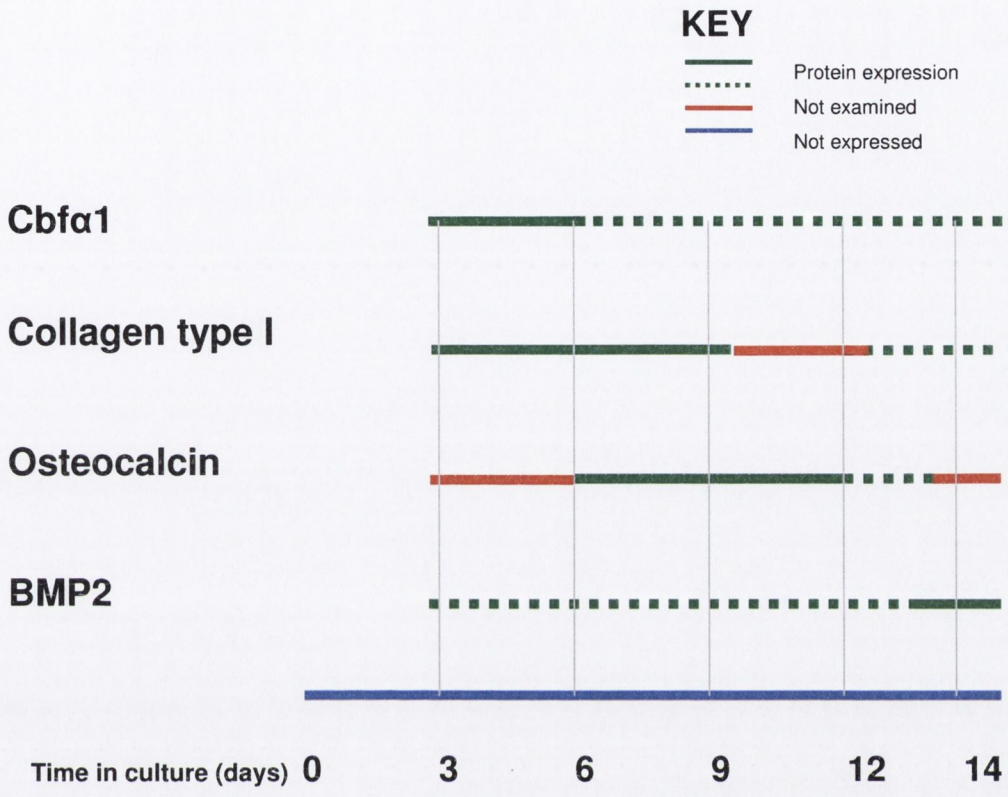


Figure 5.7 Summary of strain-induced protein expression over time

Strain-induced protein expression was examined as a function of time. The early osteogenic marker Cbfα1 was expressed following 3 and 6 days of strain. Collagen type I was expressed following 3, 6, and 9 days, but not 12 days of strain. Osteocalcin was not expressed after 3 days but demonstrated an increase in expression between 6, 9 and 12 days (measured by immunocytochemistry), which was not seen at 14 days (measured by western blot). BMP2 was examined only at 14 days and was found to be expressed.

5.2.8 SACCs are involved in strain-mediated Cbfα1, collagen type I and osteocalcin expression

The osteogenic protein expression profile of the MSC response to tensile mechanical strain has been demonstrated above. To investigate the mechanisms of mechanotransduction of the strain stimulus, the role of stretch activated cation channels on the cell membrane were examined. Since it has been demonstrated that Cbfα1, collagen type I and osteocalcin were all expressed after 6 days of mechanical stimulation, this time-point was chosen to investigate the mechanism of MSC osteogenic differentiation.

To examine the role of SACCs in strain-mediated Cbfα1, collagen type I and osteocalcin expression, cells were cultured in control and strained conditions for 6 days in the absence and presence of the SACC blocker gadolinium chloride (Gd^{3+}; 10μM). Protein expression was assessed by fluorescence microscopy in fixed cells. Figure 5.8 represents Cbfα1 (A), collagen type I (B) and osteocalcin (C) staining in (i) control, (ii) strained, (iii) control with Gd^{3+} and (iv) strain with Gd^{3+} conditions.

An increase in Cbfα1 immunoreactivity is demonstrated following mechanical stimulation for 6 days (Figure 5.8 A ii, iv), however, this increase in less marked in cells that were strained in the presence of Gd^{3+} (Figure 5.8 A iv). Quantification of Cbfα1 immunoreactivity (Figure 5.8A v) reveals that mechanical stimulation for 6 days significantly increases the fluorescence intensity from 15.91 ± 1.04 AFU (mean ± SEM) in unstrained controls to 20.11 ± 1.04 AFU following a 6 day 2.5% strain stimulation ($p<0.05$, Students paired t-test; $n=4$); in the presence of Gd^{3+} immunoreactivity intensity was comparable to control levels. Immunoreactivity was comparable in cells that were strained in the presence or absence of Gd^{3+}.

An increase in collagen type I immunoreactivity is demonstrated following mechanical stimulation for 6 days (Figure 5.8B ii, iv). Immunoreactivity was decreased in cells that were strained in the presence of Gd^{3+} when compared to cells that were strained in the absence of Gd^{3+}.Quantification of the intensity of immunoreactivity (Figure 5.8B v) revealed that the fluorescence intensity in cells that were stimulated with a 6 day 2.5% strain significantly decreased from 22.9 ± 0.4 AFU to 20.9 ± 0.8 AFU in cells strained in the presence of Gd^{3+} ($p<0.05$, Students paired t-test; $n=4$).

An increase in osteocalcin immunoreactivity is demonstrated following mechanical stimulation for 6 days (Figure 5.8C ii, iv), however, this increase in less marked in cells that were strained in the presence of Gd^{3+} (Figure 5.8C iv). Quantification of the intensity of immunoreactivity (Figure 5.8C v) revealed that mechanical stimulation for 6 days significantly increases the fluorescence intensity from 17.79 ± 1.94 AFU in unstrained controls to 24.37 ± 2.29 AFU following a 6 day strain stimulation ($p<0.05$, Students paired t-test; $n=4$), however, in the presence of Gd^{3+} immunoreactivity intensity was comparable to control levels. Immunoreactivity was comparable in cells that were strained in the presence or absence of Gd^{3+}. This finding provides evidence that SACCs are involved in mechanotransduction of the tensile strain stimulus that induces Cbfα1, collagen type I and osteocalcin expression after 6 days.

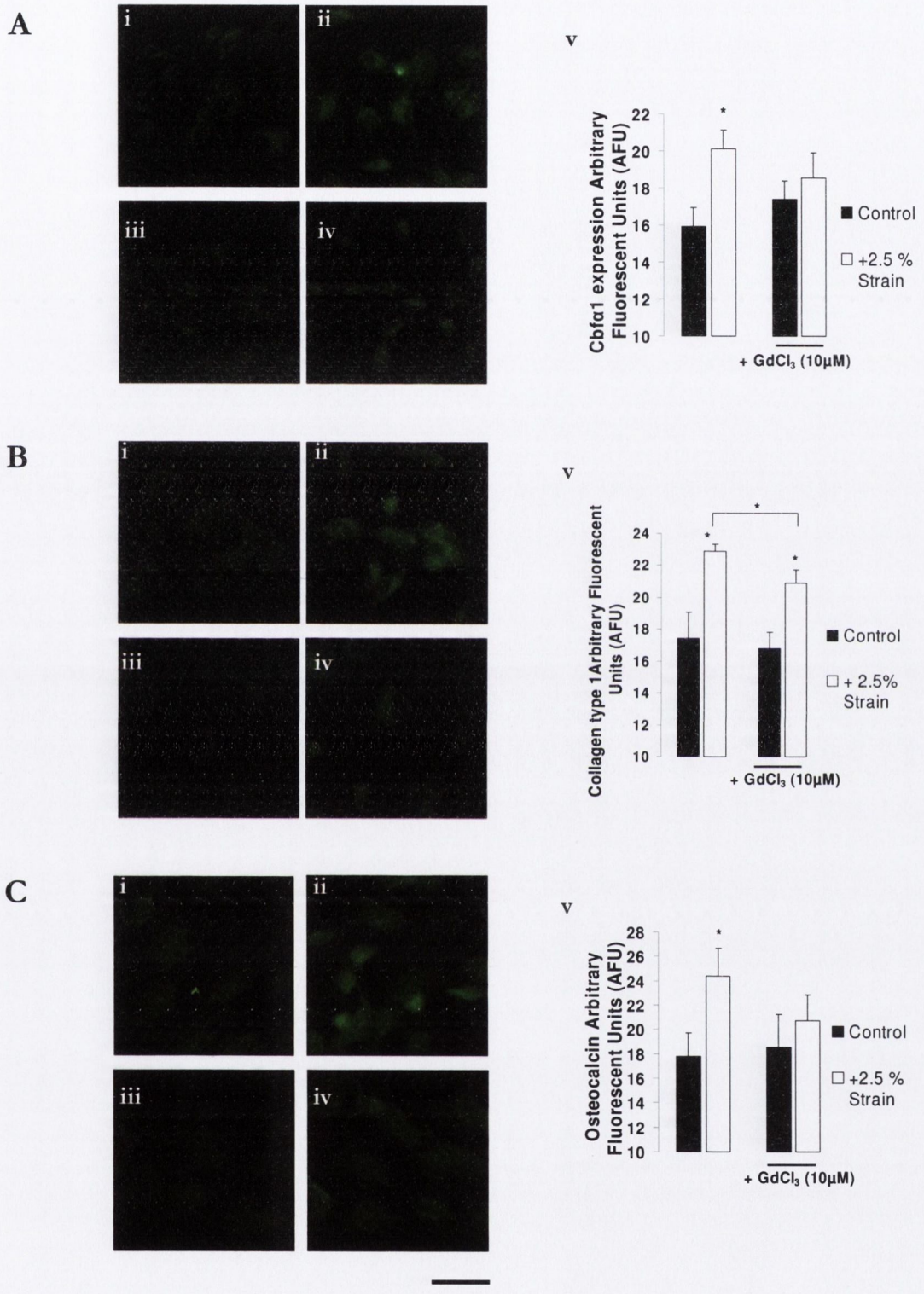
Figure 5.8 SACCs are involved in strain-mediated Cbfα1, collagen type I and osteocalcin expression

MSCs seeded onto collagen-coated silicone were grown (i, iii) statically or (ii, iv) exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz for 6 days in the absence (i, ii) or presence (iii, iv) of the SACC blocker Gd^{3+}. Cells were fixed and the expression of Cbfα1 (A), collagen type I (B) and osteocalcin (C) was assessed by immunofluorescence.

A: MSCs exposed to 2.5% strain for 6 days (ii) displayed more intense Cbfα1 immunoreactivity compared to unstrained cells (i). In the presence of gadolinium chloride (iii, iv), the increase in Cbfα1 immunoreactivity in MSCs exposed to 2.5% strain was less marked. Expression of Cbfα1 was quantified by measuring the grey level intensity of the fluorescent images (v). A significant increase in Cbfα1 fluorescence intensity was measured in cells exposed to mechanical strain compared to unstrained controls. In the presence of gadolinium chloride no significant increase was observed. Results are expressed as mean ± SEM for 100 cells in 5 independent observations, Students paired t-test *$p<0.05$. Scale 50μm.

B: MSCs exposed to 2.5% strain for 6 days (ii) displayed more intense collagen type I immunoreactivity compared to unstrained cells (i). In the presence of gadolinium chloride (iii, iv), the increase in collagen type I immunoreactivity in MSCs exposed to 2.5% strain was less marked. Quantification of the fluorescent images demonstrates that the intensity of collagen type I expression that is measured upon 2.5% mechanical strain for 6 days significantly decreased when cells are strained in the presence of gadolinium chloride. Results are expressed as mean ± SEM for 100 cells in 5 independent observations, Students paired t-test *$p<0.05$. Scale bar is 50μm.

C: MSCs exposed to 2.5% strain for 6 days (ii) displayed more intense osteocalcin immunoreactivity compared to unstrained cells (i). In the presence of gadolinium chloride (iii, iv), the increase in osteocalcin immunoreactivity in MSCs exposed to 2.5% strain was less marked. Expression of osteocalcin was quantified by measuring the grey level intensity of the fluorescent images (v). A significant increase in osteocalcin fluorescence intensity was measured in cells exposed to mechanical strain compared to unstrained controls. In the presence of gadolinium chloride no significant increase was observed. Results are expressed as mean ± SEM for 100 cells in 7 independent observations, Students paired t-test *$p<0.05$. Scale bar is 50μm.


A
i
ii
iii
iv
v
Cbfα1 expression Arbitrary Fluorescent Units (AFU)
22
20
18
16
14
12
10
*
Control
+2.5 % Strain
+ GdCl3 (10μM)
B
i
ii
iii
iv
v
Collagen type 1Arbitrary Fluorescent Units (AFU)
24
22
20
18
16
14
12
10
*
*
*
Control
+ 2.5% Strain
+ GdCl3 (10μM)
C
i
ii
iii
iv
v
Osteocalcin Arbitrary Fluorescent Units (AFU)
28
26
24
22
20
18
16
14
12
10
*
Control
+2.5 % Strain
+ GdCl3 (10μM)

5.2.9 Strain-induced BMP2 expression occurs via p38 signalling

To examine the roles of the MAP kinase p38, PI3-kinase and ERK in a 14 day 2.5% strain induced BMP2 expression, cells were cultured in control or strained conditions in the absence or presence of the p38 inhibitor SB 203580 (10μM), the PI3-kinase inhibitor LY 294002 (2μM) or the ERK inhibitor U0126 (2μM). Expression levels of BMP2 protein were measured by western immunoblot using a polyclonal antibody raised against the synthetic peptide corresponding to amino acids 45-60 of BMP2 protein, and band widths were quantified using densitometry (Figure 5.9). Expression levels of the housekeeping protein GAPDH were also measured by western immunoblot, and BMP2 expression in control and strained conditions was normalised to GAPDH levels as a representation of the total number of cells in each sample.

Figure 5.9A demonstrates that following a 14 day 2.5% strain stimulus, BMP2 expression demonstrated a 5.58 ± 2.09 fold increase (mean ± SEM) over control levels; however in the presence of the p38 inhibitor SB 203580 (10μM) this was significantly decreased to 1.3 ± 0.15 ($p<0.05$, Wilcoxon signed rank test, n=6 observations; Figure 5.9A). Similarly, Figure 5.9B demonstrates that following a 14 day 2.5% strain stimulus, BMP2 expression demonstrated a 7.89 ± 1.67 fold increase (mean ± SEM) over control levels; however in the presence of the PI3-kinase inhibitor LY 294002 (2μM) this significantly decreased to 2.15 ± 0.4 ($p<0.05$, Wilcoxon signed rank test, n=6 observations; Figure 5.9B). In contrast, Figure 5.9C demonstrates that following exposure to a 2.5% strain stimulus for 14 days there is a reduction in the fold increase of strain-induced BMP2 expression when cells were strained in the presence of U0126 (2μM) but not significantly so. Thus BMP2 expression demonstrated a 9.09 ± 1.42 fold increase (mean ± SEM) in strained conditions and a 4.93 ± 1.09 fold increase while strained in the presence of U0126 (2μM) ($p=0.06$, Wilcoxon signed rank test, n=5 observations). These findings indicate that p38 and PI3-kinase are involved in strain-induced BMP2 expression in MSCs, however this is not significantly influenced by ERK signalling.

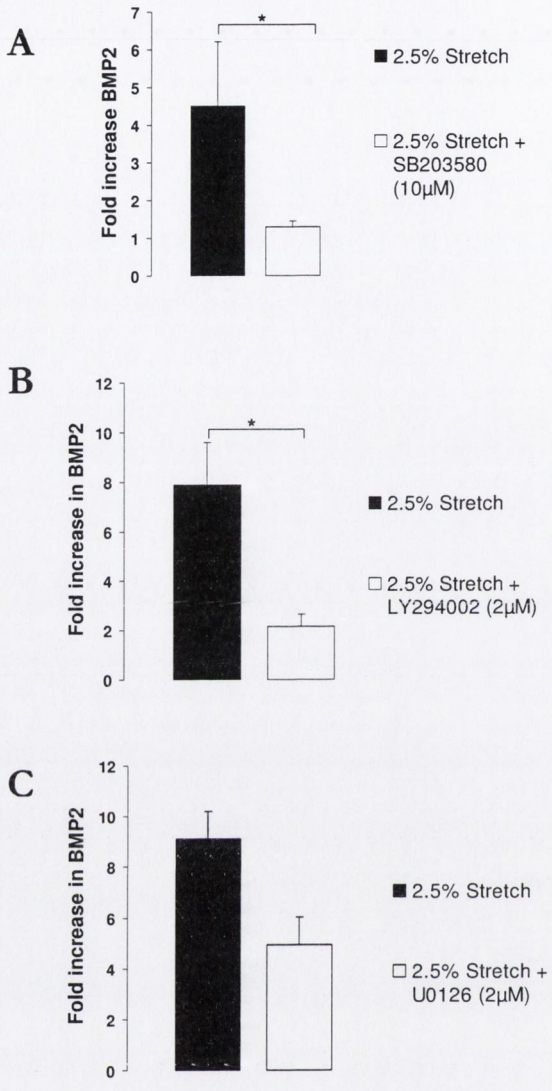
Figure 5.9 Strain mediated BMP2 expression occurs via p38 and PI3-kinase but not ERK signalling.

The role of p38, pI3-kinase and ERK on BMP2 expression in MSCs following a 14 day 2.5% strain was examined. Following 14 days in static culture or exposed to 2.5% tensile mechanical strain in the absence or presence of the p38 inhibitor SB 203580 (10μM), the PI3-kinase inhibitor LY 294002 (2μM), or the ERK inhibitor U0126 (2μM), MSCs were harvested and analysed for expression of BMP2 using western immunoblotting. BMP2 expression was normalised to the housekeeping protein GAPDH expression, and results are presented as fold increases over control expression levels.

A: In the presence of the p38 inhibitor SB 203580 (10μM), the strain-induced fold increase in BMP2 expression was significantly reduced. Results are expressed as mean ± SEM for 6 independent observations, Wilcoxon signed rank test, *$p<0.05$.

B: In the presence of the pI3kinase inhibitor LY 294002 (2μM), the strain-induced fold increase in BMP2 expression was significantly reduced. Results are expressed as mean ± SEM for 6 independent observations, Wilcoxon signed rank test, *$p<0.05$.

C: In the presence of the ERK inhibitor U0126 (2μM), there is a marked reduction in strain-induced fold increase in BMP2 expression however this failed to reach statistical significance. Results are expressed as mean ± SEM for 5 independent observations.


A
Fold increase BMP2
0
1
2
3
4
5
6
7
*
2.5% Stretch
2.5% Stretch + SB203580 (10μM)
B
Fold increase in BMP2
0
2
4
6
8
10
12
*
2.5% Stretch
2.5% Stretch + LY294002 (2μM)
C
Fold increase in BMP2
0
2
4
6
8
10
12
2.5% Stretch
2.5% Stretch + U0126 (2μM)

5.2.10 Effect of strain on p38 and ERK activation

To examine the effect of a 14 day strain on p38 activation, cells were cultured in control or strained (2.5%) conditions for 14 days. MSCs were harvested and phosphorylated/non-phosphorylated p38 protein expression assessed by western immunoblot using an antibody that recognises p38 phosphorylated at thr-180 and tyr-182, and a polyclonal antibody raised against the recombinant full length p38 protein of human origin in conjunction with densitometry (Figure 5.10A). Figure 5.10A demonstrates that exposure to a 2.5% strain stimulus for 14 days reduces phosphorylated p38 in MSCs but not significantly so. Thus phospho-p38 expression was 1.31 ± 0.35 arbitrary units (mean ± SEM) in control and 1.30 ± 0.31 arbitrary units in strained cells ($p=0.6$, Wilcoxon signed rank test, $n=6$ observations).

To examine the effect of a 14 day strain on ERK activation, cells were cultured in control or strained (2.5%) conditions for 14 days. MSCs were harvested and phosphorylated/non-phosphorylated ERK protein expression assessed by western immunoblot using an antibody specific for ERK 1 phosphorylated at Threonine 202 and Tyrosine 204 and ERK 2 phosphorylated at Thronine 185 and Tyrosine 187, and a polyclonal antibody raised against a peptide mapping the C-terminus of ERK2 of rat origin in conjunction with densitometry (Figure 5.10B). Figure 5.10B demonstrates that exposure to a 2.5% strain stimulus for 14 days reduces phosphorylated ERK in MSCs but not significantly so. Thus pERK expression was 1.39 ± 1.00 arbitrary units (mean ± SEM) in control and 0.71 ± 0.35 arbitrary units in strained cells ($p=0.4$, Wilcoxon signed rank test, $n=5$ observations) These findings indicate that 2.5% tensile strain for 14 days does not affect the levels of phosphorylated p38 or phosphorylated ERK2 MSCs.

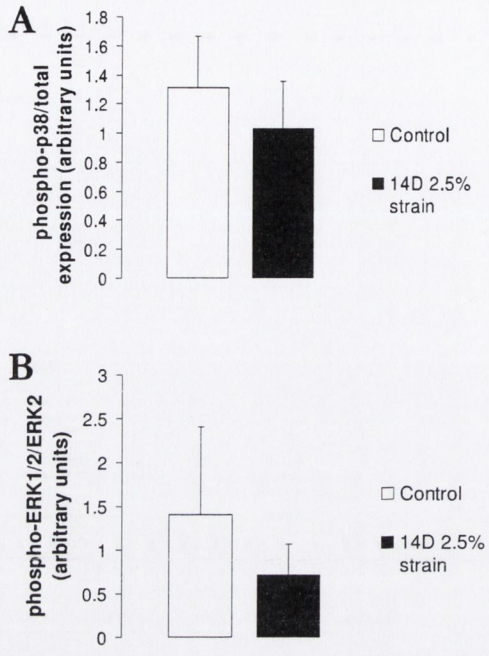


Figure 5.10 Effect of strain on p38 and ERK activation

The effect of a 14 day 2.5% strain on p38 and ERK activation in MSCs was examined.

A: Following 14 days in static culture or exposed to 2.5% tensile mechanical strain MSCs were harvested and analysed for expression of the active (phosphorylated) and inactive (non-phosphorylated) form of p38 using western immunoblotting. Phospho-p38 expression was normalised to the non-phosphorylated form of p38. There was little change in phospho-p38 activity following a 14 day 2.5% strain; however, this was not statistically significant. Results are expressed as mean ± SEM for 6 independent observations.

B: The effect of a 14 day 2.5% strain on ERK activation in MSCs was examined. Following 14 days in static culture or exposed to 2.5% tensile mechanical strain MSCs were harvested and analysed for expression of the active (phosphorylated) and inactive (non-phosphorylated) form of ERK using western immunoblotting. Phospho-ERK expression was normalised to the non-phosphorylated form of ERK.

There was a marked reduction in phospho-ERK activity following a 14 day 2.5% strain; however, this is not a statistically significant change. Results are expressed as mean ± SEM for 5 independent observations.

5.3 Discussion

Much research has emphasised the relationship between mechanical stimulation and tissue response, and more specifically between cellular mechanosensing, mechanotransduction and cell structure and function (Silver and Siperko 2003). Cell differentiation in the skeletal system occurs during embryological skeletogenesis and during fracture repair; in both environments mechanical factors mark the tight regulation of the differentiation process. Mature osteoblasts respond to mechanical stimulation through expression of bone-specific proteins (Carvalho et al. 1998; Harter et al. 1995), and mechanobiological models of tissue differentiation based on the biophysical stimuli within regenerating tissues, predict progressive transitional tissue differentiation appropriate for the mechanical properties of the ECM (Prendergast et al. 1997). Thus, this study investigated the hypothesis that mesenchymal cell fate, specifically osteogenic tissue differentiation, could be regulated, in the absence of biochemical stimulation, by mechanical strain via SACCs and intracellular signalling associated with the MAPK family members ERK and p38, and the PI3-kinase enzyme.

Continuous cyclic tensile strain of 2.5% at 0.17Hz for 3 and 6 days increased expression of the osteogenic specific transcription factor Cbfα1, as determined by fluorescence immunocytochemistry. An increase in collagen type I expression was also observed following a continuous 2.5% strain at 0.17Hz for 3, 6, and 9 days but not after 12 days of continuous stimulation. Under the same strain condition, osteocalcin expression was increased in MSCs following 6, 9 and 12 days of stimulation, however no change in osteocalcin expression was detected following a 14 day strain when examined using the Western blot technique. While this was unexpected, the profile of protein regulation is indicative of a strain-induced osteogenic response. Upon investigating growth factor expression in response to long-term tensile mechanical stimulus, western blotting identified increased levels of BMP2 in cells exposed to a 14 day 2.5% tensile mechanical stimulus, a further indication of strain-induced osteogenic differentiation.

The increase in expression of Cbfα1 following strain at the early time-points of 3 and 6 days suggests commitment of the MSCs towards an osteochondral lineage since during embryonic development, Cbfα1 expression precedes osteoblast differentiation. The concomitant increase in collagen type I expression at 3 and 6 days is confirmation of terminal commitment to the osteogenic lineage, as collagen type I is associated with osteoblast development, and not chondrocyte

development. A number of studies report an upregulation of Cbfα1 in the early osteogenic differentiation of periodontal ligament pre-osteoblast cells by *in vitro* (Ziros et al. 2002) and *in vivo* (Kawarizadeh et al. 2005) mechanical stimulation; and mechanical strain significantly increases Cbfα1 in human bone marrow stromal cells following 4 days of stretching stimulation (Jagodzinski et al. 2004). In a separate study, another type of non-invasive mechanical stimulation called extracorporeal shock wave, linked Cbfα1 activation to osteogenic cell maturation (Wang et al. 2002). The present study therefore suggests that mechanical stimulation may promote the osteogenic differentiation of MSCs by targeting the crucial Cbfα1 transcription factor.

Given that collagen type I is the major organic component in skeletal tissue and the synthesis of collagen type I is sustained in terminally differentiated osteoblasts (Mackie 2003), it was unexpected to observe a down-regulation in expression after 12 days of strain. The expression of collagen type I is upregulated in the early phase of osteoblastic differentiation (Wiesmann et al. 2006) and bone collagen architecture has a high dependency on mechanical strain distribution (Takano et al. 1999). In other studies, mechanical strain sustained an upregulation of collagen type I expression in MSCs up to 14 days (Altman et al. 2002; Juncosa-Melvin et al. 2007; Park et al. 2004; Wiesmann et al. 2006). However, only Park et al examined continuous uniaxial strain and strain application was not examined beyond 2 days (Park et al. 2004). In these studies using uniaxial strain, the delivery of strain was not continuous, but followed an intermittent pattern with rests between stimulations (Juncosa-Melvin et al. 2007; Wiesmann et al. 2006). Thus, the down regulation of collagen type I with sustained tensile stimulation observed in this study may reflect the development of a strain-tolerence of MSCs. All cells of the body are dynamic entities, and often arrange themselves so as to minimise adverse effects of their microenvironment. During cyclic tensile loading, this is seen morphologically with alignment of the cells perpendicular to the direction of strain. It is possible that a parallel mechanism is activated that affects protein expression following a continuous repeated stimulus, and that the down-regulation of collagen type I expression is a marker of tolerance or a desensitisation to the particular strain pattern.

It is known that bone cells desensitise rapidly to mechanical signals (Robling et al. 2001), and bone remodelling is driven by dynamic rather than static loading. A recent long term *in vivo* study determined that mechanical loading is more effective

in enhancing bone biomechanical and structural properties if the loads are applied in discrete bouts, separated by recovery periods, than if the loads are applied in a single session (Robling et al. 2002). Other studies describe a desensitisation of osteoblasts to mechanical stimulation much earlier (following 1 hr) as reflected in the pattern of ERK phosphorylation (Jansen et al. 2004; Rubin et al. 2002). Therefore, it is possible that in this study, the downregulation of collagen type I expression following long-term strain may be an MSC reflection of the 'diminishing returns' phenomenon i.e. as the duration of the loading bout increases without interruption, the osteogenic response tends to saturate. This has been described by Turner, (Turner 1998) for bone cells in accommodation to a customary mechanical loading environment, making them less responsive to routine loading signals, particularly when the loading stimulus is of low magnitude (Gross et al. 2004; Srinivasan et al. 2002).

In a previous study carried out in this laboratory, osteocalcin expression was unchanged in response to a 3 day strain. In this study however, an increase in expression was observed following 6 days of continuous strain, and this was sustained until 12 days of strain, but not after 14 days when examined with the Western immunoblot technique. A recent study reports an osteocalcin gene response to an intermittent strain regimen in combination with a collagen type I substrate, that peaked after 4 days (Ward et al. 2007). The expression of osteocalcin has been considered to establish the differentiated state of the osteoblast (Shea et al. 2003) since it is expressed shortly before mineralisation, marks the late stages of MSC osteogenic differentiation. The delayed down-regulation of osteocalcin could be related to distinct transcriptional regulation of each protein, and that the signalling down regulating collagen type I had yet to have an effect on osteocalcin. It is possible that the desensitisation response for osteocalcin had taken place at 14 days, however the measurements were not recorded using the same technique, and therefore it is difficult to state this definitively. While Mao and Nah (2004) report that continuous stimulation is required to sustain the differentiation response, if this is considered in the context of fracture repair, while mechanical stimulation *is* continuous, the magnitude of the environmental strains vary in accordance with the development of the repair site. Also, it is important to consider the reports that skeletal unloading inhibits proliferation and differentiation of osteoprogenitor cells *in vitro*; thus continuous strain is necessary to maintain the osteogenic phenotype (Kostenuik et al. 1997).

Therefore, it could be that continuous strain sustains MSC differentiation, only when the magnitude of strain is varied.

Members of all the major families of growth factors have been implicated in the control of osteoblast differentiation. BMP2 is expressed during fracture healing in rats (Onishi et al. 1998), and in embryological development (Cao and Chen 2005). In this study, the increase in BMP2 levels at 14 days of continuous strain represents an osteogenic growth factor response to uniaxial strain and this was regulated by p38 and PI3-kinase signalling, which is in agreement with the study by Ghosh-Choudhury et al. (2002). BMP2 is a growth factor that promotes the osteogenic phenotype and a number of studies definitively link the BMP2 induction of osteoblast differentiation to have a Cbfα1 dependency (Bae et al. 2007; Lee et al. 2000b), and since BMPs can induce Cbfα1 (Ducy et al. 1997) this is part of a positive regulatory mechanism. Given the effects of BMP2 on the elevation of osteogenic genes associated with matrix maturation and mineralisation in osteoblastic cells (Selvamurugan et al. 2007) and progenitor cells (Wang et al. 1993) has been reported, and MSC osteogenic induction by growth factors occurs via a PI3-kinase regulated autocrine BMP2 expression (Ghosh-Choudhury et al. 2002; Oreffo et al. 1999) this is a further indicator that in this study, the osteogenic phenotype is progressing through the phases of development. The finding that uniaxial cyclic tensile strain alone upregulates BMP2 is in agreement with a similar study in a 3D environment where the authors report an upregulation of BMP2 mRNA levels following the application of 10% and 12% strain (Sumanasinghe et al. 2006).

The temporal expression of osteogenic proteins observed in this study, reflects, in the main part, an osteogenic response. This has some similarities with the temporal expression of osteoblast gene products as a function of osteoblast differentiation that has been described in a number of studies using primary cultures of rodent calvaria (Owen et al. 1990). Therefore, MSCs are responsive to uniaxial strain in a manner that follows the stages of differentiation of the osteoblast, namely, early differentiation, maturation and matrix synthesis.

To investigate the detection of cyclic tensile strain by MSCs that initiates the cell fate phenotypic response, mechanosensitive SACCs were examined, since they have a sensitivity to membrane tension (Charras et al. 2004). Using the SACC blocker gadolinium chloride, this study found that SACCs were involved in the strain-induced expression of the osteogenic markers Cbfα1, collagen type I and

osteocalcin, following a 6 day strain-stimulus. Since Gd^{3+} is reported to be the most potent SACC blocker available, it was used in this study. However, it is known that Gd^{3+} is also nonselective, and its toxicity limits have been reported (Franz 1996). Gd^{3+} blocks SACCs by binding to a site in the permeation pathway for Na^{+} and Ca^{2+} (Morris 1990), but it also displaces Ca^{2+} and other ions from specific and nonspecific membrane binding sites (Yang and Sachs 1989). While Gd^{3+} can block L-type Ca^{2+} currents (Franz 1996; Lacampagne et al. 1994), it has a higher affinity for SACC than for other cation channels (Sadoshima et al. 1992). A 10 μM concentration of Gd^{3+} has demonstrated effective channel inhibition in a number of studies (Franz 1996; McBride et al. 2000).

The presence of SACC on primary bone cells has been confirmed (Charras et al. 2004), and upon investigating mechanotransduction in a bone cell line, Danciu et al. (2003) report the involvement of SACCs in strain-induced early signal transduction responses, including activation of the PI3-kinase pathway. Studies on cellular mechanotransduction in terminally differentiated cells report a role for SACC in coordinating many physiological events; however this is an underdeveloped area for undifferentiated primary progenitor cells. Therefore this finding represents a novel understanding of the mechanotransduction processes in MSCs. SACCs contribute to a wide array of cellular activities and stretch induced gene expression via SACCs has been reported in mammalian myocardium (Kent et al. 1989). Since expression of Cbfα1, collagen type I and osteocalcin was observed, albeit to a significantly lesser degree while SACCs were blocked, this is not unexpected, since many other membrane components couple mechanical stimulation with cellular response, such as integrins and G proteins (reviewed in (Sebastine and Williams 2006)). This, in combination with the specificity of Gd^{3+}, may contribute to the protein expression observed when SACC were blocked. MSCs have extensively demonstrated mechanical sensitivity (Jagodzinski et al. 2004; Juncosa-Melvin et al. 2007; Wiesmann et al. 2006) and ionic control of cell fate mechanisms (Riddle et al. 2006); therefore, this research provides a platform for further investigations into the role of SACC in the signal transduction of cell fate, in particular, gene and protein expression.

Following SACC channel opening, channel permeability results in an increase in intracellular Ca^{2+} concentration. It is postulated that the Ca^{2+} then acts as a second messenger to regulate Ca^{2+}-dependent kinases, which phosphorylate other intracellular proteins to direct the appropriate cell response to mechanical

stimulation (Lammerding et al. 2004). Changes in the mechanical environment therefore, initiate electrical and chemical changes in the cell which define the cellular response.

Upon examination of the MAPK pathways ERK, p38 and the phosphotidyl enzyme PI3-kinase in MSCs following the application of strain of 14 days, this study demonstrated an involvement of p38 and PI3-kinase, but not ERK in the strain-induced expression of BMP2. Additionally, upon examination of the phosphorylation of ERK and p38, their phosphorylation status was comparable to control levels at the 14 day time-point. This is suggestive of a role for p38 and PI3-kinase in the strain-induced autocrine regulation of BMP2 and thus MSC differentiation, but that ERK is not involved in this process. Also, the strain-induced activation of p38 and pI3-kinase must occur prior to 14 days of strain, and consequently I was unable to detect p38 or PI3-kinase activity by the 14 day time point. Future studies would monitor more time-points earlier on the application of strain, for example within the first hour to monitor immediate responses, over a 12 hour period, then following 3, 6, and 9 days to follow the distinct phases of osteoblast development (Owen et al. 1990).

The activation of PI3-kinase is in agreement with the study by Danciu et al (Danciu et al. 2003) where they found a cyclic tensile strain stimulus promotes the phosphorylation of PI3-kinase in a calcium-dependent manner in MC3T3-E1 cells. Since PI3-kinase phosphorylation is involved in cell survival and the regulation of gene expression, this further supports the finding in this study that blocking SACCs (and thus intracellular Ca^{2+}), significantly attenuated protein expression. Thus it is possible that this occurred via a PI3-kinase pathway, however further experimentation is necessary to definitively confirm this. In another study, the PI3-kinase pathway has been associated with the positive regulation of BMP2 induced MSC osteogenesis (Osyczka and Leboy 2005). In the same study, a negative regulation mechanism for ERK signalling in MSC osteogenic differentiation was reported, however the authors draw attention to the inter-species differences in response to the same stimuli, and note opposing differentiation patterns in human and rat MSCs when stimulated with BMP2 (Osyczka and Leboy 2005).

In a study examining MAPK involvement in chemically induced activation of MC3T3-E1 cells, the authors report supporting evidence for the MAPK involvement observed in this study (Suzuki et al. 2002). They report a critical role

for p38 in the differentiation of MSCs and that ERK is mainly involved in the proliferation early in the pre-differentiation phase. Thus since this study examined signalling at the late stage of differentiation only, it is possible that ERK may have an involvement in the osteogenic process, but that only p38 and PI3-kinase are involved in the mediation of growth factor activities, in the later stages. Low MAPK levels after repeated long-term strain suggest increased MAPK degradation after numerous activations due to the applied strain (Rhee et al. 2006). Thus, it is possible that the MAP kinases have been phosphorylated extensively. Many studies report the involvement of ERK in osteoblast activity (Jaiswal et al. 2000) mediated through Cbfα1 (Runx2) (Ge et al. 2007), and in the mechanical stimulation of osteoblasts (Weyts et al. 2002). It has been noted that bone marrow stromal cells do not activate MAP kinases permanently but only for a short time to induce the subsequent reactions (Rhee et al. 2006). Therefore the activation of ERK in the early response to mechanical stimulation of MSCs is an area for further investigation. This novel finding that p38 physically couples mechanical strain to autocrine BMP2 growth factor activity in MSCs further substantiates the role of extracellular mechanical stimulation in the regulation of osteogenic differentiation and the critical role MAPKs and PI3kinase cascades play in the osteoblast differentiation process.

It can be concluded from this study that mechanical forces play a significant role in the osteogenic differentiation pathway. Although our findings suggest that mechanical stimulation may induce differentiation toward an osteogenic phenotype in 2D culture, long term continuous stimulation indicated a desensitisation to tensile strain, an event which is observed in bone cells (Robling et al. 2002; Turner 1998). In summary, through the application of a continuous, low magnitude, low frequency tensile mechanical strain to MSCs seeded on a 2D collagen-coated silicone membrane, this study demonstrated that MSCs are capable of detecting, transducing and responding phenotypically to a biomechanical stimulus. The expression of Cbfα1, collagen type I, osteocalcin and BMP-2 strongly indicates the directed differentiation towards the osteogenic lineage by this pattern of stimulation. SACCs play a role as mechanosensors in this process, and are involved in the internalisation of the differentiation signal. Furthermore, p38 and pI3-kinase, but not ERK, are involved in strain-induced BMP-2 increased activity, thus it can be inferred that they play a role in

mechanotransduction of strain-induced osteogenic differentiation in MSCs (Figure 5.11).

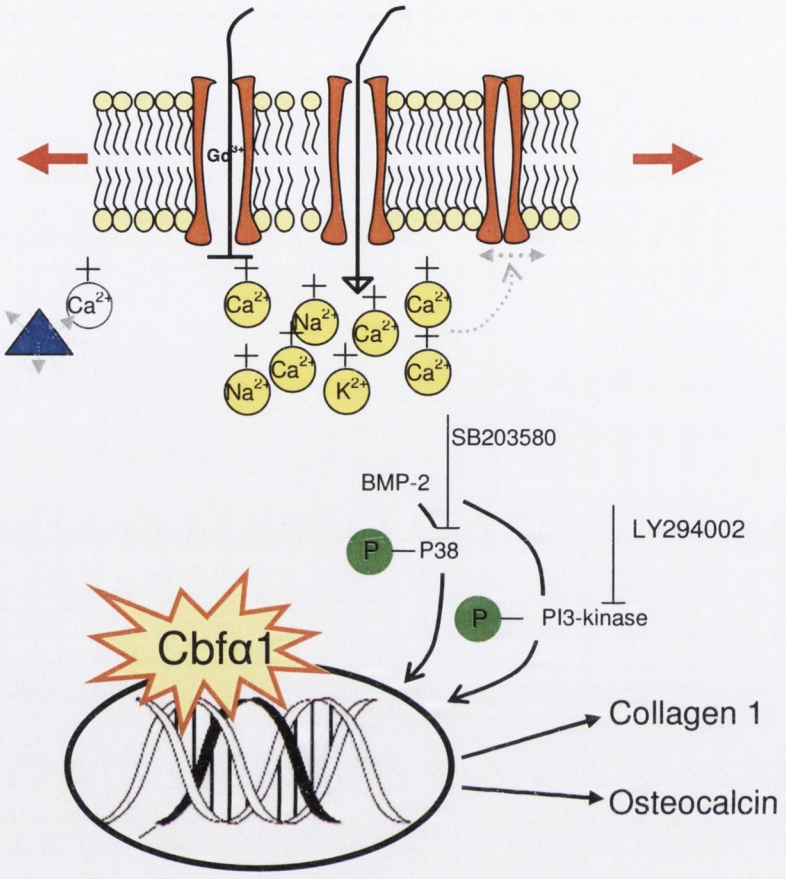


Figure 5.11 Strain-induced osteogenic differentiation in Mesenchymal Stem Cells

Following tensile mechanical stimulation, SACCs in the cell membrane become active allowing an influx of cations to the cell. These can become involved in further channel opening, or initiate signalling cascades within the cell. Strain induced the expression of the transcription factor Cbfα1, and the osteogenic proteins collagen type I, osteocalcin and BMP2. PI3-kinase and p38 were found to form part of the signalling mechanisms in strain-induced BMP2 expression, through studies whereby the pathways were blocked using pharmacological inhibitors.

To improve the sensitivity of detection and to quantitatively monitor changes in protein expression observed in response to mechanical strain, it is desirable to monitor cellular changes through mRNA expression using the reverse transcriptase polymerase chain reaction (RT-PCR) technique and this forms part of the future studies recommended from this research. The novel identification of

mechanical stimulus alone (i.e. in the absence of osteoinductive growth factors) as a regulator of MSC osteogenic differentiation, and the associated mechanotransduction pathways holds important consequences for the development of orthopaedic tissue engineering solutions by circumventing the need for the addition of growth factors, thus minimising additional regulatory issues and avoiding over-complication of the prediction of unforeseen or unexpected events.

6 Mechanisms in strain-induced mesenchymal stem cell apoptosis

6.1 Introduction

It has been shown that cyclic tensile strain is a stimulus that promotes the activation and differentiation of MSCs towards the osteogenic phenotype (chapter 5). Therefore, this mechanical stimulus is a useful tool for creating niche micro-environmental conditions for the preconditioning of stem cells for regenerative orthopaedic tissue engineering. It is known that physical force also plays a role in other cell responses such as growth and migration (Ingber 1997). It is possible then that the strain stimulus can also act as a regulator of other biological responses such as MSC proliferation and apoptosis, which have yet to be fully investigated. The maintenance of cell turnover and cell survival is central to the development of a functional tissue engineered construct and *in vivo* effect, therefore, this chapter aims to examine how continuous cyclic strain of varying magnitudes affects both the mitotic pattern of MSCs, and to investigate the effects of continuous strain on programmed cell death, with an insight into the mechanisms behind this.

Stem cells are characteristically described by their patterns of self-renewal: they are capable of proliferating while maintaining their proliferation and differentiation potential; this feature favourably selects them for tissue engineering applications. Self-renewal requires maintenance of proliferation potential and inhibition of differentiation and apoptosis (Molofsky et al. 2004). Stem cells *in vivo* are quiescent and will only divide when a stimulus is provided by the microenvironment (Moore and Lemischka 2006). For self-renewal, mitogens stimulate the cell to enter the cell cycle by inducing the expression of early response transcription factors which initiate a complex cascade in which the cell begins to replicate DNA (Satija et al. 2007). When MSCs are cultured *in vitro*, they exhibit a unique expansion capacity through their response to fetal calf serum supplemented culture conditions (Mannello and Tonti 2007). Fetal calf serum is a widely used growth supplement for cell culture because of the high levels of growth stimulatory factors and low levels of growth inhibitory factors. Following the initial seeding of a tissue engineering construct, it is desirable for the cells to populate the scaffold to generate an implantable treatment loaded with cells that are responsive to local biochemical and biomechanical cues, to proliferate or differentiate. It is therefore important to establish the mitogenic effects of continuous cyclic tensile strain that is inductive for osteogenic differentiation.

The population of progenitor cells within a scaffold in response to mitotic signals, and the onset of lineage commitment of these cells is a fine balance for tissue engineering design. On one hand it is desirable to populate an implantable scaffold with cells that will continue to divide, therefore increasing the number of cells that will lay down matrix, and on the other hand, express the phenotype of cells that will form the tissue-specific functional extracellular matrix. Cell differentiation is necessary for the matrix formation process to be initiated, which is the commitment of the progenitor cell, and marks lower self-renewal capabilities.

Since MSCs are mechanoresponsive, it is possible that the mechanical strain can regulate their self-renewal dynamics. During embryological development of the skeleton and skeletal response to injury, mesenchymal progenitor cells are involved in the initial events of formation and repair (Bruder et al. 1994; Caplan 1987). In the course of embryological skeletogenesis, endrochondral ossification initiates with progenitors of mesenchymal origin, that aggregate at sites where ossification is imminent. Following chondrogenic differentiation and chondrocyte hypertrophy, limb strain patterns are believed to define centres of ossification, and bone is formed (Nowlan et al. 2007). In this model of bone formation, hypertrophy marks a transition from proliferation to differentiation, as non hypertrophic chondroprogenitors continue to proliferate (Cancedda et al. 2000; Kronenberg 2003). Fracture repair occurs through complex progression of progenitor cells to skeletal cells including chondrocytes and osteoblasts that form a fracture callus (Kraus and Kirker-Head 2006). The local recruitment of progenitors is in response to cytokine activity following matrix disruption (i.e. stress signals). In the early stages of repair mesenchymal cells are highly proliferative (Tatsuyama et al. 2000). Proliferation of the MSCs is followed by growth factor-mediated and mechano-regulated lineage specific commitment (Dimitriou et al. 2005; Huiskes et al. 1997; Prendergast et al. 1997).

In embryonic development, hypertrophic activity is concomitant with the cessation of proliferation, followed by the onset of tissue specific cell development and gene expression. Fracture repair models parallel embryologic formation, reflecting cytokine and mechanical sensitivity for the proliferation of precursors preceding tissue regeneration (Kraus and Kirker-Head 2006). Therefore, it could be hypothesised that the onset of mechanical strain-induced

lineage specific protein expression patterns seen during development and repair, concomitantly marks a negative regulation of the self-renewal patterns of the osteochondral progenitor cells, in synergistically opposing manner.

In response to mitogenic stimuli, intermediate second messenger signalling results in the synthesis of DNA prior to the mitotic event. The synthesis of DNA can be used for the detection of proliferation. Tritiated thymidine is incorporated only in cells synthesising DNA prior to mitosis (Reichard and Estborn 1951), or promptly degraded with negligible partition of radioactivity into other components of DNA or into ribonucleic acid (Rubini et al. 1960). As the synthesis phase in most proliferative cells is five times the time needed for the mitotic division, the registration of DNA-synthesising cells reveals a larger portion of the population of potentially dividing cells than the registration of cells in any other phase of cell division (Dahl 1983). Therefore, this is a rigorous detection method for proliferation of cells in culture (Bocelli-Tyndall et al. 2006; Liu et al. 1992; Wang et al. 2006).

Proliferation dynamics during mechanical stimulation of a range of cell types associated with skeletal tissues have been examined in a number of studies, with differential responses. MSCs harvested from rats that had experienced a withdrawal of *in vivo* mechanical stimulation by hindlimb suspension demonstrate a loss in their *in vitro* proliferative capacity compared to cells harvested from control rats that had not experienced mechanical withdrawal (Kostenuik et al. 1997). Osteoblasts demonstrate a positive proliferation response to uniaxial strain, however this response has a time dependency, demonstrating reduced proliferation response to cyclic strain beyond a certain number of cycles (Kaspar et al. 2002). The requirement for mechanical loading for bone remodelling that maintains bone turnover is well established (Morey and Baylink 1978). It appears then that mechanical stimulation is favourable for the maintenance of bone and promotion of mesenchymal stem cell proliferation; however, it is clear that an optimal *in vitro* stimulation pattern exists, since reports have drawn attention to the negative regulation of MSC proliferation in response to strain and these remain to be defined.

Apoptosis, or programmed cell death, is a cellular event that occurs as part of normal development, or in response to a noxious stimulus. Cell death through apoptosis follows a highly orchestrated sequence of events. Apoptotic signalling

often converges on the caspase family. Eventually intracellular structures including cytoskeletal and associated proteins, kinases, members of the Bcl-2 family of apoptosis-related proteins, presenilins and amyloid precursor protein, and DNA-modulating enzymes (Chan and Mattson 1999) are proteolytically cleaved through an amplifying cascade of caspase activation. The morphological features of programmed cell death include membrane blebbing, nucleus fragmentation and DNA degradation. Membrane bound apoptotic bodies form and are ingested by surrounding healthy cells (Arends and Wyllie 1991; Kerr et al. 1972). No cell contents are leaked to the extracellular environment, thus avoiding further local cell damage through the absence of an inflammatory response.

The initial step for the mechanotransduction of the apoptotic signals involves cellular mechanosensors. Two mechano-sensitive ion channels that have been shown to facilitate mechanotransduction in a variety of cell types are stretch activated cation channels (SACCs) and L-type voltage activated calcium channels (VACCs) (Guharay and Sachs 1984). Activation of SACCs can allow calcium entry either directly into the cell, or indirectly through the activation of voltage sensitive channels (Charras and Horton 2002; McBride et al. 2000). In addition, voltage-sensitive L-type calcium channels can also be activated directly by mechanical stimulation (Lyford et al. 2002). Calcium influx through these channels then mobilises intracellular calcium stores to increase the intracellular calcium concentration (el Haj et al. 1999). VACCs have been identified on bone cells (Chesnoy-Marchais and Fritsch 1988) and there is considerable evidence that they participate in transduction of hormonal and mechanical stimuli. Both SACCs and VACCs have been shown to be involved in responses to strain in bone cells (Rawlinson et al. 1996).

One of the second messengers in apoptotic signalling is c-Jun N-terminal kinase (JNK). JNK, a member of the MAPK family is a stress activated protein kinase and is involved in a number of cellular responses to stimuli including UV irradiation, DNA damage, heat shock and oxidants, genotoxic stress, as well as by inflammatory cytokines (Davis 2000; Kirber et al. 2000). Following the detection of an apoptotic stimulus, persistent activation of JNK is one of the pathways that can mediate intracellular signalling that leads to cell death. JNK has been proposed as a mediator of cell death in response to mechanical strain (Zhang et al. 2007) in a number of cell types (Arnoczky et al. 2002; Hsieh and Nguyen 2005).

Another facet of the apoptotic machinery within the cell is the calpain system. Calpains are calcium-dependent cytosolic proteases which are involved in cellular processes including cell migration proliferation and differentiation, as well as apoptosis, dependent on their level of activation. Physiologically, the activities of calpains are sensitive to the endogenous protein inhibitor calpastatin, autolytic processing, and intracellular [Ca^{2+}]. Calpains can become overactivated under extreme conditions that result in sustained elevation of Ca^{2+} levels. Substrates for calpain include cytoskeletal, plasma-membrane associated proteins, signal transduction proteins and transcription factors (Goll et al. 2003; Molinari and Carafoli 1997; Suzuki et al. 1992; Wang 2000). While traditionally associated with necrotic cell death, recent research has identified the involvement of calpain in apoptosis in some cell types (Syntichaki et al. 2002; Wang 2000). Calpain has been shown to underlie apoptotic events in neurones (Lai and Cheng 2002; Tuan et al. 2003) and glia (Shields et al. 2000). It is thought that proteolysis mediated by the caspase and calpain systems might have common roles in mediating cell death. Calpain might facilitate apoptotic cell death by aiding caspase-3 in the proteolysis of cellular proteins. It seems that calpain and caspase share a number of dual substrates and a few related substrates (Wang 2000).

The effect of stretch on apoptosis has been examined *in vitro* on a number of cell types from organs whose normal physiological function involves the exposure to cyclical mechanical strain. In culture, osteoblast cells subjected to mechanical stretch undergo apoptosis dependent on their differentiation stage. Strain is apoptotic in young osteoblast cultures, whereas proliferation occurs in osteoblasts that are differentiated (Weyts et al. 2003). Additionally, in the pre-osteoblast cell line MC3T3-E1, cyclic stretch causes phosphorylation of JNK, which appears to be dependent on influx of extracellular calcium via SACCs (Danciu et al. 2003). In human patellar tendon fibroblasts, mechanical stretch activates JNK, and the stretched fibroblasts undergo apoptosis (Skutek et al. 2003), however, this response is reversed with sustained mechanical stretch. JNK is also activated in tenocytes in response to cyclic strain, via calcium mechanotransduction (Arnoczky et al. 2002). Thus, these findings suggest that mechanical stretch can activate both anti- and pro-apoptotic pathways, depending on cell state. Examination of MSC apoptosis has revealed that it follows caspase-dependent apoptosis, and exhibits many of the hallmark features (Raz et al. 2006). Several studies have shown that functional intrinsic and extrinsic pathways exist embryonic, fetal and perinatal

progenitor cells (D'Sa-Eipper and Roth 2000; Kennea et al. 2005). Apoptosis through caspase-3 activity has been observed in MSCs that were prevented from adhering to their extracellular matrix (anoikis) (Feng et al. 2007) and MSCs undergo caspase-dependent apoptosis in response to hypoxia and serum deprivation (Zhu et al. 2006).

The aim of this study was to assess the effect of mechanical strain on the proliferative patterns and viability of MSCs and to characterise the mechanotransduction signalling pathways that underlie the strain-induced apoptosis in this cultured cell system. Apoptosis is detected using the TUNEL technique. TUNEL detects DNA strand breaks through labelling of free 3'-OH DNA ends generated by DNA fragmentation in apoptosis (Bursch et al. 1990). The TUNEL system provides an accurate quantitative assessment of cell death by providing a colourimetric DAB based system to distinguish cells containing fragmented DNA (Gavrieli et al. 1992).

The mechanosensitive ion channels SACCs and VACCs were examined for their roles as mechanosensors and in mechanotransduction of the strain signal through the ion channel blockers gadolinium chloride and nicardipine. Given that JNK is a stress activated MAPK and that phosphorylation has been associated with apoptotic cell death (Tournier et al. 2000; Wada and Penninger 2004), the activation of JNK during strain-mediated apoptotic MSC death was assessed using fluorescence immunocytochemistry. The role of JNK in the mechanotransduction of events that lead to DNA fragmentation was then assessed using the JNK inhibitor D-JNK1. D-JNK1 is a protease resistant synthetic peptide based on the cytoplasmic inhibitor JNK-binding domain (JBD) of the (Dickens et al. 1997). D-JNK1 peptide inhibits the interaction between JNK and its substrates, blocking activation of the transcription factor c-Jun by JNK (Bonny et al. 2001). Caspase-3 activation is a downstream target of JNK phosphorylation and is part of the execution cascade through which most apoptotic signalling converges to mediate the hallmark morphological features of apoptosis. Therefore, the activity of caspase-3 during cyclic tensile strain was examined by immunofluorescence detection of the cleaved protein. Furthermore, the activity of calpain during cyclic strain was examined using a pharmacological inhibitor with enzymatic detection, and its role as a mediator of DNA fragmentation during strain-mediated apoptosis.

6.2 Results

6.2.1 Mechanical strain of 2.5% and 10% reduces MSC proliferation

To determine the influence of a 2.5% mechanical strain on MSC proliferation dynamics, the rate of mitosis was monitored through quantification of new DNA formation detected by the uptake of tritiated (^{3}H-) thymidine. MSCs were exposed to mechanical strain of 2.5% for 1, 2 and 3 days and pulsed with ^{3}H-thymidine during the last 24hrs of mechanical stimulation. Cells were harvested onto a filtermat, and radioactive counts were detected through scintillation counting (Figure 6.1Ai). Following 1 day of 2.5% strain, ^{3}H-thymidine uptake was comparable to that in control cells that had not been exposed to stretch. To control for cell number in each sample, counts were normalised to the total number of cells in the population, determined by the DNA Hoechst assay. Thus, the average ^{3}H-thymidine incorporation when normalised to total cell number was 0.13 ± 0.03 cpm/cell (mean ± SEM) for the control group and 0.12 ± 0.02 cpm/cell in the strained group. However, following 2 days of 2.5% strain, ^{3}H-thymidine (^{3}H[TdR]) uptake was significantly decreased from 0.09 ± 0.02 cpm/cell in the control group to 0.04 ± 0.01 in the strained group ($p<0.05$, Students paired t-test; n=11). Similarly, after a 3 day 2.5% strain stimulation, ^{3}H-thymidine uptake was significantly decreased from 0.06 ± 0.02 cpm/cell in the control unstrained group to 0.02 ± 0.007 cpm/cell in cells exposed to mechanical strain ($p<0.05$, Students paired t-test; n=8).

MSC proliferation during the 2 days following cell-seeding onto the collagen-coated silicone was also monitored. Using that data with the data above, a rate of proliferation over a 4 day period could be determined for control and mechanically stimulated cells (Figure 6.1Aii). The rate can be calculated as the slope of the line for both control and strained curves for ^{3}H[TdR] incorporation between 2 and 5 days in culture. The slope of the proliferation curves for both control and mechanically stimulated cells is a negative value indicating a decrease in the rate of proliferation in both groups. For mechanically stimulated cells the absolute value of the slope is 0.0412 and 0.0262 for control cells, indicating that the rate of proliferation is decreasing faster in strained cells, compared to unstrained controls. This finding demonstrates that mechanical stimulation of

2.5% for longer than 1 day significantly reduces the rate of MSC proliferation in a time-dependent manner.

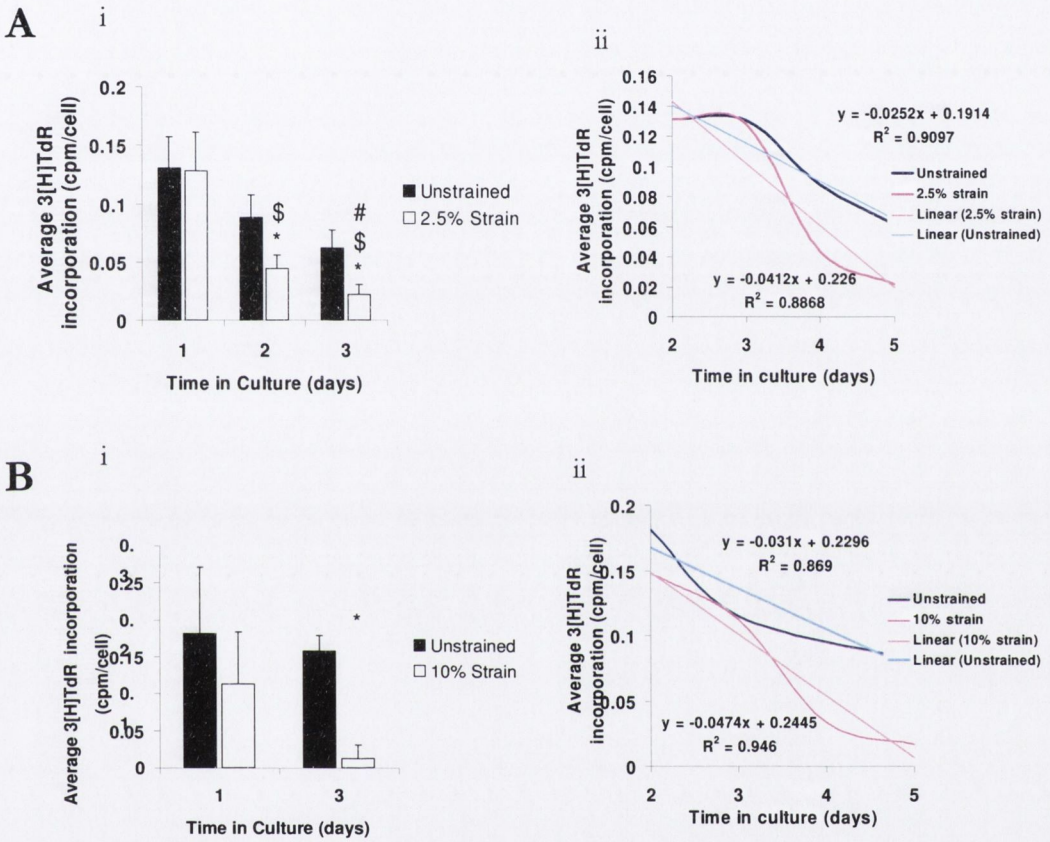
To determine the influence of a 10% mechanical strain on MSC proliferation dynamics, the rate of mitosis was monitored through quantification of new DNA formation detected by the uptake of tritiated (^{3}H-) thymidine during mechanical stimulation of progressive durations. MSCs were exposed to mechanical strain of 10% for 1 and 3 days and pulsed with ^{3}H-thymidine during the last 24hrs of mechanical stimulation. Cells were harvested onto a filtermat, and radioactive were counts detected through scintillation counting (Figure 6.1Bi). Following 1 day of 10% strain, ^{3}H-thymidine uptake was comparable to that in control cells that had not been exposed to stretch. Thus, the average ^{3}H-thymidine incorporation when normalised to total cell number was 0.18 ± 0.09 cpm/cell (mean ± SEM) for the control group and 0.11 ± 0.07 cpm/cell in the strained group. However following 3 days of 10% strain stimulation, ^{3}H-thymidine uptake was significantly decreased from 0.16 ± 0.01 cpm/cell in the control unstrained group to 0.01 ± 0.004 cpm/cell in cells exposed to mechanical strain ($p<0.05$, Students paired t-test; n=8). Figure 6.1B (ii) demonstrates that the slope of the proliferation curves for both control and mechanically stimulated cells is a negative value indicating a decrease in the rate of proliferation in both groups. For mechanically stimulated cells the absolute value of the slope is 0.0412 and 0.0262 for control cells, indicating that the rate of proliferation is decreasing faster in strained cells, compared to unstrained controls. This finding demonstrates that mechanical stimulation of 10% for longer than 1 day, significantly reduces the rate of MSC proliferation in a time-dependent manner.

Figure 6.1 Cyclic tensile strain of 2.5% and 10% reduces MSC proliferation

MSCs seeded on collagen-coated silicone were exposed to 1, 2 and 3 days of cyclic tensile mechanical strain (0.17Hz). During the final 24hrs of strain application, cells were pulsed with ^{3}H-thymidine (2.0 μCi/ml). Cell proliferation was assessed by measuring ^{3}H-thymidine uptake through scintillation counting.

A: (i) Mechanical strain of 2.5% for 1 day had no effect on MSC proliferation; however, 2.5% strain for 2 and 3 days significantly reduced MSC proliferation in MSCs, compared to unstrained controls. Results are expressed as mean ± SEM for 8-11 independent observations, Students paired t-test, *$p<0.05$ strain v control; \$$p<0.05$ strain v 1 day strain; #$p<0.05$ strain v 2 day strain. The rate of proliferation was calculated over 4 days (ii). Proliferation decreased with time in both control and mechanically stimulated cells, however, the rate of decrease was greater in cells exposed to mechanical strain.

B: (i) Mechanical strain of 10% for 1 day had no effect on MSC proliferation; however, 10% strain for 3 days significantly reduced MSC proliferation in MSCs, compared to unstrained controls. Results are expressed as mean ± SEM for 4 independent observations, Students paired t-test, *$p<0.05$ strain v control. The rate of proliferation was calculated over 4 days (ii). Proliferation decreased with time in both control and mechanically stimulated cells, however, the rate of decrease was greater in cells exposed to mechanical strain.


A
i
Average 3[H]TdR incorporation (cpm/cell)
0.2
0.15
0.1
0.05
0
1
2
3
Time in Culture (days)
$
*
#
$
*
Unstrained
2.5% Strain
ii
Average 3[H]TdR incorporation (cpm/cell)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
2
3
4
5
Time in culture (days)
y = -0.0252x + 0.1914
R2 = 0.9097
y = -0.0412x + 0.226
R2 = 0.8868
Unstrained
2.5% strain
Linear (2.5% strain)
Linear (Unstrained)
B
i
Average 3[H]TdR incorporation (cpm/cell)
0.3
0.25
0.2
0.15
0.1
0.05
0
1
3
Time in Culture (days)
*
Unstrained
10% Strain
ii
Average 3[H]TdR incorporation (cpm/cell)
0.2
0.15
0.1
0.05
0
2
3
4
5
Time in culture (days)
y = -0.031x + 0.2296
R2 = 0.869
y = -0.0474x + 0.2445
R2 = 0.946
Unstrained
10% strain
Linear (10% strain)
Linear (Unstrained)

6.2.2 Mechanical strain induces DNA fragmentation in cultured MSCs

To determine whether mechanical strain has a detrimental impact on MSC viability, its ability to induce DNA fragmentation in cultured MSCs was assessed. DNA cleavage into oligonucleosomal fragments is a distinguishing feature of apoptosis (Lee et al. 1993) that can be readily detected using the colorimetric TUNEL system. MSCs were exposed to mechanical strains of 2.5%, 5%, 7.5% and 10% for 3 days. Cells were fixed and analysed for levels of DNA fragmentation using TUNEL (Figure 6.2). At 2.5% strain, DNA fragmentation was not altered compared to unstrained control cells. Thus, the average percentage of DNA fragmentation was 5.41 ± 1.68% (mean ± SEM) for the unstrained control group and 8.56 ± 2.09% in the strained group (n=5). At 5% strain, the percentage of cells displaying DNA fragmentation was comparable to unstrained control cells, with 6.86 ± 1.61% of cells in the unstrained control group and 4.98 ± 0.94% of cells in the strained group displaying DNA fragmentation (n=5). At 7.5% strain however, DNA fragmentation significantly increased from 11.51 ± 3.63% in the unstrained control group to 20.33 ± 4.65% following a 7.5% strain stimulation ($p<0.05$, Students paired t-test; n=5). Similarly, at 10% of strain, 12.66 ± 7.78% of cells in the control unstrained group displayed DNA fragmentation and this was significantly increased to 47.09 ± 13.9% of cells displaying DNA fragmentation following a 3 day 10% strain stimulus ($p<0.05$, Students paired t-test; n=6). This finding demonstrates that mechanical strain induces apoptosis in cultured MSCs in a magnitude-dependent manner. Sample TUNEL images displaying the apoptotic effect of mechanical strain on MSCs are shown in Figure 6.2B.

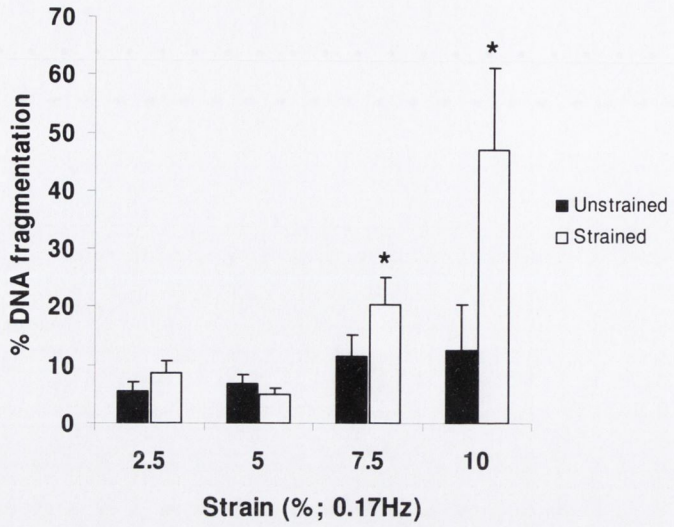
Figure 6.2 Strain-mediated apoptosis is magnitude-dependent.

MSCs were exposed to a 3 day cyclic tensile mechanical strain (2.5% - 10%; 0.17Hz). Cell viability was assessed by measuring DNA fragmentation by the TUNEL assay.

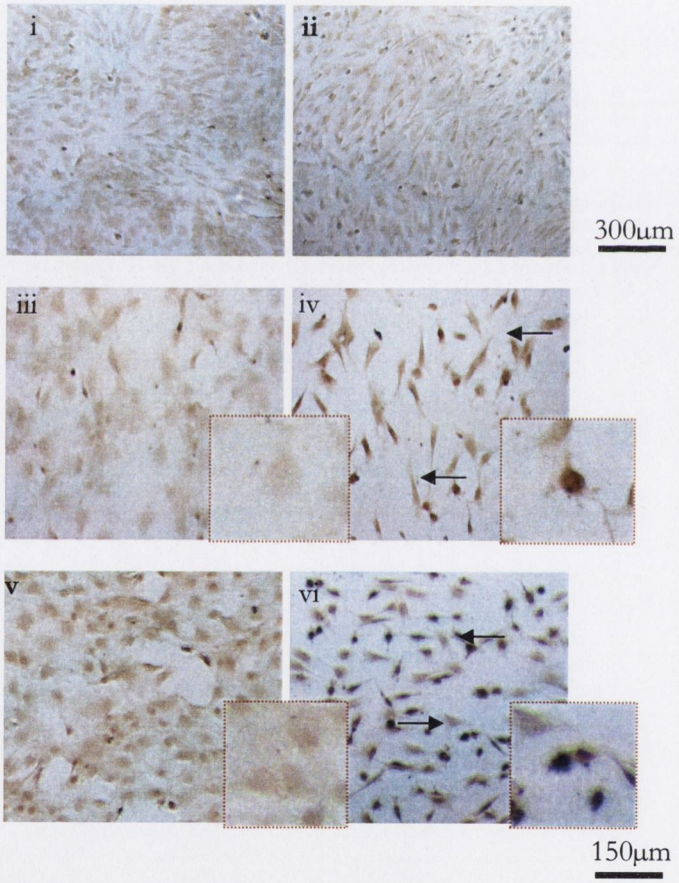
A: Mechanical strains of 2.5% and 5% for 3 days had no effect on MSC DNA fragmentation (% TUNEL +ve cells); however, strains of 7.5% and 10% for 3 days significantly increased DNA fragmentation in MSCs. Results are expressed as mean ± SEM for 5/6 independent observations, Students paired t-test, *$p<0.05$.

B: Light microscopic images of TUNEL staining of (i, iii, v) control MSCs, (ii) 2.5%, (iv) 7.5% and (vi) 10% strained MSCs. Arrows indicate representative apoptotic TUNEL positive cells. Scale bar as indicated.

A


70
60
50
40
30
20
10
0
% DNA fragmentation
*
*
Unstrained
Strained
2.5
5
7.5
10
Strain (%; 0.17Hz)

B


i
ii
300μm
iii
iv
v
vi
150μm

6.2.3 Strain of 7.5% and 10% induces p-JNK expression after 3 days.

c-Jun N-terminal kinases (JNKs) are stress activated protein kinases that act in the signal transduction coupling of cellular stresses, including physiological stimuli (Hamada et al. 1998), to apoptosis (Hochedlinger et al. 2002). Since DNA fragmentation, a morphological marker of apoptosis, has been observed following 7.5% and 10% tensile mechanical strain, these strain magnitudes were chosen to examine the influence of strain on JNK activation.

Following a 3 day strain of 7.5% at 0.17Hz, cells were fixed and probed for the expression of phospho-JNK (p-JNK) using immunofluorescence. Tensile mechanical strain of 7.5% at 0.17 Hz for 3 days promoted the expression of p-JNK in MSCs. Figure 6.3A demonstrates that MSCs in static culture display low p-JNK immunoreactivity (i); immunoreactivity was increased in mechanically stimulated cells (ii). Quantification of the grey level intensity (Figure 6.3A iii) demonstrates that p-JNK immunoreactivity significantly increased from 17.25 $\pm$ 0.98 AFU (mean $\pm$ SEM) in unstrained controls to 24.74 $\pm$ 2.52 AFU following a 3 day strain stimulation ($p<0.05$, Students paired t-test; $n=6$).

Following a 3 day strain of 10% at 0.17Hz, cells were fixed and probed for the expression of p-JNK using immunofluorescence. Tensile mechanical strain of 10% at 0.17 Hz for 3 days promoted the expression of p-JNK in MSCs. Figure 6.3B demonstrates that MSCs in static culture display low p-JNK immunoreactivity (i) and immunoreactivity was increased in mechanically stimulated cells (ii). Quantification of the grey level intensity in control and strained cells (Figure 6.3B iii) demonstrates that p-JNK immunoreactivity significantly increased from 10.91 $\pm$ 2.24 AFU (mean $\pm$ SEM) in unstrained controls to 24.12 $\pm$ 1.87 AFU following a 3 day strain stimulation ($p<0.05$, Students paired t-test; $n=4$). These data indicate that a 3 day tensile strain stimulation of 7.5% and 10% promotes the expression of p-JNK in MSCs.

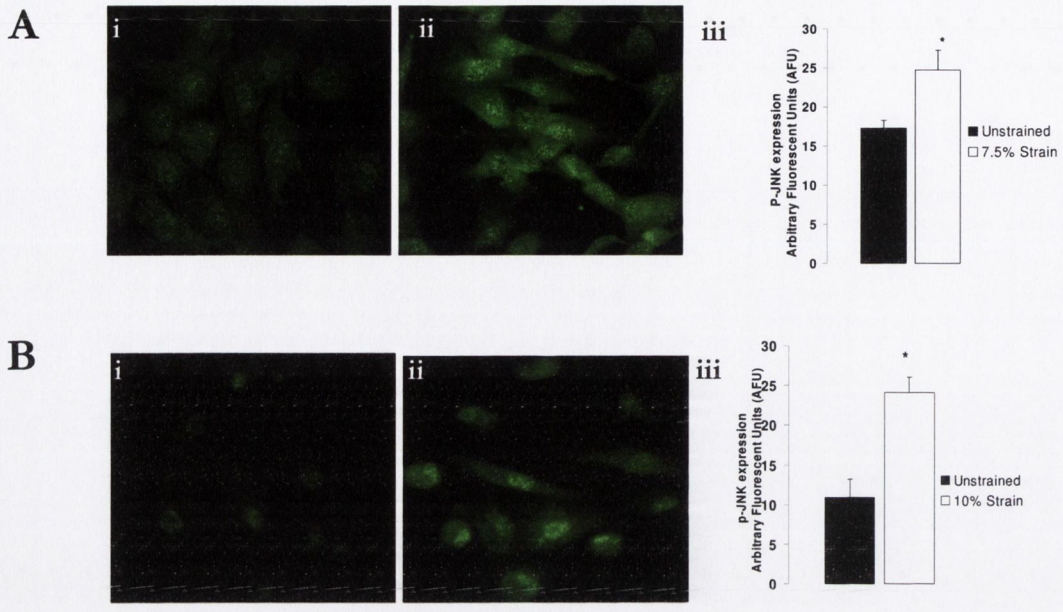


Figure 6.3 JNK is phosphorylated and after 3 days of 7.5% and 10% strain

MSCs seeded onto collagen-coated silicone were grown statically or exposed to cyclic mechanical tensile loading of 7.5% and 10% at 0.17 Hz for 3 days. p-JNK activity was assessed on fixed cells by immunofluorescence.

A: MSCs exposed to 7.5% strain for 3 days (ii) displayed more intense p-JNK immunoreactivity compared to unstrained cells (i). Expression of p-JNK was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in p-JNK fluorescence intensity was measured in cells exposed to 7.5% mechanical strain for 3 days compared to unstrained controls. Results are expressed as mean ± SEM for 4 independent observations, Students paired t-test *$p<0.05$. Scale bar is 50μm.

B: MSCs exposed to 10% strain for 3 days (ii) displayed more intense p-JNK immunoreactivity compared to unstrained cells (i). Expression of p-JNK was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in p-JNK fluorescence intensity was measured in cells exposed to 10% mechanical strain for 3 days compared to unstrained controls. Results are expressed as mean ± SEM for 4 independent observations, Students paired t-test *$p<0.05$. Scale 50μm.

6.2.4 Strain of 7.5% and 10% induces active caspase-3 expression after 3 days.

One of the early biochemical hallmarks of apoptosis that precedes the morphological features of apoptotic cells is the activation of caspase-3, known to cleave key structural, nuclear and signalling proteins (Blatt and Glick 2001). DNA fragmentation occurs in response to tensile mechanical strains of 7.5% and 10% (Figure 6.2); therefore, to identify the mechanisms of strain-induced DNA fragmentation, caspase-3 activity was examined using an antibody that detects the cleaved (active) form of caspase-3.

Following a 3 day strain of 7.5% at 0.17Hz, cells were fixed and probed for the expression of active caspase-3 using immunofluorescence. Tensile mechanical strain of 7.5% at 0.17 Hz for 3 days induced the activation of caspase-3 in MSCs. Figure 6.4A demonstrates that MSCs in static culture display low active caspase-3 immunoreactivity (i); immunoreactivity was increased in mechanically stimulated cells (ii). Quantification of the grey level intensity in control and strained cells (Figure 6.4A iii) demonstrates that active caspase-3 immunoreactivity significantly increased from 11.95 $\pm$ 1.34 AFU in unstrained controls to 23.82 $\pm$ 1.82 AFU following a 3 day strain stimulation ($p<0.05$, Students paired t-test; $n=6$).

Following a 3 day strain of 10% at 0.17Hz, cells were fixed and probed for the expression of active caspase-3 using immunofluorescence. Tensile mechanical strain of 10% at 0.17 Hz for 3 days induced the activation of caspase-3 in MSCs. Figure 6.4B demonstrates that MSCs in static culture display low active caspase-3 immunoreactivity (i); immunoreactivity was increased in mechanically stimulated cells (ii). Quantification of the grey level intensity in control and strained cells (Figure 6.4B iii) demonstrates that active caspase-3 immunoreactivity significantly increased from 8.68 $\pm$ 1.86 AFU (mean $\pm$ SEM) in unstrained controls to 20.3 $\pm$ 2.75 AFU following a 3 day strain stimulation ($p<0.05$, Students paired t-test; $n=5$). These data indicate that a 3 day tensile strain stimulation of 7.5% and 10% promotes the expression of active caspase-3 in MSCs.

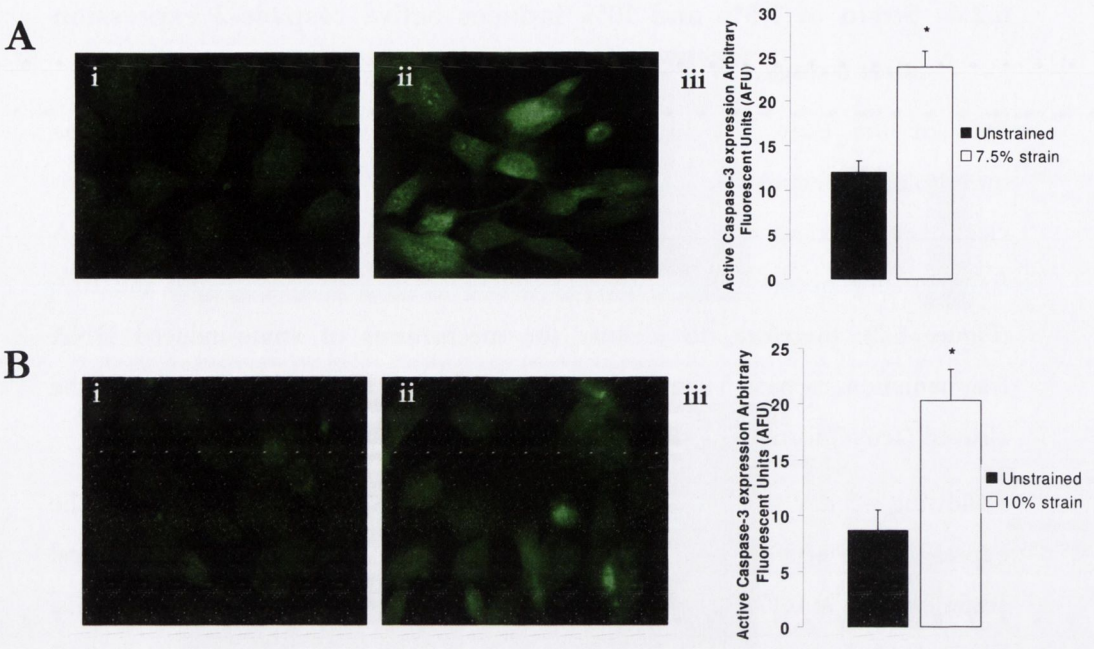


Figure 6.4 Caspase is active after 3 days of strain at 7.5% and 10%

MSCs seeded onto collagen-coated silicone were grown statically (i) or exposed to cyclic mechanical tensile loading of 7.5% at 0.17 Hz for 3 days (ii). Caspase-3 activity was assessed on fixed cells by immunofluorescence.

A: MSCs exposed to 7.5% strain for 3 days (ii) displayed more intense active caspase-3 immunoreactivity compared to unstrained cells (i). Expression of active caspase-3 was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in active caspase-3 fluorescence intensity was measured in cells exposed to 7.5% mechanical strain for 3 days compared to unstrained controls. Results are expressed as mean ± SEM for 5 independent observations, Students paired t-test *$p<0.05$. Scale bar is 50μm.

A: MSCs exposed to 10% strain for 3 days (ii) displayed more intense active caspase-3 immunoreactivity compared to unstrained cells (i). Expression of active caspase-3 was quantified by measuring the grey level intensity of the fluorescent images (iii). A significant increase in active caspase-3 fluorescence intensity was measured in cells exposed to 10% mechanical strain for 3 days compared to unstrained controls. Results are expressed as mean ± SEM for 5 independent observations, Students paired t-test *$p<0.05$. Scale bar is 50μm.

6.2.5 JNK is involved in strain-mediated MSC DNA fragmentation

Mechanical strain causes a maximal increase in DNA fragmentation following a 3 day stimulation of 10% strain (Figure 6.2) therefore this strain magnitude was used to determine if JNK was involved in directly mediating strain-induced DNA fragmentation. Following JNK inhibition by exposing MSCs to D-JNK inhibitor 1 (1μM) in both static and mechanically stimulated culture conditions, the TUNEL technique was used to assess the levels of strain-induced DNA fragmentation after a 3 day stimulation (Figure 6.5). In control cells, 14.54 ± 6.54% (mean ± SEM) of cells displayed fragmented DNA in the nucleus (TUNEL positive). This was significantly increased to 60.08 ± 12.34% in cells exposed to 10% mechanical strain for 3 days ($p<0.01$ ANOVA, Newman-Keuls post-hoc, n=4 observations). While treatment of cells with D-JNK inhibitor 1 alone for 3 days had no effect on MSC viability (9.42 ± 3.62% MSCs with fragmented DNA), mechanical stimulation in the presence of D-JNK inhibitor 1 failed to increase DNA fragmentation and significantly reduced the strain-induced increase in DNA fragmentation (60.08 ± 12.34% to 17.0 ± 3.12% TUNEL positive MSCs; ($p<0.01$ ANOVA, Newman-Keuls post-hoc, n=6 observations)). This finding suggests that JNK is directly involved in strain-induced DNA fragmentation.

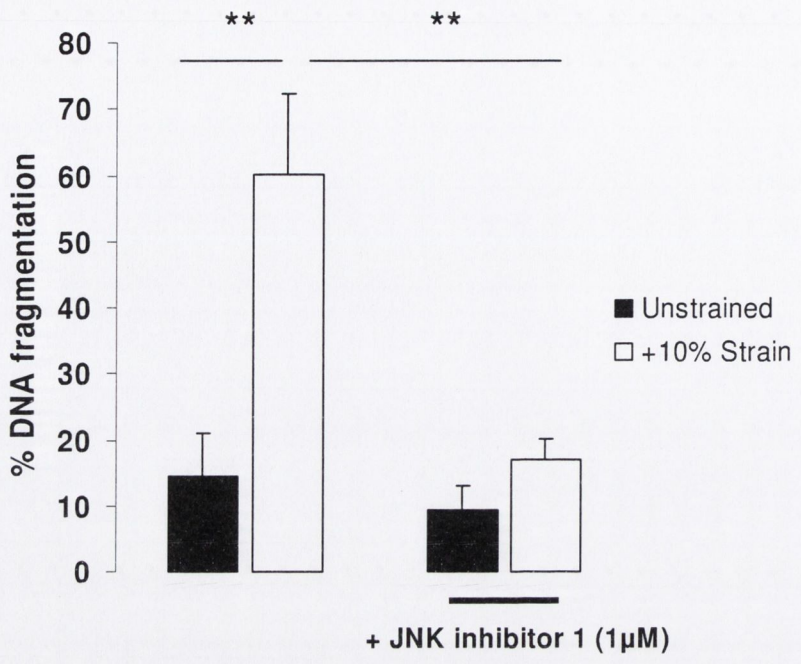


Figure 6.5 Strain mediated DNA fragmentation occurs via JNK activation

MSCs were exposed to a 3 day cyclic tensile mechanical strain of 10% (0.17 Hz) in the presence or absence of the JNK inhibitor D-JNK inhibitor 1 (1μM). Cell viability was assessed by measuring DNA fragmentation by the TUNEL assay.

Mechanical strain of 10% for 3 days significantly increased DNA fragmentation (% DNA fragmentation) in MSCs that were exposed to mechanical strain. This was prevented when cells were strained in the presence of D-JNK inhibitor 1 and the strain-induced increase in DNA fragmentation was significantly decreased. Results are expressed as mean ± SEM for 4 independent observations **$p<0.01$, ANOVA Newmal-Keuls post-hoc. These data indicate a role for JNK in strain-mediated MSC apoptosis.

6.2.6 Stretch activated cation channels are not involved in strain-mediated MSC apoptosis

The gating selectivity of SACC for cations is known to be dependent on membrane tension (Guharay and Sachs 1984). In strain-induced apoptosis, a physical tensile strain becomes a chemical signalling cascade through as yet, an unknown mechanism of mechanotransduction. Since SACC have demonstrated responsiveness to mechanical stimulation, I decided to investigate their involvement in the coupling of the apoptotic physical stimulus to second messenger activation and DNA fragmentation. The action of SACCs can be blocked with the lanthanide gadolinium chloride.

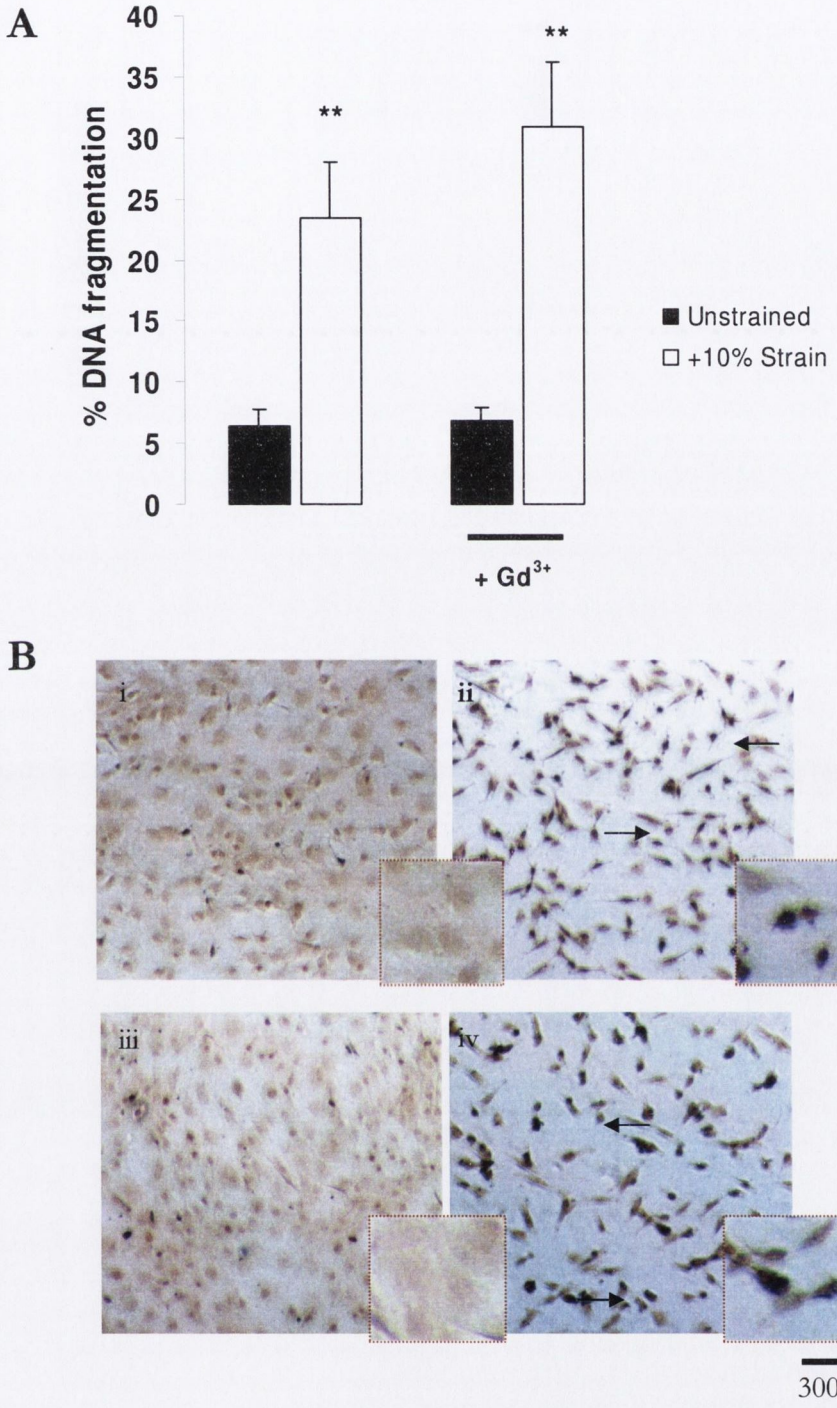
Mechanical strain causes a maximal increase in DNA fragmentation following a 3 day stimulation of 10% (Figure 6.2) therefore this strain magnitude was used to determine if SACCs were involved in strain-induced DNA fragmentation. Following a 3 day 10% strain in the absence or presence of the SACC blocker gadolinium chloride (Gd^{3+}; 10μM), the TUNEL technique was used to assess the levels of DNA fragmentation (Figure 6.3A). In control cells, 6.40 ± 1.36% (mean ± SEM) of cells displayed fragmented DNA in the nucleus (TUNEL positive). This was significantly increased to 23.48 ± 4.46% in cells exposed to 10% mechanical strain for 3 days ($p<0.05$ ANOVA, Newman-Keuls post-hoc, n=6 observations). While treatment of cells with Gd^{3+} alone for 3 days had no effect on MSC viability (6.91 ± 1.06% MSCs with fragmented DNA), it did not prevent the strain-induced increase in DNA fragmentation (30.85 ± 5.28% TUNEL positive MSCs; $p<0.01$ ANOVA, Newman-Keuls post-hoc, n=6 observations). This finding suggests that SACC play no role in the mechanotransduction of strain-induced DNA fragmentation. Representative TUNEL images of MSCs are shown in Figure 6.6B.

Figure 6.6 SACCs are not the primary mechanosensors in strain-induced DNA fragmentation.

MSCs were exposed to a 3 day cyclic tensile mechanical strain of 10% (0.17 Hz) in the absence or presence of the SACC blocker Gd^{3+} (10μM). Cell viability was assessed by measuring DNA fragmentation by the TUNEL assay.

A: Mechanical strain of 10% for 3 days significantly increased DNA fragmentation (DNA fragmentation) in MSCs that were exposed to mechanical strain. When cells were strained in the presence of Gd^{3+} (10μM), the significant increase in DNA fragmentation was retained, suggesting that mechanotransduction leading to DNA fragmentation induced by cyclic strain, occurs through mechanosensors that are distinct from SACCs. Results are expressed as mean ± SEM for 5 independent observations **$p<0.01$, ANOVA, Newman-Keuls post-hoc.

B: Light microscopic images of TUNEL staining of (i) control MSCs, (ii) 10% strained MSCs (iii) control + Gd^{3+} (10μM) MSCs and (iv) 10% strained MSCs in the presence of Gd^{3+} (10μM). Arrows indicate representative apoptotic TUNEL positive cells. Scale bar is Scale bar is 300μm.


A
40
35
30
25
20
15
10
5
0
% DNA fragmentation
**
**
Unstrained
+10% Strain
+ Gd3+
B
i
ii
iii
iv
300um

6.2.7 Stretch activated cation channels are not involved in strain-induced JNK phosphorylation

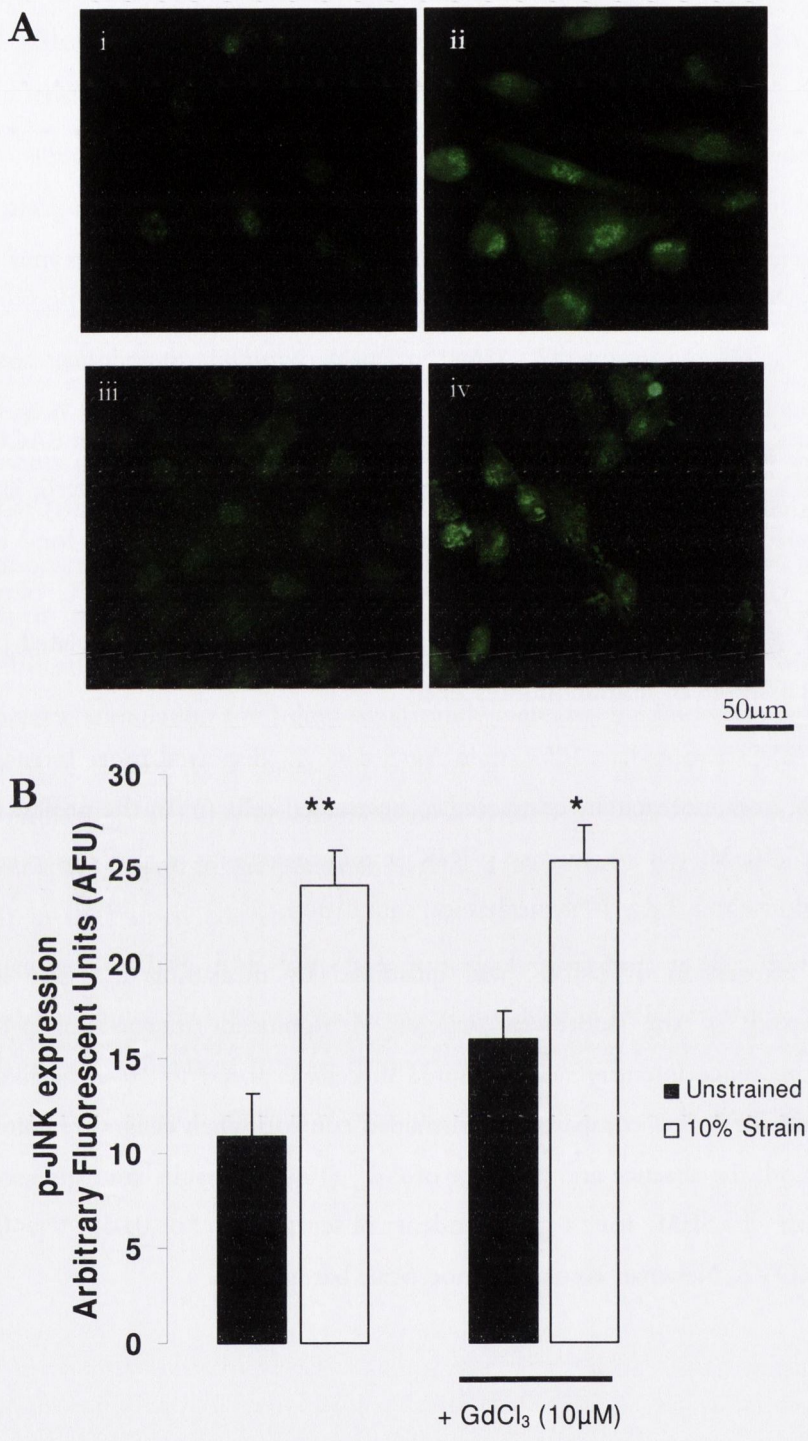
JNK activation has bee reported to occur via calcium dependent mechanotransduction (Arnoczky et al. 2002). To investigate the role of SACC in JNK activation at strain magnitudes that induce DNA fragmentation (Figure 6.3) MSCs were strained at 10% for 3 days in the absence and presence of the SACC blocker gadolinium chloride (Gd^{3+}; 10μM). Phosphorylated-JNK (p-JNK) expression was assessed by fluorescence microscopy in fixed cells. Figure 6.7A represents p-JNK staining in (i) control, (ii) 10% strained, (iii) control with Gd^{3+} and (iv) 10% strain with Gd^{3+} conditions. An increase in p-JNK immunoreactivity is demonstrated following 10% mechanical stimulation for 3 days (ii), a pattern which is reflected when cells were strained in the presence of Gd^{3+} (iv). Quantification of the intensity of immunoreactivity (Figure 6.7B) reveals that mechanical stimulation for 3 days significantly increases the fluorescence intensity from 10.91 ± 2.24 AFU (mean ± SEM) in unstrained controls to 24.12 ± 1.87 AFU following a 3 day strain stimulation ($p<0.01$, ANOVA, Newman-Keuls post-hoc; n=4), and in the presence of Gd^{3+}, from 15.93 ± 1.54 AFU (mean ± SEM) in unstrained controls to 25.41 ± 1.88 AFU following a 3 day strain stimulation ($p<0.05$, ANOVA, Newman-Keuls post-hoc; n=4). This finding provides evidence that the mechanosensors involved in JNK activation following a 3 day strain stimulus of 10% are distinct from SACCs.

Figure 6.7 Strain-induced JNK activation is not dependent on SACC

MSCs seeded onto collagen-coated silicone were grown statically (i, iii) or exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz for 3 days (ii, iv) in the absence (i, ii) or presence (iii, iv) of the SACC blocker Gd^{3+}(10μM). Cells were fixed and the expression of phosphorylated-JNK was assessed by immunofluorescence.

A: MSCs exposed to 10% strain for 3 days (ii) displayed more intense p-JNK immunoreactivity compared to unstrained cells (i). In the presence of Gd^{3+}(10μM) (iii, iv), intense p-JNK immunoreactivity is also observed in response to a 3 day 10% mechanical stimulation.

B: Expression of p-JNK was quantified by measuring the grey level intensity of the fluorescent images. A significant increase in p-JNK fluorescence intensity was measured in cells exposed to 10% mechanical strain for 3 days compared to unstrained controls when cells were cultured in both the absence and presence of Gd^{3+} (10μM). Results are expressed as mean ± SEM for 4 independent observations, *$p<0.05$, **$p<0.01$, ANOVA, Newman-Keuls post hoc. Scale bar is 50μm.


A
i
ii
iii
iv
50μm
B
p-JNK expression
Arbitrary Fluorescent Units (AFU)
30
25
20
15
10
5
0
**
*
Unstrained
10% Strain
+ GdCl3 (10μM)

6.2.8 Stretch activated cation channels are not involved in strain-induced caspase-3 activation

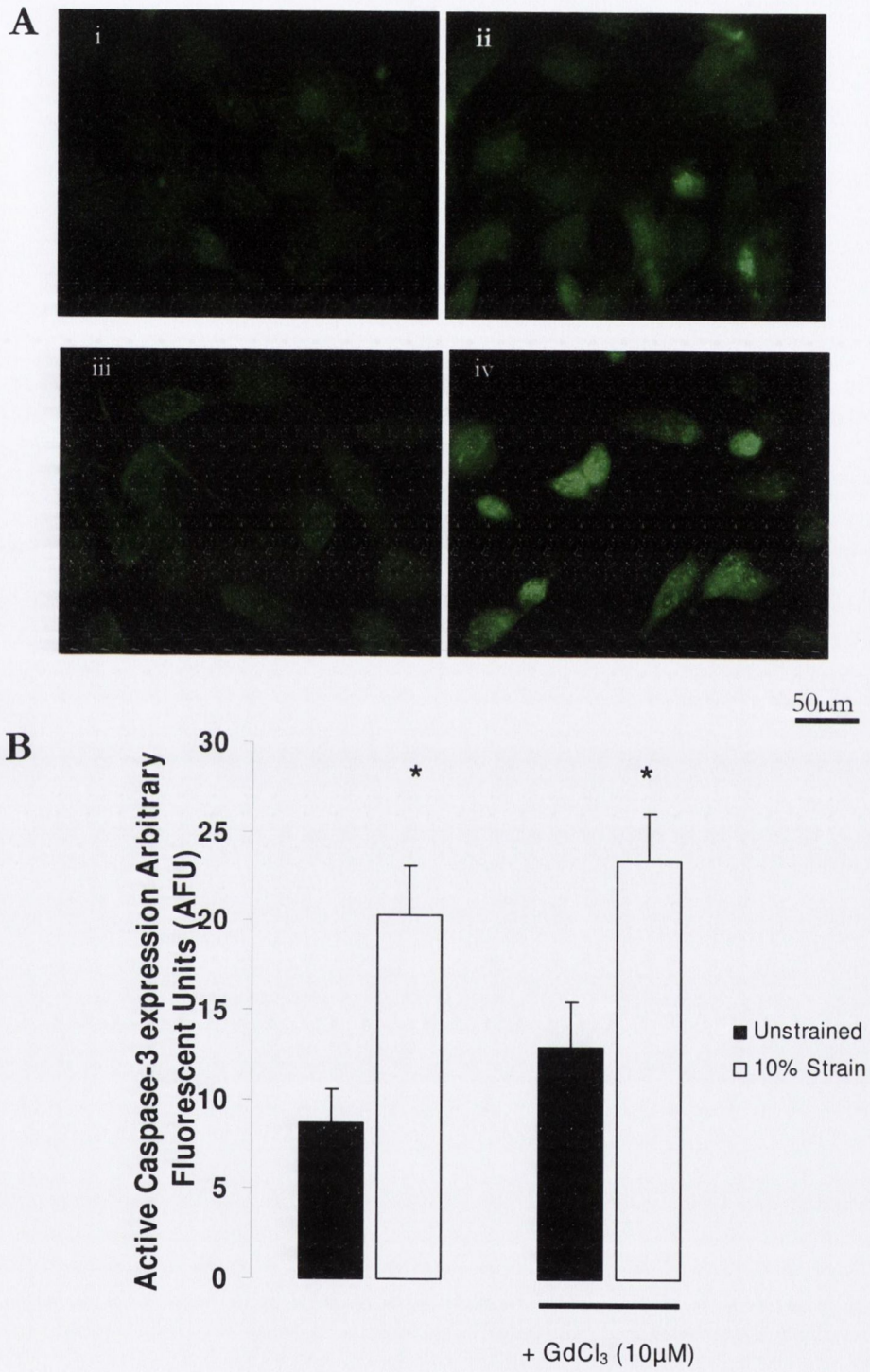
The role of SACCs in signalling pathways that activate caspase-mediated apoptosis has previously been identified (Liao et al. 2003). In this study, the activation of caspase-3 during strain-induced DNA fragmentation has been observed (Figure 6.3), therefore, it was decided to examine the role of SACCs as mechanosensors involved in the cleavage of caspase-3. To investigate the role of SACCs in the cleavage of caspase-3 at strain magnitudes that induce DNA fragmentation, MSCs were strained at 10% for 3 days in the absence and presence of the SACC blocker gadolinium chloride (Gd^{3+}; 10μM). Active caspase-3 expression was assessed by fluorescence microscopy in fixed cells using an antibody specific for the cleaved form of caspase-3. Figure 6.8A represents active caspase-3 staining in (i) control, (ii) 10% strained, (iii) control with Gd^{3+} and (iv) 10% strain with Gd^{3+} conditions. An increase in active caspase-3 immunoreactivity is demonstrated following 10% mechanical stimulation for 3 days (ii), a pattern which is reflected when cells were strained in the presence of Gd^{3+} (iv). Quantification of the intensity of immunoreactivity (Figure 6.8B) reveals that mechanical stimulation for 3 days significantly increases the fluorescence intensity from 8.68 ± 1.86 AFU (mean ± SEM) in unstrained controls to 20.3 ± 2.75 AFU following a 3 day strain stimulation ($p<0.05$, ANOVA, post-hoc; n=5), and in the presence of Gd^{3+}, from 12.90 ± 2.59 AFU (mean ± SEM) in unstrained controls to 23.35 ± 2.73 AFU following a 3 day strain stimulation ($p<0.05$, ANOVA, Newman-Keuls post-hoc; n=5). This finding provides evidence that the mechanosensors involved in caspase-3 activation following a 3 day strain stimulus of 10% are distinct from SACC.

Figure 6.8 Strain-induced caspase-3 activation is not dependent on SACC

MSCs seeded onto collagen-coated silicone were grown statically (i, iii) or exposed to cyclic mechanical tensile loading of 2.5% at 0.17 Hz for 3 days (ii, iv) in the absence (i, ii) or presence (iii, iv) of the SACC blocker Gd^{3+} (10μM). Cells were fixed, and the expression of active caspase-3 was assessed by immunofluorescence.

A: MSCs exposed to 10% strain for 3 days (ii) displayed more intense active caspase-3 immunoreactivity compared to unstrained cells (i). In the presence of Gd^{3+} (10μM) (iii, iv), intense active caspase-3 immunoreactivity was also observed in response to a 3 day 10% mechanical stimulation.

B: Expression of active caspase-3 was quantified by measuring the grey level intensity of the fluorescent images. A significant increase in caspase-3 fluorescence intensity was measured in cells exposed to 10% mechanical strain for 3 days compared to unstrained controls when cells were cultured in both the absence and presence of Gd^{3+} (10μM). Results are expressed as mean ± SEM for 5 independent observations, *$p<0.05$, ANOVA, Newman-Keuls post hoc. Scale bar is 50μm.


A
i
ii
iii
iv
50µm
B
Active Caspase-3 expression Arbitrary Fluorescent Units (AFU)
30
25
20
15
10
5
0
*
*
Unstrained
10% Strain
+ GdCl3 (10µM)

6.2.9 Voltage activated calcium channels are involved in strain-mediated MSC DNA fragmentation

Investigation into the role of SACC in transduction of the mechanical stimulus leading to DNA fragmentation has revealed that there are other non-SACC mechanosensors present at the cell membrane, that are involved in the detection and transduction of the strain stimulus that initiates an apoptotic response. Since studies have identified voltage activated calcium channels to have mechanosensitive properties (Lyford et al. 2002), I decided to investigate their role in this apoptotic response. Mechanical strain causes a maximal increase in DNA fragmentation following a 3 day stimulation of 10% (Figure 6.2) therefore this strain magnitude was used to determine if Ca^{2+} channels were involved in strain-induced DNA fragmentation. Following a 3 day 10% strain in the absence or presence of the L-type VACC blocker nicardipine (0.5μM), the TUNEL technique was used to assess the levels of strain-induced DNA fragmentation after a 3 day stimulation (Figure 6.9A). In control cells, 8.93 ± 1.31% (mean ± SEM) of cells displayed fragmented DNA in the nucleus (TUNEL positive). This was significantly increased to 22.18 ± 4.6% in cells exposed to 10% mechanical strain for 3 days ($p<0.01$ ANOVA, Newman-Keuls post-hoc, n=6 observations). While treatment of cells with nicardipine alone for 3 days had no effect on MSC viability (7.39 ± 1.26% MSCs with fragmented DNA), mechanical stimulation in the presence of nicardipine failed to increase DNA fragmentation and significantly reduced the strain-induced increase in DNA fragmentation (22.18 ± 4.6% to 12.23 ± 4.53% TUNEL positive MSCs; ($p<0.05$ ANOVA, Newman-Keuls post-hoc, n=6 observations)). This finding suggests that VACCs are involved in strain-induced DNA fragmentation. Representative TUNEL images of MSCs are shown in Figure 6.9B.

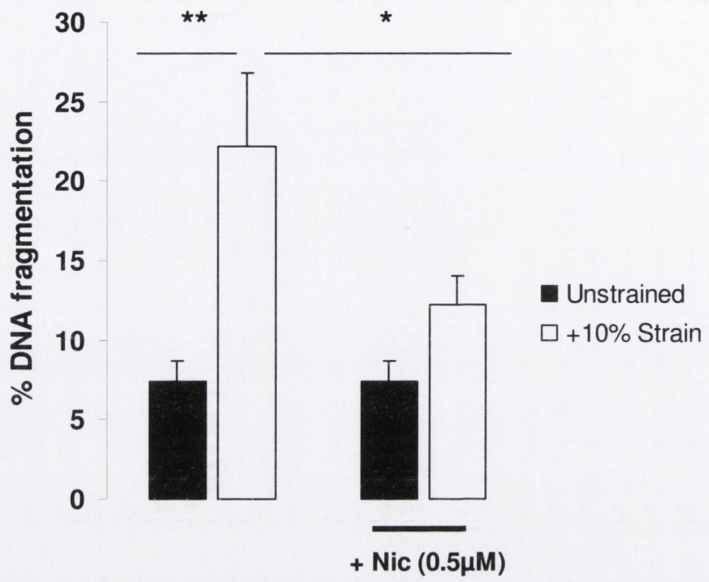
Figure 6.9 Ca^{2+} channels are involved in strain-mediated DNA fragmentation

MSCs were exposed to a 3 day cyclic tensile mechanical strain of 10% (0.17 Hz) in the absence or presence of nicardipine (0.5μM). Cell viability was assessed by measuring DNA fragmentation by the TUNEL assay.

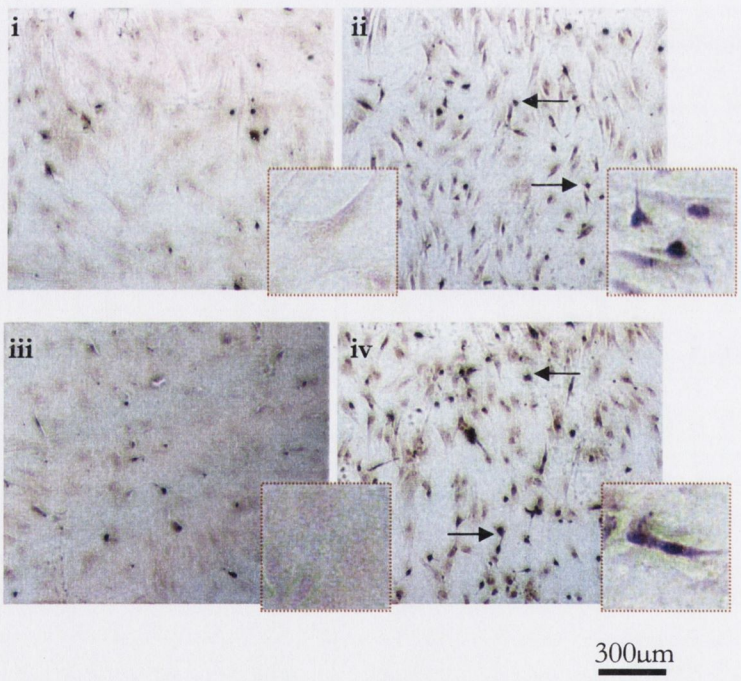
A: Mechanical strain of 10% for 3 days significantly increased DNA fragmentation (% DNA fragmentation) in MSCs that were exposed to mechanical strain. This was prevented when cells were strained in the presence of nicardipine (0.5μM), and the strain-induced increase in DNA fragmentation was significantly decreased. Results are expressed as mean ± SEM for 6 independent observations *$p<0.05$, **$p<0.01$ ANOVA Newman-Keuls post-hoc.

B: Light microscopic images of TUNEL staining of (i) control MSCs, (ii) 10% strained MSCs (iii) control + nicardipine (0.5μM) MSCs and (iv) 10% strained MSCs in the presence of nicardipine (0.5μM). Arrows indicate representative apoptotic TUNEL positive (+ve) cells. These data indicate a role for L-type voltage activated calcium channels in strain-mediated MSC apoptosis. Scale bar is 300μm.

A


30
25
20
15
10
5
0
% DNA fragmentation
**
*
Unstrained
+10% Strain
+ Nic (0.5μM)

B


i
ii
iii
iv
300um

6.2.10 Calpain is involved in strain-mediated MSC DNA fragmentation

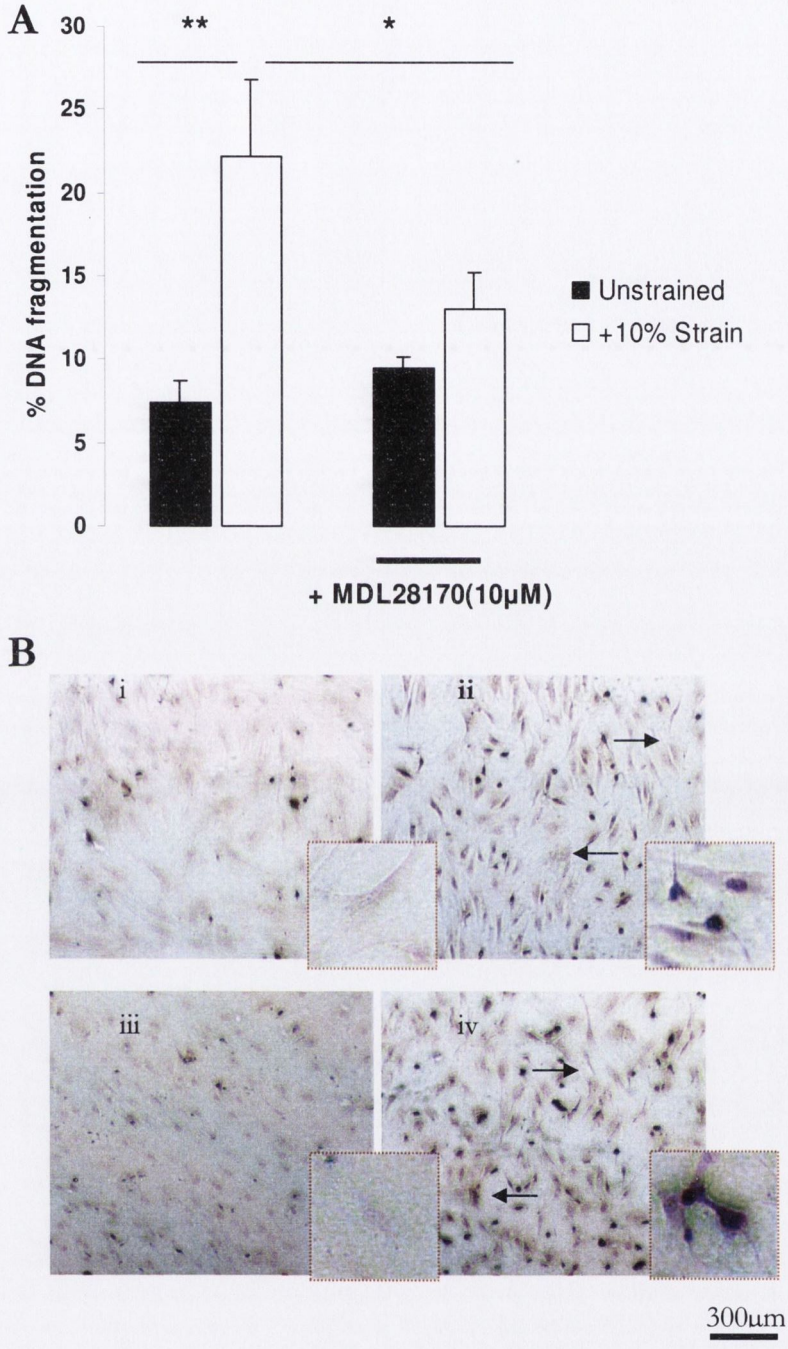
The role of the L-type voltage gated calcium channels in limiting MSCs apoptosis to 10% mechanical stimulation has been demonstrated (Figure 6.9). Calpain is a Ca^{2+}-dependent protease. Reports suggest that calpain is activated in response to physical stresses on the cell (Ray et al. 2000). Mechanical strain causes a maximal increase in DNA fragmentation following a 3 day stimulation of 10% (Figure 6.2) therefore this strain magnitude was used to determine if the calpain was activated in response to strain and involved in directly mediating strain-induced DNA fragmentation (Figure 6.10). Following calpain inhibition with MDL 28170 (10μM), the TUNEL technique was used to assess the levels of strain-induced DNA fragmentation after a 3 day mechanical stimulation (Figure 6.10A). In control cells, 8.93 ± 1.31% (mean ± SEM) of cells displayed fragmented DNA in the nucleus (TUNEL positive). This was significantly increased to 22.18 ± 4.6% in cells exposed to 10% mechanical strain for 3 days ($p<0.01$ ANOVA, Newman-Keuls post-hoc, n=6 observations). While treatment of cells with MDL 28170 alone for 3 days had no effect on MSC viability (9.49 ± 1.69% MSCs with fragmented DNA), mechanical stimulation in the presence of nicardipine failed to increase DNA fragmentation and significantly reduced the strain-induced increase in DNA fragmentation (22.18 ± 4.6% to 13.01 ± 5.29% TUNEL positive MSCs; ($p<0.05$ ANOVA, Newman-Keuls post-hoc, n=6 observations)). This finding suggests that calpain is directly involved in strain-induced DNA fragmentation. Representative TUNEL images of MSCs are shown in Figure 6.10B.

Figure 6.10 Strain mediated DNA fragmentation occurs via Calpain signalling

MSCs were exposed to a 3 day cyclic tensile mechanical strain of 10% (0.17 Hz) in the absence or presence of the calpain inhibitor MDL 28170 (10μM). Cell viability was assessed by measuring DNA fragmentation using the TUNEL assay.

A: Mechanical strain of 10% for 3 days significantly increased DNA fragmentation (% TUNEL +ve cells) in MSCs that were exposed to mechanical strain. This was prevented when cells were strained in the presence of MDL 28170 (10μM) and the strain-induced increase in DNA fragmentation was significantly decreased. Results are expressed as mean ± SEM for 6 independent observations $p<0.05$, ANOVA Newman-Keuls post-hoc.

B: Light microscopic images of TUNEL staining of (i) control MSCs, (ii) 10% strained MSCs (iii) control + MDL 28170 MSCs and (iv) 10% strained MSCs in the presence of MDL 28170. Arrows indicate representative apoptotic TUNEL positive (+ve) cells. These data indicate a role for calpain in strain-mediated MSC apoptosis. Scale bar is 300 μm.


A
30
25
20
15
10
5
0
% DNA fragmentation
**
*
Unstrained
+10% Strain
+ MDL28170(10μM)
B
i
ii
iii
iv
300um

6.2.11 Calpain activity in MSCs during 10% cyclic tensile strain

Since calpain has been identified as playing a role in strain-induces MSC apoptosis, it was decided to examine calpain activity during application of the mechanical stimulus. Therefore, cells were strained for 2 days in the absence or presence of MDL 28170, prior to monitoring the activity of the protease. To examine calpain activation, cells were homogenised in lysis buffer and calpain activity determined by monitoring the cleavage of the fluorescently labelled calpain substrate, to its fluorescent product. Figure 6.11 shows that exposure of MSCs to 10% mechanical strain increased calpain activity from 48.07 ± 18.64 pmol AFC produced/mg protein/min (mean ± SEM) in control cells to 231.84 ± 149.48 pmol AFC produced/mg protein/min in cells that were exposed to 10% mechanical strain. Cells treated with MDL 28170 alone for 2 days displayed a level of calpain activity comparable to control values (35.92 ± 7.68 pmol AFC produced/mg protein/min). Furthermore exposure of cells to 10% mechanical strain in the presence of MDL 28170 prevented the strain induced increase in calpain observed after 2 days of strain (74.52 ± 38.44 pmol AFC produced/mg protein/min). While this is not a statistically significant result, it presents a trend for increased calpain activity during mechanical strain that is blocked when using a calpain inhibitor.

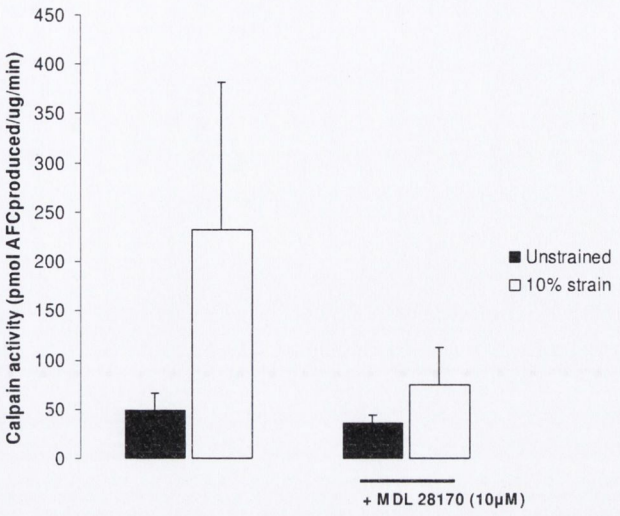


Figure 6.11 Calpain activity during 10% cyclic strain

MSCs were exposed to 10% cyclic strain for 2 days in the presence or absence of calpain inhibitor MDL 28170 (10μM). Cells were lysed and calpain activity was assessed by the cleavage of the fluorogenic AFC substrate. Exposure of MSCs to 10% strain for 2 days increased calpain activity and this was abolished by MDL 28170 (10μM), indicating the involvement of the protease calpain during apoptosis at 10% strain. Exposure of cells to the calpain inhibitor alone had no effect on calpain activity. Results are expressed as a mean ± SEM for 6 independent observations.

6.3 Discussion

The aim of this study was to investigate the effects of cyclic mechanical strain on MSC proliferation dynamics and MSC viability, and to characterise the intracellular mechanotransduction mechanisms that underlie strain-mediated apoptosis in MSCs. Continuous cyclic tensile strain of both 2.5% and 10% reduced the rate of synthesis of new DNA in MSCs reflecting a decrease in MSC proliferation in response to strain. MSC DNA fragmentation occurred in a magnitude-dependent manner, with significant DNA fragmentation occurring at strains at and above 7.5% and maximal apoptosis occurring at 10% of strain, reflecting an apoptotic response profile to mechanical stimulation. Cyclic strain induced phosphorylation of JNK after 3 days; the ability of strain to induce DNA fragmentation was blocked by a JNK inhibitor, indicating that strain-mediated DNA fragmentation occurs via JNK activation. Strain increased the activity of caspase-3, suggesting that strain-mediated apoptosis progresses via a caspase-dependent mechanism. Strain-mediated DNA fragmentation was unaffected by blocking SACCs with gadolinium chloride suggesting that SACCs are not pertinent in this pathway. In contrast, when L-type VACCs were blocked by nicardipine, the strain-induced activation of apoptosis was abrogated, indicative of a role for VACC as a mechanosensor in the initiation of the pro-apoptotic mechanotransduction signal. Given that activation of the L-type voltage-activated Ca^{2+} channel in response to 10% strain would be expected to increase intracellular concentration Ca^{2+}, we sought to examine the role of the calcium-activated protease, calpain, in this strain-induced apoptotic cascade. In the presence of the calpain inhibitor MDL 28170, strain-induced apoptosis was prevented suggestive of a calpain-dependent mechanism of strain-induced apoptosis.

MSCs characteristically retain a self-replicative property, through largely unknown mechanisms that suppress cell differentiation or apoptosis (Molofsky et al. 2004). Mesenchymal progenitor cells in their niche are quiescent, but responsive to signalling for proliferation, for example. During cell proliferation, DNA is replicated as part of the cell cycle. The measurement of DNA synthesis is a reliable indicator of cell proliferation in organotypic and monolayer culture (Dahl 1983), therefore, in this study, proliferation during cyclic tensile strain was detected by measuring the uptake of ^{3}H-thymidine (Bocelli-Tyndall et al. 2006; Wang et al. 2006).

Results in the current study indicate that mechanical stretch has no effect on MSC proliferation following 24 hrs of stimulation and significantly reduces proliferation following 48 and 72 hrs of stimulation, without a magnitude dependency. However it is difficult to determine whether reduced rate of proliferation is a direct response to strain or as a consequence of part of the population beginning to enter commitment to the osteogenic lineage or a feature of the combination of both. Differentiation status following 3 days of strain is strongly supported by protein markers discussed in Chapter 5. In a recent study, MSC proliferation was examined 6hrs following the application of cyclic stretch (duration: 15, 30, 60 min; magnitude: 2%, 4%, 8%; frequency: 1 Hz) (Song et al. 2007). The authors report an increase in metabolic activity, reflective of an increase in the number of cells, and an increase in the activity of the c-fos gene, which is an index for cell proliferation (Angel and Karin 1991). Using a similar strain regimen, van Griensven et al (Skutek et al. 2003) also report the stimulatory effect of short-term (15 min; 5% 1Hz) mechanical stimulation on MSC proliferation. The authors go on to examine longer stimulations over longer time periods (8 h/day; 3 days) and report a reduction in MSC proliferation when compared to static controls (Skutek et al. 2003). Upon investigating differentiation in that study, only cells that were exposed to long-term strain were capable of persistent induction of osteogenic differentiation (Skutek et al. 2003). Simmons et al (Simmons et al. 2003) found a decrease in proliferation in response to a continuous 3% equibiaxial strain of 0.3Hz for up to 3 days. Proliferation was based on cell counts and therefore apoptosis was unaccounted for. However the strain magnitude was low, cells remained viable, and they also report strain-induced advanced osteogenic differentiation (Simmons et al. 2003).

A series of recent studies published by a group investigating bone-marrow derived and adipose-derived MSC strain responses for vascular tissue engineering report that strain negatively regulates MSC proliferation patterns following 6/7 days of cyclic strain at 1Hz in both 2D and 3D environments (Hamilton et al. 2004; Lee et al. 2007; Nieponice et al. 2007). In the 2D studies the measure for proliferation was cell counting through visual inspection, making it difficult to corroborate as the method has the possibility of subjectivity, counts were not normalised and no provisions were made for cell loss through apoptosis in response to strain (Hamilton et al. 2004; Lee et al. 2007). However the 3D study confirms the proliferation trend by additionally assessing metabolic activity and normailsing to

cell number (Nieponice et al. 2007). Nevertheless, the finding that mechanical tensile strain causes a decrease in the rate of proliferation is consistent with the proliferation trend reported in these results, as lower cell numbers would be expected in the sample proliferating at the lower rate when assayed at the same time-point. Each of these studies, report lineage specific protein expression, indicating commitment of MSCs, albeit smooth muscle related protein markers. Inspection of the proliferation patterns then reveals that divergent trends are reported when considerably different strain regimens promote the response, the dominant parameter being the duration of strain application.

Since only long term strain regimens were applied for this study, and these result in lineage specific protein marker up-regulation, the question then is, what are the proliferation dynamics concomitant with the onset of cell differentiation? Hence, we see the evidence supporting the differential mitotic responses of MSCs to strain with the emerging trend that short term stimulations promote proliferation at the expense of differentiation, and long-term application of strain negatively regulates proliferation while cell differentiation is promoted. In a study by Pratap (2003) we find the most compelling evidence to support the cell commitment/cell growth relationship. That study examines the effect of Cbfα1 expression, a marker of osteogenic commitment, on the cell cycle dynamics and they conclude that Cbfα1 promotes osteoblast maturation at a key developmental transition by supporting exit from the cell cycle and activating genes that facilitate bone cell phenotype development. Therefore, since this research has identified the upregulation of Cbfα1 (Chapter 5) and in this study, the cell cycle response concomitant with Cbfα1 expression is toward reduced growth rates, in combination, reflect bone cell phenotype development in agreement with osteogenic development reported in previous studies (Koike et al. 2005; Pratap et al. 2003).

Two physiological processes in which mesenchymal progenitor cells are involved in osteogenic development are in the embryological development of the skeleton, and during fracture repair (Bruder et al. 1994; Caplan 1987) – both of which are stimulated by mechanically active environments. The proliferation dynamics of cells from the mesenchyme have been well characterised during skeletal formation in the embryo and skeletal tissue healing following fracture damage (Brighton et al. 1991). Prior to bone formation in both of these physiological processes, events

are marked by proliferation of the progenitor cells and the cessation of proliferation marks the onset of tissue specific development. It is therefore possible that this pattern seen in embryological skeletal development and bone regeneration is reflected in the *in vitro* mechanical stimulation of progenitor cells that leads to expression of early bone markers.

An interesting observation from the pattern of static proliferation was the trend towards reduced proliferation rates with time. It is known that during *in vitro* MSC culture, following a 2 day lag phase, cells proliferate exponentially in the log phase, however the observation in this study suggest an opposing trend. It is suggested that this is due to the dimensional constraints imposed by the 60x10mm substrate. Cell to cell contact may have been achieved soon after seeding and therefore the normal sub-confluent environment that would exist when growth patterns are observed would not have applied. It is known that seeding density affects expansion of MSCs *in vitro* (Bruder et al. 1997; Colter et al. 2000; Javazon et al. 2001). Additionally, it is worth noting that the MSCs continue to proliferate in both static and stimulated conditions, albeit at a negative rate.

Apoptosis, or programmed cell death, is a normal physiological process in which a cell actively participates in its own destruction in a way that circumvents an inflammatory response (Arends and Wyllie 1991; Kerr et al. 1972). This process is characterised by distinct biochemical and morphological changes to the cell, including the internucleosomal cleavage of DNA (Ziegler and Groscurth 2004). The ability of cyclic strain to induce DNA fragmentation was assessed by TUNEL staining.

The use of mechanical stimulation is emerging as a successful approach for *in vitro* pre-conditioning for tissue engineering applications, through the control of cellular proliferation and in directing differentiation towards appropriate cell types (Holtorf et al. 2006). However, the optimal mechanical conditions that will stimulate optimal cell scaffold development whilst maintaining cell viability have yet to be completely defined (Satija et al. 2007). This study examined the relationship between tensile strain and mesenchymal stem cell viability and the physical stimulus to the apoptotic response through examining the mechanotransduction pathways that were activated.

The sensitivity of MSCs to sustained cyclic tensile strain was shown to have a threshold dependency. For a number of cell types, previous work supports the

relation between strain magnitude and apoptotic injury, however the threshold limits are not consistent and this is thought to be cell specific (Birukov et al. 2003). The finding that cell survival is maintained up to strains of 5% is in agreement with the study of Plotkin et al (2005), where the authors report a protective effect of strains up to 5%, due to extracellular-regulated kinase (ERK) activation in osteocytes (Plotkin et al. 2005). The promotion of bone remodelling gene expression in response to sustained low strains (0.25 – 2%) has been observed in bone stromal cells (Rubini et al. 1960), and an absence of apoptosis in foetal osteoblast cultures had been reported in response to strains up to 2.5% (Weyts et al. 2003). The apoptotic response in MSCs in this study that was associated with larger strains have also been observed in retinal pericytes (Suzuma et al. 2007) where 10% cyclic tensile strain induced apoptosis via JNK activation, and in smooth muscle cells where 10% cyclic strain leads to apoptosis via Bax/Bcl-x_L (Morrow et al. 2005). The strain magnitude dependency of cell apoptosis has also been demonstrated in vascular endothelial cells (ECs) (Liu et al. 2003). In that study, the threshold limits were elevated from those described above, however cell survival was reported for strains that are physiological for ECs (6% and 10%) and apoptosis was stimulated by higher, potentially pathological levels of cyclic stretch (20%). In contrast, cell survival has been reported in MC3T3-E1 osteoblasts exposed to uniaxial strain of 20% (Danciu et al. 2003), however, in that study the maximum exposure time was 60 min. Thus, the impact of strain on cell viability is dependent upon both magnitude and duration of exposure.

In this study, through blocking the cell membrane VACCs using the selective L-type Ca^{2+} channel blocker, nicardipine, the apoptotic effect of sustained 10% cyclic strain was abolished. L-type Ca^{2+} channels have previously been shown to have mechanosensitive properties (Lyford et al. 2002) and intracellular Ca^{2+} fluxes in response to mechanostimulation of L-type Ca^{2+} channels have been linked to strain-mediated apoptosis in neo-natal cardiomyocytes (Liao et al. 2003). Mechanisms of mechanosensation in VACC have yet to be fully defined, however, research has recently identified the von Willebrand factor A (VWA) type domain on the extracellular sequence of the alpha (2) delta ($\alpha 2\delta$) subunit of the VACC (Olesen et al. 1988). Since this domain mediates binding to proteins such as collagen, found in the extracellular matrix, it follows that the $\alpha 2\delta$ subunit may be involved in the detection of extracellular mechanical events and link

subsequent activation of the channels. SACCs have also shown to be activated in response to physical stimuli (Guilak et al. 1999) however the present study reveals that apoptosis in response to tensile strain is independent of signalling via SACCs in MSCs.

JNK is activated in response to a variety of cellular stresses and extracellular signals (Nishina et al. 2004) and in apoptotic cells, morphological features are often preceded or concomitant with JNK activation (Cosulich and Clarke 1996). However, JNK activation has been reported to have both cell survival (Rubini et al. 1960) and apoptotic effects (Arnoczky et al. 2002). JNK phosphorylation has been identified in this study, sustained after 3 days of cyclic tensile strain. This study demonstrates that inhibition of JNK activation during 10% cyclic strain offers cell protection suggesting a role for JNK in strain-mediated apoptosis of MSCs. Phosphorylation of JNK has been associated with mechanical stretch (Hsieh and Nguyen 2005; Nguyen et al. 2006; Suzuma et al. 2007) and the cell survival or death response is dependent on whether JNK activation is transient or sustained, which is regulated by the magnitude of applied stretch. Large strains have shown to evoke sustained JNK activation, that has consequently lead to apoptotic cell death (Arnoczky et al. 2002), whereas low strains have stimulated sustained JNK activation while simultaneously promoting expression of positive bone remodelling genes (Rubini et al. 1960). JNK activation through cyclic strain occurs as a consequence of calcium-dependent mechanotransduction (Arnoczky et al. 2002), therefore JNK activation in this study may be downstream of the L-type calcium channel which we have also demonstrated to be involved in this strain-induced apoptotic event.

Calpains are Ca^{2+} dependent proteases and target many cytoskeletal proteins (Molinari and Carafoli 1997). Extensive studies on spinal cord injury have revealed calpain activation through rises in intracellular Ca^{2+} in response to trauma (Ray et al. 2000). Cell death through a loss of structural integrity and destabilisation of the cellular architecture follows. Calcium activation of calpain and subsequent inhibition of apoptosis by a calpain inhibitor has been demonstrated in pancreatic islet cells (Chang et al. 2004) and Sharma et al (Sharma and Elisseeff 2004) have linked apoptosis through calcium-activated calpain via modulation of caspase-3 activity. In this study, calpain has been implicated in strain-mediated apoptosis since application of 10% strain in the presence of a

calpain inhibitor prevented strain-mediated cell death. Given that L-type Ca^{2+} channels have been shown to be active during mechanical stimulation of MSCs, it is therefore possible that changes in intracellular Ca^{2+} concentrations that follow induce conformational changes in calpains that lead to their activation. In neurons, Ca^{2+} influxes associated with permeability changes following tensile forces have been linked to cytoskeletal degradation via spectrin proteolysis through calpain activity (Buki and Povlishock 2006) and calpain inhibition has previously demonstrated protection against UV-induced cell death and reduces spectrin degradation (McCollum et al. 2002). Thus it is possible that in this study, MSC apoptosis that follows strain-mediated calpain activation is a consequence of a loss in cytoskeletal integrity through calpain-mediated spectrin proteolysis. A recent study has identified the involvement of calpain in the JNK stress response pathway (Tan et al. 2006) therefore the activation of JNK observed in response to mechanical strain may then be influenced by the calcium-mediated calpain activation as well as direct activation from free intracellular calcium. While DNA fragmentation was inhibited when calpain activation was blocked, analysis of the activity of calpain revealed that it was unchanged compared to in response to strain both in the absence and presence of the calpain inhibitor. However, there is a trend which suggests higher levels of calpain activity during strain, and this trend is not seen in the presence of the calpain inhibitor. Thus, further analyses of the activity of calpain during strain is required before discounting its activity in response to strain, possibly by using an more sensitive technique such as an immunocapture-based biochemical assay.

In summary, this study demonstrated that MSCs are mechanoresponsive in terms of proliferation and apoptosis. Proliferation decreases with increasing duration of cyclic strain, and this is concomitant with the onset of osteogenic differentiation. Continuous uniaxial mechanical strain applied to mesenchymal stem cells in monolayer at a magnitude greater than 7.5% promotes apoptosis in a manner involving the L-type VACC, whereas SACC are not involved. JNK is activated in strain-mediated apoptosis and inhibition prevents the apoptotic response. Finally apoptosis occurs via caspase-3 and calpain dependent mechanisms (Figure 6.12). Further understanding of the consequences of mechanical strain on MSC viability, and the intracellular signalling events that underlie such affects, will assist in the development of tissue engineering strategies in which mechanical priming of

MSCs is used to encourage optimal formation of tissue engineered constructs, in a manner that limits cell loss through apoptosis.

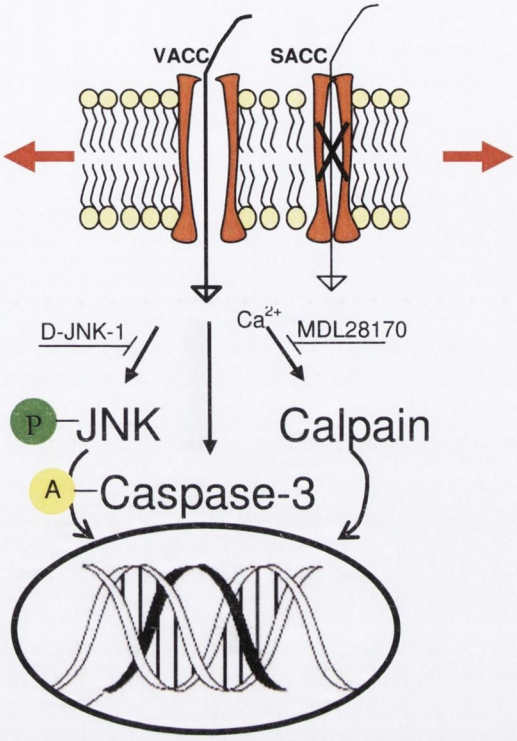


Figure 6.12 Mechanotransduction in strain-mediated apoptosis

Apoptosis is induced in MSCs stimulated by 7.5% strain and greater. This occurs via voltage activated calcium channels but not stretch activated cation channels; the calcium sensitive apoptotic pathways associated with JNK and calpain signalling are active during strain-induced apoptosis, as demonstrated by inhibition of these molecules; ultimately, the caspase pathway becomes activated and DNA fragmentation occurs.

7 Final Discussion

7.1 General Discussion

Tissue engineering aims to address the functional restoration of damaged or diseased tissues, through the multidisciplinary integration of sciences that can provide cells, scaffolds and molecular signalling to form a biocompatible construct that will actively integrate in the host. The recent development of the tissue engineering approach has been significantly facilitated by the discovery and knowledge of mesenchymal stem cells which have the capability of multilineage differentiation. In addition, these cells possess many other features that make them an appropriate cell source for tissue engineering applications (Barbash et al. 2003; Bocelli-Tyndall et al. 2007; Metheny-Barlow et al. 2004; Nauta and Fibbe 2007). Sourcing, isolation and the lack of ethical considerations for their use contribute to their widespread selection as cells for tissue engineering applications (Le Blanc and Pittenger 2005).

MSCs are responsive to many external stimuli, and biological factors such as properties of the extracellular matrix, physical force as well as growth factors and cytokines can regulate their differentiation. The biochemical induction of MSCs (e.g osteogenic factors) is a powerful approach for tightly regulated lineage specific differentiation. While this holds merits as a useful tool for the retrospective functional characterisation of the MSC population, the use of chemical inducers is not suitable for tissue engineering (Satija et al. 2007). Firstly, the *in vitro* chemical osteogenic induction of MSCs involves incubation with a synthetic glucocorticoid. Secondly, other factors include ascorbic acid and β-glycerol phosphate, and these conditions are unlikely to reflect the physiological signals MSCs receive to induce osteogenesis *in vivo* (Chamberlain et al. 2007). Thirdly, broad tissue exposure to the cocktail may lead to undesirable responses at other sites. Finally, continuous stimulation is required for cells to perform their function efficiently (Mao and Nah 2004; Song et al. 2006), and coupled with the short half-life of most of these factors, the need for large doses would involve considerable cost implications (Corsi et al. 2007). Therefore, the *in vivo* delivery of appropriate concentrations of osteoinductive factors to maintain constant stimulation would not be realistic. For biochemical induction reflective of *in vivo* sites of osteogenesis, genetically modified cells or the use of recombinant proteins involved in osteogenic differentiation such as BMPs (Schuckert et al. 2006) are possible approaches. However, their use involves high costs, and has been associated with side-effects, such as immune responses (Conese et al. 2007).

There is significant evidence that physical factors may be used to improve or accelerate tissue regeneration and repair *in vitro* (Butler et al. 2000), and mechanical stimulation from the environment is necessary for normal development. To attain directed differentiation of MSCs, biomechanical factors are appropriate stimuli for tissue engineering applications. Since the only stimulation they receive is physical force, and this can be accurately controlled. Mechanical stimulation has the benefit of minimising the level of cellular intrusive intervention, and through scaffold design or as an intrinsic feature of most *in vivo* microenvironments, the stimulus can be maintained beyond *ex vivo* cultivation, upon implantation. Furthermore, the osteogenic cellular responses to mechanical signals occur much quicker than when induced using growth factors (Oreffo et al. 1999; Sumanasinghe et al. 2006; Ward et al. 2007). Therefore, the control of MSC osteogenic differentiation by mechanical stimulation forms the incentive for this research.

As with the introduction of any new clinical treatment, rigorous examination of the quality, efficacy and safety of their use is essential for approval by regulatory bodies. To this end lies an area for development before the introduction of mesenchymal stem cells as cell-based therapies, as the mechanisms of their action that contribute to tissue regeneration both *in vitro* and *in vivo* have yet to be fully understood. Herein lies an intrinsic consensus among tissue engineering researchers for the introduction of clinical based applications: that is to maximise the impact of tissue engineering in regenerative medicine, for approaches that use mesenchymal stem cells as a cell source, most progress will be made upon gaining an understanding of intrinsic resident stem cell behaviour and the environmental cues, needed to activate and regulate these cells (Barrilleaux et al. 2006; Chamberlain et al. 2007; Giordano et al. 2007; Gurtner et al. 2007; McGonagle et al. 2007; Satija et al. 2007; Watt and Hogan 2000). Through the experimental investigation of mechanobiology, this can be achieved; and this is where the state of the art lies in mechanobiology for MSC-based tissue engineering.

This research addressed how the fundamental cell fate decisions of proliferation, differentiation and cell death are induced and regulated by mechanical stimulation, and provided insight to some of the mechanisms of mechanotransduction in these processes. Cells were stimulated in tension, through cyclically deforming their substrate, which was a 2D collagen-coated silicone membrane. Briefly, the cellular substrate was characterised mechanically and topographically, and an optimal

seeding density was established. Cell attachment was favourable on a collagen-coated membrane that had been exposed to UV irradiation. Interestingly, this was not the most mechanically stiff material; nor had it the most topography. Thus, the favourable properties of this substrate are attributed to substrate chemistry, which forms part of the further investigations that lead from this research. The marrow stromal cell population was phenotypically characterised as a homogeneous mesenchymal cell population, and the osteogenic potential of these cells was demonstrated on a collagen-coated silicone substrate. MSCs demonstrated osteogenic differentiation on collagen coated silicone following incubation with the osteogenic growth factors dexamethasone, β-glycerophosphate, and L-ascorbic acid-2-phosphate. Trends towards the involvement of p38 and pI3-kinase but not ERK intracellular signalling were identified. The onset of osteogenic markers was moderately delayed from that reported in other studies, and it is thought that this could be reflective of the order of magnitude difference in the concentration of dexamethasone that was used this study compared to other studies; however all the hallmark features of growth factor–induced osteogenesis were observed.

The mechanosensitivity of MSCs was demonstrated, and it was shown that tensile strain is a potent regulator of osteogenic differentiation, as assessed by the temporal expression of Cbfα1, collagen type I osteocalcin and BMP2. Stretch-activated cation channels (SACCs) are involved in the mechanosensing of strain-induced osteogenesis, and strain-mediated osteogenesis activates autocrine BMP2 synthesis, which is dependent on p38 and pI3-kinase but not ERK intracellular signalling. ERK signalling has been a common feature of the intracellular reaction to mechanical signals in particular, but also many other extracellular stimuli. Further investigations will re-examine the role of ERK in the strain-induced mechanotransduction of osteogenic differentiation, using different time points and other approaches e.g. siRNA to examine its role. The effects of stretch are mediated through membrane calcium channels also (el Haj et al. 1999; Lyford et al. 2002) and since the effects of blocking SACCs were a marked reduction on osteogenic protein expression and not a complete block, it is likely that other mechano-sensitive elements are involved in transduction. Therefore, it is appropriate to investigate the involvement of VACC, G-proteins, integrins and other mechanosensitive proteins, to completely define the detection and initial transduction of tensile strain. The rate of MSC proliferation is reduced in response to continuous cyclic tensile strain, and this trend compliments the

finding of strain-induced onset of osteogenic differentiation (Owen et al. 1990). Upon examining cell apoptosis in response to mechanical stimulation, it was found that tensile strain of 7.5% and above can induce MSC apoptosis, and this occurs via L-type voltage activated calcium channels, but not stretch-activated cation channels. The intracellular signalling in strain-induced apoptosis occurs via JNK, and caspase-3 and calpain are the intracellular proteases by which apoptosis proceeds.

Mesenchymal stem cells reside in their niche environment in a quiescent state. Their ubiquitous expression profile throughout a multitude of tissues, is an indication firstly of the plasticity of these cells, and secondly, their importance as a resident population to support cell turnover, or to react in the occurrence of injury. This potential is reflected in the scope for clinical applications involving these cells. That their activation is driven in response to certain physiological and pathological needs, suggests that they inherently retain a high responsive capacity. It is through the intelligent exploitation of this property that will make the most significant contribution to their use in tissue engineering applications. However, the success of future clinical applications depends critically upon a thorough understanding of the biology of these cells. The ability to induce a response from MSCs towards commitment to the osteogenic lineage demonstrated in this research represents a powerful tool for MSC based osteogenic tissue engineering. The most significant findings lie in the mechanical induction of osteogenesis and its mechanistic control, in particular BMP-2 regulation, as well as having identified the boundary conditions for strain-related apoptosis, which has relevance for the design and use of bioreactors, and the associated detection and intracellular signalling involved in the strain related activation of apoptosis.

7.1.1 Differentiation

The experimentation in this study identifies that it is possible to control differentiation by applying just one of the extracellular aspects that exist in the MSCs microenvironment – mechanical strain, in the absence of all other inductive factors. In addition to the expression of osteogenic proteins, the change in proliferation dynamics bears functional relation to the development of the osteoblastic phenotype. In a detailed examination of the relationship between cell proliferation and the temporal gene expression that characterises the developmental sequence of bone cell differentiation, Owen et al. (Owen et al. 1990) associate the down-regulation of proliferation with the onset of genes

associated with extracellular matrix maturation. Mineralisation of extracellular matrix contributes to the cessation of proliferation, since the process itself immobilises osteoblasts by embedding them in the mineralising osteoid (Coelho and Fernandes 2000; Stein and Lian 1993). Thus a reciprocal and functionally coupled relationship exists between proliferation and differentiation (Owen et al. 1990). This *in vitro* model mirrors the *in vivo* events of embryological development of the skeleton, and during fracture repair (Bruder et al. 1994; Caplan 1987). During both of these physiological processes, the cessation of proliferation marks the onset of bone formation. Thus the behaviour of osteoblast forming mesenchymal stem cells in response to mechanical stimulation in this study is in agreement with this reciprocal model of osteoblastic differentiation and strongly supports the regulation of MSC osteogenesis by mechanical strain.

Examination of the protein expression regulation by mechanical strain, determined temporal expression of the transcription factor Cbfα1, collagen type I, osteocalcin, and an autocrine regulation of the osteogenic growth factor BMP-2. BMP-2 is a growth factor central to bone formation, and its regulation in bone development has a close relationship with Cbfα1 (Bae et al. 2007; Ducy et al. 1997; Lee et al. 2000b), possibly as part of a positive regulatory mechanism. Its potency in the osteogenic process makes it a target for growth factors for physiologically relevant osteogenic tissue engineering. This study found that the strain-induced autocrine of regulation BMP-2 was dependent on p38 and PI3-kinase. Additionally, when SACCs were blocked, strain-induced osteogenic protein expression was less marked. Thus, it was inferred that the strain-induced osteogenic profile involves SACCs and occurs via p38 and PI3-kinase dependent intracellular signalling. The ability of mechanical strain to up-regulate osteogenic transcription factors and bone matrix associated proteins is highly significant and has important implications for tissue engineering, however the greatest insights to be gained from these findings are in the mechanistic control of the osteogenic differentiation of MSCs from a purely biomechanical stimulus.

Once cells are removed extracorporeally for *in vitro* culture to study their physiology, while conditions are optimised, the environment is rarely replicative of *in vivo* conditions, and it can be argued that in this study, while it is physiologically relevant to use strain as an osteoinductive stimulus, the absence of growth factors and cytokines that are present in the cellular microenvironment make this model of osteogenic differentiation unrealistic. These results demonstrate that the

application of a mechanical stimulus *is* sufficient to drive lineage differentiation towards the osteogenic route and reveal that it does this through a mechanism that induces an appropriate growth factor environment, reflected in the synthesis of the BMP-2 growth factor. Thus, once stimulated, MSCs self-regulate an appropriate growth factor environment through autocrine mechanisms demonstrated in this study by the up-regulation of BMP-2, therefore more closely replicating the *in vivo* conditions for bone development and growth in their *in vitro* environment. The induction of an autocrine regulatory mechanism for BMP-2 has possibly the most far-reaching significance among the osteogenic profile identified in this study, as it is a major target for the sustaination of the osteogenic phenotype. Additionally it identifies that a combination of biomechanical signals with biochemical signals is not necessary for the creation of an *in vitro* cellular environment that reflects both the mechanical and chemical physiological conditions that surround MSC osteogenic induction *in vivo*. It also provides an example of the bioactive/trophic properties of MSCs since it is known that they secrete a broad spectrum of macromolecules that serve to structure regenerative environments in fields of tissue injury (Caplan 2007). The self-regulation of osteogenic differentiation is possible through this stimulus, however - should the goal be to influence proliferation, then this study also identifies that this pattern of mechanical stimulation is not appropriate and that growth factor supplementation may be more appropriate for cell division.

This insight into the mechanisms of osteogenic differentiation has important consequences for contributing to the understanding of how they are regulated and how they can be controlled. Since so much emphasis is placed upon the importance of this if MSCs are to be considered for use in clinical applications this research positively contributes to the development of the knowledge pool on the regulatory mechanisms of MSCs and therefore positively contributes to their consideration for use in clinical applications.

7.1.2 Apoptosis

Having identified the significance of mechanical conditioning for tissue engineering applications, this research then turned to defining the control of cell viability in response to mechanical stimulation, as cell viability is obviously a critical determinant for the success of any tissue engineering application. Thus the

identification of boundary conditions for a continuous tensile mechanical stimulation that would minimise cell death through apoptosis to be 5% strain or less, can contribute significantly to bioreactor design. The use of bioreactors to facilitate *ex vivo* tissue generation has developed to address all aspects of the tissue engineering life cycle from cell seeding to nutrient diffusion to promoting matrix deposition (Martin et al. 2004), however without knowledge on boundary conditions for cell viability, design and stimulation patterns are often inadvertently applied that impact on the viability of the cells. Bioreactor considerations are currently a major target area for strategies in tissue engineering, with an emphasis being placed on the control of structural and functional aspects of design being considered as advantageous to address accuracy in tissue engineering (Freed et al. 2006). Thus the findings of this study can have an important bearing on strain control in bioreactor design, and also in the design of stimulation patterns for the regulation of cell fate. Additionally, this research has defined the signalling events at the cell membrane and intracellularly during strain-induced cell death thus identifying potential therapeutic targets to mitigate the effects of inevitable apoptotic injury as a consequence of the tissue engineering process. As in the case with cell differentiation, the identification of the mechanisms of strain mediated apoptosis will help contribute to the much needed pool of information on the biology of stem cells, for their consideration in clinical applications. The lack of published work on apoptotic signalling pathways in MSCs make direct comparisons of apoptotic mechanisms difficult. However it is clear the mechanical stretching directly activates intracellular signalling pathways which in turn lead to programmed cell death. This knowledge of strain-related apoptosis can be applied to improve tissue engineering strategies, particularly in the field of bioreactor design. Understanding the mechanisms of which MSCs undergo apoptosis in response to physical force can contribute to the development of methodologies to increase their survival in environments particularly where they experience tensile mechanical signals.

When mechanically induced differentiation proliferation and cell death are considered together, these results strongly suggest that there is an optimal level of stimulation where proliferation can be balanced with differentiation, and differentiation can be directed without loosing cell numbers through mechanically associated apoptosis. Definition of the precise mechanical parameters for this is the work of another study. From this study, we know more about how MSCs contribute to the development of tissue engineered constructs, and have an

understanding of how the chemical and mechanical microenvironment influences their differentiation, proliferation and programmed cell death. This can contribute to the optimisation of the delivery of strain stimuli and to bioreactor design for delivery of the stimulus.

7.1.3 Experimental considerations

In the following section, a number of points are addressed from particular areas of the research that raise questions.

Firstly, the investigations of MSC behaviour in response to strain were carried out on cells of rat origin, thus the question must be addressed as to the relevance of this for the advance of tissue engineering strategies for human applications. It is known that for MSCs, in certain instances, interspecies differences exist. For example, MSCs do not express the same surface molecules in all species. Human and rat MSCs have been shown to be CD34 -, while some papers report variable expression of CD34 on murine MSCs (Peister et al. 2004). However, significantly, a recent study comparing the osteogenic differentiation capacity of human and rat cells in response to osteoinductive factors, reported that no differences exist (Zavan et al. 2007). In addressing the relevance between species of the strain magnitude values proposed to have biological effects, it is known that the mechanical properties of bone tissue are similar among mammals. For example, the Young's modulus of rat femoral bone is similar to that of human femoral bone, so the major difference between rats and humans is size (Robling et al. 2006). Therefore, since the cell fates examined are such fundamental cell events, and that tissue mechanical properties are similar between species it is anticipated that both the mechanical control of rat MSC fate and the magnitudes of strain applied are fully conserved (Kronenberg 2003) and therefore relevant for human studies also.

Another aspect to address is the reactive fluid shear stress that is present by design when cyclically straining the MSCs. Fluid shear forces that occur as a consequence of a moving body in a liquid were minimised by using a volume of culture medium that just covered the cells; however, turbulent flow will still exist. It is known that bones cells are particularly sensitive to shear stimuli, particularly for bone remodelling (Burger and Klein-Nulen 1999). Also, shear stress has been found to activate JNK in endothelial cells, but only transiently, and cell apoptosis

did not follow (Richards et al. 1999). Shear strains have in fact been reported to favour cell survival (Li et al. 2005). Also, Mao and Nah (2004) discuss that the deformation of cellular structures that is brought about by fluid flow, is by definition strain. In chapter 5, examination of the effect of fluid flow found that it was not sufficient to induce protein expression changes that were observed after the application of tensile strain. Because the membranes were deformed minimally at low frequency (Brown et al. 1998; Simmons et al. 2003), it was therefore assumed that the primary stimulus involved in all cell fate responses was tensile matrix deformation, transduced to the cell by surface receptors including VACCs and SACCs, rather than the coincident fluid shear stimulus.

It is acknowledged that the magnitude of strain experienced by cells is diminished from that which is applied to the substrate (Charras and Horton 2002; Plotkin et al. 2005). Recently, Wall et al. (2007) reported that the strain experienced by tenocytes during uniaxial strain was 63% of the magnitude of substrate strain applied, however within different areas of individual cells, strains exceeded the membrane strain applied. It can therefore be extrapolated that overall, the strain experienced by cells is less than that applied to the substrate, however within cells, a range of strains exist that may follow a Gaussian distribution centring on or below the magnitude of substrate strain applied.

The pattern of delivery of mechanical stimulation by cyclic tensile strain can be defined by a number of parameters: continuous or intermittent, magnitude, frequency and duration. For this research, a continuous tensile mechanical stimulus was chosen. The strain was applied at a frequency of 0.17 Hz, and at magnitudes between 2.5% and 10% for periods of 1 – 14 days. The application of a continuous strain has been cited as necessary to maintain cell phenotype (Williams 1998) and another paper discusses that multiple cycles of change in force magnitude are significant in that bone and cartilage cells respond more readily to rapid oscillation in force magnitude than to a constant force (Mao and Nah 2004). So while the force applied was continuous, it was not constant as it followed a sinusoidal pattern of stress and relaxation. However, in most studies, strain is applied with rest periods (Juncosa-Melvin et al. 2007; Wiesmann et al. 2006), and it has also been discussed that as the duration loading increases without interruption, the osteogenic response tends to saturate, (Turner 1998), making cells less responsive to routine loading signals, particularly if the loading stimulus is of low magnitude (Gross et al. 2004; Srinivasan et al. 2002). In the

study on strain mediated differentiation, the cessation of collagen type I up-regulation could have been a saturation response. For consistency, and within time constraints, intermittent loading was not applied; however in consideration of these responses it forms an interesting future study.

The magnitudes of strain applied in this study are approximately 10 – 30 fold higher than strain magnitudes measured by strain gauges on the bone surface of mammals during a variety of activities (ε = 0.3%; (Rubin and Lanyon 1984). However, the MSCs reside in the bone marrow, and are not embedded on the surface of bones, and while they migrate within the cortical bone network, due to the complex microarchitecture of bone matrix, the exact strain magnitude to which cells are exposed *in vivo* is unknown (Charras et al. 2004). In addition, the bone marrow stroma is significantly more compliant and therefore is capable of non-destructively transmitting higher forces than actual bone structures. Charras and Horton (2002) identified that 2.5% strain causes an increase in cytosolic calcium 50% in osteoblast cells and it has been reported that strains on the order of 1%–3% elongation are needed to obtain a cellular response *in vitro* (Burger and Klein-Nulend 1999). Therefore, while the strains used in this study in appear high compared to those measured *in vivo* we do not have enough information to state if they are physiological or not; since they are within a range observed to obtain cellular responses, they are considered appropriate for the examination of mechanotransduction on MSCs. The frequency of a typical stride is 1Hz or higher therefore this study examines responses at a frequency which is an order of magnitude lower (0.17Hz) than that which is physiological. However, since the stimulus was continuous, higher frequencies were not appropriate but could form part of the future study that was to examine the effects of the intermittent application of strain.

The variation in response of mechanically sensitive cells to the type of mechanical load that is applied such as fluid shear, tension or compression is significant, since opposite effects can by induced by 2 different types of stimuli (Mullender et al. 2004). This study used tensile mechanical stimulation, to most closely mach *in vivo* bone loading. For bone tissue engineering, mechanical forces like linear stretching or pressure correlate most closely with the physiological conditions and, therefore, are most widely used in connection with osteogenic tissue engineering. In their discussion on mechanical stimulation, Mao and Nah (2004) define the deformation of cellular structures following fluid flow as strain, and

continue to state that all mechanical forces are thus commonly experienced as strain (Mao 2002) and furthermore that all exogenous forces are transmitted in biological tissues as strain, and following mechanotransduction activity, become cellular and gentetic responses (Mao and Nah 2004). Additionally, while compressive forces act on the majority of skeletal tissues during loading, a range of stimuli is experienced by the cells since they are 3D entities and are not necessarily aligned to experience compressive stimulus upon loading. Thus to examine the effects of tension is a suitably physiological approach to use in the investigation of mechanoregulation in MSCs. Brown et al. compared different cell strain devices with regard to the occurrence of fluid shear stresses and found that the distribution of reactive normal stresses was not homogenous (Brown et al. 1998). In this study, efforts to mitigate these effects included geometric regularity of the substrate, careful grip-to-grip alignment, and the use of an elastic silicone substrate. However, stress concentrations in the substrate immediately next to the grips was an inherent local strain (Brown 2000) and the strip was always cut a few millimetres inside the clamped area to eliminate cells that may be affected by this.

To examine the involvement of ion channels and signalling pathways, this study used established pharmacological blockers and inhibitors for each pathway examined. While these were carefully selected to be as specific as possible, concentrations were selected as appropriate from literature and appropriate control groups were included to monitor the effects of each drug alone, it is known that it is not always possible to achieve a 100% specific target, and it is possible the drugs added have other effects on cellular metabolic processes. For example, gadolinium (Gd^{3+}) is a blocker of SACCs and was used to examine their role in strain-induced differentiation and apoptosis since is the most potent SACC blocker available. However, it is known that Gd^{3+} is also nonselective, and its toxicity limits have been reported (Franz 1996). While Gd^{3+} can block L-type Ca^{2+} currents (Franz 1996; Lacampagne et al. 1994), it has a higher affinity for SACCs than for other cation channels (Sadoshima et al. 1992) Since 10 μM concentration of Gd^{3+} has demonstrated effective channel inhibition without toxicity (Franz 1996; McBride et al. 2000), this concentration was used. As an alternative to examine specific pathways a siRNA approach may be useful for future studies. The introduction of a siRNA specific for the target signalling molecule ensures a continuous and efficient protein knockout phenotype in the infected cell, thereby providing a model where the effects of its absence can be monitored.

7.1.4 The development of mechanoregulation

This research has addressed many of the fundamental questions surrounding the mechanoreglation of cell fate however it has also raised several questions. What is the mechanism at the proliferation/differentiation switch? Could it be connected to Cbfα1 expression? Does another pattern of strain stimulus induce another lineage commitment? Was there up-regulation of any other lineage markers? How is phenotypic commitment maintained? Can it become reversed? To address these would allow further exploitation of the MSC-mechanical strain relationship to facilitate the therapeutic use of stem cells. In particular, a pressing issue would be to define a particular stage of commitment where a sufficiently large majority of the population were terminally committed so as the risk of *in vivo* teratoma formation from transplanted MSCs is eliminated. It is thought that this will only occur if the implanted cells contain no proliferating cells, only lineage committed cells.

While it is clear that the outcomes of mechanical loading of MSCs in this study cannot have a direct effect on tissue engineering since it was carried out in a 2D environment and on a silicone membrane, the principles of mechanotransduction that have been deciphered form insights into cell signalling and the safety parameters defined for the maintenance of cell viability in bioreactor culture have far reaching consequences. This system is a model from which the biology of mechanoregulation can be defined. It is true that the cells may react differently on a 3D environment, but these cell fates are such fundamental cellular events that their regulation as defined from this research can is likely to be applicable in all environments and across species. One possibility for the application of these mechanically induced osteogenic cells is to use the mechanical stimulation as a pre-conditioning tool; the 2D environment could be used as an inductive tool for cells that were to be subsequently seeded onto a scaffold for a tissue engineering application. The mechanical stimulation may sufficiently prime and direct scaffold development without further stimulation, and the construct could be implanted immediately.

7.1.5 Conclusion

Successful tissue engineering depends on a thorough knowledge and understanding of the biological aspects involved. Perhaps the most significant and pressing aspect in tissue engineering with mesenchymal stem cells today is the translation of research from the laboratory to the clinic (Hunziker et al. 2006). This research directly addresses the sixth principle of the functional tissue engineering paradigm outlined by members of the U.S. National Committee on Biomechanics that states '.....establishing how physical factors influence cell activity in bioreactors.....' (Butler et al. 2000) which addresses the mechanical aspects of tissue engineering design. Thus, this research has major implications for the creation of successful tissue engineering constructs for load bearing tissue applications. When set back into its multidisciplinary context and used in combination with the 'state-of-the-art' in scaffold and bioreactor design (Freed et al. 2006), the results of this can positively impact on the progression of functional tissue engineering and bring the introduction of tissue engineered based regenerative medicine one step closer to a clinical reality.

This research has demonstrated a suitable system upon which the mechanobiology of cells can be investigated, has clearly defined mechanical strain as an epigenetic factor in the osteogenic differentiation of MSCs as well as delineating some of the intricacies of how mechanical forces induce cell fates. Through gaining an insight to the understanding of how intrinsic mesenchymal stem cell behaviour is regulated by environmental cues also contributes to the development of scaffolds to promote constructive remodelling (Badylak 2007).

Tissue engineering specifically in the control of MSC differentiation stands to gain most from minimally invasive interventions on a cellular level, in terms of the accurate description of the cell as an entity for regulatory approval. This is where mechanical regulation can stand out amongst alternative inductive signalling such as genetic manipulation or biochemical treatments. Therefore, through the development of a minimally invasive system for the control of cell fate and the identification of the mechanisms through which these occur, this research positively contributes to progress on the fundamental biological pool of knowledge that can provide support for the translational advancements of MSCs as therapies in the future.

The findings herein also have consequences for the field of theoretical mechanobiology as it provides an experimental validation of a current theoretical

model of mechanobiology (Prendergast et al. 1997), and can contribute to their development by adding another layer of complexity but accuracy through the introduction an autocrine growth factor induced mechanism which further controls the development of the phenotype. Furthermore, this research provides useful numerical boundary values for strain magnitude parameters in the control of apoptosis to integrate in the development of computational models in bioengineering research.

These novel results demonstrate the potent effect of mechanical signals on MSC biological response and suggest mechanisms by which mechanical forces might regulate developmental, regenerative, and apoptotic processes in tissue homeostasis. Knowledge of mechano-responsive behaviours could lead to bridging the gap between laboratory research and the clinic and expand various orthopaedic therapeutic possibilities. Further investigations of these signalling pathways may aid in the identification of novel therapeutic targets for the control of MSC differentiation and apoptosis.

7.2 Future experiments

This study has provided significant insights to the mechanoregulation of mesenchymal stem cells, and also provides a platform for addressing several important questions that have risen during the course of the study, and as a consequence of the knowledge that has been gained from the results.

The regulation of stem cell renewal by mechanical strain is a particularly interesting topic in tissue engineering, since opposing effects have been reported. There is importance in tissue engineering for having control over this mechanism as cell proliferation is both desirable to populate tissue engineering scaffolds, and undesirable in the context of tumour formation following implantation. The decrease in the proliferation response following continuous strain stimulation identified in this study warrants further investigation to uncover the mechanisms of this, so that they can be exploited in directing the desirable proliferation response for specific stages of the tissue engineering process. Nucleostemin has recently been identified as a nuclear protein involved in MSC proliferation that becomes down-regulation upon induction of differentiation (Kafienah et al. 2006). It is proposed that the combination of mechanical strain with monitoring the

expression of this protein provides an interesting approach towards deciphering the molecular control of MSC proliferation in response to biophysical conditions.

In this study, cells were continuously stimulated with a strain of constant frequency and magnitude. In explaining some of the responses to strain, in particular long term strain, the existence of response saturation (Turner 1998) is a possibility for a diminished cellular response under an unchanged stimulation. Thus, for long term stimulations in particular, and to promote matrix maturation, and intermittent stimuli of varying magnitude/frequency is appropriate for a future investigation.

The effects of fluid flow on the MSC response has have been examined in this study; however, for some responses it cannot be discounted. Therefore, as a future study, the computational examination of the magnitude of the shear forces that exist on the cells in this model, would contribute greatly to the determination of their propensity to inadvertently affect the strain responses.

In the examination of the molecular mechanisms of differentiation, activity of the signalling molecules p38 and pI3-kinase was not detected at the 14 day time points. Additionally, the activation of ERK in response to strain was not detected in this study however it has been reported in other studies to be activated during strain and in osteogenesis (Rhee et al. 2006; Simmons et al. 2003). Insights to a more specific temporal pattern of signalling will benefit from more frequent investigations and at earlier time points.

Another study in the interests of translating this 2D model to a more clinically relevant tissue engineering application would be to examine similarities/differences in responses in a 3D environment. Additional technical investigations include, for the substrate properties, to prepare the collagen using a technique whereby the helical structure is retained, to examine mRNA expression following strain using RT-PCR, and to include immunocapture-based biochemical assays for improved detection of enzymatic activity.

Through building upon the novel cell biology uncovered in this research, the intricacies of mesenchymal stem cell dynamics will be of an appropriate standard to consider their clinical use.

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IX APPENDIX I – FACS RAW DATA

SAMPLE	CD90+	CD45+
1	96.89	6.34
2	96.2	4.68
3	96.54	1.86
4	95.03	0.05
5	97.89	1.28
6	97.32	2.16
7	98.3	2.17
Mean	96.88143	2.648571
Stdev	1.097777	2.139645
SEM	0.414921	0.80871

X APPENDIX II – SOLUTIONS

Cell Culture Solutions

70% EtOH (100ml)
70ml EtOH
30ml H_2O

PBS
Na_2HPO_4 (80mM)
NaH_2PO_4 (20mM)
NaCl (100mM)

Tris-buffered saline (TBS; pH 7.4)
TrisHCl (20mM)
NaCl (150mM)

Trypsin Solution
0.3mg trypsin/ml PBS

Supplemented Dulbeccos Modified Eagle Medium
Fetal Calf Serum 10%
Penicillin/Streptomycin 2%
Glutamine 0.5%
Glutamax 0.5%
Non-essential amino acids 1%

Osteogenic DMEM
Dexamethasone (68nM)
β-glycerophosphate (10mM)
Ascorbic acid-2-phosphate (0.05mM)

Cell Harvesting Solutions

Lysis Buffer, pH .4 (Harvesting of total culture protein)
HEPES (25mM)
MgCl2 (5mM)
EDTA (5mM)
DTT (5mM)
PMSF (2mM)
Leupeptin (10μg/ml)
Apoprotein (10μg/ml)
Pepstatin (10μg/ml)

Hoechst Buffer, pH 7.4
Tris base (10 x 10mM)
Na_2EDTA (1mM)
NaCl (1M)

SDS-PAGE solutions

Phosphate buffered saline-Tween 20(PBS-Tween), pH 7.4
Na2HPO4 (80mM)
NaH2PO4 (20mM)
NaCl (100mM)
Tween 20 (0.1%)

Tris buffered saline-Tween 20(TBS-Tween), pH 7.4
Tris-HCl (20mM)
NaCl (150mM)
Tween 20 (0.1%)

Sample buffer, pH 7.4
Tris-HCl (20mM)
Glycerol (10%)
SDS (10%)
B-Mercaptoethanol (5%)
Bromophenol Blue (0.05%)

Stacking gel (4% pH6.8)
Acrylamide/bis-acrylamide (30% stock, 13% (v/v), dH2O 60% (v/v), Tris-HCl (0.05M, 25% (v/v))
SDS (10%w/v stock, 1% (v/v))
APS (10%w/v stock, 0.5% (v/v))
TEMED 0.5% (v/v)

Separating Gel (10%, pH 8.8)
Acrylamide/bis-acrylamide (30% stock, 33% (v/v), dH2O 40% (v/v), Tris-HCl (0.05M, 25% (v/v))
SDS (10%w/v stock, 1% (v/v))
APS (10%w/v stock, 0.5% (v/v))
TEMED 0.05% (v/v)

Separating Gel (12%, pH 8.8)
Acrylamide/bis-acrylamide (30% stock, 40% (v/v), dH2O 33% (v/v), Tris-HCl (0.05M, 25% (v/v))
SDS (10%w/v stock, 1% (v/v))
APS (10%w/v stock, 0.5% (v/v))
TEMED 0.05% (v/v)

Electrode Running Buffer
Tris Base (25mM)
Glycine (200mM)
SDS (0.1%)

Transfer Buffer pH 8.3
Tris base (25mM)

Glycine (192mM)
Methanol (20%)
SDS (0.05%)

Fluorogenic Assay Solutions

Lysis Buffer, pH 7.4
HEPES (25mM)
MgCl2 (5mM)
EDTA (5mM)
DTT (5mM)
PMSF (2mM)
Leupeptin (10µg/ml)
Apoprotein (10µg/ml)
Pepstatin (10µg/ml)

Incubation Buffer
HEPES (50mM)
Dithiothreitol (10mM)
Glycerol (20%)

Experimental Solutions

Collagen solution
Rat tail collagen type I (1%)

Papain solution
Papain in papain buffer (0.1%)

FACS Buffer pH 7.4
PBS
Heat inactivated fetal calf serum (2%) (v/v)
Sodium azide (0.09%)(w/v)

Blocking Buffer
TBS
BSA (2%)
Triton-X100 (0.1%)
Heat-inactivated horse serum (20%)

Paraformaldehyde pH 7.4
dH_20
paraformaldehyde (4%)

XI APPENDIX III– PUBLICATIONS

- Kearney, E. M, Prendergast, P. J., Campbell, V.A. *'Mechanisms of strain-mediated mesenchymal stem cell apoptosis.'* **Journal of Biomechanical Engineering** 2008 Accepted.
- Kearney, E. M, Farrell, E., Prendergast, P. J., Campbell, V.A. *'Tensile strain as a regulator of mesenchymal stem cell osteogenesis.'* Submitted to **Annals of Biomedical Engineering** 2008.
- Kearney, E. M, Farrell, E., Prendergast, P. J., Campbell, V.A. *'The influence of mechanical strain on adult rat mesenchymal stem cells.'* **European Society of Biomechanics thematic workshop in Mechanobiology of Cells and Tissue Regeneration**, Leuven Aug 2005
- E.M. Kearney, E. Farrell, P. J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat mesenchymal stem cells.'* Presented at the **European Tissue Engineering Society**, Munich, Aug 2005
- Kearney, E M; Farrell, E; Prendergast, P J; Campbell, V A *'The influence of mechanical strain on adult rat mesenchymal stem cells.'* Presented at the 52nd **Orthopaedic Research Society** Chicago, Feb, 2006
- E.M. Kearney, P.J. Prendergast, V.A. Campbell. *'Mechanical strain as a regulator of adult rat marrow stromal cell dynamics.'* Presented at the 1st **UK Mesenchymal Stem Cell** York, April 2006
- E.M. Kearney, E. Farrell, P.J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat marrow stromal cell dynamics.'* Presented at the 5th **World Congress in Biomechanics**; Munich, Jul 2006
- Kearney, E M; Prendergast, P J; Campbell, V A *'Differentiation of mesenchymal stem cells in response to strain: a possible role for stretch activated cation channels.'* Presented at the 53rd **Orthopaedic Research Society** meeting, San Diego, Feb, 2007
- E. M. Kearney, E. Farrell, P. J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat mesenchymal stem cell viability and osteogenic potential.'* Presented at the Biomedical section of the **Royal Academy of Medicine** Meeting Jan 05.
- E. M. Kearney, E. Farrell, P. J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat mesenchymal stem cell viability and osteogenic potential.'* Presented at the 11th **Bioengineering in Ireland** (11), Dublin, Jan 2007
- E. M. Kearney, E. Farrell, P. J. Prendergast, V.A. Campbell. *'Osteogenic differentiation of mesenchymal stem cells seeded on a silicone membrane.'* Presented at the Biomedical section of the **Royal Academy of Medicine** Meeting Jun 05.

- E. M. Kearney, P. J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat mesenchymal stem cell viability and osteogenic potential.'* Presented at the Biomedical section of the **Royal Academy of Medicine** Meeting Jan 06.
- E. M. Kearney, P. J. Prendergast, V.A. Campbell. *'The influence of mechanical strain on adult rat mesenchymal stem cells.'* Presented at **Bioengineering in Ireland** (12), Galway, Jan 2006