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The Influence of Age on Cyclic Tensile Strain-Induced Differentiation and Apoptosis in Mesenchymal Stem Cells

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Thesis submitted to the University of Dublin, Trinity College in partial fulfilment of the requirements for the degree of

Doctor in Philosophy

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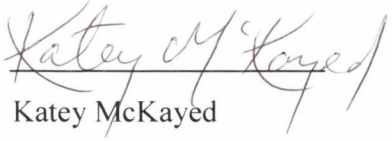

Katey McKayed

I Abstract

Mesenchymal stem cells, or MSCs, are adult progenitor cells capable of differentiating into specialised skeletal cells such as chondrocytes, osteocytes and adipocytes. Under the appropriate conditions, these cells can be manipulated *in vitro* for the development of new skeletal tissue such as bone or cartilage. Thus, interest has been focused on MSCs as a promising cell source for use in tissue engineering, which holds considerable potential in the field of regenerative medicine for developing therapies to treat osteochondral disease. When isolated from a donor source, the cells retain their multipotent capacity *in vitro* and can be induced along a specific lineage depending on the environment in which they reside and the conditions to which they are exposed. However, there is a reported reduction in the differentiation potential of these cells with increasing age. This thesis focused on elucidating a mechanical stimulus that enhances mesenchymal stem cell differentiation and deducing the means by which the stimulus is transduced within the cell, termed mechanotransduction. The response of MSCs, isolated from young and aged rats, to cyclic tensile strain and the possible correlation between changes in mechanosensitivity and age-related changes in the cell were investigated.

MSCs were isolated from young and aged rats and culture expanded for 3 weeks followed by assessment of several cellular characteristics. There was an age-related reduction in the rate of proliferation and the expression of mechanosensitive proteins, integrin receptor subunit α2 and cytoskeletal actin in these stem cells. There was also an increase in lipid peroxidation with age, which is associated with increased membrane rigidity. Following exposure to 10% cyclic tensile strain, apoptosis was induced, and this was significantly greater in cells isolated from aged rats compared to young. Application of 2.5% cyclic tensile strain for a period of 7 days induced both an osteogenic and chondrogenic response in young and aged MSCs. The osteogenic response to strain was weaker in aged MSCs compared to young cells and this was accompanied by a similar decrease in pERK expression in aged MSCs, whilst all other mechanosensors increased in response to strain, with no effect of age. This could indicate mechanotransduction during ostegenesis occurs via the ERK pathway, which is

affected by increasing age. In this study, mechanotransduction occurs via an RGD-independent mechanism since blockade of integrin activity using an RGDS peptide did not affect the strain-induced differentiation potential.

This thesis demonstrates that alterations occurring in the mesenchymal stem cell membrane with increasing age may be contributing to an altered mechanoresponse to tensile strain. A high rate of strain (10%) has been shown to elicit an apoptotic response in young and aged stem cells. However, aged cells were more vulnerable to strain-induced apoptosis, which could be attributed to a reduced expression of mechanosensors in the cell membrane and their propensity to upregulate signalling pathways associated with cell survival. Additionally, increased membrane stiffness associated with greater lipid peroxidation of the cell membrane may also contribute towards an altered response in aged cells. The differentiation potential of both young and aged MSCs has also been highlighted in this thesis. In response to 2.5% strain, MSCs were capable of differentiating along both osteogenic and chondrogenic lineages. This has serious implications for the field of tissue engineering and further investigation is necessary to optimise directed differentiation of these cells. However, these findings suggest that aged MSCs are capable of being mechanically primed for potential use in tissue engineering applications which would greatly benefit the treatment of osteochondral disease amongst the aged population.

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"The noblest pleasure is the joy of understanding."

- Leonardo da Vinci (1452 – 1519)

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2 Materials and Methods

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V Abbreviations

^{o}C	Degrees celcius
1^{o}	Primary
2^{o}	Secondary
2-D	Two dimensional
3-D	Three dimensional
α	Alpha
Ab	Antibody
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATP	Adenosine Triphosphate
β	Beta
BMP	Bone morphogenetic protein
BrdU	5-Bromo-2'-deoxy-uridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Cbfα-1	Core binding factor alpha-1
cDNA	Complimentary deoxyribonucleic acid
CFU	Colony forming unit
CO_2	Carbon dioxide
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylinodole
ddH_2O	Double de-ionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotidetriphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetra-acetic acid
ERK	Extracellular signal-related kinase

EtOH	Ethanol
FBS	Foetal bovine serum
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
$GdCl_3$	Gadolinium chloride
GTPases	Guanosine triphosphates
H_2O	Water
H_2O_2	Hydrogen peroxide
hr	Hour(s)
HEPA	High efficiency particle air
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
Hz	Hertz
IgG	Immunoglobulin G
ILK	Integrin-linked kinase
JNK	c-jun N-terminal kinase
kDa	Kilo Dalton
mA	Milliamp
MAPK	Mitogen activated protein kinase
M	Molar
mg	Milligram
MHC II	Major histocompatibility complex II
min	Minute
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
nm	Nanometre
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Phosphatidylinositol
PMSF	Phenylmethylsulphonyl fluoride
RGDS	Arginine-Glycine-Aspartic Acid-Serine
ROCK	Rho-associated kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SACC	Stretch-activated cation channel
s-DMEM	Supplemented DMEM
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
SMC	Smooth muscle cell
TBS	Tris-buffered saline
TBS-T	TBS-Tween
TdT	Terminal deoxynucleotidyl transferase
TEMED	N, N, N-N-tetramethylenediamine
TGF-β	Transforming growth factor-β
Tris-HCl	Trizma-hydrochloride
TUNEL	TdT-mediated-UTP-nick end labelling
UV	Ultraviolet light
VACC	Voltage-activated cation channel
µl	Microlitre
µm	Micrometre/Micron
µM	Micromolar

Chapter 1 Introduction

1.1 Introduction

Musculoskeletal health is a key determinant in the quality of life for the aging population. According to United Nations statistics, the percentage of people above the age of 60 has increased since the 1950's and continues to grow faster than any other age group due to a transition in fertility rates and mortality. As life expectancies for men and women increase, improved healthcare is essential to ensure successful aging and enhanced musculoskeletal health is pivotal for overcoming age-associated diseases such as osteoporosis and arthritis. There is a necessity for better therapies to treat such diseases as current therapies involve invasive and complex surgeries in older people which maintain to fail at an undesirable level.

Tissue engineering holds great potential in the field of regenerative medicine for treating osteochondral disease. Mesenchymal stem cells (MSCs) are undifferentiated progenitor cells that can differentiate into specialised skeletal cells such as chondrocytes, osteocytes, adipocytes and tendon cells (Pittenger *et al.* 1999). Under the appropriate conditions, these cells can be manipulated *in vitro* for the development of skeletal tissue. As such, interest has been focused on MSCs as a valuable cell source for use in the engineering of skeletal tissues such as bone and cartilage. When isolated from a donor source, the cells retain their multipotent potential *in vitro* and can be induced along specific lineages depending on the environment in which they reside and the conditions the cells are exposed to.

To date, the majority of successes in regenerative medical research have been restricted to young patients/donors, most commonly resulting from sporting injuries (Saris *et al.* 2009, Gudas *et al.* 2003). It is essential to extend such successes to include the aged population, with implications for overcoming osteochondral diseases which are prevalent amongst this age group. The current challenge lies in elucidating the optimum conditions required for the differentiation of MSCs along the osteogenic and chondrogenic routes and priming techniques to harness the full potential of these cells for use in tissue engineering applications. Research in this area is aimed at developing the

optimum *in vitro* environment for tissue engineering of bone and cartilage for use in regenerative medicine in treating osteochondral defects.

1.2 Mesenchymal Stem Cells

Many adult tissues contain populations of stem cells that have the capacity for renewal following trauma, disease or during aging. These cells can be found within a specific tissue itself or in other tissues that serve as stem cell reservoirs (Pittenger *et al.* 1999). MSCs are a multipotent progenitor cell, capable of differentiating into the mesenchymal tissues of the skeletal system. These tissues include bone, cartilage, muscle, tendon, ligament and adipose. MSCs can be used for the regeneration of these mesenchymal tissues through the principles and practices of tissue engineering (Caplan 2007, Chamberlain *et al.* 2007, Pittenger *et al.* 1999). These cells can be isolated from a variety of tissues, most commonly from the fat pad and bone marrow, which represent a small percentage of MSCs but they are easily expandable *in vitro* (Fox *et al.* 2007). MSCs can replicate and proliferate as undifferentiated cells while maintaining the potential to differentiate into lineages of mesenchymal tissues, via the mesengenic process (Figure 1.1). Their multipotency allows them to give rise to different cell and tissue types in response to various environmental factors or stimuli.

Mesenchymal stem cells were first described as bone-forming progenitors from the stromal fraction of rat bone marrow by Friedenstein *et al.* (1966). Whole bone marrow was placed in plastic culture dishes and it was reported that the adherent cells appeared to be heterogenous and could segregate into colonies. *In vivo* assays of these colonies demonstrated a high ability for self renewal and multipotentiality from a heterogeneous population of stem or progenitor cells and that their differentiation *in vitro* can be modified at the colony level (Owen & Friedenstein 1988). Further studies that built on Friedenstein's work established that these were multipotential cells capable of differentiating into osteoblasts and chondrocytes (Chamberlain *et al.* 2007).

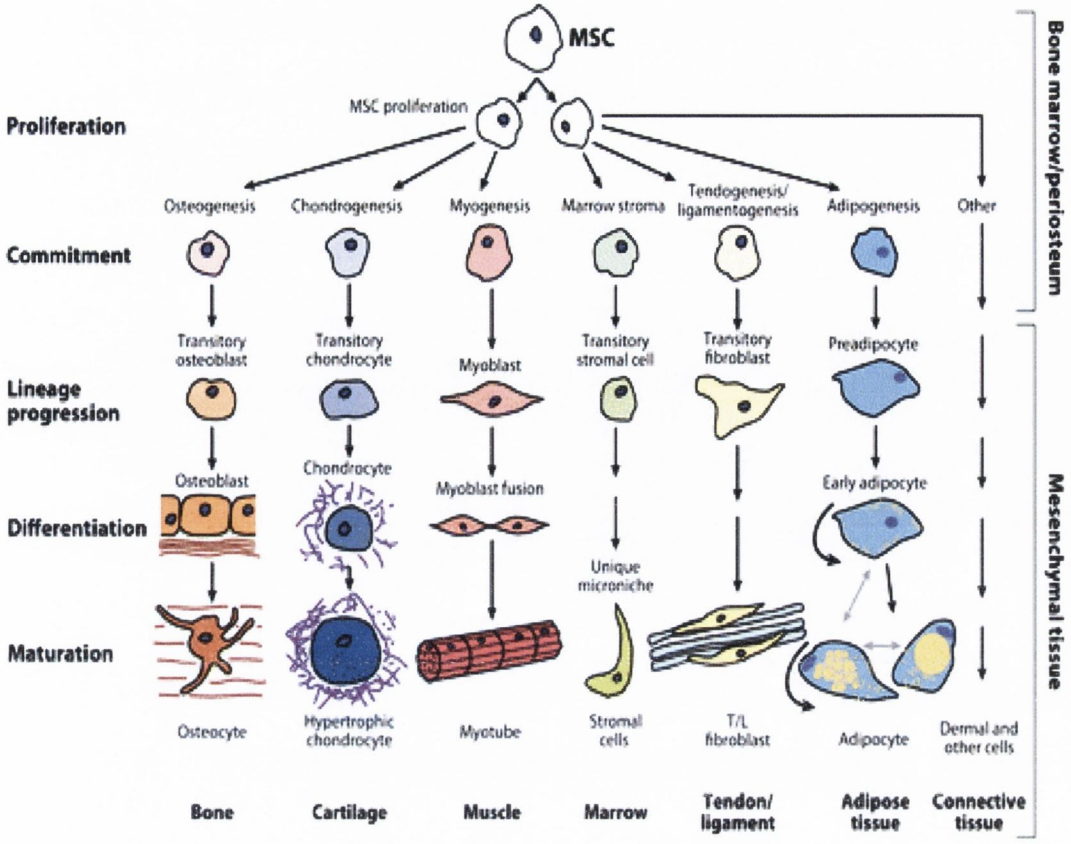


Figure 1.1: The mesengenic process - adult bone marrow multipotent progenitor cells are capable of proliferating and differentiating into distinctive end-stage cell types, such as those that fabricate specific mesenchymal and connective tissues (adapted from Singer & Caplan 2011).

In adults, skeletal tissues are constantly required to rejuvenate themselves, a process that involves the death of differentiated cells and their replacement with newly differentiated cells. The new cells arise in a multistep sequence or lineage that originates from MSCs located in the bone marrow (Caplan, 2005). Therefore, MSCs play an essential role in the maintenance and regeneration of adult skeletal tissues that are submitted to normal physiological turnover or following the occurrence of an injury. For example, damage to articular cartilage has serious consequences due to the lack of vasculature causing a poor intrinsic repair capacity so cartilage is therefore unable to self repair. All stem cells share a fundamental property whereby they have the ability to balance the cell-fate decision between self-renewal and differentiation (Djouad *et al.* 2007), which is

determined by the microenvironment in which they reside. Essentially, the function of adult MSCs *in vivo* is to supply replacement units for the differentiated cells that naturally expire or succumb to injury or degenerative disease such as osteoporosis or arthritis. However, the frequency of stem cell-generated replacement cells decreases with increasing age (Bernardo *et al.* 2009) and Caplan (2007) reports low titres of human MSCs in elderly individuals as assessed by colony forming units (CFUs). Thus with increased age there appears to be an increased susceptibility to irreparable damage to skeletal tissue as peak production of these replacement units decreases.

1.2.1 MSC Isolation and Characterisation

The purity, yield and differentiation characteristics are quite different for stem cell preparations isolated from different tissues (Caplan 2007). Human MSCs were first identified in postnatal bone marrow and later in a variety of other tissues, including muscle, connective tissue and adipose tissue. Amniotic fluid and placenta have been found to be rich sources of MSCs (In 't Anker *et al.* 2004) whereby both fetal and maternal MSCs can be isolated from human placenta (Bernardo *et al.* 2007). MSCs can also be isolated from a number of other sources including umbilical cord blood (Lee *et al.* 2004, Hou *et al.* 2003), synovial fluid (Jones et *al.* 2004), and the fat pad (De Ugarte et *al.* 2003), however they are most commonly extracted from the stromal fraction of the bone marrow. While MSCs represent a minor fraction of the total nucleated cell population in bone marrow, they can be seeded and enriched by standard cell culture techniques (Barry & Murphy 2004). Undifferentiated MSCs exhibit a fibroblast-like morphology and they express a characteristic pattern of cell-surface antigens (Stolzing & Scutt 2006, Deans & Mosely 2000). Although, unique identifying markers have not yet been recognised for MSCs, leading to many inconsistencies surrounding their identification. They are therefore often identified simply by assessing the proliferative capacity of a culture preparation into colony forming units, differentiation potential into multiple lineages of mesenchymal tissue (Sethe *et al.* 2006, Pittenger *et al.* 1999) and the ability to adhere to plastic in culture.

Traditional isolation methods rely on the fact that MSCs selectively adhere to plastic surfaces. Alternatively, the cells can undergo centrifugation over a Percoll gradient, separating cell populations based on their density (Dazzi *et al.* 2006). These methods are relatively non-specific and as such, the need to isolate and further analyse MSCs requires more recently developed strategies based on cell surface markers. An approach that is now increasingly being used relies on the sorting of bone marrow populations by flow cytometry, based on the reactivity of MSCs to certain antibodies (Jackson *et al.* 2007, Jones *et al.* 2006). Since no specific markers have been identified for MSCs, it is common practise to eliminate non-mesenchymal stem cells from cultures by testing against positive markers for haematopoietic stem cells (HSCs), such as CD34, CD45 and CD11b. Simultaneously, markers that are present on MSCs, including CD90, CD73 and endoglin CD105, can be tested to support the positive identification of these cells. More than 95% of an MSC population must present several of these positive markers whilst lacking expression of the HSC markers (Dominici *et al.* 2006). Valuable information can also be gained by analysing the expression of cell surface molecules on MSCs. They express a broad spectrum of cell adhesion molecules, important in cell binding and homing interactions, including integrins α1, α2, α3, β1, β2 and β4 (Barry & Murphy 2004, Majumdar *et al.* 2003). Although cell markers solely exclusive to MSCs have yet to be elucidated, identification and evaluation of markers and characteristics described above provide moderately conclusive evidence for the isolation of positive MSC populations in cell cultures.

1.3 MSC Proliferation

The development of multicellular organisms follows numerous predetermined molecular and cellular pathways culminating in the formation of an individual consisting of billions of cells, each with distinct responsibilities. Furthermore, the cells, tissues and organs must be maintained throughout life as well as requiring repair following trauma or disease. Cellular development is achieved through a series of pre-programmed events including cellular proliferation, lineage commitment, lineage progression, lineage expression, cellular inhibition, and

regulated apoptosis. Sequential development through these programmed events leads to the formation of differentiated cells, tissues and organs of an individual. Although the majority of cells progress through this developmental sequence to form an individual, a few cells depart this developmental field and become reserve precursor cells, such as MSCs. These cells are responsible for the continual maintenance and repair of tissues and organs throughout life (Young & Black 2004). The specific environmental cues necessary to initiate the proliferation of MSCs *in vivo* are not understood (Pittenger 1999) but several studies have described different stimuli that induce or increase the rate of proliferation *in vitro*. With the increasing number of clinical protocols using hMSCs there is a need for serum supplements other than foetal bovine serum (FBS). Serum-free media have indeed been investigated, but none have thus far been demonstrated to support the proliferation of hMSCs in the absence of growth factors (Shahdadfar *et al.* 2005). Low magnitude mechanical signals including tensile strain have been shown to increase MSC proliferation (Luu *et al.* 2009, Song *et al.* 2007) as well as hypoxic conditions, which induces an extended lag phase prior to increasing proliferation (Grayson *et al.* 2006). There is an age-associated bone loss due to the deterioration of the marrow-based stromal cell population which may hinder the ability of any intervention to harness the potential of stem cells to treat disease (Duque 2003). Aging animals exhibit a considerable reduction in stem cell population and regenerative capacity, while simultaneously showing a predisposition toward adipogenesis (Astudillo *et al.* 2007, Liu *et al.* 2007). Thus, age is a determinant of the stem cell population viability, and may function toward a reduced regenerative capacity (Luu *et al.* 2009).

1.4 MSC Differentiation

The present challenge in utilising MSCs in tissue engineering lies in designing a suitable microenvironment that provides instructive signals to induce cellular differentiation in order to create functionally organised skeletal tissues such as bone or cartilage. While MSCs are considered suitable candidates for cell-based tissue engineering applications due to their intrinsic capability to self-renew and

differentiate, there remains a lack of true understanding regarding the molecular mechanisms that govern their stem cell potential (Baksh *et al.* 2004). Research is currently centred on determining the conditions and treatments that will optimise and enhance differentiation of MSCs along either the osteogenic or chondrogenic routes. Under appropriate *in vitro* conditions MSCs can differentiate along either of these lineages, a process which has been described and characterised by multiple laboratories (Kearney *et al.* 2010, McMahon *et al.* 2008, Djouad *et al.* 2007, Sumanasinghe *et al.* 2006, Barry *et al.* 2001, Pittenger *et al.* 1999). Stimuli that have been shown to promote MSC differentiation *in vitro* include growth factors, hypoxia and application of mechanical force.

1.4.1 Osteogenic Differentiation

Osteogenic differentiation can be achieved by culturing cells in the presence of growth factors, including β-glycerol phosphate, dexamethasone and ascorbic acid. In response to these growth fsctors, MSCs form aggregates and express increased levels of alkaline phosphatases and calcium deposition (Chamberlain *et al.* 2007, Pittenger 1999, Jaiswal *et al.* 1997). Differentiation along the osteogenic route has been shown to be regulated by members of the mitogen-activated protein kinase (MAPK) family, including extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (Jaiswal *et al.* 2000). Another regulator of osteogenic differentiation is the application of mechanical force, including forces that mimic *in vivo* conditions as well as experimental forces. In these cases, MSCs are seeded onto or within appropriate substrates to which some form of mechanical force is applied. It has previously been suggested that differentiation is influenced by mechanical stimuli, since MSCs transplanted into cartilage defects in rabbit knee joints showed substantial differences in mechanical properties of reparative tissues caused by exposure to different local mechanical environments (Wakitani *et al.* 1994). Studies have since shown that application of cyclic tensile strain of different magnitudes can induce osteogenic differentiation in MSCs in the presence or absence of inductive growth factors (Kearney *et al.* 2010, Zhang *et al.* 2008, Ward *et al.* 2007, Jagodzinski *et al.* 2004). The application of uniaxial cyclic stretching at a magnitude of 10% has been shown to upregulate collagen I

and III expression while simultaneously decreasing F-actin expression in cells (Zhang *et al.* 2008). In a study by Ward *et al.* (2007), it was demonstrated that focal adhesion kinase (FAK) signalling controls strain-enhanced osteogenic differentiation via an ERK1/2 pathway, as measured by expression levels of osteogenic marker genes and subsequent matrix mineralisation. Cyclical stretching and dexamethasone have both highlighted an enhancement of the osteogenic commitment of MSCs but in the short term, cyclic stretching is a more potent differentiation stimulus compared to dexamethasone (Jagodzinski *et al.* 2004).

1.4.2 Chondrogenic Differentiation

To promote chondrogenic differentiation, isolated MSCs are often cultured in the presence of chondrogenic growth factors such as transforming growth factor-β (TGF-β), dexamethasone and ascorbic acid. The cells develop a multilayered, matrix-rich morphology and histological analysis highlights an upregulation of chondrogenic markers such as collagen II and proteoglycans (Chamberlain *et al.* 2007, Jackson *et al.* 2007, Pittenger *et al.* 1999). Culturing MSCs in a hypoxic or low oxygen environment has proven successful in enhancing their differentiation into chondrocytes. It is suggested that hypoxia promotes the chondrogenic differentiation of MSCs in part by activating transcription factor Sox-9 via a hypoxia-inducible factor (HIF)-1α-dependent mechanism. The hypoxic environment increases HIF-1α nuclear accumulation and its transactivation through Akt and p38 activity. Knockdown of HIF-1α prevents the hypoxia-induced increase in chondrogenesis providing evidence for a role of HIF-1α in the enhancement of cartilage formation within a low oxygen microenvironment (Kanichai *et al.* 2008, Robins *et al.* 2005). Mechanical force has also been shown to influence chondrogenic differentiation in MSCs in the presence or absence of inductive growth factors (Amos 2008, McMahon *et al.* 2008, Angele *et al.* 2004, Huang *et al.* 2004). However, almost all investigations into force and chondrogenesis of MSCs use compression as the investigative tool. There are extremely few observations of the effect of tensile strain on MSC chondrogenesis. McMahon *et al.* (2008) examined the application of 10% cyclic tensile loading for

7 days on adult MSCs seeded within a collagen I-glycosaminoglycan (GAG) scaffold and treated with TFG-β1. It was found that 10% strain significantly increases the rate of GAG synthesis, which is attenuated in the presence of a stretch-activated ion channel inhibitor demonstrating the involvement of these ion channels in the mechanotransduction pathway, coupling cyclic tensile loading to matrix synthesis. Furthermore, chick limb bud MSCs seeded within a tubular scaffold have been exposed to 5% circumferential tensile strain by inserting a balloon catheter into the lumen of the scaffold and inflating the tube. Results indicate that embryonic stage 22 MSCs follow established expression patterns of cartilage development whereas stage 25 cells produce a more fibrous tissue and exhibit signs of differentiation in response to tensile strain (Amos 2008). These studies indicate the influence and potential of mechanical forces on MSC differentiation. However, a clear mechanobiological explanation for osteochondral repair has yet to be elucidated (Kelly & Prendergast 2004), highlighting the necessity to further investigate the cause, effect and activation of cell signalling pathways involved in mechanotransduction.

1.5 Tissue Engineering

Tissue engineering is a multidisciplinary field of regenerative medicine that encompasses aspects of cell biology and engineered constructs for the development of novel therapeutics to treat skeletal injuries or defects. It is the process of creating functional three-dimensional tissues using cells combined with scaffolds or devices that facilitate cell growth, organisation and differentiation in response to the appropriate signals (Figure 1.2). The development of models based on the integration between cells and biomaterials could lead to a better understanding of the basic mechanisms of bone and cartilage physiology. The models should faithfully mimic the desired tissues; resemble their organisation, their mechanical properties and their physiological response to different stimuli (Tortelli & Cancedda 2009). Tissue engineering arises from the problem that cell-based therapies rely on an intact scaffold (connective tissue) of the diseased or injured tissue. In cases where there is widespread fibrosis or scar tissue, cell-based therapies can fail due to the lack of a blood supply and/or the lack of a

microenvironment including receptors and biological mediators to support cell differentiation, proliferation and function (Koch *et al.* 2009).

In general, tissue engineering involves the *in vitro* seeding and attachment of cells within a scaffold. These cells then ought to proliferate, migrate and differentiate into the specific desired tissue type while secreting extracellular matrix components required to form the tissue. The type of scaffold used is crucial to enable the cells to behave in the appropriate manner to produce tissues and organs of the desired shape and size (Sachlos & Czernuszka 2003).

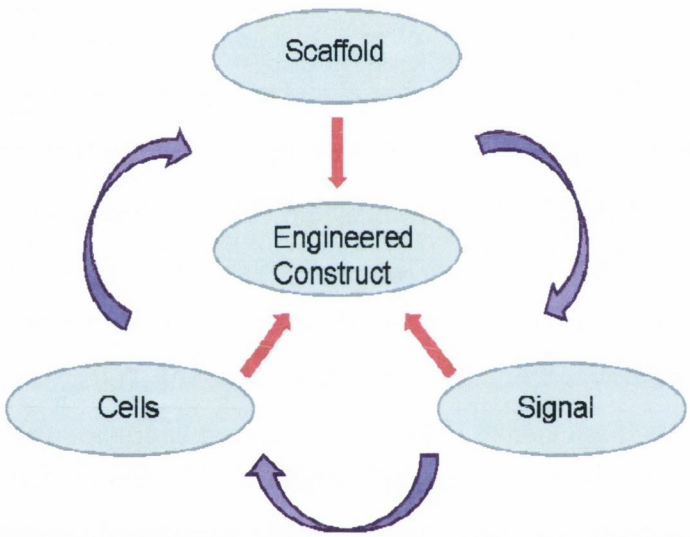


Figure 1.2: Tissue engineering triad - A successfully engineered construct encompasses cells capable of growing within a scaffold and their response to both substrate and extracellular signals.

In recent years, MSCs have generated a significant amount of interest in this area of regenerative medicine due to their ability to expand many-fold in culture while retaining their growth and multipotency. Classical stem cell therapy involves the transplantation of autologus or allogeneic stem cells into patients either through a local delivery or by systemic infusion (Barry & Murphy 2004). There is evidence

to suggest that MSCs are immune privileged, making them an advantageous cell type for allogenic transplantation. They express low levels of major histocompatibility complex (MHC) I and no MHC II or human leucocyte antigen (HLA) II (Chamberlain *et al.* 2007), therefore reducing the risk of complication and rejection in terms of transplantation (Jackson *et al.* 2007). The major histocompatibility complex is a genomic region that functions in both the immune system and in autoimmunity. It has also been reported that MSCs exhibit immunosuppressive properties including a modulatory effect on T cell functions (Bartholomew *et al.* 2002). With their relative ease of expansion, immunologic characteristics and ability to differentiate into various cell types *in vitro*, MSCs are regarded as a promising source of cells for use in osteochondral tissue engineering applications (Chamberlain *et al.* 2007, Tuan 2006).

1.5.1 Substrate Design

The main objective of tissue engineering is to create therapeutic constructs *in vitro*, which are capable of replacing or repairing the functions of a tissue damaged by disease or injury (Robu *et al.* 2011). In order to achieve this, cells are cultured on a suitable biomaterial either for priming purposes or for actual implantation. The first stage of bone or cartilage tissue engineering begins with the design and fabrication of the appropriate substrate such as a porous three-dimensional (3-D) scaffold, which should be fabricated from a highly biocompatible material that does not elicit an immunological response (Hutmacher 2000). Several pre-requisites have been identified as crucial for the production of a tissue engineering scaffold: interconnecting pores of appropriate size to favour tissue integration and vascularisation, composition by a material with controlled biodegradability or bioresorbability so that new tissue will eventually replace the scaffold, appropriate surface chemistry to allow for cellular attachment, differentiation and proliferation, mechanical properties should match the intended site of implantation and the scaffold should not induce any adverse response (Sachlos & Czernuszka 2003, Hutmacher 2001). Cell-based clinical therapies using MSCs adopt the following approaches; tissue engineering strategies that incorporate MSCs into 3-D scaffolds to replace *in vivo* tissue as

described above and cell replacement therapy whereby defects can be healed by replacing host cells with allogeneic donor cells (Caplan 2005). The use of 3-D scaffolds is critical to tissue engineering applications as they allow the *in vitro* development of living tissue constructs that can be implanted at specific defective sites for clinical repair (Grayson *et al.* 2006). Two-dimensional (2-D) substrates are also employed in tissue engineering applications for experimental testing and cell priming prior to implantation strategies. In this case, the substrate ought to be biocompatible to allow for cell attachment, differentiation and proliferation or have a coating mimicking the extracellular matrix environment to promote these cellular functions.

1.5.2 Mesenchymal Stem Cells in Tissue Engineering

Bone marrow derived MSCs comprise just one of numerous stem cell types that are studied in research and clinical trials but further exploration is required to elucidate optimum treatments and procedures. MSCs have been investigated with great interest and persist in their employment in developing novel therapies to treat skeletal defects caused by injury or disease. Their abundant successes to date merit the value of MSCs and the requirement for ongoing research into the use of these cells in tissue engineering applications. For example, MSCs have successfully been used to treat defective healing at fracture sites, both alone and in combination with scaffolds, leading to the repair of large bone defects (Quarto *et al.* 2001). Cultured MSCs have also been administered to humans, via bone marrow transplant, to treat a condition called osteogenesis imperfecta which causes osteoblasts to produce defective collagen I, leading to osteopenia, bone fracture and bone deformities. Results show new dense bone formation, increased bone mineral content and reduced frequency of bone fracture in all patients, without provoking an immune response (Horwitz *et al.* 1999). In 2002, Wakitani *et al.* expanded autologous MSCs *in vitro*, by embedding them in collagen gels that were re-implanted into areas of articular cartilage defect in patients suffering from osteoarthritis, leading to formation of hyaline cartilage tissue formation. In more recent times, clinical use of culture-expanded MSCs with porous hydroxyapatite scaffolds has been reported in the treatment diaphyseal segmental

defects in the tibia, humerus and in 2 separate ulnar fractures. Autologous bone marrow-derived MSCs were expanded *in vitro* and loaded in hydroxyapatite macroporous ceramic scaffolds. An external fixator was employed to maintain stability of the seeded grafts and the fracture defects. Progressive integration of the implants with the surrounding bone was observed as well as progressive new bone formation inside the bioceramic pores and vascular ingrowth. All patients experienced recovery of limb function as bone formation progressed and the implants were eventually completely integrated into the existing bone (Maracci *et al.* 2007). Bone marrow aspirates, isolated from the iliac crest, contain progenitor cells that can be used to heal nonunions. A nonunion occurs when a broken bone is unable to heal fully. Marrow was aspirated from both anterior iliac crests and then injected into 60 non-infected atrophic nonunions of the tibia. The volume of mineralized bone formation was determined by comparing pre-operative computerised tomography (CT) scans with scans performed 4 months following the injection. Bone union was obtained in 53 patients and the number of progenitors injected into the nonunion sites of the 7 patients where bone union was not obtained were significantly lower those in the patients who obtained bone union. The volume of the mineralized callus measured at 4 months on the CT scans of the patients who had union ranged from 0.8 to 5.3cm^3. Although percutaneous autologous bone-marrow grafting is now established as an effective and safe method for the treatment of an atrophic tibial diaphyseal nonunion, its efficacy may be related to the number of progenitors in the graft (Hernigou *et al.* 2006).

Highlighting the importance of mechanical loading in stem cell-based therapies for cartilage repair in strengthening neocartilage properties and maintaining the cartilage phenotype, the effect of dynamic compressive loading on chondrogenesis was studied, in addition to the production and distribution of cartilage specific matrix and the hypertrophic differentiation of human MSCs encapsulated in hyaluronic acid (HA) hydrogels. Following 70 days in culture, dynamic compressive loading increased the mechanical properties, glycosaminoglycan (GAG) and collagen contents of HA hydrogel constructs in a seeding density dependent manner. Furthermore, loading promoted a uniform spatial distribution of cartilage matrix in HA hydrogels leading to significantly

improved mechanical properties compared to non-compressed free swelling constructs (Bian *et al.* 2012). This study shows the potential in priming MSCs *in vitro* for use in treating cartilage defects. Tissue engineering based successes and the potential of MSC based therapies are not exclusive to treatment of musculoskeletal defects. Isolated murine MSCs have been injected into healthy adult myocardium, which undergo neo-angiogenesis close to the injection site within one week of transplantation. The transplanted cells differentiate into cardiomyocytes, endothelial cells and smooth muscle cells indicating that cultured MSCs can successfully engraft into healthy tissue and differentiate into numerous cell types in vivo (Gojo et al. 2003).With such promising potential, it is of critical importance to investigate and determine the best techniques for isolation, expansion, conditioning and delivery of MSCs for their employment in tissue engineering strategies.

However, there is currently a lack of consensus and cases of conflicting reports regarding the effect of donor age and cell processing on MSC function. A number of studies have previously shown no age-related differences in differentiation of MSCs; although, amongst these groups several have found changes in proliferation, attachment, senescence or self-renewal in mouse, rat, and human MSCs (Kretlow *et al.* 2008, Mareschi *et al.* 2006, Bellows *et al.* 2003). Scharstuhl *et al.* (2007) investigated whether the availability of bone marrow derived MSCs and their capacity to differentiate is age-related and whether the cells are affected by osteoarthritis etiology; including age-associated joint trauma history and joint dysplasia. It was found that sufficient MSCs can be isolated, expanded and differentiated along the chondrogenic lineage, regardless of age or the cause of osteoarthritis. Further investigation is required to improve our understanding on the effect of age on MSC isolation, characterisation and differentiation potential if cells from aged donors are to be successfully used in treating osteochondral defects via a tissue engineering approach.

1.6 Mechanotransduction

Physical forces, including; gravity, tension, compression, pressure, and shear stress, are known to influence growth and remodelling in all living tissues and their effects are exerted at the cellular level. Cell growth, differentiation, secretion, movement, signal transduction, gene expression and death are all modified by applying mechanical stresses directly to cultured cells (Ingber 1997). Mechanotransduction describes the mechanism by which cells translate a mechanical signal into a biochemical response through specialised molecules that alter their biochemical state of activity upon mechanical distortion, thus converting mechanical energy into biochemical energy. The means in which cells sense mechanical stimulation, however, remains largely unknown (Han *et al.* 2004). Thus, mechanobiology centers on identifying the mechanosensitive molecules and associated cellular components involved in this process. These include stretch-activated ion channels, integrins, cadherins, cytoskeletal filaments, nuclei, extracellular matrix (ECM), and various other structures and signalling molecules which contribute to the mechanotransduction response (Ingber 2006). There is an undeniable effect of mechanical force on the differentiation of MSCs, however the mechanisms controlling this remain uncertain. Several mechanobiological models have been developed that hypothesise a relationship between the mechanical environment and tissue differentiation (Kelly & Prendergast 2004). Prendergast *et al.* (1997) propose an *in vivo* model of mechanoregulation (Figure 1.3) which encompasses several biophysical stimuli involved in the regulation of tissue differentiation. During movement of the fluid phase by mechanical loading, fluid velocity is produced in skeletal tissue. At high velocities there will be a reduced differentiation potential since fluid flow acts to enhance cellular deformation. It is the mechanical properties within the tissue that establish the pattern of differentiation. For instance, collagen synthesis would reduce motion causing a reduction in shear strain and relative velocity, which would in turn allow for ossification or bone formation to occur.

The response to mechanical force is presumed to occur via a number of possible mechanotransduction pathways. There are many different cellular responses to such forces, and many different mechanisms by which the force is transduced to

mediate a response. Considering the remarkable breadth of mechanosensitive events it is unlikely that any one or even a few mechanotransducers can account for all of these events. It is generally accepted that like soluble ligand-induced signal transduction, mechanotransduction initiates at the local force–membrane interface, or focal adhesions, by inducing conformational changes of membrane-bound proteins, followed by a cascade of diffusion- or translocation-based signalling in the cytoplasm (Na *et al.* 2008). Resultant changes in protein conformation can modify the protein's function to transduce information.

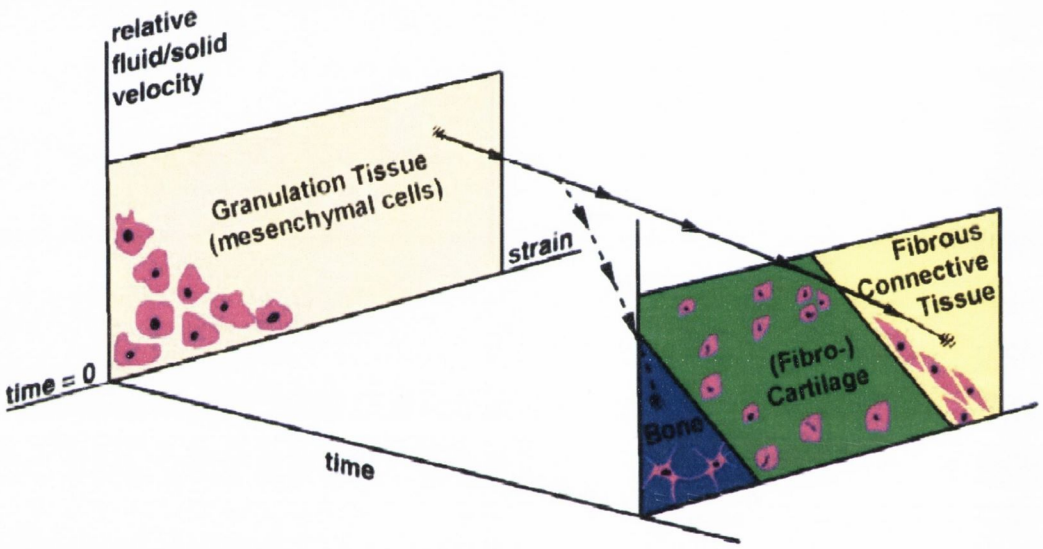


Figure 1.3: A mechanoregulation model - indicating the hypothesised interaction between biophysical stimuli and cell differentiation (adapted from Prendergast *et al.* 1997).

Force-induced effects on conformation represent a general mechanism by which enzymatic activity or protein interactions can be modified to mediate signalling (Chen 2008, Orr *et al.* 2006, Huang *et al.* 2004). Such signalling pathways include transduction via plasma membrane receptors including integrin receptors, G-protein coupled receptors and stretch activated ion channels. Of particular interest is the activation of mechanosensitive integrin receptors, present on the MSC plasma membrane, which interact with the cytoskeleton of the cell triggering further downstream signalling cascades in response to a mechanical stimulus.

Some of the particularly well-described subcellular sites for sensing mechanical forces are stretch-modulated ion channels and focal adhesions. Other putative sites for mechanosensing include the nucleus itself, and the cytoskeleton (Chen *et al.* 2008, Han *et al.* 2004).

Integrins can trigger signal transduction cascades and induce focal adhesion formation as a result of ECM ligand binding and associated changes in receptor conformation. They achieve this by activating the small GTPase Rho and stimulating its downstream target Rho-associated kinase (ROCK), which promotes actin filament polymerisation, inducing cytoskeletal contraction. Mechanotransduction is also mediated by integrin-associated, receptor-like tyrosine phosphatase and changes in the binding of the cytoskeletal link protein, actin filament-associated protein which induces activation of Src kinases (Ingber 2006, Han *et al.* 2004, Riveline *et al.* 2001). Upon activation, integrins associate with Src through its SH3 domain which exposes the Src kinase domain and activates Src. Activation of Src can regulate interactions between integrins and the cytoskeleton, cause dissolution of actin stress fibres and release of mechanical tensile stress (Wang *et al.* 2005, Fincham *et al.* 2000, Felsenfeld *et al.* 1999). Force-induced dynamic changes in Src activity have been reported (Wang *et al.* 2005), and Na *et al.* (2008) have shown that stress-induced Src activation occurs rapidly in the cytoplasm and depends on the microfilament and microtubule integrity, substrate rigidity and cytoskeletal prestress.

Cellular functions influenced by mechanotransduction such as proliferation, differentiation and apoptosis are also regulated by intracellular calcium-mediated processes. Changes in calcium concentration in the cytosol occur through the activity of several calcium transporters that mobilise calcium intracellular stores and allow influx from extracellular sources. Transient receptor potential canonical (TRPC) channels participate in calcium homeostasis. A recent report suggests that rabbit bone marrow-derived MSCs express genes encoding TRPC1, 2, 4 and 6 and that TRPC1 plays a role in cellular proliferation (Torossian *et al.* 2010). Furthermore, TRPV4, a member of a subfamily of TRP channels has been identified as a mechanically sensitive ion channel involved in the regulation of chondrogenic differentiation of MSCs (Guilak *et al.* 2009, Liedtke *et al.* 2007,

Muramatsu *et al.* 2007). A role for calcium ion signalling has been highlighted in bone mechanobiology. *In vitro* studies have shown that intracellular calcium in osteoblastic cells can be rapidly increased in response to fluid flow and this can be suppressed by the stretch-activated cation channel (SACC) blocker gadolinium chloride. Gadolinium chloride has previously been demonstrated in this laboratory to reduce the induction of collagen I expression associated with osteogenic differentiation, thus identifying a role for SACC as mechanosensors in strain-induced MSC differentiation (Huang & Ogawa 2010, Kearney *et al.* 2010). Uniaxial strain is reported to evoke an influx of extracellular calcium and induce phosphorylation of proteins associated with cell proliferation, survival and regulation of gene expression in osteoblasts. Such proteins include members of the phosphoinositide 3-kinase (PI3K) pathway and JNK. Both calcium channels and SACC are required for osteoblast protein phosphorylation in response to stretch providing evidence for the dependence of the PI3K pathway on intracellular calcium concentration in cells exposed to tensile strain (Danciu *et al.* 2003). However, a role for L-type voltage-activated calcium channels (VACC) in strain-induced apoptosis has also been recognised such that blockade of these channels by nicardipine abrogates the activation of apoptosis, indicating a role for VACC in the initiation of this mechanotransduction pathway. Part of this response is dependent on the calcium-activated protease, calpain, since the presence of the calpain inhibitor MDL 28170 prevents the strain-induced apoptosis observed (Kearney *et al.* 2008).

The study of aging in cellular mechanotransduction has emerged in recent years for its potential role in mediating cellular function and dysfunction. The possibility that the cell's capacity to sense, process, and respond to mechanical stimuli is altered with aging and that these changes may be involved in the etiology of aging-associated disease is currently under investigation (Wu *et al.* 2011). The MAPK associated proteins are often linked with signalling cascades activated by integrin activation (Katsumi *et al.* 2004). Rice *et al.* (2005) investigated the effect of age on MAPKs as critical components for regulating numerous mechanotransduction-related cellular responses in the rat aorta. It was reported that aging alters uniaxial strain-induced MAPK and the 70 kDa ribosomal S6-kinase (p70S6k), confirming mechanoregulation of the MAPKs but

highlighting the differential effect of age on mechanotransduction. Fluid flow-induced calcium signalling has also been shown to be age dependent in osteoblasts. Cells from young rats exhibited increased basal intracellular calcium activity and are more responsive to fluid flow than osteoblasts isolated from aged rats. Ultimately, understanding mechanotransduction pathways and how they are influenced by age and mechanical stimulation may help elucidate the etiologies of osteochondral diseases such as osteoporosis (Donahue *et al.* 2001).

1.6.1 Integrins

Integrins are a superamily of cell adhesion receptors that bind to ECM ligands and cell surface ligands. These receptors derive their name from their function of integrating the extracellular and intracellular environments by binding ligands outside the cell to cytoskeletal components and signalling molecules inside the cell. Integrins are transmembrane heterodimers consisting of α and β subunits which are non-covalently associated and completely distinct from each other. In vertebrates there are numerous variations of the subunits in existence; 18 α subunits and 8 β subunits, which form 24 known αβ pairs (Luo *et al.* 2007). Briefly, integrins bind ECM ligands and associate with intracellular actin filaments through a number of cytoskeletal linker proteins to mechanically connect intracellular and extracellular structures. Each element of this link from the ECM through the integrin-mediated adhesions to the cytoskeleton transmits forces that may originate from both intracellular contractile forces and forces applied from outside the cell. Cells also sense the physical properties of their environment through forces exerted on integrin-mediated adhesions. These forces activate a network of signalling pathways and genetic programs to direct cell fate and behaviour (Schwartz 2010) and it is for this reason that integrins are often described as mechanoreceptors.

Integrin expression in MSCs was first studied by Pittenger *et al.* (1999), who identified the presence of α1, α2, α3, aα, αv, β1, β3 and β4. Each α and β subunit contains a large extracellular domain, of approximately 80–150 kDa, a single transmembrane α-helix and a short, cytoplasmic domain of 10–70 residues

(Wegener & Campbell 2008). The variety in subunit composition contributes to diversity in ligand recognition, binding to the cytoskeleton and coupling to downstream signalling pathways. The general topology of integrins includes an extracellular, globular, N-terminal ligand-binding head domain, which represents an α and β subunit interface, standing on two extended C-terminal legs, which connect to the transmembrane and cytoplasmic domains of each subunit (Figure 1.4). Half of integrin α subunits contain a ~200 amino acid domain known as the inserted (I) domain, or von Willebrand factor A domain. The α I domain is the major ligand-binding site when present. The N-terminal region of α subunits contains a folded β-propeller domain, which houses the α I domain. An inserted (I) domain on the β subunit is homologous to the α I domain, except that it contains two additional segments; one of which forms the interface with the β-propeller and the other, known as the specificity-determining loop (SDL), plays a role in ligand binding. The region C-terminal to the β-propeller in the α subunit comprises the leg, the upper part of which contains the thigh domain with the lower leg consisting of the calf-1 and calf-2 domains. There is a small calcium-binding loop between the thigh and calf-1 domains which is the α subunit genu; the key pivot point for conformational change in the α subunit. As for the β subunit, the β I domain is inserted into the hybrid domain, which forms the upper part of the β subunit leg. The hybrid domain is in turn inserted in the plexin/semaphorin/integrin (PSI) domain. The remainder of the β leg consists of four integrin epidermal growth factor–like (EGF) domains and a β tail domain (Evans *et al.* 2009, Luo *et al.* 2007, Kim *et al.* 2003).

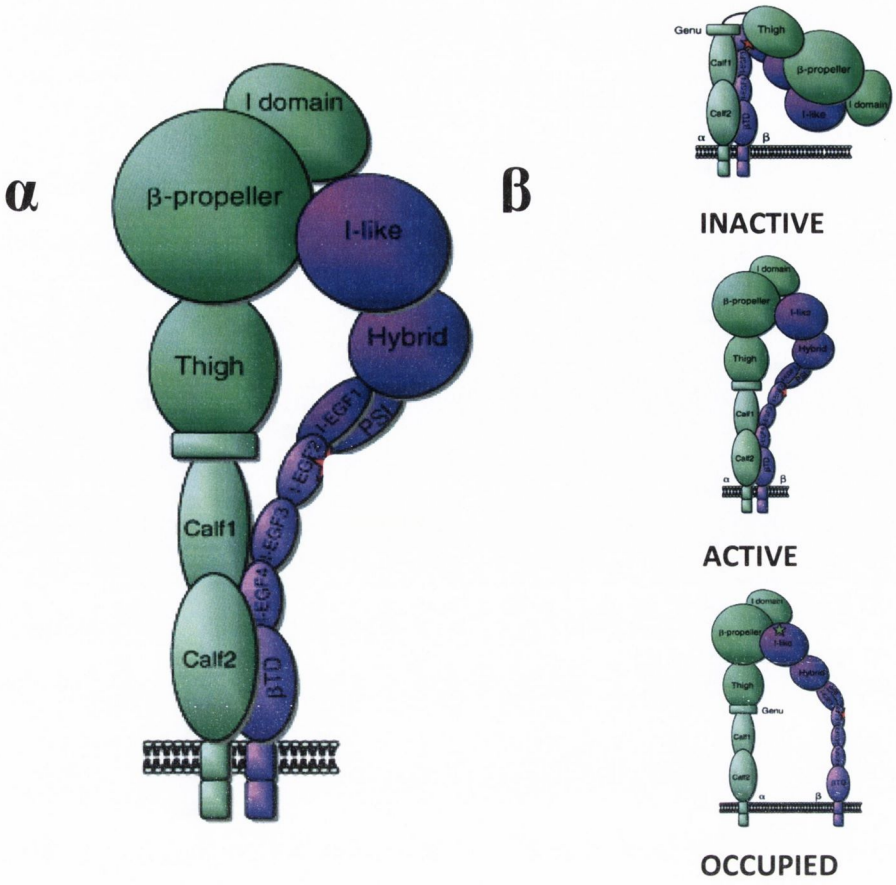


Figure 1.4: General structure of integrin receptors and their conformational stages (adapted from Evans *et al.* 2009).

Integrin mediated adhesion encompasses a cascade of partially overlapping events: cell attachment, spreading, cytoskeletal organisation, and focal adhesion formation (Lebaron & Athanasiou 2000). Initially, the cell contacts the surface and ligand binding occurs, which leads to actin organisation into microfilament bundles known as stress fibers. Finally, formation of focal adhesions occurs, linking the ECM to the actin cytoskeleton. During the process of cell adhesion, integrins are employed in a physical capacity anchoring the cell as well as in signal transduction through the cell membrane (Hersel *et al.* 2003, van der Flier & Sonnenberg 2001). The binding of talin to the β subunit's cytoplasmic tail has been established as playing a key role in integrin activation (Calderwood 2004). Talin also binds to actin and various cytoskeletal and signalling proteins, thus linking activated integrins directly to intracellular signalling pathways and the

cytoskeleton (Harburger & Calderwood 2009). Leading on from the activation of the β-integrin tail, conformational changes are propagated across the plasma membrane to the integrin's extracellular domains, increasing their affinity for ligands (Luo *et al.* 2007). The binding of individual integrins to a ligand, which can lead to formation of small clusters of integrins, forms an initial talin-mediated connection between the cytoskeleton and the ECM. The forces that are transmitted through such adhesions contribute to the reinforcement of the ECM-cytoskeleton link and to the recruitment of additional cytoskeletal and signalling proteins. As the adhesions mature a multiprotein complex assembly forms at the cytoplasmic face of clustered, ligand-bound integrins. It is these focal adhesion complexes that connect integrin receptors to the actin cytoskeleton and transmit signals into the cell. There remains considerable variability in the molecular composition of integrin-containing adhesions, and it remains poorly understood how their dynamic assembly and turnover are determined, and how the clustering of integrins and binding of ECM proteins triggers signalling (Harburger & Calderwood 2009, Ginsberg et *al.* 2005). Ligand interaction induces significant allosteric changes in integrins that extend to their intracellular domains (Luo *et al.* 2007). These conformational changes could facilitate the recruitment of intracellular adaptor or signalling proteins that crosslink and cluster integrins (Takodoro *et al.* 2003). Alternitavely, interaction with matrix-immobilized ligands can lead to integrin assembly into complexes in a matrix-dependent manner prior to intracellular protein recruitment (Zaidel-Bar *et al.* 2004). Integrin clustering could be naturally dependent on the factors that control adhesion, including ligand chemistry, matrix stiffness, and cell stiffness (Paszek *et al.* 2009)

As previously mentioned, the combination of α and β subunits determines the ligand specificity of the integrin. However, some integrins are highly promiscuous in that they can bind to numerous extracellular ligands. In an attempt to elucidate small recognition sequences, the tri-peptide motif RGD (arginine-glycine-aspartic acid) was identified as a minimal essential cell adhesion peptide sequence in fibronectin (Pierschbacher & Ruoslahti 1984). Since the identification of RGD other cell adhesive sites have been identified in many other ECM proteins; vitronectin, fibrinogen, von Willebrand factor, collagen, laminin, osteopontin, viral and bacterial proteins and snake venoms (neurotoxins and disintegrins;

Ruoslahti 1997). About half of the 24 known integrins have been shown to bind to ECM molecules in an RGD dependent manner: α3β1, α5β1, α8β1, αIIbβ3, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α2β1 and α4β1 (Pfaff 1997). Other significant cell adhesion motifs have been identified apart from RGD, thus this sequence is not deemed the universal cell recognition motif for all integrins, but it is exceptional in terms of its broad distribution and usage (Hersel et al. 2003). As it is such a small structure, the RGD site can be easily reproduced with peptides, which is how the site was originally identified. Short peptides containing the RGD sequence can mimic cell adhesion proteins in two ways: when coated onto a surface they promote cell adhesion, whereas in solution they act as snares, preventing adhesion (Ruoslahti 1996, Figure 1.5). Synthetic peptides containing the RGD sequenceare capable of competing with adhesion proteins for its receptor thereby inhibiting cell attachment and migration of both normal and tumor cells on substrates coated with the adhesion proteins (Pierschbacher and Ruoslahti 1984). These peptides are a valuable tool for the analysis of cell adhesion phenomena. For example, RGD-containing cell adhesion peptides were employed in a study to determine the role of cell adhesion receptors in the penetration of tumor cells through amniotic membrane tissue. The cell lines A375M, A375P and RuGli were seeded onto the membranes and were able to traverse the amnion in 72h. However, a marked decrease in the invasion was observed in the presence of RGD-containing peptides with all three cell lines. The RGD peptides inhibited the attachment of the cells to fibronectin, vitronectin and collagen I. This study highlights how RGD-containing synthetic peptides can inhibit *in vitro* tumor cell invasion through a human amniotic membrane and this inhibition was found to be concentration-dependent, nontoxic and correlated with the ability of the peptides to interact with matrix adhesion receptors. The effect was also specific in that control peptides had little or no effect on invasion (Gehlsen *et al.* 1988). The potential to develop RGD-based therapeutics that function as either agonists or antagonists was proposed by Craig *et al.* (1995), whereby as an agonist the peptide could promote the interaction of cells and tissues with artificial matrices or as an antagonist, control the nature of cell-cell and cell-ECM interactions

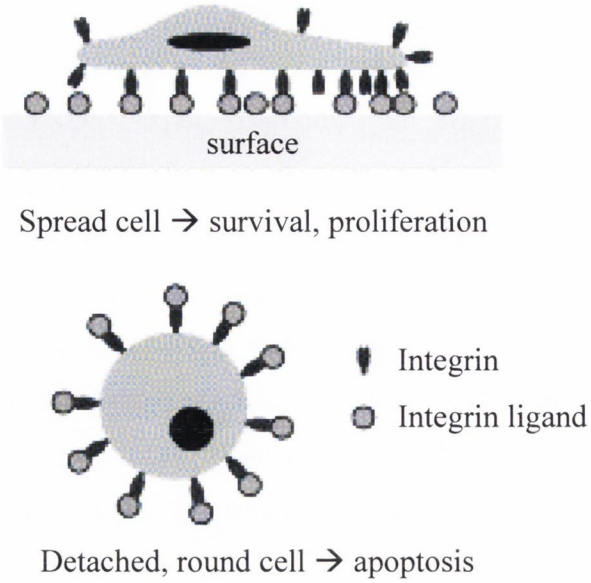


Figure 1.5: Effects of integrin ligand RGD - immobilised ligands serve as agonists of the ECM, while non-immobilised ligands act as antagonists, leading to cell detachment (adapted from Hersel *et al.* 2003).

As adhesion molecules, integrins can be dynamically regulated by a process known as inside-out signalling, whereby stimuli received by cell surface receptors for chemokines and cytokines initiate intracellular signals that impinge on the cytoplasmic domains of integrins and alter adhesiveness toward extracellular ligands. In addition, outside-in signalling consists of ligand binding which transduces signals from the extracellular domain to the cytoplasm in the classical outside-in direction (Luo *et al.* 2007). Integrins of the βl-type are predominantly responsible for binding to the ECM. Such integrins share a common βl-subunit but varying α-subunits. Each integrin of this group preferentially binds certain matrix components: αlβ1 and α2βl to collagen and laminin, α3β1, α4βl, and α5βl to fibronectin and α6βl and α7βl to laminin (Shakibaei *et al.* 1995). Integrins function as traction receptors that can detect and transmit alterations in mechanical force acting on the ECM. Integrins are known to adopt three major conformational states: inactive (low affinity), primed or active (high affinity), and ligand occupied (Figure 1.4). It is proposed that interaction with ligands in the ECM causes a conformational change followed by clustering in the plasma

membrane that transduce signals to the interior of the cell and form supramolecular structures known as focal adhesions. These adhesions link directly to actin filaments in the cytoskeleton, directing the formation of actin stress fibres and overall organisation of the cytoskeleton (Connelly *et al.* 2008, Takada *et al.* 2007, Frelinger *et al.* 1990).

Following adhesion through actin and talin, paxillin and vinculin are recruited, making the transition from an early adhesion to focal complex formation. A loss of cytoskeletal tension results in rapid dissociation of vinculin from the focal complex, suggesting that force is required for complex development and maintenance. Focal complexes then develop into larger focal adhesions, which extend further from the cell periphery via a Rho-dependent mechanism. Rho triggers many target molecules that, in turn, initiate cascades of downstream events. One of the immediate Rho targets, Rho-associated kinase (ROCK or Rho-kinase) is necessary for focal contact formation (Katsumi *et al.* 2004, Galbraith *et al.* 2002, Riveline *et al.* 2001). Focal adhesion complexes function as signalling centres assuring substrate adhesion and targeted location of actin filaments and signalling components within the cell, rendering them essential for establishing cell migration and maintaining cell growth and survival (Figure 1.6; Docheva *et al.* 2007, Brakebusch & Fassler 2003). Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase, is activated upon integrin ligation to the ECM at sites of focal adhesions. It acts as a phosphorylation-regulated signalling scaffold and is important for adhesion turnover, Rho-family GTPase activation, cell migration and cross-talk between growth-factor signalling and integrins (Mitra *et al.* 2005). FAK is a key regulator of integrin-mediated signals from the ECM to the cytoskeleton and downstream signalling molecules. It is due to upstream conformational changes in focal adhesion complexes that result in phosphatase-dependent activation of tyrosine kinases such as FAK that mediate mechanotransduction (Salasznyk *et al.* 2007, Riveline *et al.* 2001). When integrin clustering occurs, the autophosphorylation of FAK generates docking sites for SH2-domain-containing proteins, including Src kinases, which in turn become activated and phosphorylate FAK, promoting its kinase activity and its interaction with other proteins (Mitra *et al.* 2005). The Src family of protein kinases are rapidly activated in response to integrin-ligand interactions and their activity

contributes to reinforcing initial integrin-mediated adhesions by activating downstream kinases and other adaptor proteins (Ginsberg *et al.* 2005)

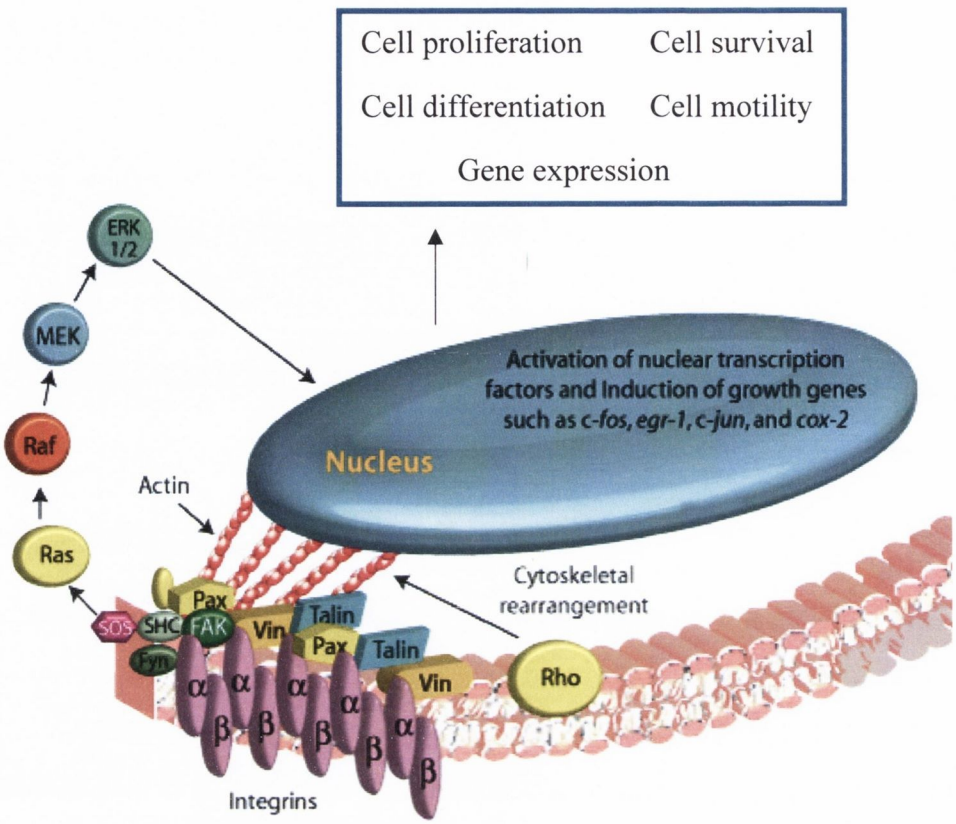


Figure 1.6: Integrin receptors expressed in the plasma membrane function in cell growth, survival and differentiation (adapted from www.sciencemag.org).

Recent evidence suggests that signals generated by integrin ligation to bone marrow ECM proteins regulate MSC commitment along the osteogenic lineage (Salasznyk et al. 2007). When MSCs deposit increased amounts of ECM proteins fibronectin and collagen I onto thin-film poly(lactide-co-glycolide) (PLGA) substrates, integrin expression profiles change accordingly with increases observed in α2β1 and α5β1 at various time points. The MAPK and PI3K pathways are also activated in MSCs cultured on these substrates, and their inhibition significantly inhibits osteogenic differentiation. These reports indicate that initial ECM deposition, ensuing matrix remodelling, and the resultant integrin expression profiles influence osteogenesis in MSCs (Kundu et al. 2009). During

chondrogenic differentiation of MSCs, the expression of the fibronectin receptor α5β1 rose while receptors for the collagens (α1β1, α2β1, α3β1) are weakly expressed by undifferentiated MSCs but are subsequently activated during differentiation. Intracellular signalling components integrin-linked kinase (ILK) and CD47 show increased expression with ongoing differentiation.

Integrin-mediated signalling appears to play a role in the generation and maintenance of chondrogenic differentiation which might be transduced by ILK and CD47 (Goessler et al. 2008). ILK is an essential protein that performs a major role as a signalling scaffold at sites of integrin adhesions. It forms a heterotrimeric complex with the LIM-domain protein PINCH and the actin- and paxillin-binding protein parvin. The formation of this complex precedes, and is essential for, precise targeting of its components to integrin-mediated adhesions (Harburger & Calderwood 2009, Legate et al. 2006). ILK and its interactive proteins have been implicated in the process of cytoskeletal connection and communication with the ECM as they provide a molecular scaffold for the assembly of proteins such as paxillin, which bridge the ECM via integrins to actin. It functions by binding to the β subunit cytoplasmic domain and has also been shown to possess serine/threonine kinase activity and to phosphorylate signalling proteins such as Akt1 (Lange *et al.* 2009, Wu & Dedhar 2001, Hannigan *et al.* 1996). Integrin receptors and their associated proteins, such as actin and FAK, are integral in the signal transduction pathways activated by mechanical stimuli. It is critical to investigate their expression and functionality in young cells as well as with age to better understand mechanotransduction and whether it is altered with age and how this might affect strain-enhanced differentiation, mediated via these mechanosensitive proteins.

1.6.2 Actin (Cytoskeleton)

The cell cytoskeleton provides a mechanical framework to support and maintain cell shape. There are three main components to the cytoskeleton: actin filaments, microtubules and intermediate filaments. All three components are organised into networks that resist deformation and can reorganise in response to externally

applied forces (Fletcher & Mullins 2010). Actin occupies a variety of assembly formations providing the basic framework for cell shape, motility and intracellular organisation. As a cause of its functional diversity, actin has the intrinsic capability to assemble and disassemble filaments as required such as in response to changes in force (Schwartz 2010, Schoenenberger *et al.* 2002). It is well established that the cytoskeleton also participates in mechanotransduction. When exposed to mechanical forces cells will reorganise their actin cytoskeleton and focal adhesions, and subsequently align accordingly (Liu *et al.* 2010, Katsumi *et al.* 2004). When exposed to shear stress, Src is rapidly activated at remote cytoplasmic sites, which is dependent on cytoskeletal prestress. Here, microtubules are the necessary structures that transmit the stresses to activate Src. Such rapid signal transduction through the prestressed cytoskeleton is a unique feature of mechanotransduction (Na *et al.* 2008). The cytoskeleton can in a sense recall the history of cellular mechanical stress, with its sharp responses principally dependent on the organisation of cell–matrix adhesions and the cytoskeleton following previous mechanical stimulations (Asparuhova *et al.* 2009).

The small Rho-family GTPases regulate actin assembly and contraction in response to mechanical cues mediated by cell shape by causing alterations in the activity of RhoA. The Rho GTPases play a crucial role in regulating cytoskeletal dynamics and are required for normal cell proliferation and differentiation. Active Rac1 is required for the formation of new matrix adhesions or focal complexes while active RhoA is required for stress fibre formation. The generation of tensile stress promotes maturation of focal complexes into focal adhesions where necessary (Asparuhova *et al.* 2009). The kinase ROCKII is an important effector of RhoA that mediates actin cytoskeletal tension and stress fibre formation by activating myosin light chain kinase, which in turn, activates the dimerised motor protein myosin II (Riddick *et al.* 2008). The activation of RhoA, ROCK II and the cytoskeletal tension that accompanies their activation are important factors in MSC fate, for example during chondrogenic differentiation. However, progenitor cells with constitutively active RhoA or ROCKII are able to undergo osteogenic differentiation downstream of soluble factors; inhibition of this pathway results in adipogenic differentiation (Arnsdorf *et al.* 2009, Woods & Beier 2006, Woods *et al.* 2005, McBeath *et al.* 2004). Small GTPases are regulated by a variety of

external signals, for example, ligand-engaged integrins in matrix adhesions influence signalling toward the activation of Rac1 and RhoA. Alterations in small GTPase activity in response to external mechanical stimulation lead to complex remodelling of the actin cytoskeleton as well as of cell–matrix adhesions (Figure 1.7). Adherent cells utilise cell–matrix adhesions to continuously sense structural and mechanical features of their environment, and a major response is a Rac1- and RhoA-dependent change in actin cytoskeletal dynamics. Consequentially, the actin cytoskeleton remodels cell mechanosensors in response to incoming signals. Cytoskeletal reorganisation regulated by small GTPases is essential for this adaptation to physical stimuli (Asparuhova *et al.* 2009).

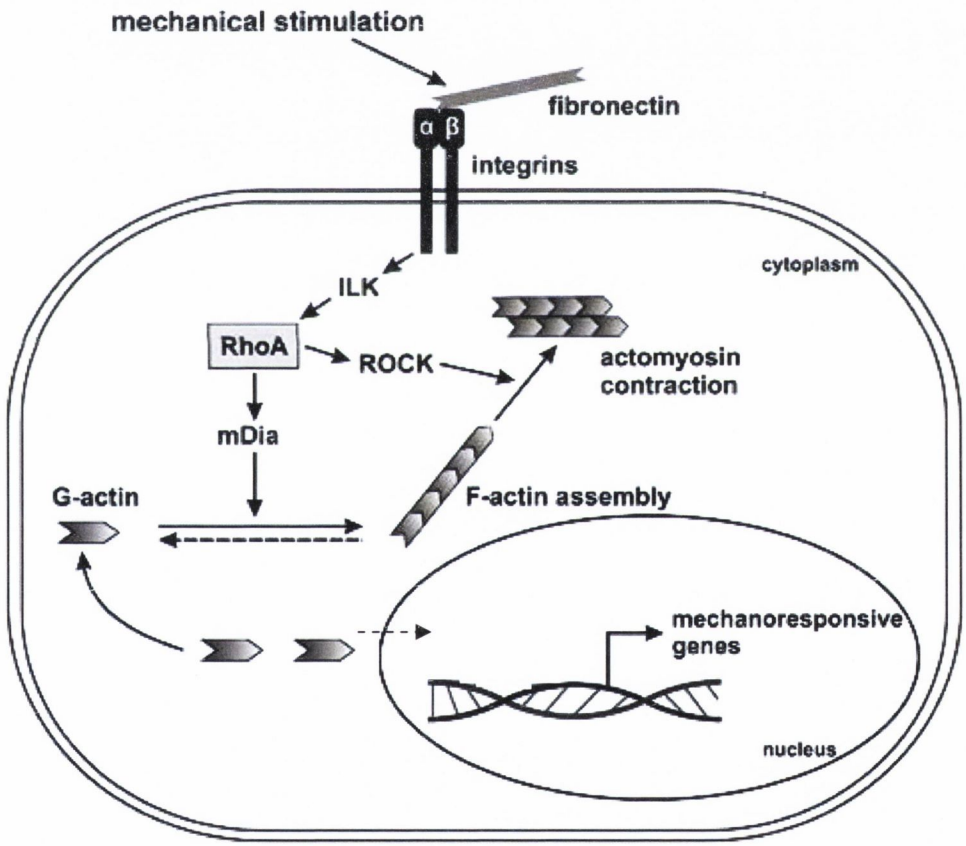


Figure 1.7: Mechanically-induced RhoA-actin signalling via direct transmission of the stimulus through integrins, ILK, RhoA, ROCK, actin and the nucleus, resulting in altered gene expression (adapted from Asparuhova *et al.* 2009).

There is evidence to suggest that human MSCs alter their cytoskeletal components during differentiation. Human MSCs were differentiated into chondrocytes and osteoblasts and treated with Cytochalasin D (CytoD) to temporarily disrupt the cytoskeleton prior to and following chemically-induced differentiation. Removal of CytoD from the media of cytoskeleton disrupted cells showed a recovery of cytoskeletal structures. It was also found that disruption of the cytoskeleton had marked effects on hMSCs and hMSC-chondrocytes, compared to the limited cytoskeleton disruption in hMSC-osteoblasts (Yourek *et al.* 2007). Conversely, Rodriguez *et al.* (2004) reported a notable change in cytoskeletal organisation in human MSCs during osteogenic differentiation. Actin cytoskeleton was altered from a large number of thin, parallel microfilament bundles spanning the cytoplasm in undifferentiated MSCs to a few thick actin filament bundles in a cortical organisation in differentiated cells. Disruption of the cytoskeleton with CytoD treatment reduced the expression of differentiation markers but under osteogenic culture conditions, a reversible reorganisation of microfilaments was observed. These findings indicate that changes in the dynamic of the actin network formation may be crucial in supporting osteogenic differentiation of human MSCs.

1.6.3 Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a central component of the integrin-signalling pathway, which functions to transmit signals from the extracellular matrix into the cytoplasm (Schaller 2001). It is a cytoplasmic protein tyrosine kinase that discretely localises at points of focal adhesions (Schlaepfer *et al.* 1999, Schaller 1996). It is activated by phosphorylation at specific tyrosine residues, which then stimulate downstream signalling, including the ERK1/2 pathway, leading to a variety of cellular responses (Ward *et al.* 2007). In other words, FAK serves as a scaffold for the assembly of complexes of signalling molecules that initiate downstream signalling events, including activation of MAPKs leading to potential stem cell differentiation (Salasznyk *et al.* 2007). FAK contains an N-terminal FERM domain, a central kinase domain, proline-rich regions and a C-terminal focal-adhesion-targeting (FAT) domain that interacts with paxillin and talin in the

focal adhesion complex (Harburger & Calderwood 2009). The prominent site of autophosphorylation is tyrosine 397 which is located just above the kinase domain and acts as a docking site for the SH2 domains of several proteins including the Src family of tyrosine kinases (Schaller *et al.* 1994). Cell adhesion activation of FAK signalling occurs at integrin co-localisation in focal adhesions. The actin cytoskeleton is also required for tyrosine phosphorylation of FAK, as evidenced by abolished tyrosine phosphorylation in response to stimuli including integrin adhesion when treated with CytoD (Schlaepfer *et al.* 1998). Members of the Rho family of GTP-binding proteins were previously described as key regulators of the actin cytoskeleton. It has been proposed that activation of Rho induces tyrosine phosphorylation of FAK, whereas inhibition of Rho blocks the tyrosine phosphorylation in response to certain stimuli (Schaller 2001, Wang *et al.* 1997). This would suggest that tyrosine phosphorylation of FAK may be regulated in part by Rho-dependent alterations of the actin cytoskeleton.

There are a number of downstream biochemical pathways that can be activated by FAK such as the PI3K and MAPK pathways. FAK activation can support tyrosine phosphorylation of Shc, which in turn recruits Grb2 and SOS into a complex promoting the activation of Ras and the MAP kinase pathway including ERK (Figure 1.8; Schlaepfer & Hunter 1998). The role of FAK in the activation of MAPK pathways due to integrin association remains uncertain. Integrins can exploit other signalling pathways to induce MAPK activity, since MAPK activation can be dissociated from FAK tyrosine phosphorylation (Barberis *et al.* 2000). Alternatively, the expression of FAK can also augment adhesion-dependent MAPK activation. It has been demonstrated that inhibition of FAK signalling obstructs MAPK activation in response to integrin-mediated signalling (Schlaepfer *et al.* 1999). Thus, under certain conditions FAK can function as a main regulator of MAPK activation in response to integrin stimulation, whereas under other conditions alternative signalling pathways to MAPK prevail.

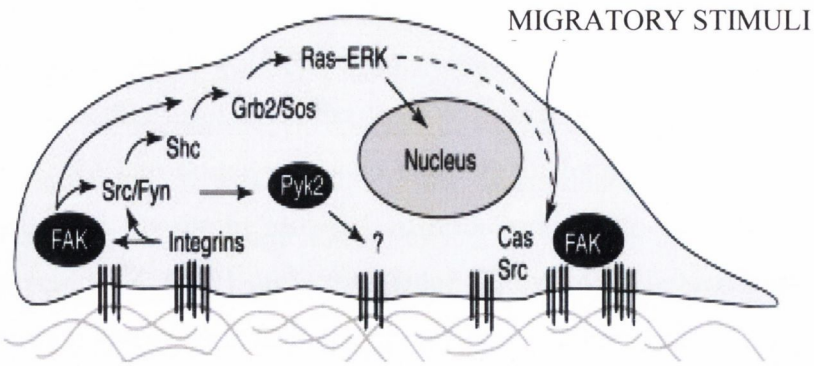


Figure 1.8: Integrin stimulation of FAK promotes Src-family activation, Grb2 binding to Shc and signalling to the Ras–ERK cascade (adapted from Schlaepfer & Hunter 1998).

FAK signalling has been implicated in MSC differentiation such that conditional inactivation of FAK abolishes the mechanically induced osteogenic response. In this study physical forces triggered the expression of Sox9 and Runx2, transcription factors essential for the commitment of stem cells to a skeletogenic lineage. As a result of FAK deletion, the bone marrow cells no longer up-regulated skeletogenic genes and collagen fibrils remained disorganised (Leucht *et al.* 2007). It is understood that particular tyrosine residues control osteogenic differentiation via an ERK1/2 pathway during strain, implicating FAK as a critical role player in strain-enhanced differentiation (Ward *et al.* 2007). When cultured on ECM of different stiffnesses MSCs have been shown to possess diverse lineage. Inhibition of FAK and ROCK, a recognised mechanotransducer of matrix stiffness during osteogenesis, leads to decreased expression of osteogenic markers through downregulation of ERK1/2 signalling. Furthermore, integrin α2 was shown to be upregulated on stiffer matrices during osteogenic differentiation, and its deletion reduced the osteogenic phenotype through ROCK, FAK, and ERK1/2. Together, these reports indicate that matrix rigidity affects the osteogenic potential of MSCs through mechanotransduction events that are mediated by α2

(Shih *et al.* 2011). When comparing the expression, basal activation, and the ability of force to activate FAK in the aorta of adult and aged rats, it was found that age significantly altered the basal phosphorylation status of FAK and the focal adhesion associated protein paxillin. Application of aortic intraluminal pressure increased the phosphorylation of FAK, Src and paxillin in adult rats while aortic loading in the aged animals failed to induce FAK phosphorylation. This study suggests that FAK and focal adhesion-associated proteins are mechanically regulated and furthermore, that FAK mechanotransduction is altered with aging (Rice *et al.* 2007).

1.7 Cellular Aging

The body's regenerative potential decreases with age which may contribute to aging of adult stem cells. However, it is unclear if adult stem cells undergo functional and molecular changes with age or whether aging is caused by extrinsic environmental factors without affecting the stem cell pool (Wagner *et al.* 2009, Giangrecco *et al.* 2008). MSCs face long replicative histories, thus accumulating genetic damage as well as being subjected to damage from intracellular and extracellular sources such as reactive oxygen species (Fehrer & Lepperdinger 2005). Free radical-derived reactive oxygen species (ROS) are constantly generated in most living tissues and can potentially damage DNA, proteins and lipids. There is evidence to suggest that oxidative stress can cause lipid peroxidation, damage to membrane lipids, which has been linked to an increased rigidity in various cell types (Ochoa *et al.* 2003). Oxidative stress occurs if ROS reach abnormally high concentrations (Droge 2003). ROS can be produced extracellularly or by the cells themselves, the main intracellular sources being peroxisomes, lipoxygenases and mitochondrial respiratory chain reactions (Stolzing & Scutt 2006). ROS and other free radical emissions by cells are often taken as an indicator and cause of *in vivo* and *in vitro* aging (Sethe *et al.* 2006). Excessive oxidative stress caused by increased ROS levels can induce stress-related senescence pathways regulated by p53/p19 leading to cellular senescence or apoptosis. DNA damage accumulates as a consequence of endogenous or exogenous attacks, such as oxidative stress, and activates checkpoint responses

that are mediated by the p53 and p16-Rb pathways which result in apoptosis or cellular senescence. If these events occur in adult stem cells, they alter tissue homeostasis, a phenomenon that might contribute to aging (Figure 1.9; Pelicci 2004).

Little is known about the effects of age on MSCs and whether age-related characteristics such as lipid peroxidation occur in these cells, causing consequences in their efficiency as maintenance and repair cells as well as their potential for use in tissue engineering applications. *In vitro* aging of cells is dependent on the number of cell divisions occurring in culture. Following approximately 20 population doublings, cells enlarge, become more granular and decrease their proliferation rate. Ultimately, they irrevocably cease cell division although they remain metabolically active and can be maintained in this state for long periods of time (Wagner *et al.* 2009). Cells in this state are referred to as senescent cells, exhibiting a complex phenotype that entails changes in function and replicative capacity (Sethe *et al.* 2006). Senescent cells are understood to secrete degradative enzymes, inflammatory cytokines and growth factors that stimulate tissue aging (Krtolica & Campisi 2003). It has been reported that cells isolated from aged donors exhibit a decrease in proliferation potential and accelerated cellular senescence compared with cells isolated from young donors (Stenderup *et al.* 2003).

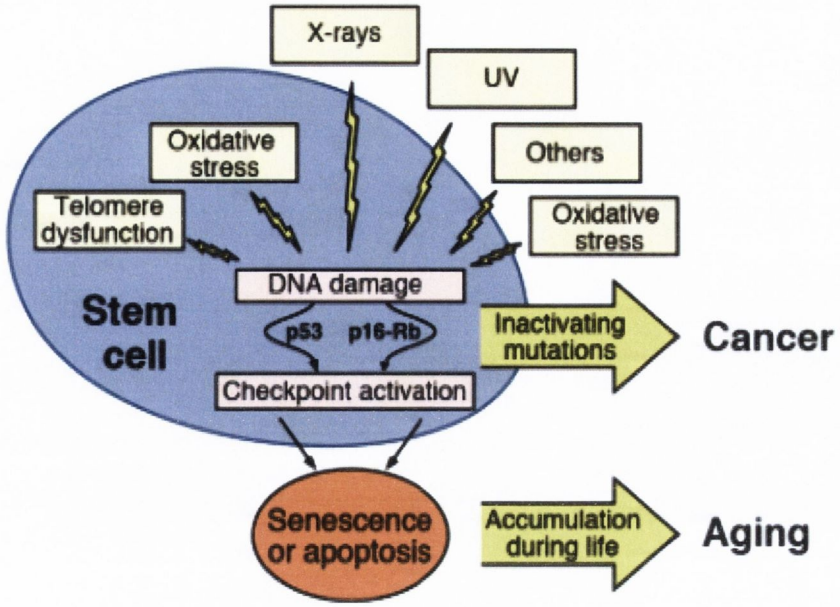


Figure 1.9: Mechanisms associated with cellular aging - oxidative stress, p53 signalling which can lead to DNA damage (adapted from Pelicci 2004).

An important parameter to consider with regard to *in vitro* aging is the effect of passage number on the differentiation capability of MSCs, particularly with regard to decreased proliferation and the tendency towards cellular senescence in cells isolated from aged donors (Kretlow *et al.* 2008). A study by Stenderup *et al.* (2003) investigated the combined effects of donor age and *in vitro* passages on MSC differentiation. They reported a decrease in osteoblastic differentiation with increased passage number for young and aged donors but found no effect on differention when comparing the two age groups. Similarly, Scharstuhl *et al.* (2007) reported an independency of age in the chondrogenic potential of human MSCs. Other groups have reported intrinsic alterations occurring in MSCs with aging such as reduced cell numbers, reduced differentiation potential, increased oxidative damage and an altered response to mechanical stimulation (Zhou *et al.* 2008, Rice *et al.* 2007, Stolzing & Scutt 2006). It is unclear whether MSCs retain their differentiation potential irrespective of age or age-related alterations in cellular characteristics. Further investigation is required into *in vivo* and *in vitro* aging of MSCs to fully understand the potential of these cells for employment in regenerative medicine techniques.

1.7.1 Lipid Peroxidation

Lipid peroxidation is viewed as the major process producing damage from oxygen radicals which increase with age, thus leading to the lipid peroxidation theory of aging. Lipid peroxidation generates a complex variety of products, some of which react with protein and DNA and are therefore toxic and mutagenic. In phospholipid membranes, the molecules that reduce peroxyl radicals to hydroperoxides are either vitamin E or some other molecule of fatty acid. If another molecule of fatty acid reduces the peroxyl radical, a new carbon-centered radical is generated which propagates the fatty acid oxidation. Through this radical chain process, one oxidising agent can cause many molecules of fatty acid to become oxidised. Vitamin E reduces peroxyl radicals which normally breaks the radical chain and slows the rate of lipid peroxidation (Marnett 1999). Malondialdehyde is a naturally occurring product of lipid peroxidation which has been used for many years as a biomarker for lipid peroxidation because of its reaction with thiobarbituric acid (TBA; Figure 1.10) to form an intensely colored chromagen (Janero 1990). There is a proposed link between peroxidation of membrane lipids and the deterioration that is characteristic of the aging process such that age-related membrane lipid peroxidation causes fluidity changes in the membrane; mediated through MDA. The change in fluidity in turn disrupts vital functions, such as the maintenance of selective permeability to ions or signal transduction (Chen & Yu, 1994, Rikans & Hornbrook 1997). Free radicals react with membrane fatty acids and phospholipid components to form lipid peroxides, causing irreversible damage to membrane fluidity, causing irreparable harm to the cell's integrity (Pratico 2002).

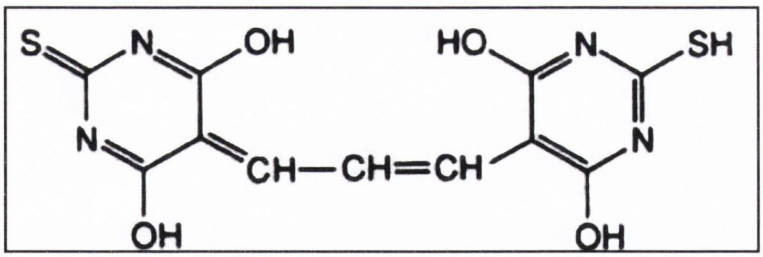


Figure 1.10: MDA forms a 1:2 adduct with TBA which can be measured fluorometrically and colorimetrically.

1.8 Apoptosis

Although there is vast potential for MSCs, both realised and in the future of regenerative medicine, current limitations include the small number of cells that can be harvested from a primary source and the inconsistencies that arise in proliferation and differentiation capabilities across individuals and different age groups. A better understanding of the intrinsic gene regulation and the external environmental stimuli and cues governing MSC fate will enhance both the understanding of MSC biology and the ability to promote survival, self-renewal and readiness to differentiate (Pricola *et al.* 2009). Apoptosis is an extensively regulated mechanism of programmed cell death active in development and the regulation of cells in tissues under normal physiological or pathological conditions. First described by Kerr *et al.* (1972), apoptosis is a mechanism of controlled cell deletion, which compliments and opposes mitosis in regulating animal cell populations. It is an active, intrinsically controlled phenomenon, either initiated or inhibited by numerous environmental stimuli, both physiological and pathological. External events that induce apoptosis include oxidative stress and high rates of mechanical strain (Kearney *et al.* 2008, Simon *et al.* 2000). Although there are a wide variety of stimuli and conditions which can trigger apoptosis, not all cells will necessarily die in response to the same stimulus (Elmore 2007). Aging is often associated with an enhanced occurrence of and susceptibility to apoptosis in several types of cells including neurons (Li *et al.* 1997), cardiomyocytes (Kajstura *et al.* 1996), lymphocytes (Phelouzat *et al.* 1996) and

chondrocytes (Adams & Horton 1998). Apoptosis plays an important role in the aging process in that several types of cells exhibit enhanced apoptosis with aging under normal physiological conditions and enhanced vulnerability to apoptosis by insults. It is considered that the incidence of apoptosis in aging depends on the level of accumulated injury and involvement in the activation of apoptosis-related cytokine signalling (Higami & Shimokawa 2000).

Apoptosis occurs in two distinct stages, the first comprises nuclear and cytoplasmic condensation and breaking down of the cell into numerous membrane-bound fragments. These apoptotic bodies are then taken up by surrounding cells where they are rapidly degraded by lysosomal enzymes (Figure 1.11; Kerr *et al.* 1972). Apoptosis is initiated by two main pathways, the extrinsic or death receptor pathway which occurs via transmembrane receptors and the intrinsic activation of mitochondrial pathways but current evidence suggests that the two pathways are linked and that molecules in one pathway can influence the other (Elmore 2007, Igney & Krammer 2002). Upon stimulation of death receptors such as by tumor necrosis factor α (TNF- α), caspase-8 is activated which in turn can activate downstream caspases 3, 6 and 7. Caspases are a group of intracellular protein-cleaving enzymes that mediate cleavage events through an amplifying cascade cleaving one another in sequential steps. Once caspases are initially activated, an irreversible commitment towards cell death occurs. To date, ten major caspases have been identified and are broadly categorised based on their function: initiators (caspase-2,-8,-9,-10), executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5; Cohen 1997). Caspase-8 activation can result in mitochondrial pathway activation which leads to the release of cytochrome C which binds and activates apoptotic protease activating factor (Apaf)-1 and pro-caspase-9, forming an apoptosome (Hill *et al.* 2004).

The extrinsic and intrinsic pathways both terminate at the point of the execution phase, often considered as the final pathway of apoptosis. Activation of the execution caspases begins this final phase of apoptosis by activating cytoplasmic endonuclease, that degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspases -3, -6, and -7 function as executioner caspases but caspase-3 is considered to be the most important effector as it is

activated by any of the initiator caspases (caspase-2, -8, -9, or -10) and induces cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies. Finally, it is the phagocytic uptake of apoptotic cells that is the last component of apoptosis (Elmore 2007). Both types of apoptotic pathway inevitably lead to cleavage of cell proteins and subsequent DNA fragmentation and cell death (Brodie & Blumberg 2003, Kumar 1999, Salvesen & Dixit 1997).

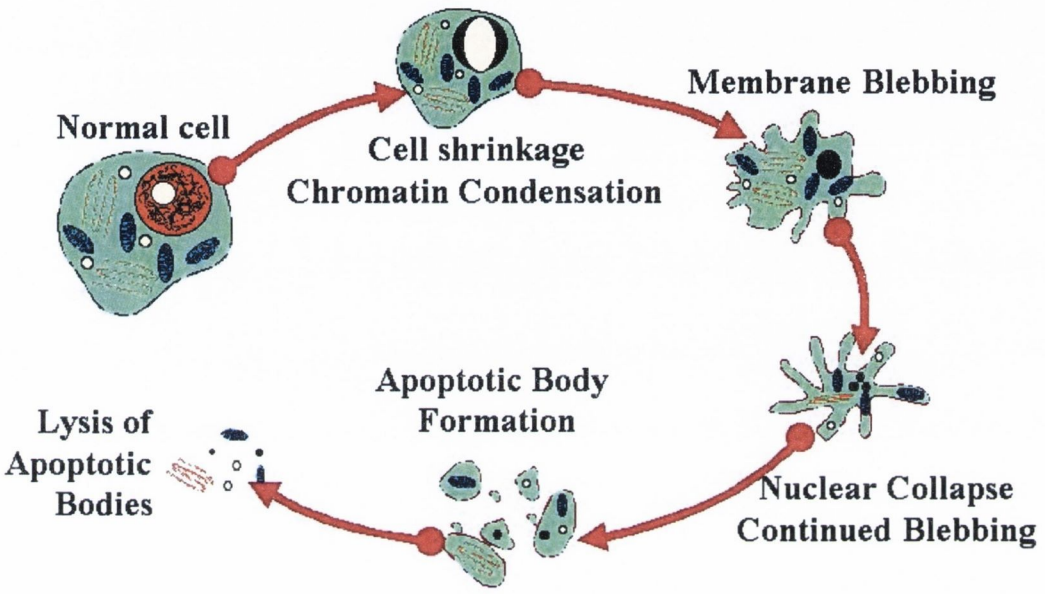


Figure 1.11: Events leading to apoptosis - nuclear and cytoplasmic condensation, cell breakdown, phagocytosis of apoptotic bodies and lysosomal breakdown (adapted from www.microbiologybytes.com).

1.8.1 Mesenchymal Stem Cell Apoptosis

Apoptosis of MSCs has been reported under various conditions including serum deprivation (Bhakta *et al.* 2005), and exposure to tensile strain (Kearney *et al.* 2008) and hydrogen peroxide (Wei *et al.* 2010). Hydrogen peroxide induced apoptosis of MSCs occurs via endoplasmic reticulum (ER) and mitochondrial pathways rather than the extrinsic apoptosis pathway. There is transient activation of p38 and JNK in response to hydrogen peroxide with no effect on the ERK1/2 pathway. p38 is involved in the regulation of early apoptosis of MSCs while JNK

is associated with late apoptosis (Wei *et al.* 2010). Kearney *et al.* (2008) examined the effect of cyclic tensile strain on MSC viability. It was found that strain rates greater than 7.5% promote apoptosis in young MSCs mediated by voltage activated calcium channels and the calpain and JNK pathways. To gain a true insight into the mechanism of strain-induced apoptosis requires determination of the point of communication between signal transduction in response to mechanical stimuli and activation of pro-apoptotic pathways. An age-related increase in apoptosis occurs in human MSCs, whereby upregulation of the p53 pathway with age may play a critical role in mediating this increase. It is proposed that such intrinsic alterations in MSCs with aging may contribute to the process of skeletal aging (Zhou *et al.* 2008). Integrin-mediated cell anchorage and signalling can regulate cell survival processes and prevent cells entering a form of apoptosis known as anoikis, which is triggered by degenerate cell–ECM interactions (Stupack 2005). A novel study by Popov *et al.* (2011) established the first cellular and molecular analysis demonstrating the role of collagen I-binding integrins in the survival of MSCs in a collagen I-rich environment. Their major finding was that the deficiency of $\alpha 2$ or $\alpha 11$ integrin in hMSCs initiates apoptosis via the Akt pathway, while the deficiency of $\alpha 1$ integrin is tolerable for hMSC survival on collagen I. The major cellular changes observed included reduced adhesion, spreading and motility on collagen I; increased apoptosis associated with mitochondrial leakage and Bax upregulation; and influenced integrin-mediated signalling to Akt, FAK and ERK.

1.9 Study Aims

Mesenchymal stem cells are regarded as a promising cell source for use in tissue engineering applications due to their multipotential capability, high proliferative capacity and ease of expansion in culture. The various studies discussed previously clearly indicate the potential of MSCs as a cell source to treat osteochondral defects. Essentially, they can be isolated from a donor, primed or differentiated *in vitro* via biochemical and biophysical stimuli for introduction to defective sites for the acquisition of novel, healthy tissue. However, the exact mechanism of how MSCs respond to stimuli such as mechanical force remains

uncertain. There is also a necessity to fully understand how MSCs sense and transduce mechanical signals such that optimum conditioning environments can be isolated and utilised for successful tissue engineering strategies. Furthermore, there is discrepancy in the literature regarding the response and potential of MSCs isolated from aged donors; whether alterations in cellular characteristics occur with increasing age and how this may affect the cell's response to an applied stimulus. It is hoped that determining the true potential of both young and aged MSCs for skeletogenic differentiation, mediated by mechanical strain, will have significant implications for the use of these cells in regenerative medicine to treat osteochondral injury and disease within the young and aged population.Therefore, the overall objective of this thesis is to provide an improved understanding of mesenchymal stem cell physiology in terms of aging and to deduce the mechanoresponse of these cells to tensile strain.

- The first objective is to investigate the effect of age on MSC characteristics such as proliferation, mechanosensor expression and membrane lipid peroxidation. It is hypothesised that alterations in cell physiology with age will alter the mechanoresponse to tensile strain.

- The second objective is to examine the response of MSCs to cyclic tensile strain in terms of viability, in order delineate how a mechanical stimulus regulates MSC fate with increasing age.

- In addition to examining the effect of tensile strain on mechanotransduction, the third objective is to determine the significance of integrin-mediated signalling in MSCs during the response to tensile strain and whether this is affected by age.

- The final objective of this thesis is to determine the effect of tensile strain on MSC differentiation and associated signal transduction pathways with the aim of defining how strain regulates MSC differentiation. Furthermore, it is hoped to establish potential differences in the response to strain that are hypothesised to occur with increasing age.

Chapter 2 Materials and Methods

2.1 Cell Culture

2.1.1 Aseptic technique

All materials used during cell culture were sterile and all procedures were carried out aseptically in a laminar flow hood (Astec-Microflow laminar flow workstation, Florida, U.S.A.) to prevent exposure to bacterial or fungal contaminants.

2.1.2 Sterilisation of equipment

All glassware, non-sterile plastic equipment and deionised distilled water (ddH_2O) were wrapped in aluminium foil and autoclave tape and autoclaved at 121°C for 20min (Priorclave Ltd., Model #EH150, London, England). Dissection instruments were baked at 200°C overnight in a hotbox oven (Sanyo-Gallenklamp Hotbox Oven, Model #OHG050, Loughborough, England). Bioreactors were also baked for several hours at 200°C. All equipment was sprayed with 70% alcohol before being placed into the laminar flow hood. Plastic consumables such as falcon tubes, petri dishes and culture flasks (Sarstedt, Wexford, Ireland) were purchased sterile and only opened within the laminar flow hood.

2.1.3 Sterilisation of reagents

All media and reagents were sterile filtered using a 0.2μm cellulose acetate membrane syringe filter (Millipore, Ireland) and were aliquoted under sterile conditions where necessary.

2.1.4 Sterilisation of the culture environment

All culture work was carried out in the laminar flow hood where air is drawn into the top of the hood and passed through a pre-filter and then a HEPA (high

efficiency particle air) filter. The downward flow of sterile air creates a positive pressure inside the hood, preventing the entry of airborne pathogens and contaminants. The laminar flow hood is also equipped with an ultraviolet (UV) light to further aid sterilisation as necessary. Powder free disposable latex gloves, sprayed with alcohol, were worn during all culture procedures and all equipment was sprayed liberally with 70% alcohol before being placed into the hood. Gloves were changed regularly to avoid infection of cultures and the hood was wiped down with alcohol on a regular basis.

2.1.5 Waste disposal

All hazardous material (lab gloves, plastics, sharps and carcasses) were separated into appropriated UN approved primary packaging and brought to the hazardous material facility (Trinity College, Dublin). The waste was then sent for disposal in accordance with Irish and EU legislation.

2.2 Isolation and culture of mesenchymal stem cells

2.2.1 Animals

Male adult Wistar rats (young 3-5 months; approximately 200-300g and aged 22-26 months; approximately 700-750g) were used in this study. The animals were bred at Banton and Kingman (UK) and Charles River (France). Animals were maintained in a 12 hour light/dark cycle at an ambient temperature of 20-24°C. Food and water were available *ad libitum*. Animals were sacrificed by CO_2 asphyxiation by placing them in a CO_2 gas chamber for 3min then retrieved for dissection.

2.2.2 Dissection

The work area and equipment were sprayed with 70% alcohol prior to dissection. The rat was placed on its dorsal surface and the area around the hind limbs was sprayed liberally with 70% alcohol before removing the fur. The hip joint was dislocated and exposed by cutting through the leg muscle. The leg was cut free from the body, with caution to avoid severing the femoral artery. The same process was applied to the remaining hind limb. Both amputated legs were sprayed liberally with 70% alcohol. The femur was dislocated from the tibia at the knee joint and was pulled free from the surrounding muscle. Any remaining muscle was carefully dissected from the femur. The femur was placed into a small petri dish of pre-warmed (37°C) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, England), supplemented with 10% Foetal Bovine Serum (FBS; Gibco BRL, Dublin, Ireland), 2% penicillin/streptomycin (Gibco BRL, Dublin, Ireland), 1% non-essential amino-acids (Gibco BRL, Dublin, Ireland), 0.5% L-Glutamine (Gibco BRL, Dublin, Ireland)and 0.5% Glutamax (Gibco BRL, Dublin, Ireland). To obtain the tibia, the foot was bent towards the knee joint and the Achilles tendon, along with several other tendons, was severed on the near side of the foot. The knee cap was removed and with it all adherent muscle present. The tibia was placed in supplemented DMEM (sDMEM) in the small petri dish and transferred into the laminar flow hood.

2.2.3 MSC isolation and culture

Extraction of the bone marrow was carried out in the laminar flow hood immediately after dissection. Bones were cut at both epiphyses using a 12.5cm bone cutter and the marrow was flushed into a 50ml Falcon tube (Sarstedt, Wexford, Ireland) using a 25 gauge needle (Beckton Dickinson BD Microlance, Ireland) and a 10ml syringe (BD Plastipak, Ireland) filled with approximately 5ml pre-warmed (37°C) sDMEM. After all bones were flushed through, the final suspension of extracted bone marrow cells was centrifuged (Sigma Laborzentrifugen GmbH, model *Sigma 2-16K,* Germany) at 650g for 5min at 22°C. The supernatant was discarded and the pellet was re-suspended in 10ml pre-

warmed sDMEM. This 10ml suspension was serially passed through 16, 18 and 20 gauge needles three times to obtain a single cell suspension. On the third passing though the 20 gauge needle the suspension was retained in the syringe and filtered by passing it through a 40μm sterile nylon cell strainer (BD Falcon, UK) into a sterile petri dish to remove any cell clumps or bone fragments. The plated cells were incubated in a humidified chamber (Binder, CB series, South Korea) for 30min at 37 °C and 5% CO_2. Following incubation, the supernatant was removed using a sterile Pasteur pipette and placed in a sterile 50ml Falcon tube. The petri dish was rinsed twice with pre-warmed sDMEM and additional supernatant was added to the Falcon tube. Adherent cells were discarded. Cell density within the suspension was determined using a haemocytometer (Reichert, USA) and was diluted accordingly with pre-warmed sDMEM to achieve approximately $5x10^7$ cells per ml of sDMEM. The suspension was added to two sterile T75 culture flasks (Sarstedt, Wexford, Ireland). The flasks were incubated for 24 hours at 37°C and 5% CO_2. Following incubation, the culture medium was discarded to remove non-adherent cells and the flasks were rinsed with pre-warmed sDMEM before adding a further 10ml sDMEM. Culture medium was replaced every three to four days while cells were maintained in a humidified incubator at 37°C, 5% CO_2 and 95% humidity.

2.2.4 Cell passaging

Confluency was assessed by visual inspection under an inverted microscope (Nikon TMS, Japan). Upon reaching 80-90% confluency, cells were passaged at lower densities into new culture flasks, to enhance cell growth and proliferation, while simultaneously purifying the culture from non-adherent and dead cells. Culture medium was removed and the flasks were rinsed twice with pre-warmed phosphate buffered saline (PBS; 100mM NaCl, 80mM Na_2HPO_4, 20mM $Na_2H_2PO_4$; Sigma-Aldrich, England). Approximately 5ml pre-warmed Trypsin-EDTA (Sigma-Aldrich, England) was added to each flask, to detach cells from the flask surface, and incubated for 5min at 37°C and 5% CO_2. The cultures were examined under an inverted light microscope to determine detachment from the flask surface and the flask was tapped on the side to release any cells that may

have remained attached. The suspension was poured into a sterile 50ml Falcon tube and the flask was rinsed twice with 5ml pre-warmed sDMEM, also added to the Falcon tube. The cell suspension was centrifuged at 650g for 5min at 22°C. The supernatant was discarded and the pellet re-suspended in 10ml sDMEM for aspiration through a 20 gauge needle three times to obtain a single cell suspension. The cells were re-plated into T75 culture flasks at half their density and cultures were maintained at 37°C, 5% CO_2 and 95% humidity and half the culture medium was changed every three to four days. For third and fourth passages, cells were re-plated at half their density into larger T175 flasks.

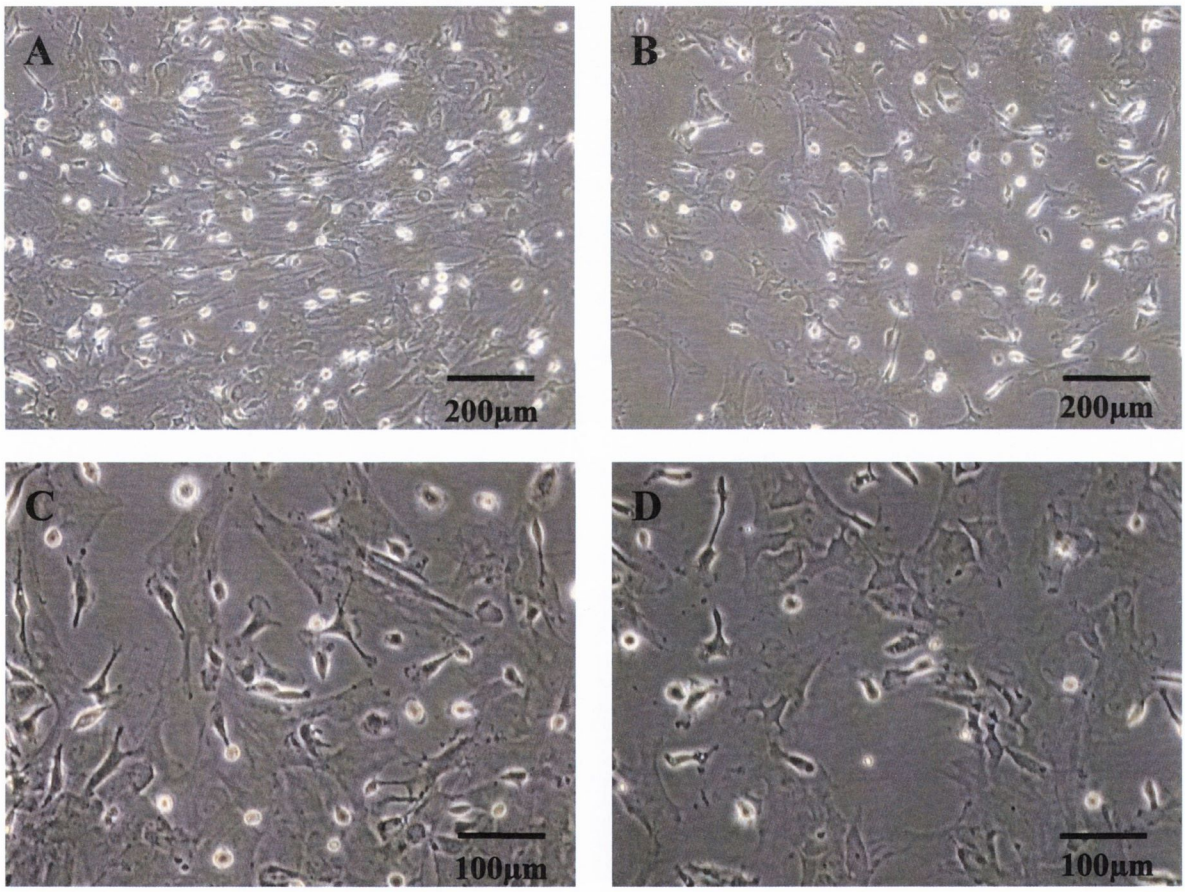


Figure 2.1: Light microscopy images of cells in culture

Young and aged MSCs 1 week after isolation, note the highly populated colony in young cells (A) and more sparse appearance of aged cells (B). At passage 3-4, cells are used for experimentation (C; young, D; aged).

2.3 Characterisation of MSCs

2.3.1 Fluorescence-activated cell sorting (FACS)

Flow cytometry was used to determine the percentage of cells expressing the CD90 epitope (positive mesenchymal stem cell marker) and CD45 (haematopoietic stem cell marker). Passage 4 cells were trypsinised, following approximately 3 weeks of expansion and centrifuged at 650g for 5min at 22°C. The supernanant was discarded and the cells were re-suspended in 2ml sDMEM. A single cell suspension was obtained by passing the pellet through a 20 gauge needle three times. A cell count was performed using a haemocytometer to determine the total number of cells present. The suspension was re-centrifuged and the cells were re-suspended in 3ml blocking buffer (50% FBS, PBS) for 20min on ice to block non-reactive sites. The cells were then centrifuged again and re-suspended in 3ml FACS buffer (PBS, 2% heat inactivated calf serum, 0.09% sodium azide, pH 7.4). A single cell suspension was obtained by aspirating the cells three times through a 20 gauge needle. This step was repeated twice to ensure the cells were washed of any remaining blocking buffer. Following the second centrifugation the cells were re-suspended in a specific volume of FACS buffer to obtain a cell density of $3x10^6$ cells/ml. Phycoerythrin (PE)-conjugated mouse anti-rat CD90 (Thy-1) monoclonal antibody (2µl) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 monoclonal antibody (2µl; BD Biosciences) were pipetted into separate FACS tubes. In addition, 2µl of PE- and FITC-conjugated mouse IgG1κ monoclonal immunoglobulin isotype controls were pipetted into separate FACS tubes (see Table 2.1). A cell suspension of 100µl was added to each FACS tube (i.e. 300,000 cells) and vortexed gently. The tubes were incubated for 45min at 4°C in darkness. The cells were then washed three times by centrifugation at 650g for 5min at 4°C, removing the supernatant and adding 200µl of FACS buffer and vortexing gently to disrupt the pellet. The cells were centrifuged a final time and re-suspended in 500µl FACS buffer. Results were acquired on a flow cytometer (CyAn™ ADP, DakoCytomation Ltd) with 488 and 633nm lasers and 20,000 events were collected for each sample. Analysis was carried out using the CyAn™ software.

Cell Type	Primary Monoclonal Antibody	Fluorescent Conjugate	Supplier
Mesenchymal Stem Cell	Mouse Anti-Rat CD90 (Thy-1)	PE	BD Biosciences, Oxford, UK
Haematopoietic Stem Cell	Mouse Anti-Rat CD45	FITC	BD Biosciences, Oxford, UK

Table 2.1: Primary antibodies and fluorochromes used in immunophenotyping

2.4 Cell proliferation assay

Cell proliferation was assayed using 5-Bromo-2'-deoxy-uridine (BrdU; Sigma Aldrich, England), a chemical which incorporates into the DNA of dividing cells. Cells grown on glass coverslips in 24-well plates were incubated in 10μM BrdU (Sigma-Aldrich, UK) for 24hr. The BrdU was diluted in normal culture medium. Following the 24hr incubation media was removed from the cells, followed by two rinses with PBS. The cells were fixed in ice-cold methanol for 10min at -20°C, followed by two rinses in PBS. The fixed cells were stored at -20°C until immunostaining with antibodies against BrdU was undertaken (see Section 2.8.1).

2.5 Lipid peroxidation/TBARS assay

Lipid peroxidation is a form of cellular damage in both plants and animals. By measuring the end products of lipid peroxidation (such as malondyaldehyde; MDA) an indication of oxidative damage can be achieved. Lipid peroxidation was identified using the thiobarbituric acid reactive substances (TBARS) assay (Cell Biolabs, Inc., San Diego, USA) in accordance with the manufacturer's instructions. TBA forms an adduct with MDA, which if present in samples can be measured spectrophotometrically or fluorometrically against an MDA equivalence

standard. The assay was performed as per manufacturer's instructions. To prepare samples for the assay, cells were re-suspended at 1-2x10^7 cells/ml in PBS containing 1xBHT (5% butylated hydroxytoluene in methanol; to prevent further oxidation of lipids during sample processing and TBA reaction). A series of MDA standards were prepared from the MDA Standard [1mM malondyaldehyde bis(dimethylacetal)] in dH_2O, ranging in concentration from 0µM - 250µM. All samples and standards were assayed in duplicate by adding 100µl of each into two separate microcentrifuge tubes. SDS Lysis Solution (100µl) was added to each tube and mixed thoroughly followed by 5min incubation at room temperature. The TBA Reagent was prepared just before use as it is only stable for 24hr. A 5.2mg/ml solution was prepared by adding the required amount of TBA to 1xTBA Diluent (2xTBA Diluent in dH_2O, pH 3.5 with Sodium Hydroxide Solution) and mixing vigorously. TBA Reagent (250µl) was added to each tube which were then closed and incubated at 95°C for 45-60min. The tubes were then removed from the heat and cooled to room temperature on ice for 5min. Samples were centrifuged at 3000rpm for 15min and the supernatant was removed for further analysis. For spectrophotometric measurement, 200µl of MDA standards and samples were transferred to a 96 well microplate, including a 0µM blank control. Absorbance readings were recorded using Genesis Lite Software (version 3.03, Life Labsystems Inc., USA) at a wavelength of 532nm in a spectrophotometric plate reader (Labsystems Multiskan RC, MTX Labsystems Inc., USA).

2.6 Substrate preparation and cell seeding

2.6.1 Silicone substrate preparation

Silicone elastomer sections (10x60mm; Specialty Manufacturing Inc., USA) were sterilised by spraying with alcohol before placing them in a petri dish. Strips were rinsed with sterile water twice and allowed to dry. A 1% collagen I solution was prepared aseptically using rat tail tendon collagen type I (Sigma-Aldrich, England) suspended in ultra-pure water. The solution was added to the petri dish and incubated for 2hr at 37°C. The collagen solution was poured off, and sections

were washed with sterile water twice and allowed to dry. The sections were then exposed to UV light in the laminar flow hood overnight.

2.6.2 Coverslip preparation

Glass coverslips (Sarstedt, Wexford, Ireland) were sterlilised by baking them in a hotbox oven at 200°C. Packages containing the sterile coverslips were sprayed with 70% alcohol before opening them in the laminar flow hood. Glass coverslips were placed inside the wells of 24-well plates (Sarstedt, Wexford, Ireland) for seeding.

2.6.3 Cell seeding

Upon reaching 80-90% confluency following approximately 21 days in culture, cells were seeded onto collagen I coated silicone substrates and glass coverslips. Culture medium was removed from the culture flasks, followed by two rinses with PBS (37°C) to remove non-adherent or dead cells. Approximately 5ml Trypsin-EDTA was added to each flask and incubated for 5min at 37°C and 5% CO_2. The cultures were examined under an inverted light microscope to determine if cells had detached from the flask surface and the flask was tapped as before to release any cells fixed to the flask surface. The suspension was added to a sterile 50ml Falcon tube and the flasks were rinsed twice with pre-warmed sDMEM and added to the Falcon tube. The cell suspension was centrifuged at 650g for 5min at 22°C. The supernatant was discarded and cells were re-suspended in 1-2ml sDMEM. The suspension was aspirated through a 20 gauge needle three times to obtain a single cell suspension. Cell density was determined using a haemocytometer and the cell suspension was diluted with sDMEM to achieve approximately $2.5x10^5$ cells per ml. From this, 500µl was pipetted evenly onto the surface of the silicone substrates and 50µl was pipetted onto each coverslip within the wells of a 24-well plate. This gave a final density of approximately $12.5x10^4$ cells per silicone substrate and $1.25x10^4$ cells per coverslip. The petri dishes and 24-well plates were incubated for 1hr at 37°C and 5% CO_2 to allow cells to adhere to the silicone

substrates and coverslips. Following incubation, a further 10-20ml sDMEM was added to the petri dishes containing the silicone substrates and 800µl sDMEM was added to each well of the 24-well plates. The silicone substrates were incubated overnight at 37°C, 5% CO_2 and 95% humidity before the commencement of mechanical strain tests. Coverslips were incubated for 1-2 days (or until they reached 80-90% confluency).

2.7 Cell Treatments

Treatments were employed to study the response of mesenchymal stem cells to various stimuli *in vitro* and during mechanical loading. These included treatment with an RGDS peptide (integrin inhibitor), and PF573228 (FAK inhibitor). Treatments were applied for testing duration only or in certain cases at varying periods of time to establish any time response of mesenchymal stem cells to these particular stimuli.

2.7.1 RGDS peptide

Cells were treated with the inhibitory peptide RGDS (5mM stock; Tocris, England) to assess its effect on integrin-mediated signalling in MSCs. A final concentration of 0.5µM RGDS (Grigoriou *et al.* 2005, Harfenist *et al.* 1988) was added to culture medium for the duration of the testing period. Medium containing RGDS was replaced every 3-4 days.

2.7.2 PF573228

Cells were treated with the FAK inhibitor PF573228 (PF228, 5mM stock; Tocris, England) to assess its effect on integrin-mediated signalling in MSCs. A final concentration of 0.05µM PF228 (Slack-Davis *et al.* 2007) was added to culture medium for the duration of the testing period. Medium containing PF228 was replaced every 3-4 days

2.8 Two-dimensional mechanical loading of MSCs

A mechanical stimulus was applied to the mesenchymal stem cells in the form of cyclic tensile strain. Mechanical rigs were used to stretch the silicone substrate that the cells were seeded to.

2.8.1 Single-station bioreactor

The single station rig (Figure 2.2) is a custom built mechanical device (Department of Mechanical Engineering, School of Engineering, Trinity College Dublin) that delivers a cyclic uniaxial stretch to the silicone substrate and the cells seeded to it. 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 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.

2.8.2 Multi-station bioreactor

The aim of this project was to determine how mechanical stimulation effects MSC differentiation and the aged response to tensile strain. In order to carry out an extensive investigation, a custom made uniaxial multi-station bioreactor (Figure 2.3; Department of Mechanical Engineering, School of Engineering, Trinity College, Dublin) was designed and fabricated to facilitate cyclic tensile loading of five samples simultaneously. The main components of the bioreactor include the clamping system, medium baths with individual wells for each sample and a motor assembly, including the cam/follower/spring assembly. Rotation of the cam is controlled by the DC motor, which drives the follower causing the spring to provide displacement. Eccentric cams with various offsets allow for a range of strains to be applied to samples.

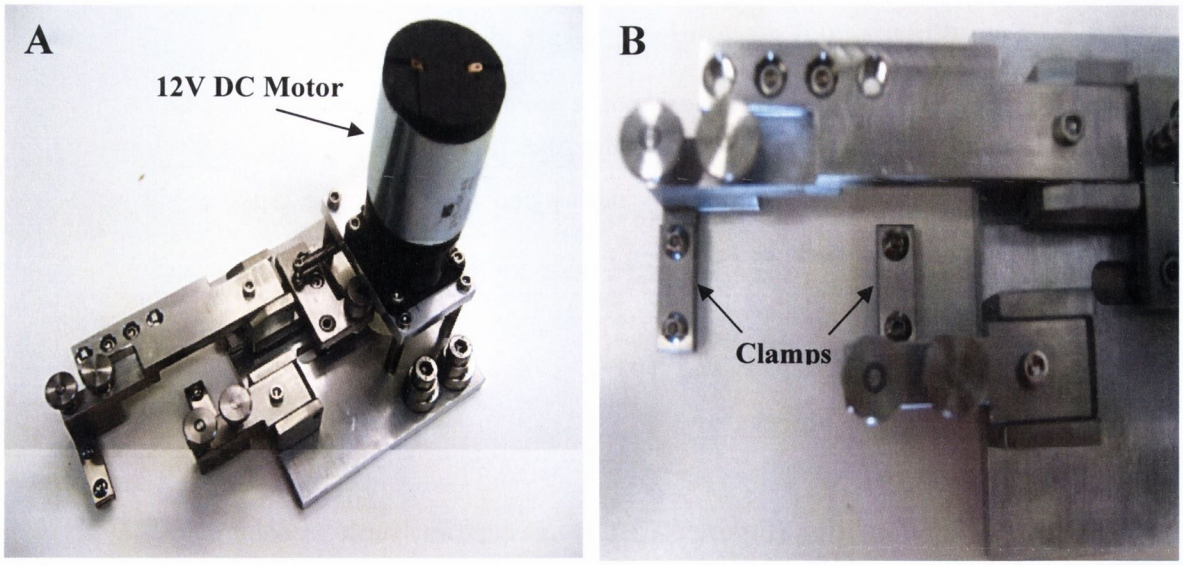


Figure 2.2: Single-station uniaxial strain device

(A) Uniaxial strain device for single substrate. (B) Clamps between which substrate is attached. The clamp on the right is attached to the stationary arm, the clamp on the left is attached to the moveable arm which causes the displacement.

2.9 Application of mechanical strain

2.9.1 Single-station bioreactor

Cyclic tensile strain was applied to the cells on a single silicone section using the mechanical 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 a sterile uncovered petri dish under the linear slide assembly. One seeded collagen-coated silicone section was carefully aligned perpendicularly across the clamps and fixed into position one side at a time so that it was firm between the clamps but with zero strain being applied. The petri dish was filled with enough sDMEM to cover the section. The apparatus was incubated at 37°C in 5% CO_2 and 95% humidity. Power connections were established between the 12V DC geared motor and the transducer. Fresh sDMEM was added to a control strip and this was also moved to the incubator. The rate of stretch was set to a frequency of 0.17Hz by adjusting the voltage from the transducer. This frequency had

previously been established within the lab in similar studies, from which this study stemmed, thus it was deemed appropriate to maintain the same experimental parameters. At this rate, the silicone section would complete one stretch-relax cycle every 6s (3s stretch–3s relax), calculated using a timer. The cells were stretched for 3 days at a magnitude of 10% or 24hr at 5% strain.

2.9.2 Multi-station bioreactor

Cyclic tensile strain was applied to the cells on five silicone substrates using the multi-station mechanical rig. The clamping frame was designed so that it could be completely detached from the motor assembly to facilitate the easy insertion of samples into the system. Samples were clamped between two stainless steel plates that are secured together with two screws. Stainless steel retaining bars allow for the frame to be held rigid during the clamping process and for the transport of the system to the rig. A separate static culturing frame was designed to hold one clamping frame and medium bath (Figure 2.3). This facilitates the static culturing of samples as clamped controls whereby zero strain is applied to the cells. The static frames contain a spring/release mechanism which when locked in place holds the clamping frame in the medium bath and when released, raises the clamping frame above the height of the bath so that both apparatus can be easily removed. The rate of stretch was set to a frequency 0.17Hz by adjusting the voltage from the transducer accordingly so that the strips would complete one stretch-relax cycle every 6s, calculated using a timer. The cells were stretched for 1 week at a magnitude of 2.5% or 24hr at 5%.

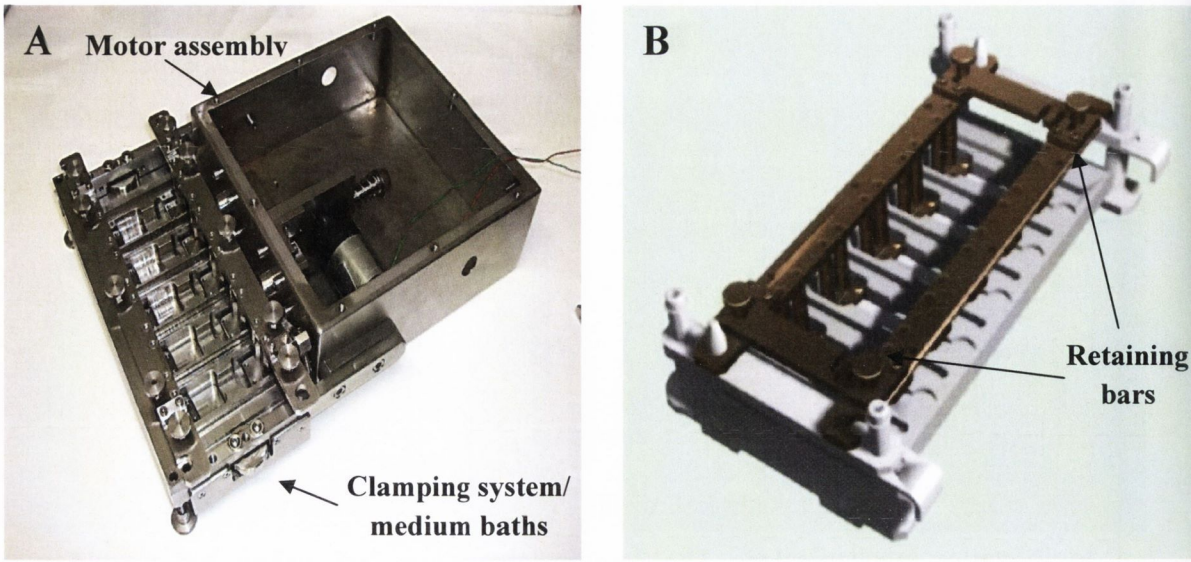


Figure 2.3: Multi-station uniaxial strain device

Multi-station uniaxial strain device for multiple substrates (A). Design image of clamping frame slotted into static culture frame with individual wells in the medium bath for each substrate (B).

2.10 Cell fixation

Cells seeded onto coverslips or silicone substrates were fixed to cease all metabolic activity and preserve the cells. Medium was removed from wells upon reaching 80-90% confluency after 2-3 days in culture or silicone substrates were removed from the rig following mechanical testing, followed by a single wash with PBS. Cells were fixed by adding 4% paraformaldehyde for 25min at room temperature (22°C). The coverslips and substrates were then washed three times with PBS and stored in PBS at 4°C until required for histological or immunocytochemical analysis.

2.11 Histological methods

Cells were examined for the expression of bone and cartilage related proteins osteocalcin and collagen II and signalling proteins integrin subunit α2, actin, pFAK and pERK by fluorescence immunocytochemistry. Markers of

mineralisation were assessed by examining calcium deposits using Von Kossa staining. Proteoglycan deposition was examined using Safranin-O staining and lipid accumulation was addressed using an Oil Red-O stain. All histological methods on mechanically loaded samples were preceeded by removing the outer 5 mm from the substrate that was clamped in the rig, dividing the remaining part of the strip into equal sections and placing each section onto a separate coverslip for staining.

2.11.1 Fluorescence immunocytochemistry

Day 1

Cells on coverslips or silicone sections were washed with PBS for 5min, then permeabilised using 0.1% Triton x100 (Sigma-Aldrich, England) in PBS for 10min. [For BrdU staining, add 500ul HCL (2M) and incubate at 37°C for 30min. Remove HCL and neutralise with 0.1M Borate Buffer (pH 8.5)].Cells were washed 3x5min in PBS and non-reactive sites were blocked with 20% blocking buffer (PBS with heat-inactivated horse/goat serum; Vector, USA) for 2hr at room temperature (22°C). The samples were incubated overnight in 100µl primary antibody (see Table 2.2) at 4°C.

Day 2

Cells were washed 3x5min in PBS, incubated with a biotinylated secondary IgG (see Table 2.2) for 1hr at room temperature (22°C) and washed 3x5min in PBS. In a darkened room, the light sensitive fluorescent dye Extravidin FITC (1:500 dilution in blocking buffer; Sigma-Aldrich, England) was added for 1hr and cells were covered to avoid any possible exposure to light then washed 6x5min in dH_2O. Hoescht stain (1:1000 dilution in PBS) was added for 7min to visualise the nuclei and cells were washed 2x5min in PBS. Coverslips were mounted, cell side down, onto glass slides in Vectashield mounting medium (Vector, USA) and sealed using clear nail varnish. Silicone samples were mounted cell side up in Vectashield and covered with a glass coverlsip prior to sealing. Slides were stored in the dark at 4°C until incorporated fluorophores were examined at 40x magnification with a confocal microscope (Zeiss 510 Meta, Germany).

Double staining

Cells on coverslips or silicone sections were washed with PBS for 5min, then permeabilised using 0.1% Triton x100 (Sigma-Aldrich, England) in PBS for 10min. Cells were washed 3x5min in PBS and non-reactive sites were blocked with 20% blocking buffer (PBS with heat-inactivated goat serum; Vector, USA) for 2hr at room temperature (22°C). The samples were incubated at 4°C overnight in 100µl primary antibody cocktail, containing both primary antibodies in blocking buffer (see Table 2.2). In a darkened room, cells were washed 3x5min in PBS, incubated with an alexa-conjugated secondary IgG (see Table 2.2) for 1hr at room temperature (22°C). Cells were covered for this incubation to avoid any possible exposure to light then washed 6x5min in dH_2O. Hoescht stain (1:1000 dilution in PBS) was added for 7min to visualise the nuclei and cells were washed 2x5min in PBS. Coverslips were mounted, cell side down, onto glass slides in Vectashield mounting medium (Vector, USA) and sealed using clear nail varnish. Silicone samples were mounted cell side up in Vectashield and covered with a glass coverlsip prior to sealing. Slides were stored in the dark at 4°C until incorporated fluorophores were examined at 40x magnification with a confocal microscope (Zeiss 510 Meta, Germany).

Image acquisition and analysis

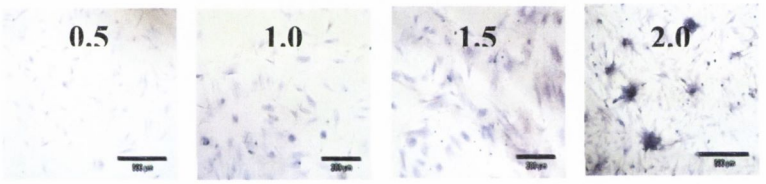
All images were acquired using a Zeiss 510 Meta confocal microscope. For each study, staining protocols were optimised prior to image analysis; optimising antibody and fluorophore dilutions, determining optimum settings for acquiring the immunofluorescent signals and measuring fluorescence in samples stained without a primary antibody. This value was subtracted from the intensity value recorded to rule out unspecific binding on the data generated. Furthermore, each cell was outlined individually prior to recording fluorescence intensity values to minimise the influence of unspecific background staining and once parameters were set for each signal, no changes were made to these settings between experiments to ensure repeatability.

Primary Antibody	Antibody Source	Secondary Antibody	Antibody Dilutions	Supplier
α2	Rabbit Polyclonal	Biotinylated Goat anti-Rabbit IgG	1^{o} 1:500 2^{o} 1:500	Millipore, Ireland
Actin	Mouse Monoclonal	Biotinylated Horse anti-Mouse IgG	1^{o} 1:1000 2^{o} 1:500	Sigma Aldrich, England
Active Caspase-3	Rabbit Polyclonal	Biotinylated Goat anti-Rabbit IgG	1^{o} 1:1000 2^{o} 1:1500	Promega, U.S.A.
BrdU (FITC-Conjugated)	Mouse Monoclonal	Biotinylated Horse anti-Mouse	1^{o} 1:500	Abcam, UK
Collagen II	Mouse Monoclonal	Biotinylated Horse anti-Mouse	1^{o} 1:500 2^{o} 1:500	Abcam, UK
	Rabbit Polyclonal	Alexa Fluor 488 Goat anti-Rabbit	1^{o} 1:300 2^{o} 1:600	Invitrogen
Osteocalcin	Mouse Monoclonal	Biotinylated Horse anti-Mouse	1^{o} 1:500 2^{o} 1:500	Abcam, UK
		Alexa Fluor 594 Goat anti-Mouse	1^{o} 1:400 2^{o} 1:700	Invitrogen
Phosphorylated-ERK	Mouse Monoclonal	Biotinylated Horse anti-Mouse	1^{o} 1:200 2^{o} 1:500	Abcam, UK
Phosphorylated-FAK	Mouse Monoclonal	Biotinylated Horse anti-Mouse	1^{o} 1:200 2^{o} 1:500	Abcam, UK

Table 2.2: Antibodies used in immunocytchemistry

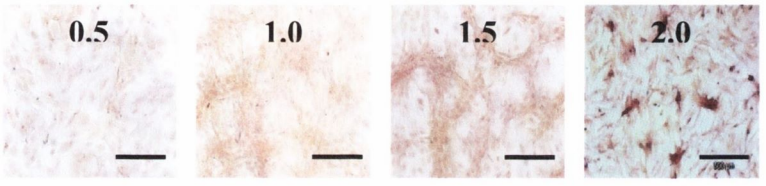
2.11.2 Von Kossa

Von Kossa staining is also used to detect extracellular calcium phosphate deposition and is used as a late marker of bone formation. Cells were incubated in 5% silver nitrate (500µl per slide in dH_2O; Sigma-Aldrich, England) under an electric lamp for 1hr at room temperature. Cells were washed five times in dH_2O (3min each) and placed in 5% sodium thiosulphate (in dH_2O , Sigma-Aldrich, England) for 2min. Cells were washed again three times in dH_2O and placed in Toludine Blue for 10sec. Cells were washed three times before imaging instantaneously (for silicone substrates). The samples were examined at 10x, 20x and 40x using phase contrast microscopy (Olympus CKX41, Japan). Following this, images were scored according to their staining intensity along a differentiation index. The index ranged from 0-2 arbitrary units, rising in increments of 0.5 units, whereby 0 represents no positive staining and 2 represents strong, intense positive staining (examples shown below).



2.11.3 Safranin-O

Safranin-O staining is used to detect proteoglycan deposition and is used as a marker of chondrogenesis. Fixed cells on silicone substrates were washed with PBS for 5min, stained with 500µl Safranin-O stain (1% in dH_2O; Sigma-Aldrich, England) for 25min then washed four times in dH_2O. Samples were examined at 10x, 20x and 40x magnification using light microscopy (Olympus CKX41, Japan). Following this, images were scored according to their staining intensity along a differentiation index. The index ranged from 0-2 arbitrary units, rising in increments of 0.5 units, whereby 0 represents no positive staining and 2 represents strong, intense positive staining (examples shown below).



2.11.4 Oil Red-O

Oil Red-O staining is used to detect lipid accumulation and is used as a marker of adipogenesis. Fixed cells on silicone substrates were washed with dH_2O for 5min, stained with 500µl Oil Red-O working solution (6ml stock in 4ml dH_2O; stock 0.6% in dH_2O; Sigma-Aldrich, England) for 1hr on an orbital shaker then washed four times in dH_2O. Samples were examined at 10x, 20x and 40x magnification using light microscopy (Olympus CKX41, Japan – see Appendix I).

2.11.5 Colorimetric TUNEL

Apoptotic cell death was identified using the DeadEnd™ Colorimetric TUNEL system (Promega Corporation, Madison, USA) in accordance with the manufacturer's instructions. Fixed sections of the silicone substrate were washed twice with PBS. Cells and nuclei were permeabilised with 0.2% Triton x100 for 5min then washed 3 times with PBS. This was followed by incubation with equilibration buffer for 10min. 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°C for 1hr. A 20X SSC solution was made up by diluting 1:10 with distilled water and added for 15min to terminate the reaction. Unincorporated nucleotides were removed by washing in PBS 3x5min. Endogenous peroxidases were blocked by adding hydrogen peroxide (1:100, distilled water) for 3–5 mins, and strips were washed 2x5min each. A solution of horseradish-peroxidase-labelled Streptavidin (1:500, PBS) was incubated at room temperature on the sections for 40min 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 (approximately 3–10mins), the sections were washed several times in dH_2O. The cells were then counterstained in methyl green (5%, dH_2O) for 10min and washed in dH_2O twice before mounting on to a glass slide. Cells were observed and imaged immediately. Observation was by 10x, 20x, and 40x magnification using light microscopy. The nuclei of TUNEL positive cells stained darker than the

negatively stained nuclei, while healthy nuclei stained pale green. TUNEL positive cells were counted and expressed as a percentage of the total number of cells in the view.

2.12 Protein harvesting

2.12.1 Sample preparation

All equipment and solutions were kept on ice during harvesting. Following 2-3 days in culture, medium was removed from the T75 flasks. For Western Immunoblotting, flasks were washed twice with ice-cold PBS and 1ml *complete* lysis buffer [10mM 2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (Tris-HCl), 50mM sodium chloride (NaCl), 10mM sodium pyrophosphate ($Na_4P_2O_7$), 50mM sodium fluoride (NaF),1mM sodium orthovanadate (Na_3VO_4), 1mM phenylmethylsulphonyl fluoride (PMSF), 50μM thre-2,3-dihydroxy-1,4-dithiolbutane (DTT), 6.25μg/ml Pepstatin A, 6.25μg/ml Apoprotin, 10μg/ml leupeptin] was added. Using a cell scraper, the bottom surface of the flask was vigorously scraped for approximately 30s in all directions to remove all adhering cells. Cell samples were stored in 2ml Eppendorf tubes at -20°C until required.

2.12.2 Protein quantification and sample equalisation

The protein concentration in cell samples was calculated according to the Bradford method (1976). Standards were prepared from a stock solution of 1000μg/ml bovine serum albumin (BSA) in dH_2O, ranging in concentration from 0μg/ml-1000μg/ml. The standards and samples (10μl) were added in triplicate into a 96-well plate (Sarstedt, Leicester, England) and 200μl of filtered BioRad dye reagent (1:5 dilution in dH_2O, BioRad Laboratories, Munich, Germany) was added to each well and incubated at room temperature (22°C) for 5min. Absorbance readings were recorded using Genesis Lite Software (version 3.03, Life Labsystems Inc., USA) at a wavelength of 595nM in a fluorometer (Labsystems Multiskan RC, MTX Labsystems Inc., USA). A standard curve was

constructed from the data (Microsoft Excel) and used to calculate the concentration of protein in each sample. Samples were then equalised to 1mg protein/ml using *stock* lysis buffer or solution and stored at -20°C until required.

2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.13.1 Preparation of sodium dodecyl sulphate (SDS) polyacrylamide gels

A polyacrylamide separating gel was used to study the proteins of interest. Gels were prepared between glass plates (10cm wide) with an approximate 1mm gap between them. To detect α2 and actin, a 10% separating gel was prepared [bis-acrylamide (30% stock) 33% v/v, dH_2O 40% v/v, Tris-HCl (1.5M, pH 8.8) 25% v/v, SDS (10% w/v stock) 1% v/v, APS (10% w/v stock) 0.5% v/v, TEMED 0.06% v/v]. Separating gel was pipetted between the glass plates, covered in isopropanol and left to set for 30min. Isopropanol was poured off and the gel was washed once with dH_2O. The separating gel was overlaid with a 4% stacking gel [bis-acrylamide (30% stock) 13% v/v, dH_2O 60% v/v, Tris-HCl (1.5M, pH 8.8) 26% v/v, SDS (10% w/v stock) 1% v/v, APS (10% w/v stock) 0.5% v/v, TEMED 0.16% v/v], followed by the immediate introduction of a 10 or 12-well comb to construct the wells, and was left to set for 30min.

2.13.2 Polyacrylamide gel electrophoresis

Equalised samples were diluted 1:2 in sodium dodecyl sulphate (SDS) sample buffer (50mM Tris-HCl, pH 7.4, 10% w/v glycerol, 4% w/v SDS, 5% v/v β-mercaptoethanol, 0.002% w/v bromophenol blue) and heated to 90°C for 5min to denature the 3-dimensional (3D) structure of the proteins. Set gels were mounted on an electrophoresis unit (Sigma-Aldrich, England) using spring clamps to hold them in place. The upper and lower reservoirs were flooded with 1x electrode running buffer [5mM 2-amino-2-(hydroxymethyl) propane-1, 3-diol (Tris-base), 3.4mM SDS, 40mM glycine] and air bubbles beneath the gels were extruded. A

pre-stained molecular weight marker (5µl, BioRad Laboratories, Munich, Germany) was added to the first well of each gel to confirm the molecular weight of protein bands. Subsequent wells were loaded with 10µl of prepared sample. Proteins were separated by applying a current of 32milliamps (mA) at maximum voltage to the gel apparatus. The migration of bromophenol blue through the gel was monitored and the current was switched off as the dye approached the bottom of the gel.

2.13.3 Semi-dry electrophoretic blotting of proteins

The gel was removed from the electrophoresis unit and washed in cold (4°C) transfer buffer (25mM Tris-base, 192mM glycine, 20% methanol v/v, 0.05% SDS w/v, pH 8.3). It was placed on top of a sheet of nitrocellulose blotting paper (0.45µm pore size; Sigma-Aldrich, England), pre-soaked in transfer buffer. The gel and nitrocellulose paper were then sandwiched between two sheets of pre-soaked filter paper (Standard Grade 3, Whatman, Kent, England), trimmed to size and placed onto the pre-wet platinum-coated titanium electrode (anode) of the semi-dry blotter (Sigma-Aldrich, England). The stainless steel lid (cathode) was moistened with transfer buffer and placed on top of the sandwich. The uncovered portion of the cathode was shielded with a thin cut-out of insulating material to ensure the applied current passed through the blot. A constant current of 225mA was applied for 75min, at maximum voltage, to transfer proteins from the gel to the nitrocellulose paper.

2.13.4 Western Immunoblotting

The nitrocellulose blotting paper was blocked for non-specific binding with 10ml of a blocking agent (see Table 2.3), diluted in TBS containing 0.05% Tween (TBS-T; 20mM Tris-HCl, 150mM NaCl, 0.05% Tween, pH 7.5), overnight in a refrigerator (4°C). The blots were washed 4x15min in TBS-T and probed for targets with a primary antibody (1°Ab; see Table 2.3) for 1hr at room temperature (22°C), then washed 2x5min and 4x10min in TBS-T. The blots were then probed

with a horse radish peroxidise (HRP) conjugated secondary antibody (2°Ab; see Table 2.3) and incubated for 2hr at room temperature (22°C) before washing 2x5min and 4x10min in TBS-T. A chemiluminescent chemical (1ml; SuperSignal, Pierce Biotechnologies, USA) was applied to each blot for 5min followed by digital imaging in a dark box recorder (Fujifilm LAS-300 Intelligent Dark Box). Images were acquired and densitometric analysis was carried out using Image Reader LAS-300 and Multi-Gauge V2.2. Re-probing blots for a second protein was carried out following initial chemiluminescent detection. Blots were incubated for 5min in a stripping solution (1:10 ReBlot™ PlusKit, Chemicon, USA) and washed 2x5min in TBS-T. The blots were then re-blocked overnight before being probed with the antibody specific for an alternate protein of interest.

Protein Target	Antibody Source	Blocking Agent	Secondary Antibody	Antibody Dilutions	Protein Band Size	Supp
α2	Rabbit Monoclonal	5% BSA	HRP Conjugated Goat anti-rabbit IgG	1° 1:500 2° 1:1000	165 kDa	Milli Irela
Actin	Mouse Monoclonal	5% BSA	HRP Conjugated Horse anti-mouse IgG	1° 1:1000 2° 1:2000	42 kDa	Sigm Aldri Engl
GAPDH	Mouse Monoclonal	5% BSA	HRP Conjugated Horse anti-mouse IgG	1° 1:500 2° 1:1000	36-40.2 kDa	Abca UK

Table 2.3: Antibodies used in Western Immunoblotting

2.14 *In vitro* analysis of messenger RNA expression

2.14.1 RNA isolation

Isolation of RNA was performed using a total RNA isolation kit (Macherey-Nagel). DNase I was prepared by adding 540µl RNase free H_2O to DNase I. RA3 buffer was prepared by adding 50ml EtOH to RA3. 70% EtOH was made by the addition of 70ml ethanol to 30ml RNase-free H_2O. RA3 buffer and cell lysis Mastermix were added to sterile tubes, followed by the addition of the MSC sample (trypsinised and pelleted). The samples were transferred through Nucleospin filter units and centrifuged at 11,000g for 1min. 350µl 70% EtOH was added to the homogenised lysate and mixed using a sterile pipette. The homogenised contents were filtered through Nucleospin RNA II column filters into sterile centrifuge tubes and the tubes were centrifuged at 8,000g for 30sec. The columns from these tubes were placed on new sterile collecting tubes. To desalt the silica membrane, 350µl membrane-desalting buffer was passed through the filter and the tubes were centrifuged at 11,000g for 1min, to dry the membrane. To digest the DNA, 95µl DNase reaction mixture was added to each sample directly onto the centre of the silica membrane of the column and incubated for 15min at room temperature (22°C). The silica membrane was washed and dried by adding 20µl RA2 buffer to each sample through the Nucleospin column, followed by centrifugation at 8,000g for 30sec. 600µl RA3 buffer was added through the Nucleospin column and the samples were centrifuged again at 8,000g for 30sec. For a final wash, 250µl RA3 buffer was added directly to the Nucleospin column and the samples were centrifuged at 11,000g for 2min. The RNA was eluted in 60µl RNase-free H_2O and centrifuged at 11,000g for 1min. To obtain a higher yield of RNA, the eluate was filtered once more through the Nucleospin filters and centrifuged for a final time at 11,000g for 1min. Columns were removed and samples were labelled and stored at -80°C until required.

2.14.2 mRNA Extraction and Reverse Transcription

Extracted mRNA from MSC samples was reverse transcribed into cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Darmstadt, Germany) according to the protocol provided by the manufacturer. 10µl RNA samples (containing 0.1µg mRNA/µl in nuclease-free H_2O) were added to sterile tubes. A 2x mastermix was then prepared containing the appropriate volumes of 10x RT buffer, 25x dNTPs, 10x random primer multiscribe reverse transcriptase (50U/µl). 10µl mastermix was added to the RNA and nuclease-free H_2O. Tubes were then incubated for 10min at 25°C followed by 2hrs at 37°C in a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland) - (See Appendix II).

2.15 Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 5.0 software.
Data are expressed as mean ± standard error of the means (SEM). Statistical analysis to compare two groups was performed using a suitable Student's t-test to determine whether significant differences existed between populations. When comparing more than two groups, statistical analysis was carried out by use of a one-way analysis of variance (ANOVA), followed by Neuman-Keuls *Post hoc* tests when significance was indicated. A p value of less than 0.05 was considered statistically significant. When comparing more than two groups or factors, statistical analysis was performed using a two-way ANOVA, followed by Bonferroni *Post hoc* tests to compare replicate means by row. All n values refer to a culture preparation derived from a single animal, except where indicated as independent observations which refer to observations made on imaging data based on several cells per image.

Chapter 3 Age-Related Alterations in Mesenchymal Stem Cells

3.1 Introduction

Mesenchymal stem cells (MSCs) are culture-adherent, multipotent progenitor cells capable of differentiating into bone, cartilage and fat. They exist in every tissue in the body and can be isolated from areas such as the bone-marrow, fat pad, synovial fluid and muscle (Caplan 2009). It is important to characterise these cells to determine the purity of the MSC populations during culture and experimental testing. Although no unique markers have been identified for MSCs to date, these progenitor populations have a distinctive set of cell surface markers which have been explored extensively. Criteria for classifying MSCs indicate that cells must be positive for surface markers CD105, CD73, and CD90 and negative for CD45, CD34, CD11b and CD19. Expression of these positive markers indicates isolation of pure bone marrow MSCs (Da Silva Meirelles *et al.* 2008).

When cultured *in vitro*, defined environmental conditions can drive MSC differentiation along specific lineages and it has been proposed that MSCs contribute to tissue repair, with therapeutic potential in repair and regeneration of several tissues (Picinich *et al.* 2007). There is evidence to suggest that these beneficial functions decrease with age, thus the process of MSC aging is of significant importance (Wilson *et al.* 2009). Intrinsic changes associated with aging in MSCs may explain altered differentiation patterns and response to stimuli. Reported age-related changes in MSCs include loss of differentiation potential, loss of proliferation potential and increases in senescent cell numbers. Markers of cellular aging that might underlie such age-related changes in MSC number and differentiation potential include changes in p53 and p21 expression, oxidative damage by reactive oxygen species (ROS) and nitric oxide (NO) and lipid peroxidation. The effects of these aging markers lead to a general loss of cell functionality and have profound physiological effects (Stolzing *et al.* 2008).

Loss of proliferation potential is often identified by a tendency towards a decrease in the number of colony forming units (CFU) with age (Kasper *et al.* 2009, Stolzing *et al.* 2006), although others report no changes (Stenderup *et al.* 2001). A plausible cause of such decreases in CFU's and an indicator of *in vitro* aging is a reduction in the cell's capacity to divide (Sethe *et al.* 2006). Baxter *et al.* (2004)

showed a decline in the replicative lifespan of human MSCs associated with donor age, causing a significant decrease in the overall growth rate of MSCs isolated from aged donors. Alterations in the rate of proliferation and overall cell number with age could dampen the efficacy of an aged-donor's cells being incorporated into a tissue-engineered construct.

Chemical species that contain one or more unpaired electrons are known as free radicals. When we oxidise any substrate with oxygen, the oxygen itself becomes reduced and is the source of free radicals (or ROS; Pratico 2002). ROS can be produced by several mechanisms, including irradiation, inflammatory processes, lipid peroxidation, and environmental pollutants (Stadtman and Berlett 1998). Disrupting the balance between the production of ROS (e.g.: superoxide and hydrogen peroxide) and antioxidant defences against them produces oxidative stress, which amplifies tissue damage by releasing pro-oxidative forms of reactive iron that drive lipid peroxidation (Gutteridge 1995). ROS and other free radical emissions by cells and tissues are often taken as an indicator and a cause of aging *in vitro* (Young *et al.* 2001) and *in vivo* (Droge 2003). Highly reactive oxygen species formed under normal metabolic conditions and during oxidative stress are able to either oxidise proteins or convert lipid derivatives to compounds that react with functional groups on proteins. Such ROS-mediated reactions cause the formation of protein carbonyl derivatives, a marker of protein damage caused by ROS, which associates oxidatively damaged protein with aging (Stadtman and Berlett 1998).

Free radicals are continuously produced *in vivo* and promptly react with their surroundings in two possible ways: firstly with other free radicals, so that their unpaired electrons join to make a covalent bond; or secondly with non-free radicals, which is the most common type of reaction. Depending on the type of substrate, different reactions can occur, including lipid peroxidation, protein oxidation, or DNA oxidation (Pratico 2002). The free radical theory of aging proposes that aging is due to the accumulation of unrepaired damage from free radical action on cellular components (Rikans & Hornbrook 1997, Harman 1956). An assumption of the free radical theory is that the free radicals contributing to the aging process are derived from oxygen, whether directly or indirectly. Lipid

peroxidation has been considered as the major process producing damage from oxygen radicals, thus the oxygen-based theory is deemed by some as the lipid peroxidation theory of aging. Stemming from this theory are several hypotheses that propose to explicate the link between peroxidation of membrane lipids and the deterioration characteristic of the aging process. One hypothesis is that age-related membrane lipid peroxidation causes fluidity changes in the membrane; mediated through the by-product malondialdehyde (MDA). The change in fluidity in turn disrupts vital functions, such as the maintenance of selective permeability to ions or signal transduction (Rikans & Hornbrook 1997, Chen & Yu, 1994). Free radicals react with membrane fatty acids and phospholipid components to form lipid peroxides, inducing irreversible impairment of membrane fluidity and plasticity, thereby causing irreversible damage to the cell's integrity (Pratico 2002). While it is evident that oxidation processes and oxidation end products play a role in MSC aging, there are limitations in their effectiveness as indicative markers of aging due to the lack of understanding regarding their propensity to regulate MSC differentiation and proliferation (Sethe *et al.* 2006).

Interactions between the cell and extracellular matrix are mediated by matrix components and cell surface receptors, including integrins. With aging there are qualitative and quantitative modifications of both matrix components and integrins and in disease conditions, some integrins appear to be differentially expressed (Labat-Robert 2001). Kasper *et al.* (2009) reported age-associated alterations to the cellular proteome of MSCs with and without osteogenic stimulation. Using high resolution 2-D electrophoresis approximately 5,000 protein spots were detected from MSC lysates. Among the proteins detected, 10 were significantly downregulated and 26 upregulated using osteogenic expansion media. In ordinary media conditions, the expression of 29 proteins was decreased and 40 proteins increased. Taken together, 14 proteins were age-dependently expressed under both media conditions. From all detected protein spots, 140 (of the 5,000) were differentially expressed under conditions of osteogenic stimulation. In contrast, from the detected age affected protein spots, 8 (of the 14) were altered following osteogenic stimulation, suggesting that a high number of age affected proteins in MSCs are also regulated during osteogenic differentiation. These proteome screening results were supported by lower actin turnover and

diminished antioxidant power in aged MSCs, respectively. It was postulated that the main reasons for the compromised cellular function of aged MSCs were a reduced responsiveness to biological and mechanical signals due to a less dynamic actin cytoskeleton and increased oxidative stress favouring molecular damage and cellular senescence. Taking these findings into account with similar differentiation potentials observed in MSC-based therapeutic approaches for older patients, studies should focus on ways to promote attracting the cells to the site of injury and mechanisms of oxidative stress protection, as opposed to simply stimulating differentiation.

There is increasing evidence to suggest disturbed cell–matrix interactions accumulating during aging (Labat-Robert 2004). A study by Hu *et al.* (1996), exploring the role of α5β1 integrin in cellular aging, compared the expression of the α5β1 integrin in early and late passage human diploid fibroblasts (HDF) and HDF derived from Werner syndrome patients. The α5β1 integrin is the receptor for fibronectin in the extracellular matrix, to which it has the highest affinity and specificity. Alterations were noted at both RNA and protein levels and in integrin-mediated functions. Senescent normal and Werner syndrome fibroblasts exhibited decreased expression of α5 polypeptides in the cell membrane, which may affect ligand binding. The membranes of senescent cells showed a decrease in functional heterodimers, and a decrease in cell adhesion and proliferation of these cells, indicating the activity of α5β1 in these functions and alterations occurring with age. The migratory ability of fibroblasts from young and aged donors has also been investigated to address age-related impairments in wound healing. Results indicate that both young and aged fibroblasts attach to plastic or collagen with similar efficacy. Although young and aged fibroblasts expressed comparable levels of the collagen receptor α2 integrin, poorly migrated aged fibroblasts exhibited a significant reduction in α2β1 function relative to fibroblasts with normal migratory abilities. Furthermore, the aged fibroblasts that exhibited poor migration showed a disordered actin cytoskeleton and reduced ability to contract collagen gels. Deficient migration by aged fibroblasts was characterised by disorganised cytoskeletal actin and reduced α2β1 function (Reed *et al.* 2001).

The aim of this study was to characterise the young and aged culture adherent cell populations following 3 weeks of expansion. This was done by quantitative identification of the positive MSC marker CD90 and negative marker CD45 using fluorescence activated cell sorting. Since there is conflict within the literature regarding age-associated changes in MSCs during aging, several characteristics of MSC biology were investigated. To examine cell proliferation, cells were incubated with the thymidine analogue BrdU followed by immunohistochemistry to identify incorporation within the nucleus. Expression of the α2 integrin and the cytoskeleton component actin were determined using immunocytochemistry and Western blotting to assess potential alterations in mechanosensitive components of the cell with age. Finally, lipid peroxidation was examined since it is associated with cellular aging and changes in membrane fluidity which could impact on MSC mechanosensitivity and therefore cell activity.

3.2 Results

3.2.1 Mesenchymal stem cell characterisation by flow cytometry

Flow cytometry was used to determine the purity of MSC populations in young and aged MSC culture preparations. It is a useful technique that quantifies cells based on the presentation of specific labelled antigens. To assess the homogeneity of cell culture preparations, cells were probed for the expression of the endoglin CD90 as a positive marker for MSCs and CD45 (haematopoietic stem cell marker) served as a negative control. Young and aged MSC populations highly expressed the CD90 epitope, whereas CD45 showed very low expression in these cell populations (overlay graph; Figure 3.1). Quantification of the raw data showed that 96.8±0.4% of the cells isolated from young rats were positive for CD90 whereas 2.1±0.8% of cells were positive for CD45. In cells isolated from aged rats 96.4±0.4% were positive for CD90 whereas 2.2±0.4% of the cells were positive for CD45 (Table 3.1, n=3, mean±SEM). These data show that following 3 weeks in culture, young and aged MSC cultures represent a homogenous CD90 positive and CD45 negative cell population and therefore can be referred to as MSC populations.

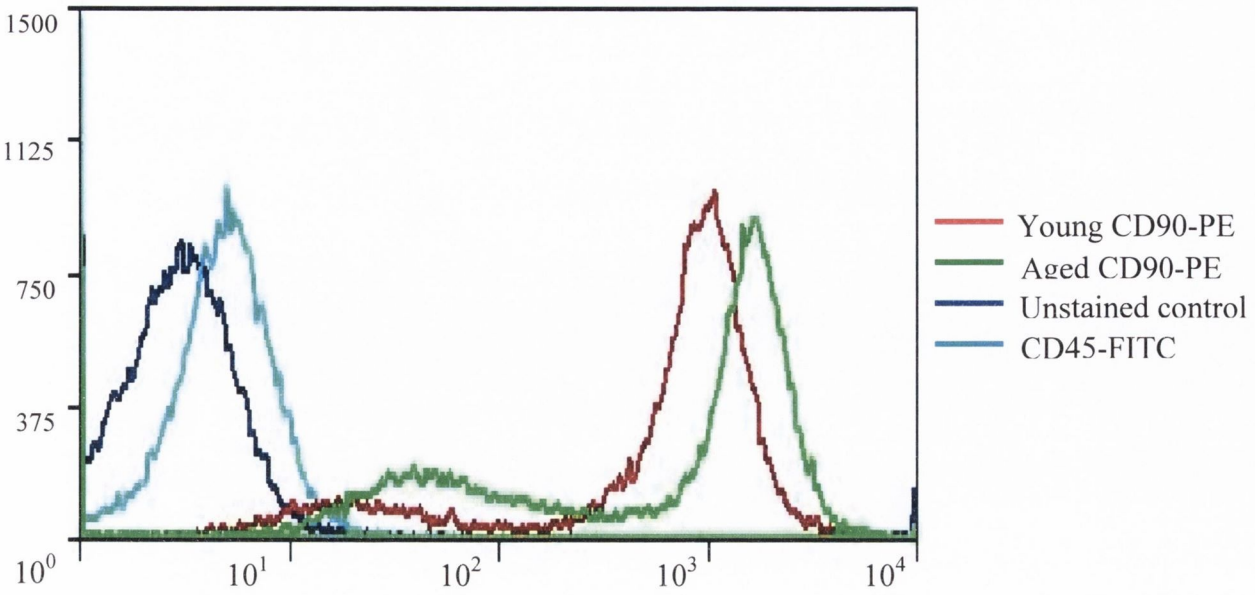


Figure 3.1: Flow cytometry analysis showing young and aged MSCs positive for CD90

Overlay graph illustrating CD90/CD45 positive cells, PE-isotype control and an unstained control. PE-CD90 was expressed at high levels in young and aged MSCs, with the majority of events taken falling at the higher end of the log scale whereas FITC-CD45 was detected at low levels, appearing at the lower end of the log scale (n=3).

Epitope	% Cells (Young)	% Cells (Aged)
CD90	96.8±0.4	96.4±0.4
CD45	2.1±0.8	2.2±0.4

Table 3.1: Quantification of CD90 and CD45 positive cells from flow cytometry analysis

3.2.2 Proliferation rate in MSCs is decreased with age

During the 3 week culture period, the MSCs isolated from aged rats appeared sparser in culture flasks, compared to MSCs isolated from young rats. Representative images showing the cells in culture highlight the difference in cell numbers between both age groups (Figure 3.2, B). To address this difference, the rate of proliferation in MSCs was assessed using the chemical 5-Bromo-2'-deoxy-uridine (BrdU), which incorporates into the DNA of dividing cells. MSCs isolated from young and aged rats were seeded onto glass coverslips in 24-well plates and were exposed to BrdU (10μM) for 24hr followed by methanol fixation and immunocytochemistry using an anti-BrdU antibody. Young MSCs expressed a significantly higher percentage of BrdU positive cells compared to aged MSCs (Figure 3.2, A), indicating a reduced rate of proliferation with age. The percentage of BrdU positive cells decreased from 72.60 ± 1.81% in young cells to 49.80 ± 3.47% in aged cells ($p<0.001$, Student's t-test, n=5, mean±SEM). Using fluorescence microscopy, cells that expressed positive immunoreactivity for BrdU (Figure 3.2, C) were counted. A percentage of BrdU positive cells was calculated against all cells present per coverslip, determined using a DAPI nuclear stain.

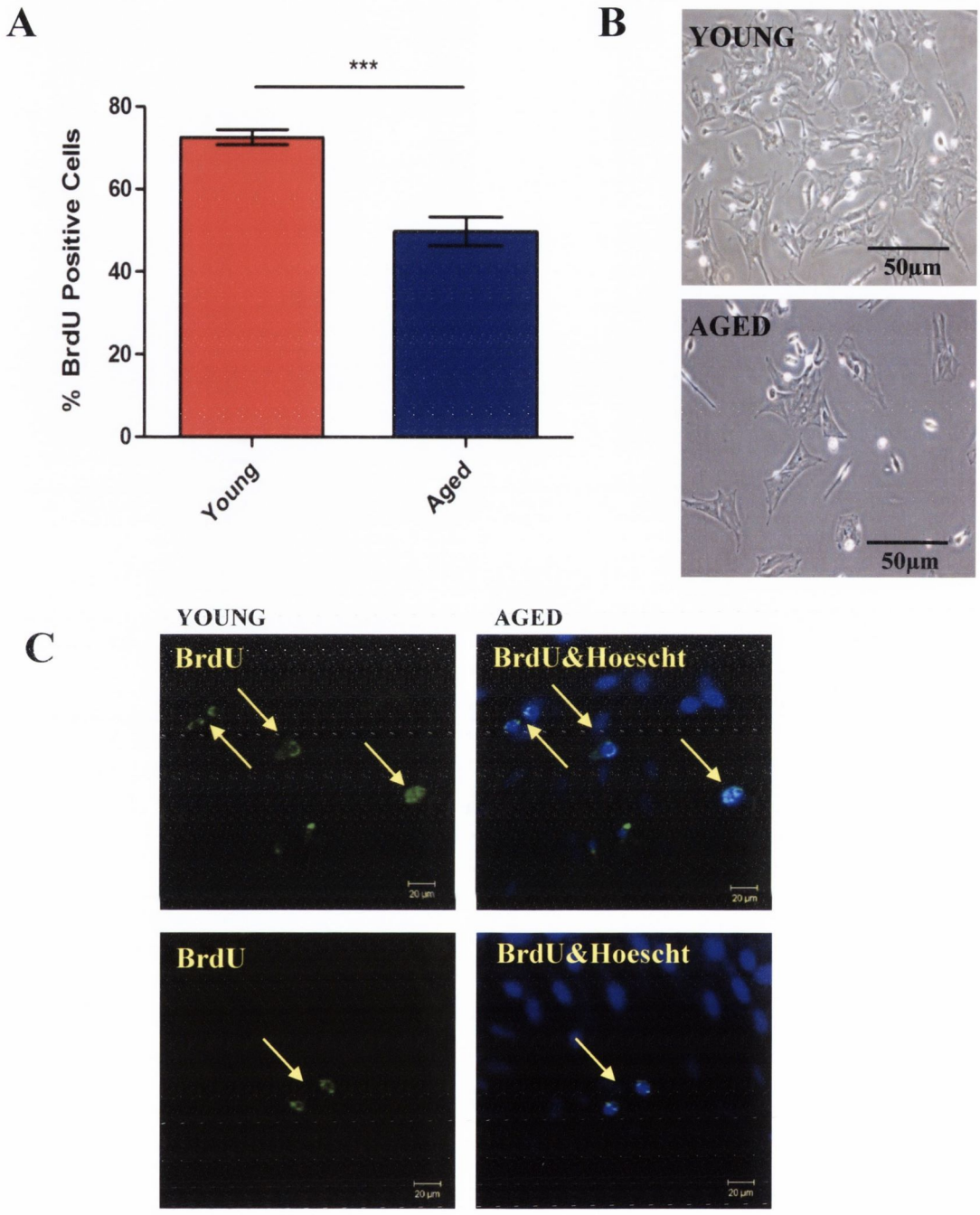


Figure 3.2: MSCs isolated from aged rats have a reduced rate of proliferation

MSCs were exposed to BrdU (10µM) for 24hrs. Cell proliferation was assessed by BrdU incorporation using immunocytochemistry for an anti-BrdU antibody. BrdU positive cells were counted and expressed as a percentage of total cells present. **A:** MSCs isolated from aged rats showed a significant reduction in BrdU incorporation (***$p<0.001$, Student's t-test, n=5 cultures). **B:** Representative images of young and aged MSCs at passage 3 in culture. **C:** Sample confocal images highlighting BrdU incorporation in young and aged MSCs and total cells present in the same field using a DAPI filter, with arrows indicating areas of BrdU incorporation. Results are displayed as mean ± SEM. Scale bar is 20µm.

3.2.3 Expression of α2 in MSCs is decreased with age

Integrins are a family of cell adhesion receptors that interact with the extracellular matrix and are involved in mechanotransduction. MSCs exhibit a number of specific integrins in the plasma membrane, including α2β1, which binds to collagen in the ECM (Miller *et al.* 2009, Takada *et al.* 2007). The expression of the α2 subunit of this receptor was assessed in young and aged MSCs by immunocytochemistry and confocal microscopy. A decrease in α2 immunoreactivity was observed in MSCs isolated from aged rats, as evidenced by a decrease in the fluorescence intensity (Figure 3.3, A). The mean fluorescence intensity significantly decreased from 181.40± 6.97 units in young MSCs to 122.20 ± 7.44 units in aged MSCs ($p<0.001$, Student's t-test, n=5). Results are presented and graphed as the mean percentage ± SEM. Distribution of α2 in the plasma membrane was often localised in dense regions around the membrane. Such regions may be indicative of focal adhesion complex formations discussed previously. Figure 3.3, B shows confocal images of α2 expression in MSCs isolated from young and aged MSCs and the localised distribution of this receptor subunit observed in aged MSCs.The distribution of the α2 integrin subunit varied amongst cells, whereby its expression was observed both in punctate areas around the plasma membrane as well as dispersed throughout the cell. Occasionally perinuclear staining was also observed.

Western Immunoblot was also used to analyse α2 expression and bands were detected at 165kDa, representing α2 expression. Bands were analysed and arbitrary units were obtained through densitometric analysis. Data is expressed as a ratio of α2 expression to the housekeeping protein GAPDH and graphed as mean ± SEM. Figure 3.4, A demonstrates that there is a significant difference in the expression of α2 between MSCs isolated from young and aged rats ($p<0.05$, Student's t-test, n=4). Arbitrary units representing α2 expression decreased from 3.79 ± 0.19 in young MSCs to 3.02 ± 0.13 units in aged MSCs. Representative western immunoblots demonstrating α2 expression are shown in Figure 3.4, B.

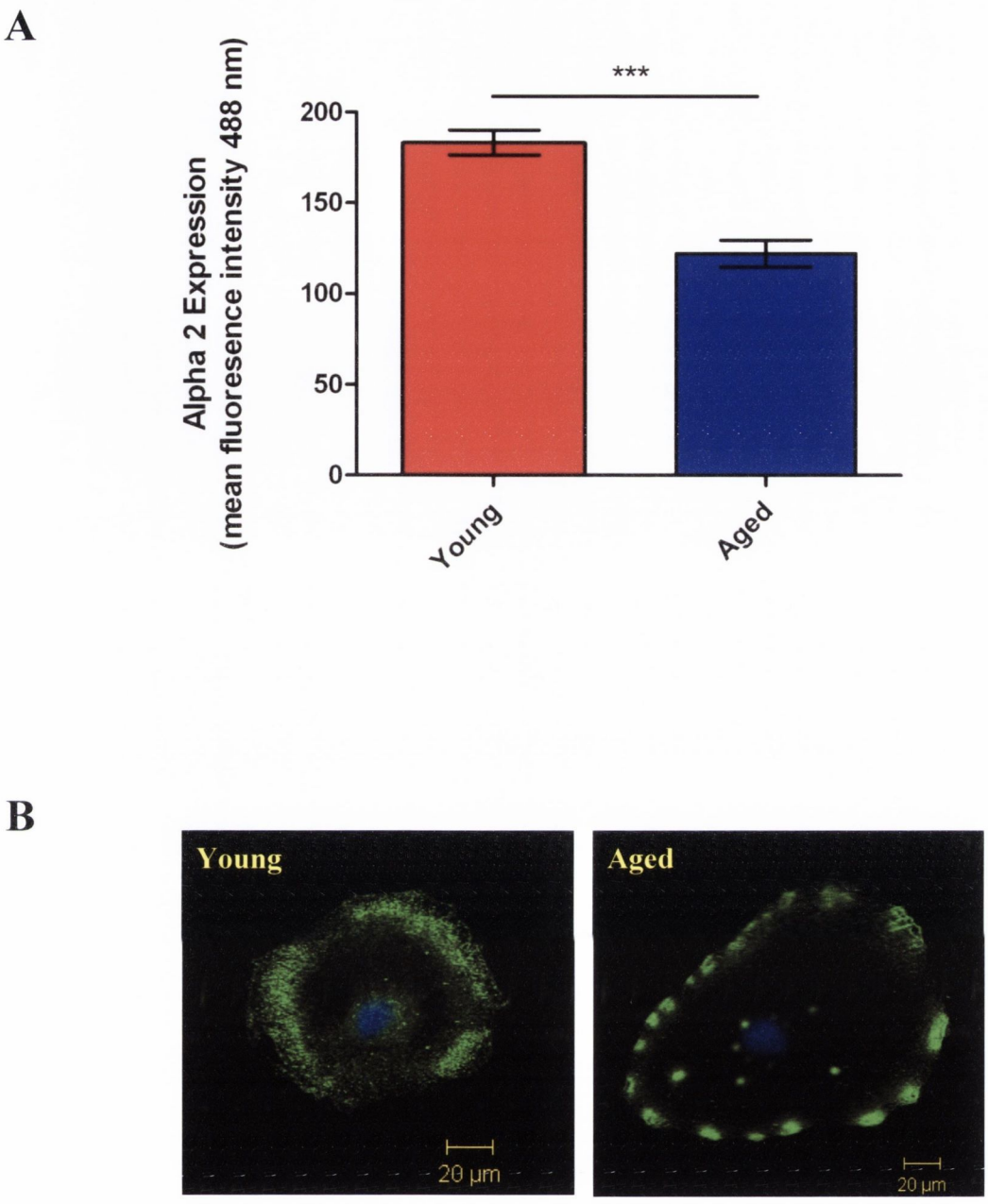


Figure 3.3: An age-related reduction in α2 expression in rat MSC cultures

MSCs were examined for the expression of the integrin subunit α2 using immunocytochemistry for an anti-α2 antibody. A decrease in expression was reflected by a decrease in mean fluorescence intensity. **A:** MSCs isolated from aged rats show a significant decrease in α2 expression (***$p<0.001$, Student's t-test, n=5 cultures). **B:** Confocal images of α2 expression in MSCs isolated from young and aged rats. Results are displayed as mean ± SEM. Scale bar is 20µm.

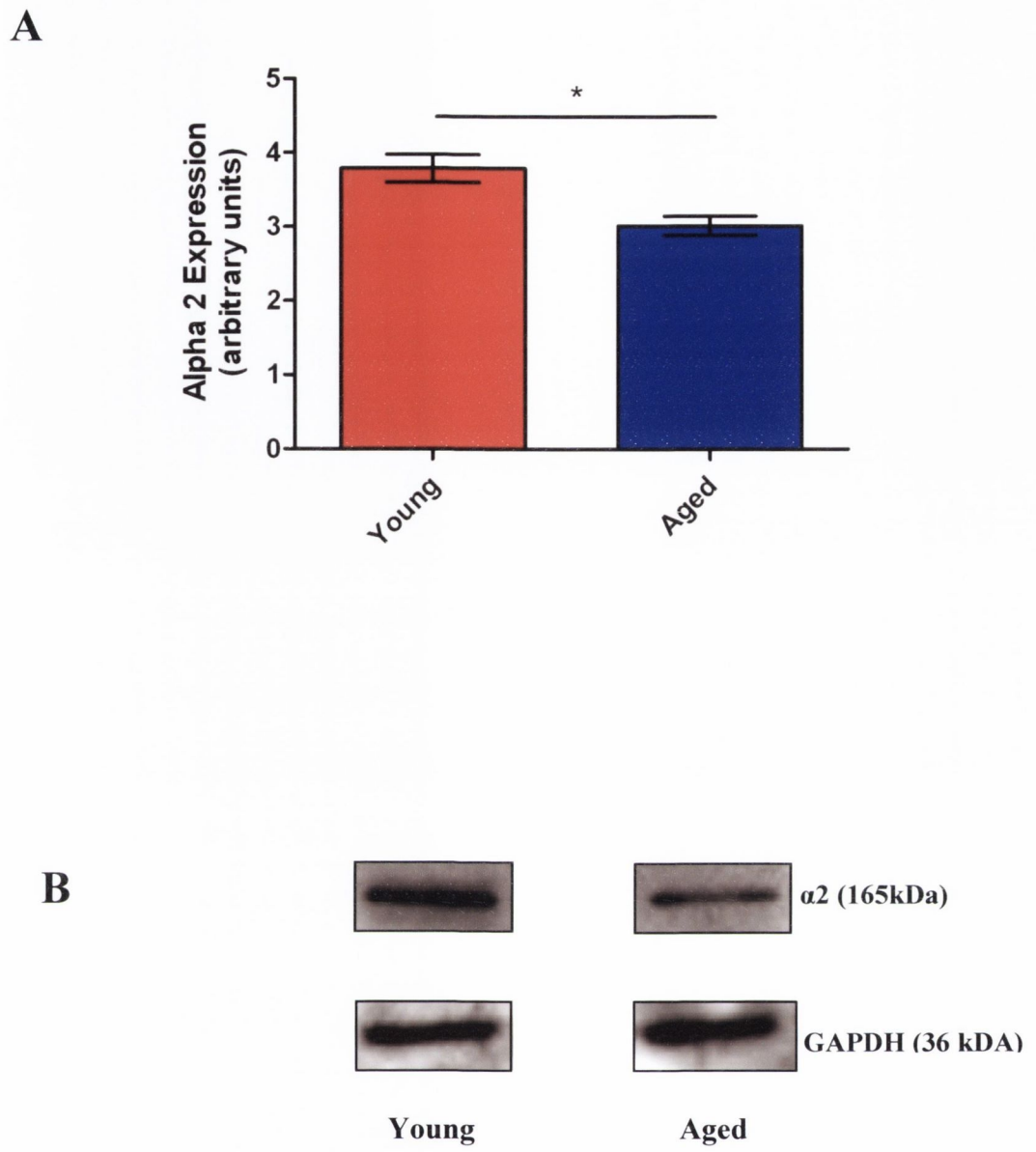


Figure 3.4: A reduction in α2 expression in MSCs isolated from aged rats

Expression of the integrin subunit α2 was further assessed by Western Immunoblot. A decrease in expression was quantified by a decrease in arbitrary units used to represent α2 expression. **A:** MSCs isolated from aged rats show a significant decrease in α2 expression ($p<0.05$, Student's t-test, n=4 cultures). **B:** Representative immunoblots of α2 and GAPDH. Results are displayed as mean ± SEM.

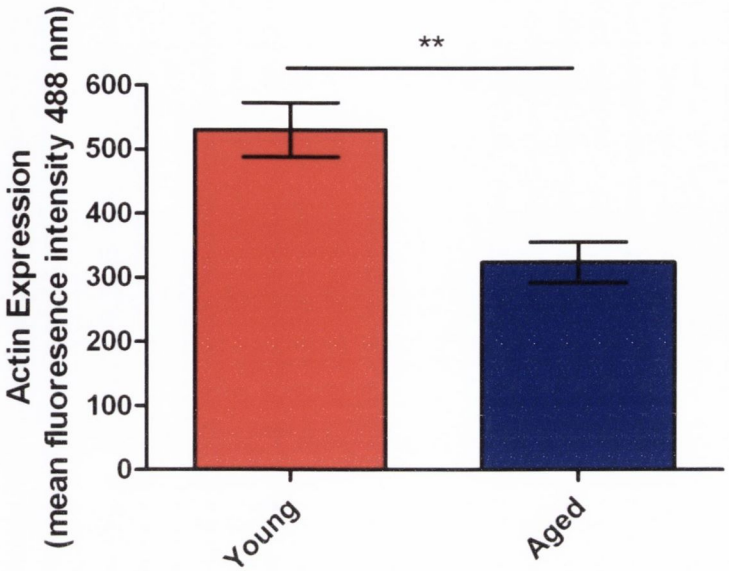
3.2.4 Expression of actin in MSCs is decreased with age

Actin is a component of the cytoskeletal network within the cell. It functions in maintaining cell shape and motility as well as playing a role in mechanotransduction via interaction with integrin receptors in the plasma membrane. Actin expression was assessed in young and aged MSCs by immunocytochemistry and confocal microscopy (Figure 3.5, A). A decrease in actin immunoreactivity was observed in aged MSCs as evidenced by a decrease in the mean fluorescence intensity, with values presented as mean ± SEM. Figure 3.5, B shows the typical staining pattern observed between young and aged MSCs in this study, whereby there was a clear decrease in the number of stress fibres visualised in aged cells compared to young. The fluorescence intensity significantly decreased from 530.30 ± 42.50 units in young MSCs to 323.50 ± 31.92 units in aged MSCs ($p<0.01$, Student's t-test, $n=5$).

Actin expression was also analysed by Western Immunoblot and bands were detected at 42kDa, representing actin expression. Bands were analysed and arbitrary units were obtained through densitometric analysis. Data is expressed as a ratio of actin expression to the housekeeping protein GAPDH and graphed as mean ± SEM. Figure 3.6, A demonstrates that there is a significant difference in the expression of actin between MSCs isolated from young and aged rats ($p<0.05$, Student's t-test, $n=4$). Arbitrary units representing actin expression decreased from 1.06 ± 0.07 in young MSCs to 0.70 ± 0.11 units in aged MSCs. Representative western immunoblots demonstrating actin expression are shown in Figure 3.6, B.

With a diverse range of functions within the cell, actin maintains the ability to reassemble its filaments to suit particular functions when necessary. Actin bundle thickness was observed using fluorescence images acquired by the confocal microscope. Using LSM Image Examiner software (Zeiss 510 Meta, Germany), a measurement tool was employed to measure bundle/strut thickness. Values obtained were averaged and graphed as mean ± SEM (Figure 3.7, A and B). There was a significant decrease in bundle thickness with age, whereby measurements decreased from 1.95 ± 0.08μm in young MSCs to 1.64 ± 0.06μm in aged MSCs.

A



B

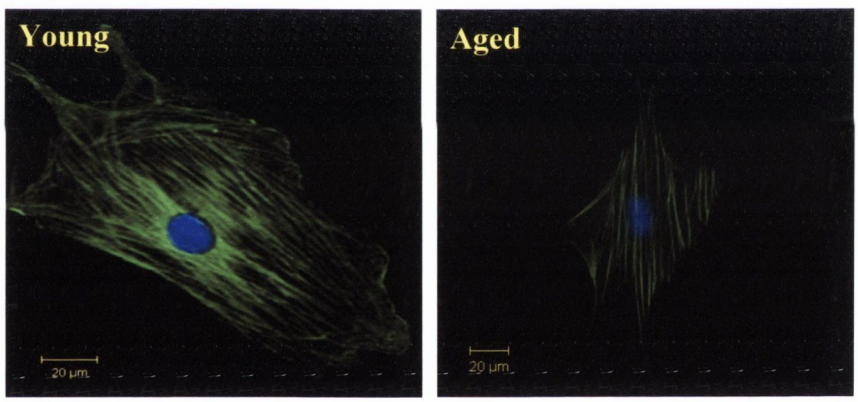


Figure 3.5: An age-related reduction in actin expression in rat MSC cultures

MSCs were examined for the expression of actin using immunocytochemistry for an anti-β-actin antibody. A decrease in expression was quantified by a decrease in mean fluorescence intensity. **A:** MSCs isolated from aged rats show a significant decrease in actin expression (**$p<0.01$, Student's t-test, $n=5$ cultures). **B:** Confocal images of actin expression in MSCs isolated from young and aged rats. Results are displayed as mean ± SEM. Scale bar is 20µm.

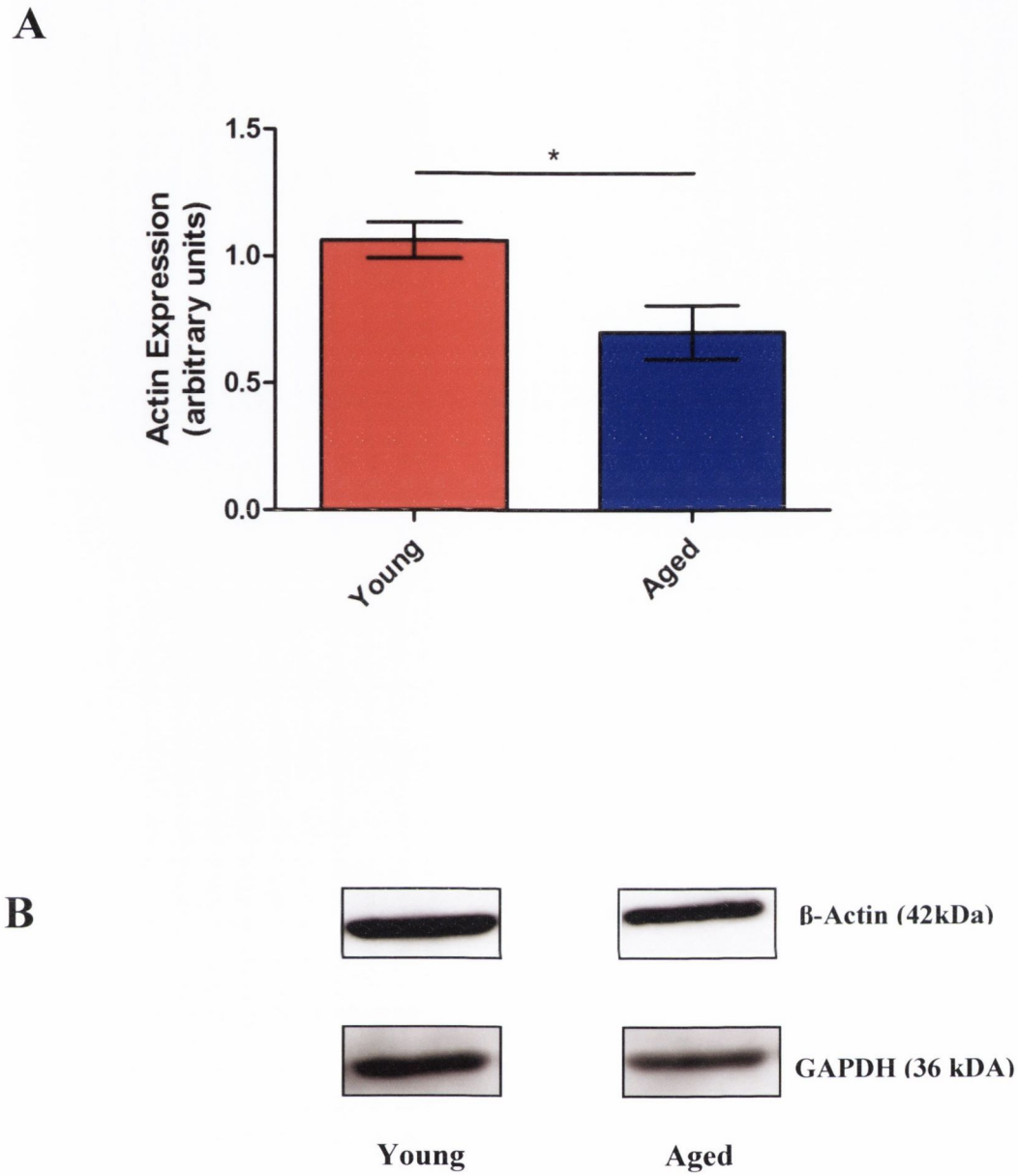


Figure 3.6: A reduction in actin expression in MSCs isolated from aged rats

Expression of actin was further assessed by Western Immunoblot. A decrease in expression was quantified by a decrease in arbitrary units used to represent actin expression. **A:** MSCs isolated from aged rats show a significant decrease in actin expression ($p<0.05$, Student's t-test, $n=4$ cultures). **B:** Representative immunoblots of actin and GAPDH. Results are displayed as mean ± SEM.

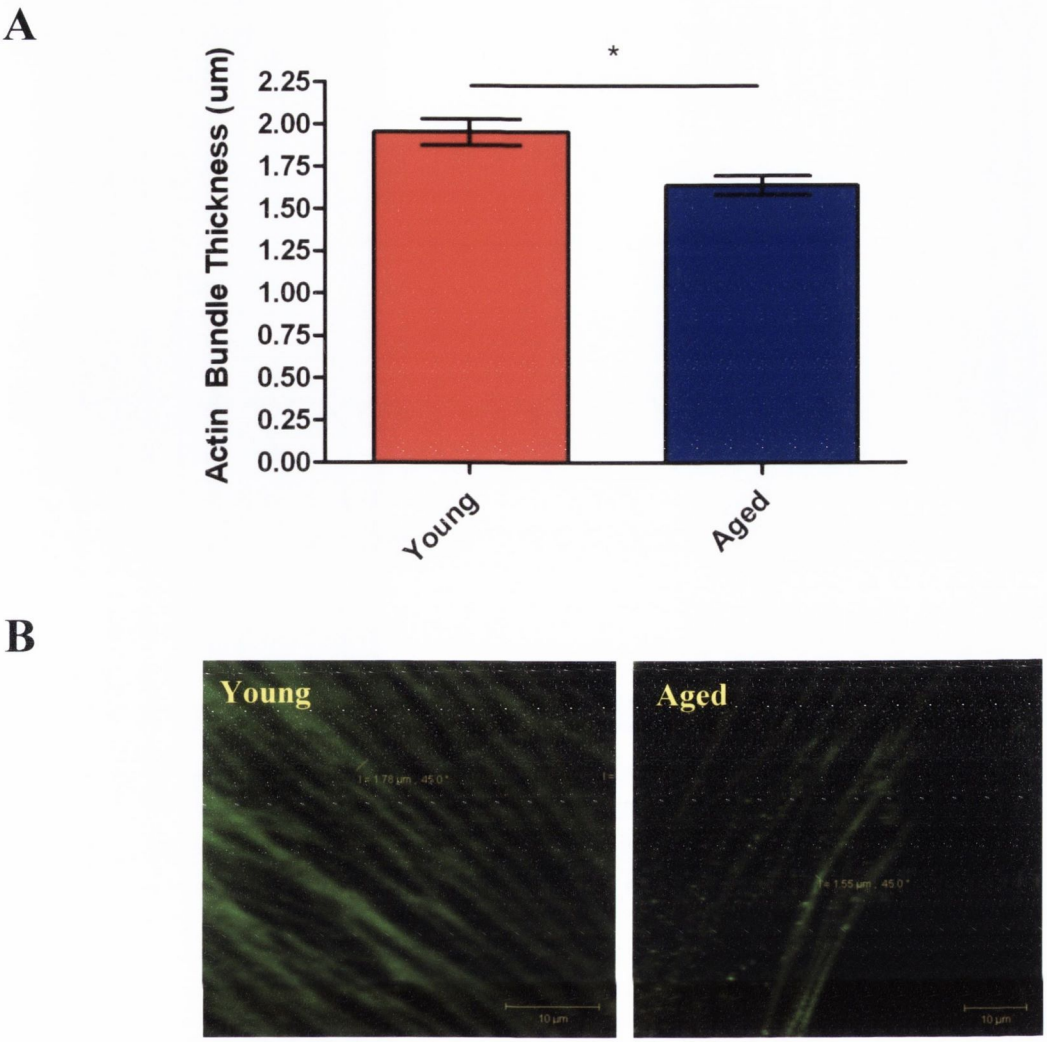


Figure 3.7: An age-related reduction in actin bundle thickness in rat MSC cultures

MSCs were examined for the expression of actin using immunocytochemistry for an anti-β-actin antibody. Actin bundle thickness was examined using a measuring tool on the LSM Image Examiner software. **A:** MSCs isolated from aged rats show a significant decrease in actin bundle thickness (*$p<0.05$, Student's t-test, n=5 cultures). **B:** Confocal images of actin expression in MSCs isolated from young and aged rats. Results are displayed as mean ± SEM.

3.2.5 Membrane lipid peroxidation in MSCs is increased with age

Membrane lipid peroxidation is a cellular process associated with aging that is caused by oxidative stress. Damage caused to the membrane lipids leads to a loss of the fluid mosaic nature of the membrane which becomes rigid in a compact state. Increased membrane rigidity with age may contribute to an alteration in the mechanoresponsiveness of MSCs. Lipid peroxidation was assessed in young and aged MSCs using a thiobarbituric acid reactive substance (TBARS) assay. Malondyaldehyde (MDA), a natural bi-product of lipid peroxidation, forms a 1:2 adduct with TBA, which can then be measured fluorometrically. The unknown MDA content in samples is determined by comparison with a predetermined MDA standard curve. An increase in lipid peroxidation was observed in MSCs isolated from aged rats, as evidenced by an increase in MDA content (Figure 3.8). There was a significant increase from 2.15±0.34 nmol MDA/g in young MSCs to 4.79±0.70 nmol MDA/g in aged MSCs ($p<0.01$, Student's t-test, $n=6$, mean±SEM).

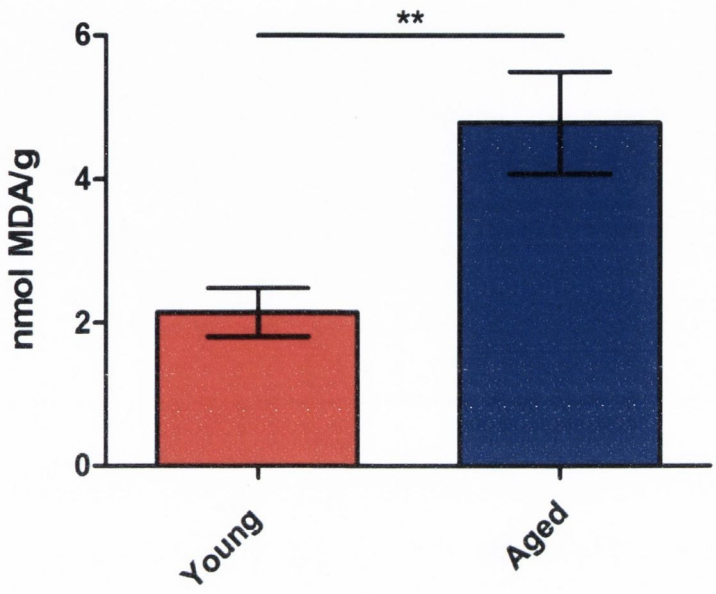


Figure 3.8: An age-related increase in lipid peroxidation in rat MSC cultures

MSCs were examined for lipid peroxidation using a TBARS assay. An increase in peroxidation of membrane lipids was quantified by an increase in MDA content in samples. MSCs isolated from aged rats show a significant increase in lipid peroxidation (**$p<0.01$, Student's t-test, $n=6$ cultures). Results are displayed as mean ± SEM.

3.3 Discussion

The aim of this study was to verify the adherent cells isolated from the bone marrow of young and aged rats as being homogenous MSC populations. Furthermore, various aspects of MSC biology were investigated to determine alterations associated with aging; this included examining cell proliferation, expression of mechanotransduction associated proteins and lipid peroxidation. It was established following approximately 3 weeks in culture that adherent cells isolated from femoral and tibial bone marrow preparations at passage 3 expressed the positive MSC marker CD90. Flow cytometry indicated that 97% of young cells and 96% of aged cells expressed CD90 as a surface marker while only 2% and 3% of young and aged cells expressed the haematopoietic marker CD45. It was concluded that both young and aged adherent cell populations were homogenous MSC preparations with a minor percentage of each expressing haematopoietic type cells. Young and aged MSCs were both shown to express integrin subunit α2 and cytoskeletal component actin by immunocytochemistry and Western immunoblot. However, there was a reduction in mechanosensory protein expression in aged MSCs. Furthermore, using a measuring tool on the confocal software revealed a decrease in actin strut thickness with age. Lipid peroxidation was increased with age due to the amplified amount of the by product malondialdehyde measured in aged cells compared to young.

Further to their ability to differentiate into various cell types, MSCs produce important growth factors and cytokines, and may provide vital cues for cell survival in damaged tissues, with or without active participation in long-term tissue repair. MSCs have become a prominent cellular candidate in attempts to heal a mounting list of tissues and have the ability to modify the response of immune cells. They are also one of the few cell sources studied that have so far been produced in the large quantities required for therapeutic applications (Le Blanc & Pittenger 2005). It is therefore of pivotal importance when investigating the therapeutic applications of these cells, that investigators are confident their findings are based on homogenous MSC populations and that other cell types do not pollute the data collected. MSCs are often characterised by their ability to proliferate in culture, a plastic adherent well-spread morphology, ability to

differentiate into multiple mesenchymal tissues under directed *in vitro* conditions and the presence of a variety of surface marker proteins (Majumdar *et al.* 1998, Pittenger *et al.* 1999). In fact, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to define human MSCs propose 3 main criteria for the positive identification of a homogenous population. Firstly, MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Secondly, ≥95% of the MSC population must express the surface antigens CD105, CD73 and CD90, as measured by flow cytometry. These cells must also lack expression (≤2%) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leukocyte antigen (HLA) class II or MHC II. Finally, the cells must be able to differentiate into osteoblasts, adipocytes and chondrocytes under standard *in vitro* differentiating conditions (Dominici *et al.* 2006). Previous work in this laboratory has confirmed homogenous MSC populations isolated from the bone marrow of young rats. In this study, a subset of positive and negative markers, namely CD90 and CD45, were selected to quantitatively identify the homogeneity of the young and aged bone marrow-derived culture preparations using FACS analysis. It was verified that young and aged populations had 96-97% positivity for CD90 and only 2-3% expression of CD45. Together with the cells' plastic adherence and distinct morphology in culture, it was concluded that defined populations of MSCs were being successfully isolated and cultured from both young and aged rats.

It is essential to consider MSC biology in terms of aging to determine the practicality of employing MSCs as a cell source for therapeutic applications in aged patients. Alterations associated with cellular aging could impact on the proliferation and differentiation of these cells; two processes pivotal in tissue engineering. It remains unclear as to what extent MSCs are subject to the causes of aging, and whether age-related changes are caused by intrinsic factors or induced by the extrinsic somatic environment (Zhou *et al.* 2008). In the current study, cells were incubated with the thymidine analogue BrdU to examine cell proliferation potential. Immunocytochemistry was employed to identify incorporation of BrdU into the DNA of dividing cells and results revealed a significant decrease in the percentage of cells positive for BrdU incorporation in aged MSCs compared to young. This finding suggests that the number of cells

undergoing cell division is lower in aged MSCs, reflecting *in vivo* effects of aging as *in vitro* conditions were the same for both young and aged cells. It has been reported by Stenderup *et al.* (2001) that the number and proliferative capacity of human MSCs are maintained during aging. In this study they estimated the cell density per colony and found a correlation between the colony size and the estimated cell density per colony, suggesting that CFU sizes reflect true cell growth. Conversely, using population doublings as a measure of proliferation Stenderup *et al.* (2003) report that cells obtained from elderly donors exhibit decreased proliferation potential and accelerated senescence compared to cells obtained from younger donors. However, MSCs exhibited morphological evidence of senescence when early-passage cells were compared with late-passage cells irrespective of donor age. In another study to determine whether there are intrinsic, age-related changes of hMSCs *in vivo*, freshly isolated MSCs and early passage MSCs were evaluated. This approach was used to avoid changes in cell behavior associated with prolonged culture periods, such as *in vitro* senescence or culture stress. There were striking age-dependent decreases in proliferation whereby the doubling time of hMSCs was greater in cells from older compared to younger subjects, and this was correlated with age. It is proposed that the increase in doubling time could be due to aging causing a uniform increase in the duration of each phase of the cell cycle. Consistent with decreased proliferation, that study found an age-related increase in p53 expression and its targets p21 and BAX, which are known to mediate this cellular function. These results indicate that there are intrinsic alterations in human MSCs with aging that may explain skeletal, cellular, and tissue aging (Zhou *et al.* 2008). The latter described studies support the findings reported here but discrepancies remain within the literature regarding the effects of aging on MSC proliferation which may be attributed to variation between studies in terms of subject type (i.e.: human or animal, murine or bovine, etc.), MSC source (e.g.: bone marrow, fat pad, synovial fluid, etc.) and method of MSC isolation.

Mechanotransduction describes how mechanical forces are translated into biochemical signals leading to functional responses by the cell. Physical stimuli play a role in regulating cell proliferation, differentiation, and apoptosis by activating various intracellular signal transduction pathways. However, it remains

relatively unknown how cells sense different types of mechanical stimulation (Han *et al.* 2004).

Integrin receptors are present on the MSC plasma membrane and interact with the cytoskeletal network of the cell triggering further downstream signalling cascades in response to a stretch stimulus for example. Other putative sites for mechanosensing include the nucleus itself, and the cytoskeleton (Chen *et al.* 2008, Han *et al.* 2004). Integrins can trigger signalling cascades and induce focal adhesion formation as a result of ECM ligand binding by activating the small GTPase Rho and stimulating its downstream target Rho-associated kinase, which promotes actin filament polymerisation, inducing cytoskeletal contraction (Ingber 2006). In this study, it was shown that there was an age-related reduction in the expression of integrin subunit α2, as measured by immunocytochemistry and Western immunoblot. There was a similar decrease observed in actin expression as well as a reduction in bundle thickness with age. In cardiomyocytes, progressive aging has been shown to influence α1, α5 and β1 integrin subunits. Although no changes were observed for α2, it is thought that the differential protein content observed for β1 is due to dimerisation occurring with other α chains, since it has the capacity to bind to several (Burgess *et al.* 2001). In the Reed *et al.* (2001) study comparing the migratory capacity in young and aged fibroblasts, significant differences were revealed in cytoskeletal organisation and α2β1 integrin function. The differences observed were correlated with the cells capacity to migrate. Aged fibroblasts that were poor migrators exhibited a disorganized actin cytoskeleton and altered alignment. Proper alignment of actin is regulated by signals transmitted from cell-surface integrins via the Rho family of GTPases (Clarke *et al.* 1998). Little is known about how cellular ageing affects the expression and function of integrins however, in the current study a reduction in α2 expression has been observed, as well as a reduction in actin expression and its arrangement. Taken together, these results indicate potentially compromised cell-matrix interactions with aging in MSCs.

Lipid peroxidation is one of the major outcomes of ROS-mediated injury to tissue. There are two broad outcomes to lipid peroxidation, structural damage to membranes and generation of secondary products such as malondialdehayde.

Membrane damage is caused by generation of fragmented fatty acyl chains, lipid-lipid cross-links and lipid-protein cross-links. Combination of these processes induces changes in the biophysical properties of membranes, leading to profound effects on membrane-bound protein activity (Montine *et al.* 2002). Lipid peroxidation has long been associated with cellular aging and it has previously been shown to increase with age in several tissue/cell types in the rat; liver, kidney, heart, brain, lung, spleen and MSCs (Stolzing *et al.* 2006, Farooqui *et al.* 1987). In a study by Choe *et al.* (1999), small unilamella liposomes were prepared and either peroxidised by Fe^{++}-ADP-ascorbic acid or loaded with cholesterol. The liposomes were incorporated into rat liver microsomal membranes and membrane fluidity was quantified by measuring changes in polarisation. Membranes exhibited a higher sensitivity to peroxidation than cholesterol such that incorporation of peroxidised liposomes induced microsomal membrane rigidity considerably more than cholesterol-loaded liposomes. Thus it was proposed that membrane fluidity can be modulated by peroxidation of membrane phospholipids, irrespective of the influences of cholesterol. This finding supports the proposal that alterations in lipid structure are more potent than compositional changes in cholesterol in promoting age-related increases in membrane rigidity. Using a TBARS assay in this study, it was found that aged MSCs exhibit a greater degree of lipid peroxidation compared to young MSCs, indicating the likelihood of increased membrane rigidity with age and altered mechanotransduction as a result of this. This would be due to alterations in membrane fluidity associated with peroxidation of membrane lipids, which disrupts vital functions necessary for signal transduction or the cell's selective permeability to various ions (Rikans & Hornbrook 1997, Chen & Yu 1994). Therefore, it is possible that aged MSCs may respond differently to an applied stimulus such as mechanical strain, which has implications for their employment in tissue engineering applications.

In summary, this study has identified the young and aged cell populations used for this research as homogenous MSC populations. Furthermore, various age-related changes in the biology of these cells have been described. These include a reduction in proliferation potential with age, diminished expression of mechanosensory proteins α2 and actin, and an increase in lipid peroxidation with

age (Figure 3.9). Together these results point to the potential for an altered mechanosensitvity in MSCs with age.

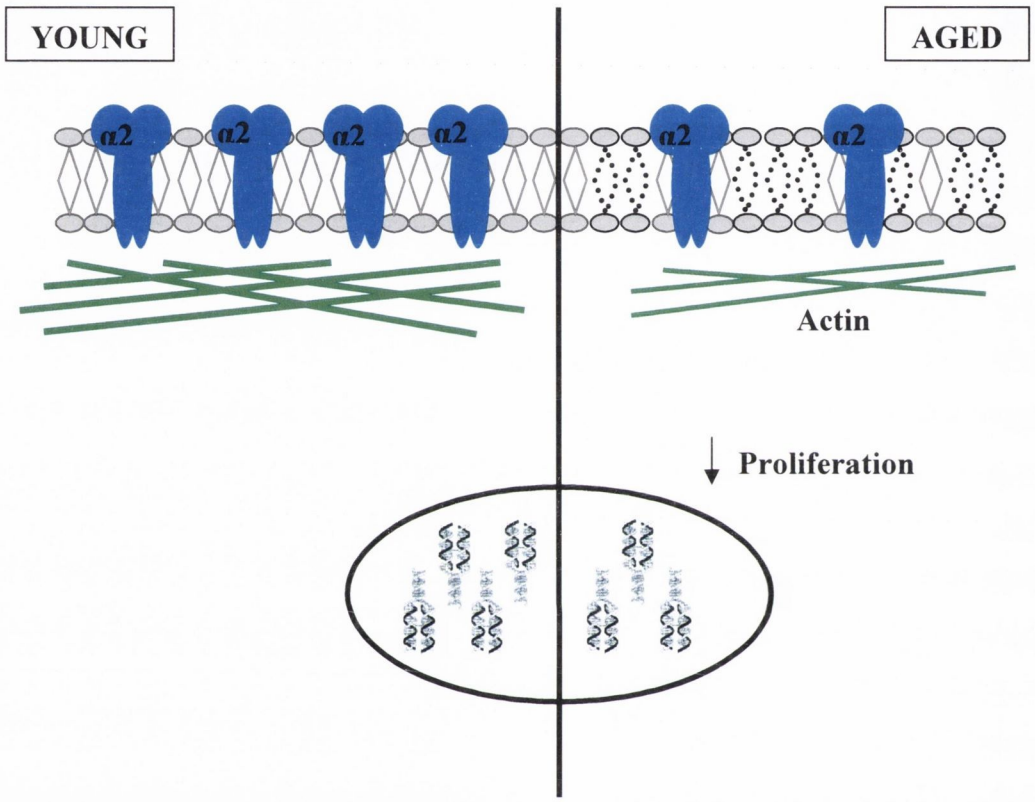


Figure 3.9: Age-related changes in MSCs; reduced α2 and actin expression, reduced proliferation and increased membrane lipid peroxidation

With increasing age in MSCs, there is a reduction in the rate of proliferation and the expression of mechanosensitive proteins; integrin subunit α2 and actin. There is also an increase in membrane lipid peroxidation in aged MSCs compared to cells isolated from young rats.

Chapter 4 Cyclic Tensile Strain-Induced Mesenchymal Stem Cell Apoptis

4.1 Introduction

Apoptosis was first described by Kerr *et al.* (1972) as a mechanism of controlled cell deletion, which appeared to play a complementary but opposing role to mitosis in regulating animal cell populations. It was suggested as being an active, inherently programmed phenomenon, initiated or inhibited by a variety of environmental stimuli, both physiological and pathological. Structural changes were described in two distinct stages; firstly nuclear and cytoplasmic condensation and breaking up of the cell into membrane-bound fragments and secondly shedding of these apoptotic bodies or uptake by other cells, where they are degraded by lysosomal enzymes. Apoptosis was observed to be involved in cell turnover in many healthy adult tissues and responsible for focal elimination of cells during normal embryonic development. It was established as a distinctive morphological process and an important basic biological phenomenon involved in cell turnover, as an active and controlled process. Aging is often associated with an enhanced occurrence of and susceptibility to apoptosis in several types of cells including neurons (Li *et al.* 1997), cardiomyocytes (Kajstura *et al.* 1996), hepatocytes (Muskhelishvili *et al.* 1995), lymphocytes (Phelouzat *et al.* 1996), renal tubular epithelial cells (Razzaque *et al.* 1999) and chondrocytes (Adams & Horton 1998). Apoptosis plays an important role in the aging process in that several types of cells exhibit enhanced apoptosis with aging under physiological conditions and vulnerability to apoptosis by insults. In stable cells that can be restored by cell proliferation, apoptosis acts to remove dysfunctional cells exhibiting homeostatic failure due to oxidative stress, glycation, and DNA damage, thereby maintaining homeostasis in the organ. Therefore, it is considered that the incidence of apoptosis in aging depends on the level of accumulated injury and involvement in the activation of apoptosis-related cytokine signalling (Higami & Shimokawa 2000).

Cyclic tensile strain has been employed for some time as an investigative tool in tissue engineering studies. It has been utilised to both mimic *in vivo* conditions and to study the effects on various cell types under *in vitro* conditions. Depending on the magnitude of strain applied, this type of mechanical stimulus can be beneficial to some cell types or detrimental to others, leading to apoptosis. In

cultured rat spinal cord cells, application of 10% cyclic tensile strain reduced viability significantly in a dose- and time-dependent manner. It was found that increasing the strain or the strain rate independently was linked with significant decreases in spinal cord cell survival. There was no clear evidence of additive effects of strain level with strain rate. Gene Ontology analysis identified 44 candidate genes which were significantly related to apoptosis and 17 genes related to the response to stimulus. Further analysis identified the upregulation of various genes, in particular of the MAPK pathway, which may be involved in this cellular response to strain (Uchida *et al.* 2010). Mechanical force is a known contributor to vascular remodelling processes. Rat vascular smooth muscle cells (VSMCs) were exposed to cyclic tensile strain *in vitro* to examine the role of the cystein protease calpain in force-induced vascular cell apoptosis and its effect on injury-induced vascular remodelling processes. This resulted in increased p53 expression and transcriptional activity as well as an increase in apoptotic VSMCs. Apoptosis was prevented by pifithrin, a p53 inhibitor, indicating the reliance of force-induced apoptosis on p53. Calpain activity was also increased by mechanical strain but prevention of calpain activation by calpeptin amplified strain-induced p53 expression and transcriptional activity, leading to a further increase in the rate of apoptotis. It was concluded that calpain counteracts mechanically induced VSMC apoptosis via p53-degradion, identifying calpain as a key regulator of mechanosensitive remodelling processes in the vascular wall (Sedding *et al.* 2008). These studies demonstrate the influence of tensile strain on cellular apoptosis but little is known about how MSC viability is regulated by strain.

Mechanical conditioning of MSCs in particular has been widely utilised as a biophysical signal to promote tissue engineering applications. Replication of *in vivo* mechanical signalling is undertaken *in vitro* to regulate cell differentiation, and ECM synthesis for compatibility with an implant target site. Previous work within this laboratory endeavoured to determine the effects of mechanical strain on apoptosis in young MSCs. Mechanotransduction associated with strain-mediated cell death was investigated by seeding MSCs onto a 2-D silicone membrane and stimulating the cells over a range of strain magnitudes for a period of 3 days. Results indicated that tensile strains of 7.5% or greater induce apoptosis in MSCs. L-type voltage-activated calcium channels coupled the applied

mechanical stress to calpain and JNK activation, leading to apoptosis through DNA fragmentation (Kearney *et al.* 2008). An investigation into the effect of tensile strain on aged MSC differentiation potential has been undertaken (Huang *et al.* 2010) but to date, no other studies have determined whether there is an apoptotic response in aged MSCs to tensile strain.

Integrin receptors are broadly recognised as transducers of mechanical stimulation. Mechanical signals transmitted from the ECM converge on cell surface integrins, associated intracellularly to actin in the cytoskeleton, at focal adhesion formations. This association occurs via a group of actin-associated proteins (i.e.: talin, vinculin, paxillin, and α-actinin) within the focal adhesion. Mechanical forces applied at the integrin or transmitted outward from the cytoskeleton, converge with biochemical signals at the focal adhesion. Therefore, focus has been directed on integrins as candidates for transducing mechanical stimuli into biochemical responses intracellularly (Ingber 1991, Alenghat & Ingber 2002). Disturbing the activity of integrin receptors could impinge on their capacity as mechanotransducers. Synthetic RGDS peptides are integrin binding sequences that inhibit integrin receptor function. They have been shown to compete with adhesive proteins for binding to integrin receptors (Chung *et al.* 2006), thus inhibiting integrin-mediated functions in different cell systems. Muller *et al.* (1997) described how fluid shear flow-induced vasodilation of coronary arteries was effectively blocked by soluble Arg-Gly-Asp (RGD) peptides which compete with ECM proteins for integrin binding. It was hypothesised that integrin binding to RGD sequences in ECM proteins is a critical step in the initiation of signalling sequences whereby shear stress activates endothelial tyrosine kinases inducing vasodilation of arterioles. Increasing shear stress was applied to isolated coronary arterioles under control conditions and in the presence of a synthetic RGD peptide, GRGDNP, to competitively inhibit integrin binding to ECM proteins containing RGD sequences. GRGDNP inhibited the shear stress–induced vasodilation in a dose-dependent manner whilst an inactive structural analogue, GRGESP, had no effect on shear stress–induced vasodilation. Application of GRGDNP significantly decreased anti-phosphotyrosine binding during shear stress, suggesting a link between tyrosine kinase activation and integrin signalling during this vasodilatory response. These results indicate that integrin-matrix

interactions, possibly at focal adhesions, are fundamentally important in downstream signalling pathways. The effects of this synthetic peptide have also been considered during the development of acute lung inflammatory responses to lipopolysaccharide (LPS) treatment and on the integrin activated MAPK pathway. RGDS was shown to inhibit LPS-induced phosphorylation of FAK, ERK, JNK and p38 in lung tissue, suggesting that RGDS attenuates the inflammatory cascade upregulated during LPS-induced development of acute lung injury. RGDS suppresses fibrinogen binding activity in alveolar macrophages and inhibits migration of these cells and neutrophils to regions of lung inflammation (Moon *et al.* 2009). Although the RGD binding motif is primarily associated with adhesion to substrates, these studies indicate that using synthetic sequences to bind to the recognition site on integrin receptors affects receptor activity and therefore signalling outcomes.

Cyclic tensile strain is employed in this project as an investigative tool for the response of young and aged MSCs to a mechanical stimulus. In this study a high rate of strain was employed to investigate its effect on apoptosis in MSCs with respect to previous findings within the laboratory, and identify alterations associated with age. Cells were seeded onto 2-D substrates; collagen I-coated silicone sections, which were then mounted into a custom built bioreactor for application of 10% cyclic tensile strain. Integrin receptors are mechanotransducers, but it is imperative to note that it is their attachment to ECM proteins that stimulate activation and mechanotransduction. Interference with integrin function using a synthetic RGDS peptide was assessed in this study. Its effect on cell attachment was identified by treating the media with RGDS at various time points and counting adherent cells remaining. To investigate whether integrin inhibition enhances or attenuates strain-mediated apoptosis, RGDS was added to the media for the duration of stretch testing.

4.2 Results

4.2.1 Caspase-3 activity is increased in MSCs exposed to 10% cyclic tensile strain and this is greater in MSCs isolated from aged rats

It has been established that high rates of strain can induce apoptosis in MSCs isolated from young rats (Kearney *et al.* 2007). However, it is uncertain if MSCs isolated from aged donors respond to strain in a similar manner to young MSCs. Cells were seeded onto sections of silicone and exposed to 10% tensile strain for a period of 3 days using a single station bioreactor. Following 3 days of strain sections seeded with MSCs were removed from the mechanical rig and fixed in 4% paraformaldehyde. Cell death in response to 10% strain was examined by immunocytochemistry for active caspase-3, a key mediator of apoptosis. Young and aged MSCs were examined for caspase-3 activity following 3 days of exposure to 10% strain. An increase in active caspase-3 immunoreactivity was observed in MSCs exposed to 10% strain, compared to static controls, as evidenced by an increase in fluorescence intensity (Figure 4.1, A). There was a significant effect of strain ($F_{1,16}$ = 145.1, $p<0.001$, two-way ANOVA) and age ($F_{1,16}$ = 11.27, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 10% strain showed a significant increase caspase-3 activity compared to static control (static control:143.30±14.02 units, 10%:328±7.62 units, $p<0.001$, n=5, mean±SEM). This was also observed in aged MSCs (static control: 176.60±9.73 units, 10%:412.40±29.52 units, $p<0.001$, n=5, mean±SEM). A significant increase in caspase-3 activation was also observed between young and aged cells exposed to 10% strain (Young 10%:328±7.62 units, Aged 10%:412.40±29.52 units, $p<0.01$, n=5, mean±SEM). Representative images (Figure 4.1, B) show an upregulation in the expression of active caspase-3 in young and aged groups exposed to 10% strain compared to unstrained control groups. These results indicate the induction of apoptosis in MSCs exposed to 10% strain, with an increased response occurring in MSCs isolated from aged rats compared to young.

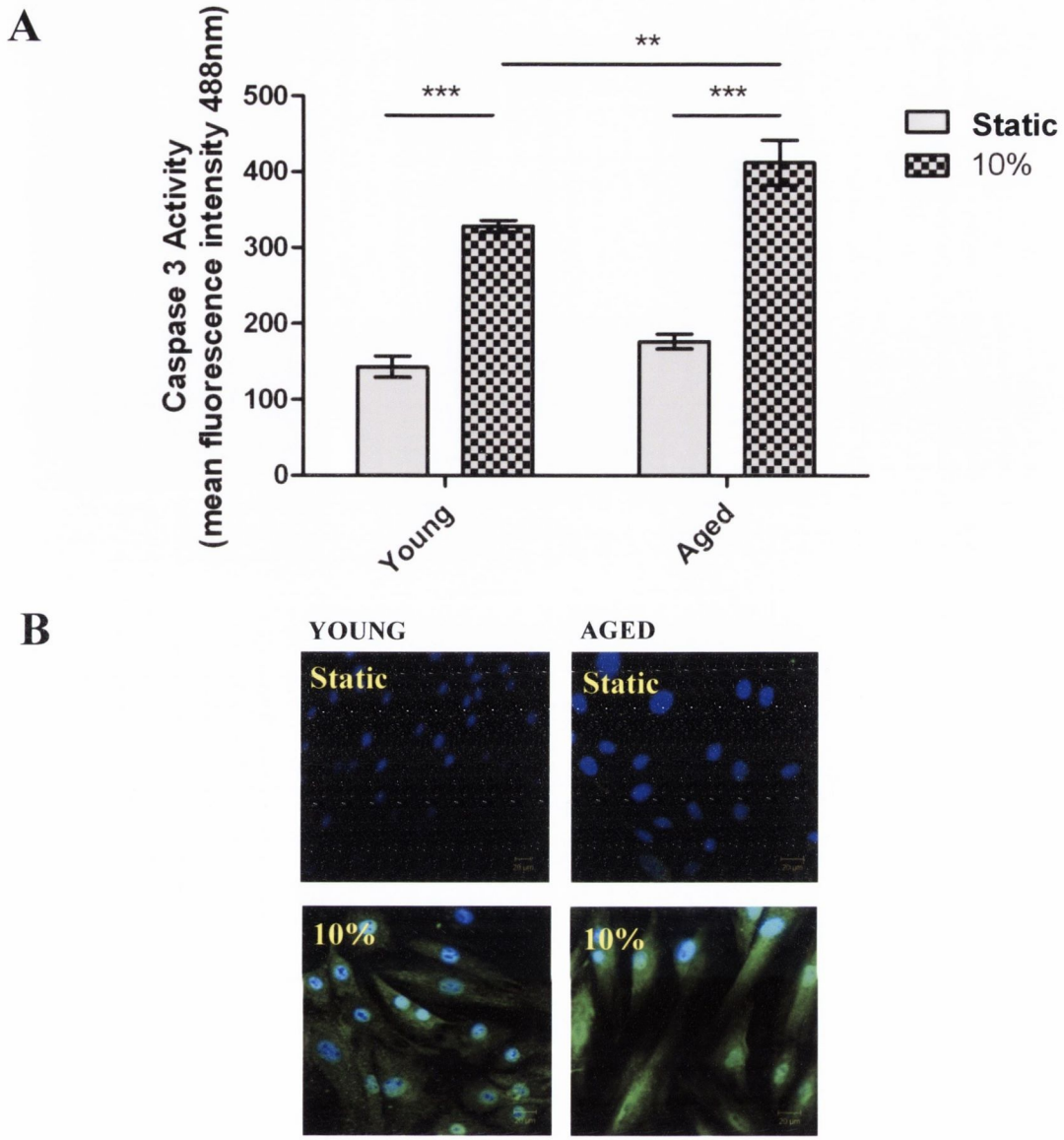


Figure 4.1: Active caspase-3 expression is increased in MSCs exposed to 10% strain for 3 days with a significantly higher increase in MSCs isolated from aged rats

MSCs were examined for the expression of active caspase-3 using immunocytochemistry for an anti-active caspase-3 antibody. An increase in expression was quantified by an increase in mean fluorescence intensity **A:** There was a significant effect of strain ($F_{1,16}$ = 145.1, $p<0.001$, two-way ANOVA) and age ($F_{1,16}$ = 11.27, $p<0.01$, two-way ANOVA) and a significant interaction ($F_{1,16}$ = 8.07, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed a significant increase in active caspas-3 expression in strained groups compared to static controls (***$p<0.001$, n=5 cultures). A significant increase was also observed in aged MSCs exposed to strain compared to young (**$p<0.01$, n=5 cultures). **B:** Confocal images of active caspase-3 expression. Results are displayed as mean ± SEM. Scale bar is 20μm.

4.2.2 DNA fragmentation is increased in MSCs exposed to 10% cyclic tensile strain and this is greater in MSCs isolated from aged rats

Cells were also analysed for levels of DNA fragmentation using the TUNEL staining technique. There was a significant effect of both strain ($F_{1,16}$ = 55.43, $p<0.001$, two-way ANOVA) and age ($F_{1,16}$ = 11.39, two-way ANOVA) and a significant interaction ($F_{1,16}$ = 8.07, $p<0.05$, two-way ANOVA), which can be seen in Figure 4.2, A. *Post hoc* tests showed that young MSCs exposed to 10% strain underwent a significant amount of apoptosis compared to static control (control:12.46±1.24%, 10%:22.43±2.13%, $p<0.01$, n=5, mean±SEM). This was also observed in aged MSCs (static control:13.62±1.92%, 10%:35.89±2.99%, $p<0.001$, n=5, mean±SEM). However, aged MSCs exposed to strain showed a significant increase in apoptotic nuclei compared to the young strain group (Young 10%:22.43±2.13%, Aged 10%:35.89±2.99%, $p<0.001$, n=5, mean±SEM). Figure 4.2, B shows representative images of TUNEL stained cells, whereby healthy nuclei appear pale green and apoptotic nuclei appear dark brown due to the occurrence of DNA fragmentation. Similar to the data obtained for caspase-3 activity, these results indicate that apoptosis is occurring in response to a high rate of strain, with an increased response observed in aged MSCs compared to young.

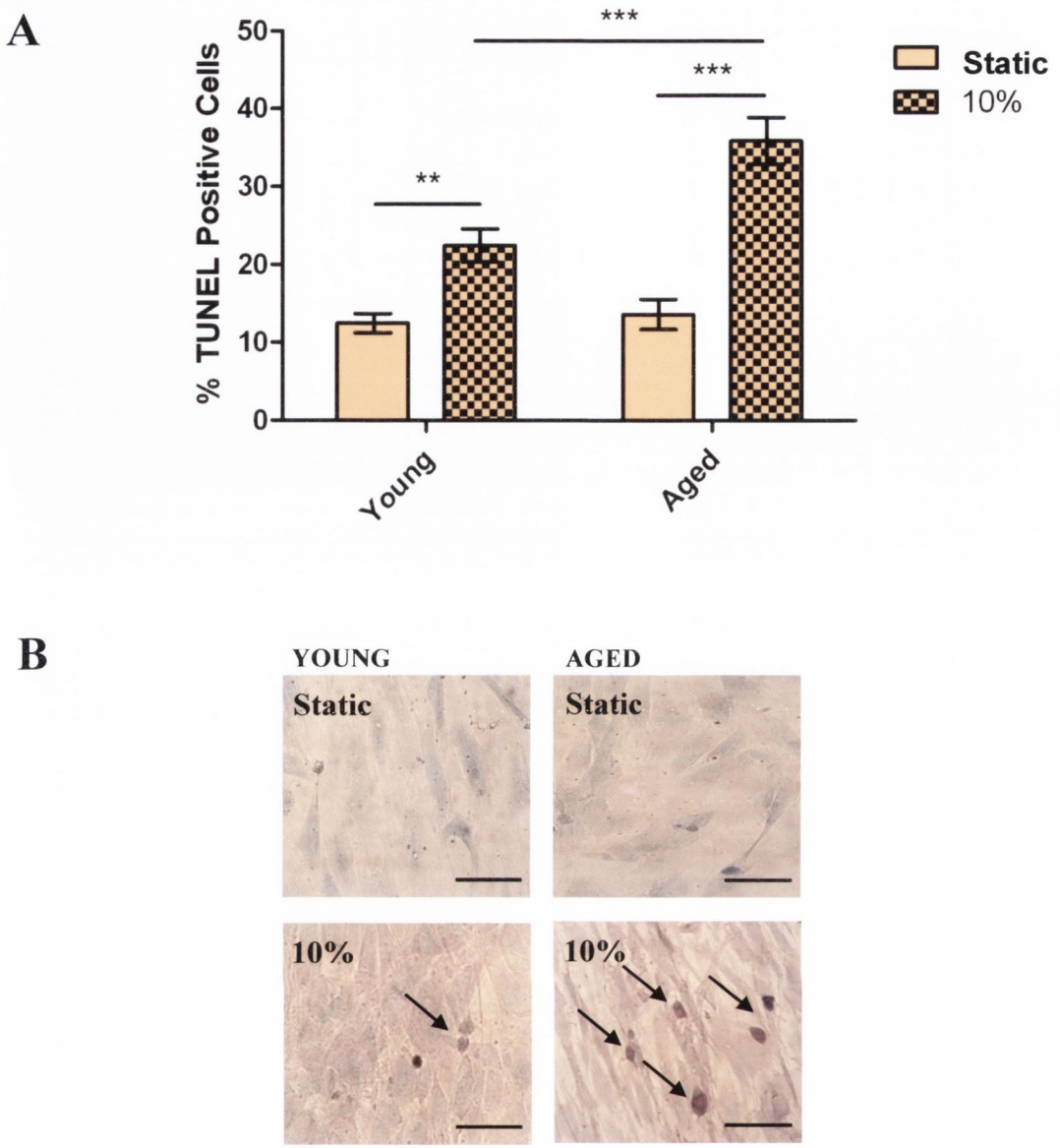


Figure 4.2: DNA fragmentation is increased in MSCs exposed to 10% strain for 3 days with a significantly higher increase in MSCs isolated from aged rats

MSCs isolated from young and aged rats were seeded onto collagen-coated sections of silicone and exposed to 10% strain for 3 days. Cell viability was assessed using the TUNEL staining technique. **A:** There was a significant effect of strain ($F_{1,16}$ = 55.43, $p<0.001$, two-way ANOVA) and age ($F_{1,16}$ = 11.39, two-way ANOVA) and a significant interaction ($F_{1,16}$ = 8.07, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed a significant increase in the percentage of apoptosis in young and aged MSCs compared to static controls (*$p<0.05$, ***$p<0.001$, n=5 independent observations). **B:** Light microscopy images of TUNEL staining. Results are displayed as mean ± SEM. Arrows indicate apoptotic nuclei and scale bar is 50μm.

4.2.3 Integrin inhibition reduces MSC cell number over time

The effect of the integrin inhibitor RGDS on cell attachment was assessed by counting the number of cells remaining adhered to silicone sections following application of the drug at various time points. Cells were treated with RGDS (0.5μM) for 6, 12, 24, 48 and 72hr, stained with a nuclear DAPI stain following fixation and were compared to 72hr untreated control. Cell numbers decreased in young MSCs exposed to RGDS for each time point when compared to untreated control (72hr control:6973±135 cells, 6hr+RGDS:4637±138 cells, 12hr+RGDS:4127±201 cells, 24hr+RGDS:3890±335 cells, 48hr+RGDS:3468±150 cells, 72hr+RGDS:3152±107 cells, $p<0.001$, one-way ANOVA, n=4, mean±SEM; Figure 4.3). There was also a significant decrease in cell numbers between 6hr treatment compared to 48 and 72hr ($p<0.01$, one-way ANOVA, n=4, mean±SEM) and between 12hr treatment and 72hr ($p<0.05$, one-way ANOVA, n=4, mean±SEM). In aged MSCs cell numbers decreased with exposure to RGDS at each of the time points when compared to untreated control (72hr control:6131±362 cells, 6hr+RGDS:4370±185 cells, 12hr+RGDS:4141±512 cells, 24hr+RGDS:3943±403 cells, 48hr+RGDS:3687±540 cells, 72hr+RGDS:2610±201 cells, $p<0.001$ and $p<0.01$, one-way ANOVA, n=4, mean±SEM; Figure 4.3). There was also a significant decrease in cell numbers between 6hr treatment compared to 72hr ($p<0.05$, one-way ANOVA, n=4, mean±SEM). These results indicate that RGDS causes a loss in cell attachment which is most effective with 72hr treatment, with no differences occurring between age groups.

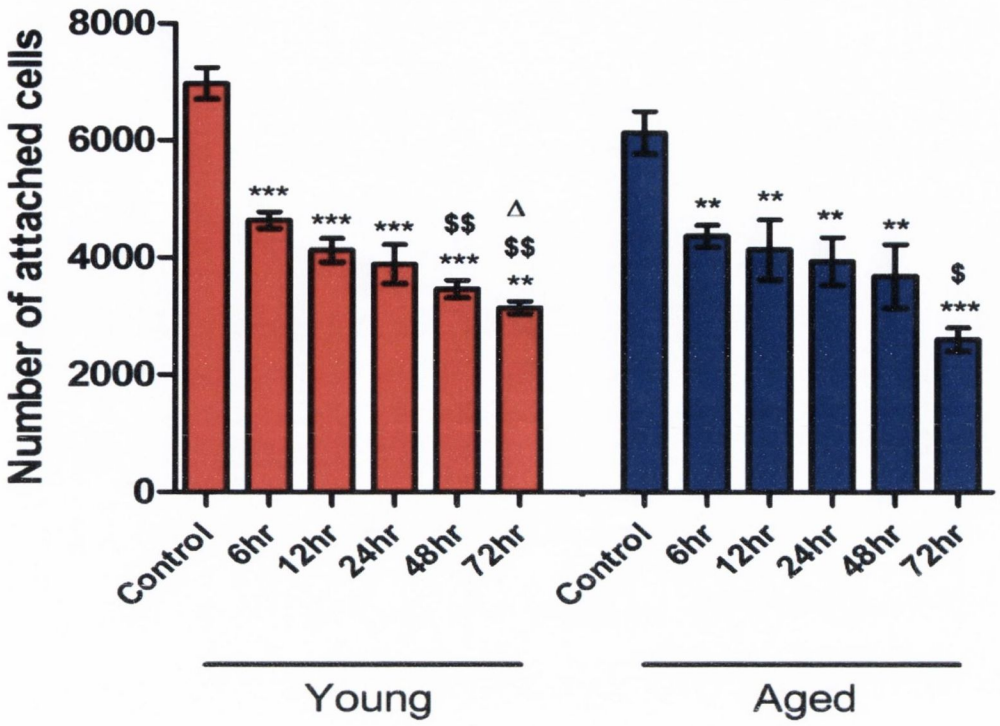


Figure 4.3: Integrin inhibition decreases cell attachment in young and aged MSCs

MSCs were examined for cell attachment using a DAPI nuclear stain. Positively stained nuclei were reported as attached viable cells and were counted. **A:** There was a significant decrease in cell numbers in young MSCs exposed to the integrin inhibitor compared to untreated control (***$p<0.001$, one-way ANOVA, n=4 cultures). Within the treated groups there was a significant decrease in cell attachment at 48 and 72hr compared to 6hr treatment ($^{\$\$}p<0.01$, one-way ANOVA, n=4 cultures) and at 72hr compared to 12hr treatment ($^{\Delta}p<0.05$, one-way ANOVA, n=4 cultures) **B:** Similarly, there was a significant decrease in cell numbers in aged MSCs exposed to RGDS compared to untreated control (***$p<0.001$, **$p<0.01$, one-way ANOVA, n=4 cultures). Within the treated groups there was a significant decrease in cell attachment at 72hr compared to 6hr treatment ($^{\$}p<0.05$, one-way ANOVA, n=4 cultures). Results are displayed as mean ± SEM.

4.2.4 Caspase-3 activity is affected by 10% cyclic tensile strain but not integrin inhibition in MSCs

It has previously been reported that 10% cyclic tensile strain induces apoptosis in young MSCs (Kearney *et al,* 2008). Here, the effect of 10% strain for a period of 3 days is assessed in the presence of an added insult; integrin inhibition. The integrin inhibitor RGDS (0.5μM) was added to the media for 3 days during strain testing. Expression of active caspase-3 was determined using immunocytochemistry and confocal microscopy. Changes in activity were observed as alterations in mean fluorescence intensity values. There was a significant effect of strain ($F_{1,8}$ = 89.33, $p<0.001$, two-way ANOVA) in young MSCs (Figure 4.4, A). *Post hoc* analysis revealed a significant increase in active caspase-3 activity in young MSCs exposed to 10% tensile strain, in the absence and presence of integrin inhibition (static control:165.58±6.00 units, RGDS:207.12±10.68 units, 10%:312.35±18.54 units, 10%+RGDS:318.38±15.86 units, $p<0.001$, n=3, mean±SEM). There was no effect of integrin inhibition applied in the absence of tensile strain.

It was shown in Figure 4.1 that 10% cyclic tensile strain also causes a significant increase in active caspase-3 expression in aged MSCs. Here the effect of 10% strain for a period of 3 days is assessed in the presence of integrin inhibition. There was a significant effect of strain (F1,8 = 110.50, $p<0.001$, two-way ANOVA) in aged MSCs (Figure 4.4, A). Post hoc analysis revealed a significant increase in active caspase-3 activity in aged MSCs exposed to 10% tensile strain, in the absence and presence of integrin inhibition (static control:165.24±17.92 units, RGDS:200.02±25.82 units, 10%:439.57±38.77 units, 10%+RGDS:390.93±16.10 units, $p<0.01$ & $p<0.001$, n=3, mean±SEM). There was no effect of integrin inhibition applied in the absence of tensile strain.

It was also shown in Figure 4.1 that aged MSCs exposed to 10% cyclic tensile strain exhibit an increased upregulation of active caspase-3 expression compared to young MSCs. Thus, the effect of tensile strain in combination with integrin inhibition was investigated in terms of age-related changes in caspase-3 activation in response to these factors (Figure 4.5, A). There was a significant effect of strain

($F_{1,8}$ = 80.45, $p<0.001$, two-way ANOVA), age ($F_{1,8}$ = 7.30, $p<0.05$, two-way ANOVA), and a significant interaction ($F_{1,8}$ = 7.38, $p<0.05$, two-way ANOVA) when comparing young and aged MSCs exposed to 10% strain. *Post hoc* analysis revealed a significant increase in caspase-3 activation in aged MSCs exposed to strain compared to young MSCs (Young 10%:312.35±18.54 units, Aged 10%:439.57±38.77 units, $p<0.01$, n=3, mean±SEM). There was a significant effect of strain ($F_{1,8}$ = 70.72, $p<0.001$, two-way ANOVA) when comparing young and aged MSCs exposed to strain in combination with integrin inhibition. *Post hoc* analysis showed a significant increase in caspase-3 activation in aged MSCs exposed to strain and integrin inhibition compared to the same treatment group in young MSCs (Young 10%+RGDS:318.38±15.86 units, Aged 10%+RGDS:390.93±16.10 units, $p<0.05$, n=3, mean±SEM). Representative images (Figure 4.4, B and C) show changes in active caspase-3 expression in response to tensile strain and integrin inhibition in young and aged MSCs. These results replicate similar findings described in Figure 4.1, indicating the induction of apoptosis in MSCs exposed to 10% strain, with an increased response occurring in aged MSCs compared to young. Integrin inhibition did not cause an increased apoptotic response in either age group.

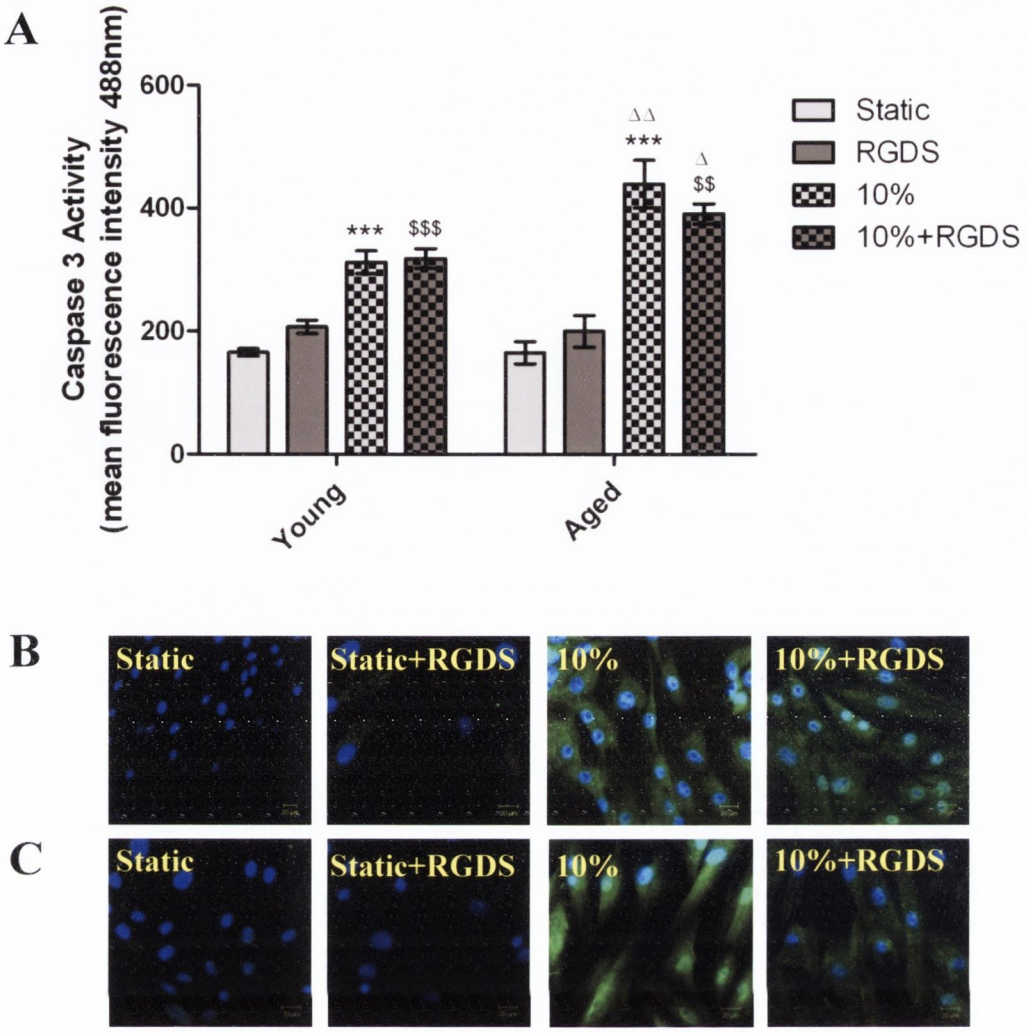


Figure 4.4: Tensile strain increases caspase-3 activity in MSCs and this response is significantly greater in aged MSCs whilst integrin inhibition is ineffective

MSCs were examined for caspase-3 activation using immunocytochemistry for an anti-active caspase-3 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8}$ = 89.33, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 110.50, $p<0.001$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in active caspase-3 activity in response to 10% tensile strain, alone or in combination with integrin inhibition (**$p<0.01$, *** $p<0.001$, n=3 cultures). There were also several differences apparent between age groups (***$p<0.001$ compares static control to tensile strain groups, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ compares combined strain and drug-treated groups to static drug-treated groups and $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$ compares young and aged tensile strain groups, n=3 cultures). **B:** Confocal images of caspase-3 expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

4.2.5 DNA fragmentation is increased in MSCs exposed to 10% cyclic tensile strain, which is greater in aged MSCs whilst integrin inhibition causes differential effects in MSCs

The effect of 10% strain on MSCs in the presence of integrin inhibition was also assessed by analysing levels of DNA fragmentation using the TUNEL staining technique. The percentage of TUNEL positive cells was determined using light microscopy and comparing the number of positively stained nuclei compared to total cells present (Figure 4.5, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 18.14$, $p<0.01$, two-way ANOVA) and drug ($F_{1,12} = 8.45$, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed a significant increase in DNA fragmentation following application of 10% tensile strain, alone or in combination with integrin inhibition (static control:13.41±1.04%, 10%:23.5±2.39%, 10%+RGDS:29.01±3.20%, $p<0.05$, n=4, mean±SEM).

In aged MSCs (Figure 4.5, A), there was a significant effect of strain strain ($F_{1,12} = 26.55$, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,12} = 8.52$, $p<0.05$, two-way ANOVA) in aged MSCs. *Post hoc* analysis showed a significant increase in DNA fragmentation following application of 10% tensile strain (static control: 16.6±0.54%, 10%:37.04±3.57%, $p<0.001$, n=4, mean±SEM). Application of integrin inhibition in combination with tensile strain caused a significant decrease in TUNEL positive cells compared to strain alone (10%:37.04±3.57%, 10%+RGDS:24.91±2.46% , $p<0.05$, n=4, mean±SEM). These results indicate the induction of apoptosis by application of 10% strain for 3 days, but this is reversed with integrin inhibition in this instance.

It was shown in Figure 4.2 that aged MSCs exposed to 10% cyclic tensile strain show an increase in the percentage of TUNEL positive cells compared to young MSCs. Here, Figure 4.5, A shows a composite graph of the effect of tensile strain in combination with integrin inhibition in terms of age-related changes in DNA fragmentation in response to these factors. There was a significant effect of strain ($F_{1,12} = 47.08$, $p<0.001$, two-way ANOVA), age ($F_{1,12} = 14.14$, $p<0.01$, two-way ANOVA), and a significant interaction ($F_{1,12} = 5.41$, $p<0.05$, two-way ANOVA)

when comparing young and aged MSCs exposed to 10% strain. *Post hoc* analysis showed that aged MSCs exposed to 10% strain showed a significant increase in the percentage of TUNEL positive nuclei compared to young (Young 10%:23.5±2.39%, Aged 10%:37.04±3.57%, $p<0.01$, n=4, mean±SEM). There was a significant interaction ($F_{1,12} = 5.41$, $p<0.05$, two-way ANOVA) when comparing young and aged MSCs exposed to 10% strain in combination with integrin inhibition. *Post hoc* analysis showed that aged MSCs exposed to 10% strain in combination with integrin inhibition showed a significant reduction in the percentage of TUNEL positive nuclei compared to strain alone (10%:37.04±3.57%, 10%+RGDS:24.91±2.46%, $p<0.05$, n=4, mean±SEM). Representative images (Figure 4.5, B and C) show changes in the percentage of TUNEL positive nuclei in response to tensile strain and integrin inhibition in young and aged MSCs. These results are similar to findings described in Section Figure 4.2, indicating the induction of apoptosis in MSCs exposed to 10% strain, with an increased response occurring in aged MSCs compared to young. Integrin inhibition did not cause an increased apoptotic response in either age group, however in aged MCSs RGDS treatment caused a reduction in strain-induced apoptosis.

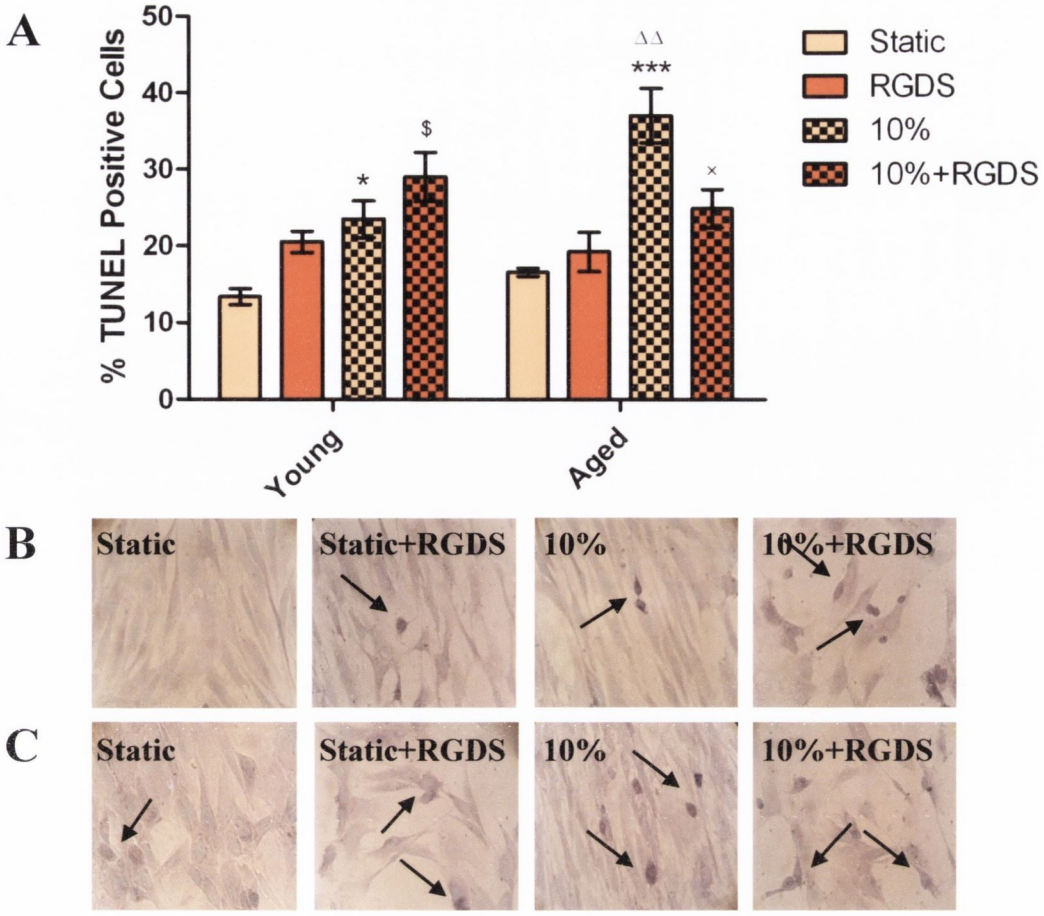


Figure 4.5: Tensile strain increases DNA fragmentation in MSCs and this response is significantly greater in aged MSCs whilst integrin inhibition attenuates this effect in aged MSCs

MSCs were examined for DNA fragmentation using the TUNEL staining technique. Apoptotic nuclei were identified by dark brown staining, healthy nuclei stained pale green. **A:** There was a significant effect of strain in both young ($F_{1,12} = 18.14$, $p<0.01$, two-way ANOVA) and aged ($F_{1,12} = 26.55$, $p<0.001$, two-way ANOVA) MSCs, drug ($F_{1,12} = 8.45$, $p<0.05$, two-way ANOVA) in young MSCs and a significant interaction ($F_{1,12} = 8.52$, $p<0.05$, two-way ANOVA) in aged MSCs. *Post hoc* analysis showed a significant increase in DNA fragmentation in response to 10% tensile strain compared to static controls (*$p<0.05$, ***$p<0.001$, n=4 cultures). Application of 10% strain in combination with integrin inhibition decreased TUNEL positive cells observed compared to 10% strain alone (*$p<0.05$, n=4 cultures) in aged MSCs. There were several differences apparent between age groups (*$p<0.05$, ***$p<0.001$ compares static control to tensile strain groups, $^{\$}p<0.05$ compares combined strain and drug-treated groups to static drug-treated groups, $^{x}p<0.05$ compares strain alone to strain in combination with integrin inhibition and $^{\Delta\Delta}p<0.01$ compares young and aged tensile strain groups, n=4 cultures). **B:** Light microscopy images of DNA fragmentation in young and C: aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

4.3 Discussion

The aim of this study was to determine the effect of a high rate of strain on MSC apoptosis and whether any age-related differences were apparent. Since integrins are identified as key transducers of mechanical signals, the effect of integrin inhibition, using an RGDS peptide, was also investigated to ascertain its effect on cell attachment and interference during tensile strain testing. It was found following application of 10% cyclic tensile strain for a period of 3 days that apoptosis was upregulated in both young and aged MSCs, as measured by increased activation of caspase-3 and an increase in the occurrence of TUNEL positive cells, indicating DNA fragmentation. Furthermore, the increase observed in aged MSCs was greater than that observed in young MSCs exposed to 10% strain, indicating increased vulnerability of aged cells to a high rate of tensile strain. Treatment with the RGDS peptide at 6, 12, 24, 48 and 72hr time points revealed a reduction in cell numbers for both young and aged MSCs, indicating an effect of integrin inhibition on cell attachment. At 72hr both age groups exhibited the least number of adherent cells remaining, compared to earlier time points however, the quantity of cells remaining were deemed sufficient to continue testing and that integrin inhibition was most effective at this time point. Strain-mediated apoptosis was investigated in the presence of the RGDS peptide and caspase-3 activation was observed to increase when MSCs were exposed to tensile strain in the presence or absence of integrin inhibition. Similar to previous experiments, there was an age-associated increase in the apoptotic response to strain. DNA fragmentation was also observed to increase in young MSCs exposed to strain with or without integrin inhibition. However, contrary to findings on caspase-3 activation, DNA fragmentation was not increased in aged MSCs exposed to strain in the presence of integrin inhibition, due to a reduction in TUNEL positive cells when compared to application of strain alone.

Harnessing differentiation potential in MSCs through the use of bioreactor-mediated priming of these cells is key to successful tissue engineering strategies. However, determining the appropriate type of mechanical stimulus and the magnitude of application are imperative in achieving a directed differentiation response towards a desired tissue type. Not only is it important to be able to direct

differentiation along a certain lineage, it is also vital to ensure experimental parameters maintain cellular viability to the utmost capacity. Apoptosis in young MSCs has been shown to occur in a magnitude-dependent manner, with cell death occurring at strains greater than 7.5% and maximally at 10% after 3 days. The mechanisms of strain-mediated apoptosis were investigated and blocking of stretch-activated cation channels (SACC) with gadolinium chloride was ineffective suggesting SACCs are not related to this apoptotic pathway. Conversely, when L-type voltage-activated calcium channels (VACCs) were blocked by nicardipine, the strain-induced activation of apoptosis was prevented, indicating a role for VACCs in the mechanotransduction pathway promoting apoptosis. An expected elevation in intracellular Ca^{2+} concentration due to VACC activation inspired an investigation into the role of the calcium-activated protease calpain in the strain-mediated apoptotic pathway. The calpain inhibitor MDL 28170 prevented apoptosis following 10% tensile strain, suggesting a calpain-dependent mechanism in strain-mediated apoptosis. In addition to this, activation of JNK was also observed during strain, which is activated in response to a variety of cellular stresses and extracellular signals (Kearney *et al.* 2008, Nishina *et al.* 2004). Similarly, high rates of tensile strain have been observed to induce apoptosis in cultured rat spinal cord cells and vascular smooth muscle cells (Uchida *et al.* 2010, Sedding *et al.* 2008, Mayr *et al.* 2002).

In the present study, elevated caspase-3 activity and increased cell numbers exhibiting DNA fragmentation identified strain-mediated apoptosis in young MSCs. It was also shown that an increased response to 10% strain was observed in aged MSCs compared to young. Huang *et al.* (2010) applied 10% mechanical strain for 48hr to test the hypothesis that mechanical loading counteracts the effects of aging by modulating the self-renewal and differentiation potential of murine adipose derived stromal cells (ADSCs). It was reported that aging reduced proliferation and increased the adipogenic potential of ADSCs. However, proliferation of both young and aged ADSCs was increased following application of 10% mechanical strain and applying pre-strain significantly reduced the expression of adipogenic marker genes in mature and aged ADSCs. Although these findings are important in terms of strain-mediated differentiation of aged MSCs, no allusion was made to the possibility of strain-mediated apoptosis in this

study. There are currently no reports describing the effects of tensile strain on aged MSC apoptosis but there is experimental evidence that aging incurs DNA damage and involves the p53 pathway and oxidative stress (Wagner *et al.* 2009, Ho *et al.* 2005). To determine intrinsic, age-related changes of hMSCs *in vivo*, freshly isolated marrow cells or early passage stromal cells were evaluated. There were definitive age-dependent decreases in proliferation and osteoblast differentiation, and increases in apoptosis and p53 pathway genes in hMSCs obtained from individuals aged over 55yrs. Consistent with the age-related decrease in proliferation and increased apoptosis, there was an increase in p53 and its targets p21 and BAX, which are known to function in cellular proliferation and apoptosis. Once activated, p53 induces p21 and BAX, which mediate different aspects of p53 action on apoptosis (Artandi & Attardi 2005). The age-related increases in p53, p21, and BAX gene expression in hMSCs observed in this study suggest that upregulation of the p53/p21 pathway may regulate age-related decreases in proliferation, and the p53/BAX pathway may mediate age-related increases in apoptosis (Zhou et al. 2008). It is possible that intrinsic changes associated with increased apoptosis may be occurring in aged MSCs. These changes would pre-dispose aged MSCs to apoptosis in the absence of tensile strain and may explain the greater increase in apoptosis observed in aged MSCs compared to young when exposed to a high rate of tensile strain.

Another possibility for the increased observation of apoptosis in aged MSCs may be the reported reduction in mechanosensory proteins α2 and actin, described previously. Reports have indicated reduced integrin expression with age in fibroblasts and cardiomyocytes causing poor migratory capacity and altered fibroblast signalling to maintain cardiac ECM (Burgess *et al.* 2001, Reed *et al.* 2001). Other studies have shown that the disruption of cell-matrix interactions leads to apoptosis in anchorage-dependent cells, which has been attributed to the loss of integrin-mediated survival signals (Meredith & Scwartz 1997). In the current study the loss of integrin-mediated survival signals could arise from the reduction in receptors present in the aged MSC membrane. Integrins directly activate survival pathways via PI3-kinase and MAPK pathways. However, in the presence of antagonists, integrins can promote apoptosis. These receptors thus act in an essential biosensory role, coordinating survival or apoptotic responses in

relation to ECM composition (Stupack & Cheresh 2002). Integrin signalling has been demonstrated to prevent apoptosis of embryonic hippocampal neurons via integrin-linked kinase (ILK) that activates Akt kinase. ILK was activated following integrin stimulation and a plasmid encoding dominant negative ILK blocked integrin-mediated Akt activation and cell survival. These results established a novel pathway for cell survival in neurons in the response to integrin receptor activation (Gary *et al.* 2003). Activation of FAK in non-neuronal cells can inhibit p53-mediated apoptosis (Ilic *et al.* 1998) and integrin signalling can also activate the PI3 kinase-Akt pathway (Lee & Juliano 2000), an anti-apoptotic pathway exploited by many cell types (Toker 2000). Since integrin activation is implicated in cell survival and signals via interaction with actin to upregulate signaling cascades, there is a distinct possibility that reduction in these proteins with age is associated with an inability to upregulate pro-survival pathways in response to an apoptotic insult such as a high rate of tensile strain.

The arginine-glycine-aspartic acid (RGD) cell adhesion sequence was first identified in fibronectin (Pierschbachar & Ruoslahti 1984). In multicellular organisms, contacts between cells and the surrounding ECM are mediated by cell adhesion receptors, amongst which the integrin family comprises the most numerous and versatile group (Hersel *et al.* 2003). A major cell adhesion recognition system encompasses proteins containing the RGD attachment site, together with the integrins that serve as receptors for them. Cell adhesive RGD sites have been identified in many ECM proteins, including vitronectin, fibrinogen, collagen and laminin. Approximately half of known integrins recognise this sequence and have been shown to bind to ECM molecules in a RGD dependent manner: $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha IIb\beta 3$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 2\beta 1$ and $\alpha 4\beta 1$ (Pfaff 1997). The integrin-binding activity of adhesion proteins can be replicated by short synthetic peptides containing the RGD sequence. Such peptides promote cell adhesion when immobilised onto a surface, and inhibit it when presented to cells in solution, reducing adhesion to substrates such as fibronectin and collagen (Grzesik & Robey 1994). Integrin-mediated cell attachment influences and regulates cell migration, growth, differentiation, and apoptosis, thus RGD peptides can be exploited to probe integrin functions in various biological systems (Ruoslahti 1996). Cells generally express more than

one type of integrin, and the expression pattern on cells is not static. They can also adopt various states of activation with different ligand binding affinities and since integrins are not exclusively involved in cell adhesion but also mediate versatile signalling events, integrin specific ligands can elicit a certain cell behaviour (Hersel *et al.* 2003).

In this study, it was found that addition of soluble RGDS peptides to the media over a range of time points resulted in a decrease in cell numbers remaining adhered to the collagen I-coated silicone sections. This loss in cell number could be due to the action of RGDS binding to specific sites on integrin receptors, thus preventing them from functioning in cell adhesion. Other cell adhesion motifs have been identified in addition to RGD, therefore ruling out the RGD sequence as the universal cell recognition motif, but it remains unique with respect to its broad distribution and usage (Hersel *et al.* 2003). Peake *et al.* (2000) describe the adhesion of bone cells to the ECM as being mediated by RGD. However, cell attachment is not always mediated through RGD, since alternative peptides are also able to facilitate adhesion. For example, attachment to laminin can involve several additional sites, including the YIGSR (Maeda *et al.* 1994) motif. Laminin YIGSR mediates cellular attachment independently of integrin receptors (Gloe *et al.* 1999). It is possible that integrin inhibition by RGDS did not cause total cell detachment in the current study due to alternative binding motifs potentially mediating cell adherence to the silicone substrate providing sufficient numbers of adherent cells to pursue the study.

The loss in cell numbers observed following RGDS treatment could also be caused by RGD-mediated apoptosis and not through the peptide's integrin inhibitory action. Buckley *et al.* (1999) reported that RGD-containing peptides are able to directly induce apoptosis without any requirement for integrin-mediated signals, by entering cells and stimulating enzymatic activity of pro-caspase-3, a pro-apoptotic protein. In previous studies investigating factors involved in fibroblast-mediated T-cell survival, peptides containing an RGD motif were able to induce apoptosis in peripheral blood T-cells reducing cell numbers by 50% in 24hr (Salmon *et al.* 1997). A similar percentage loss in cell numbers was observed in the present study following 24hr+ treatment with RGDS. It is possible that the

peptides interfere with cell-cell interactions that might be required for survival. Since numerous integrins use an RGD motif to bind to specific ECM ligands, the effects on cell survival of functional antibodies that recognise integrins on T-cells were assessed. Antibodies against integrins α2, α3, α4, α5, αV, β1, β2 and β3 were unable to support T-cell survival or induce T-cell death, indicating that RGD-containing peptides may induce apoptosis directly by supplying an active apoptotic signal in leukocytes rather than inhibiting integrin and ligand-mediated survival signals. However, this observation only occurred when cells were presented with soluble RGD peptides as opposed to peptides immobilised to a substrate. These results point toward the delivery of an apoptotic signal by RGD peptides entering the cells directly and they may not possess an integrin intracellular target. An alternative explanation, given examinations in leukocytes, could be that RGD peptides induce apoptosis through direct caspase-3 activation since pro-caspase-3 cleavage was detected in both adherent and detached cells treated with RGD peptides. These data indicate that RGD-containing peptides are able to induce apoptosis before adherent cells detach from their substrate (Buckley *et al.* 1999). Alternatively, loss of cell attachment can cause a form of apoptosis in many cell types, known as anoikis. This type of cell death can even be induced in the presence of immobilised ECM molecules when non-immobilized soluble ligands like RGD peptides are present (Hersel *et al.* 2003, Stupack *et al.* 2001). Such was the case in the present study, whereby cells were attached to the silicone substrate through collagen I interaction but soluble RGDS was added to the media, but this did not elicit an apoptotic response on its own.

Addition of RGDS to the media during application of 10% strain for 3 days resulted in a similar finding to that described previously in the absence of integrin inhibition. Active caspase-3 expression was upregulated in response to tensile strain in the presence or absence of integrin inhibition and the strain-mediated upregulation was further enhanced in aged MSCs compared to young. Integrin inhibition alone did not induce any changes in caspase-3 activity. Similar results were obtained using the TUNEL staining technique to measure DNA fragmentation. However, in aged MSCs there was no evidence of strain-mediated apoptosis in the presence of RGDS and integrin inhibition reduced the percentage of TUNEL positive cells when applied in combination with tensile strain

compared to strain alone. This potential attenuation in apoptosis could be related to an inhibition of apoptosis due to RGDS interaction with the substrate surface. Pfaff (1997) reported the presence of cell adhesive RGD sites in several ECM proteins, including collagen. Silicone substrates in this study were coated with a collagen I solution prior to cell seeding and treatment of MSCs with RGDS did not precede strain testing. Cells from the osteoblast-like cell line MC3T3-E1 attached to RGDS peptides bound to a silicone surface exhibited significant inhibition of apoptosis activation. The RGDS-modified surface increased $\alpha v\beta 3$ integrin expression, in addition to an increase in FAK expression and activated Akt. Attachment to the RGDS-modified membrane eliminated sodium nitroprusside induced apoptosis, but the surface modification was unable to assuage apoptosis mediated by treatment with free RGDS peptide (Grigoriou *et al.* 2005). The apoptotic pathway activated by free RGDS peptides in that study is unknown; however it is likely that the peptides directly interact with pro-caspase-3 (Buckley *et al.* 1999). Inhibition of PI3-kinase pathway activity eliminates RGDS-dependent resistance to apoptosis, indicating that the suppression of apoptosis is dependent on the activity of PI3-kinase (Grigoriou *et al.* 2005). In the present study there is a possibility that free RGDS peptides within the media preferentially bind to RGD motifs present in the collagen coating of silicone substrates, leaving fewer free peptides to bind to MSC integrins, which can directly activate apoptotic pathways. Moreover, there is a distinct possibility that although caspase-3 activity is upregulated in response to tensile strain in combination with integrin inhibition, it is an equal response to application of strain alone, indicating that the RGDS peptide may not be mediating the apoptotic response. Although caspase-3 activation is a key mediator of apoptosis, it is not an actual indication that apoptosis has occurred whereas TUNEL staining indentifies dead cells by DNA fragmentation. It is therefore possible that seemingly opposing findings observed in aged MSCs exposed to strain could be due to the reduction in apoptosis identified by TUNEL staining. This reduction could be due to upregulation in the activity of RGDS-mediated survival pathways, rescuing the cells from strain-induced apoptosis. Whereas caspase-3 activation indicates that the strain is causing upregulation of the apoptotic pathway but apoptosis has not necessarily occurred yet and there may be an underlying interplay between

apoptotic and survival signalling pathways mediated simultaneously by tensile strain and RGDS treatment.

In summary, this study has re-evaluated the apoptotic effect of a high rate of tensile strain on young MSCs. In addition, the increased response in aged MSCs has been identified for the first time, highlighting their enhanced susceptibility to mechanical stress (Figure 4.6). It was established that integrin inhibition, induced by treatment with an RGDS peptide, was effective at all time points ranging from 6-72hr. Integrin inhibition did not induce apoptosis in MSCs when applied alone, and showed no additive effects on strain induced apoptosis. However, there was one observation in aged MSCs that RGDS treatment applied in combination with tensile strain attenuated strain-mediated apoptosis. This opposing result may be an indication of the interplay between pro-apoptotic and pro-survival pathways induced by simultaneous application of tensile strain and integrin inhibition by RGDS treatment. This study reinforces our understanding that high rates of strain are detrimental to MSCs, especially with age. Furthermore, it proposes a link between integrin signalling and apoptosis.

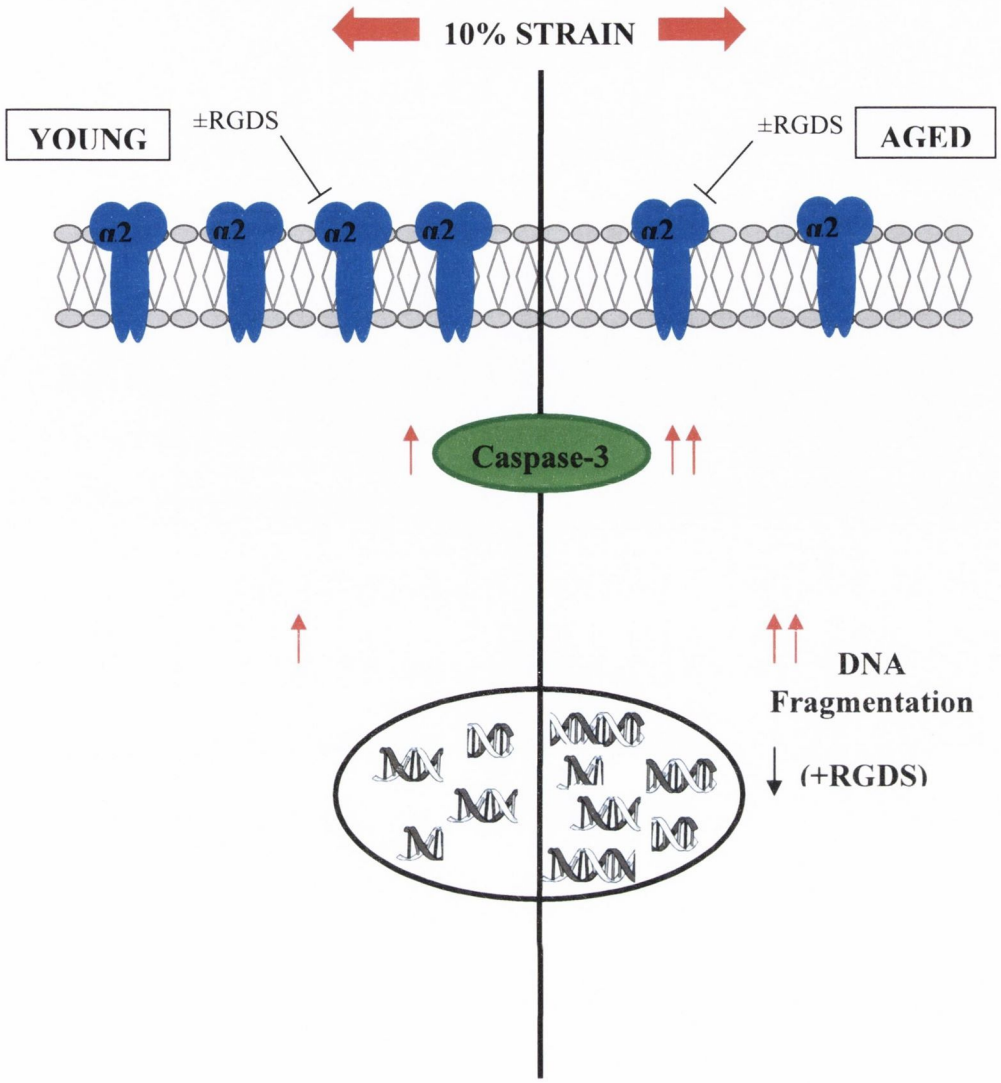


Figure 4.6: Effects of tensile strain and integrin inhibition on apoptosis in young and aged MSCs

Application of 10% cyclic tensile strain for 3 days increases caspase-3 activation and DNA fragmentation in both young and aged MSCs, and this response is further enhanced in aged MSCs (red arrows indicate upregulation induced by tensile strain). Integrin inhibition was ineffective, except in aged MSCs where it reduced the percentage of cells exhibiting fragmented DNA.

Chapter 5 The Effects of Focal Adhesion Kinase and Integrin Inhibition on Mesenchymal Stem Cell Mechanotransduction

5.1 Introduction

As the main receptors connecting the cytoskeleton to the ECM, integrins possess an intimate relationship with force. They transmit mechanical stresses across the plasma membrane, developing tractional force in the cytoskeleton which is conveyed to the ECM through the integrin, much the same way as stresses that are applied to cells from the ECM (Katsumi *et al.* 2004, Huang & Ingber 1999). Focal adhesion complex formation mediates this linkage to the actin cytoskeleton by recruiting proteins such as α-actinin, talin, vinculin and other modulators of actin dynamics (Martin *et al.* 2002). Application of force to bound integrins promotes focal adhesion assembly by activating the small GTPase Rho and stimulating its downstream target Rho-associated kinase (ROCK). This process promotes actin filament polymerisation, inducing cytoskeletal contraction (Riveline *et al.* 2001). Alterations in the cytoskeletal force balance between microfilaments, microtubules, and the ECM can feed back to modify Rho activation and focal adhesion formation, making this form of integrin mechanosignalling bi-directional (Putnam *et al.* 2003). Mechanical forces applied at the integrin or transmitted outward from the cytoskeleton, converge with biochemical signals at the focal adhesion. A more comprehensive understanding of the link between activated integrins, components of focal adhesions and other associated signalling molecules is required, whilst also acknowledging that mechanotransduction is unlikely to occur independently of other signalling pathways. Signals that stem from a mechanical stimulus and normal receptor-ligand interactions may merge into common pathways entering the nucleus, meaning that mechanical stress can modulate a cell's response to receptor-ligand interaction and likewise such interactions can modulate the response to a mechanical stimulus (Alenghat & Ingber 2002).

Integrins also function in the dynamic turnover and remodelling of focal adhesion complexes by activating focal adhesion kinase (FAK) and Src protein tyrosine kinases, including the cytoskeletal regulator paxillin. Inhibition or loss of elements in this pathway restrict adhesion complex turnover and inhibit several integrin-dependent functions (Martin *et al.* 2002). FAK tyrosine phosphorylation and activation of its catalytic activity can be induced by many stimuli including

growth factors, neuropeptides, reagents that stimulate G-protein coupled receptors and mechanical stimuli. However, the dominant mode of regulation is via integrin-dependent adhesion to the ECM and FAK is an essential component of the integrin-signalling pathway (Schaller 2001). FAK is localised to focal adhesions and is centrally connected to the regulation of cell motility and adhesion. FAK-null mice are prevented from normal embryonic development and FAK inhibition is associated with a loss of mesenchymal cell motility (Zachary 1997). The actin cytoskeleton is also necessary for FAK tyrosine phosphorylation, as experiments have shown that Cytochalasin D eradicates tyrosine phosphorylation of FAK in response to many stimuli, including integrin-dependent cell adhesion (Schlaepfer *et al.* 1998). There is evidence to suggest that Rho activation induces tyrosine phosphorylation of FAK, whereas inhibition of Rho blocks the tyrosine phosphorylation of FAK in response to certain stimuli, indicating that tyrosine phosphorylation of FAK may be directed by Rho-dependent alterations of the actin cytoskeleton. Localisation of the active subpopulation of Rho to points of adhesion could promote contractility and clustering of cytoskeletal components and FAK, leading to FAK autophosphorylation and activation of FAK signalling pathways (Schaller 2001, Flinn & Ridley 1996). FAK also associates with Src proteins promoting integrin activation of members of the Ras family of GTPases, which appear to be important for the downstream activation of extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), key regulators of gene expression and cell cycle progression (Martin *et al.* 2002).

Application of mechanical stress to cells bound to the ECM can induce a new wave of integrin activation and binding, triggering associated integrin-mediated signalling events (Ingber 2006). The tensegrity model suggests that the cytoskeleton functions in directing mechanical forces on specific signalling molecules involved in mechanotransduction, with the application of mechanical force causing changes in molecular mechanics. Direct application of force to integrins (or their subunits) results in increased cytoskeletal tension, activation of MAPKs and induction of tyrosine kinase phosphorylation (Peake *et al.* 2000, Chen & Ingber 1999, Schmidt *et al.* 1998, Wang 1998). Indeed, it has been hypothesised that the intrinsic mechanosensitivity of integrin-mediated adhesions

promotes a subset of cellular responses to externally applied mechanical forces. Increased tension within focal adhesions can elicit increased integrin clustering and FAK phosphorylation, which could mediate the effects of strain on downstream targets ERK and JNK (Katsumi *et al.* 2004). In osteoblastic cells, application of a defined physical force directly to integrin receptors using a magnetic drag force tool revealed that cyclic forces induce enhanced cytoskeletal anchorage of tyrosine-phosphorylated proteins as well as an increased activation of FAK and ERK 2. It was also observed that these findings were reliant on an intact cytoskeleton and the presence of intracellular calcium (Pommerenke *et al.* 2002). In dermal fibroblasts, p38 MAPK and ERK1/2 are significantly activated following 7 days of high frequency stretch (Nishimura *et al.* 2007). These studies demonstrate an effect of mechanical force on integrin-mediated signalling via actin, FAK and MAPKs.

In the previous chapter, the effect of the integrin inhibitor RGDS was investigated in terms of cell attachment and cell viability. In this chapter, RGDS is employed in combination with the FAK inhibitor PF573228 (PF228) to evaluate the effects of integrin and FAK protein inhibition on signalling events during cyclic tensile strain. The novel small molecule inhibitor PF228 interacts with FAK in the ATP-binding area, blocking the catalytic activity of recombinant FAK protein or endogenous FAK expressed in multiple cell lines. Treatment of cells with PF228 blocks FAK phosphorylation on tyrosine residue Tyr397, causing reduced tyrosine phosphorylation of paxillin, a known downstream effector of FAK signalling. There is also an associated decrease in cell migration and inhibited adhesion turnover previously attributed to FAK function, but little effect on cell growth or apoptosis in culture. This molecule exhibits *in vitro* selectivity towards FAK and inhibits activation of FAK in various cell lines in the range of 0.3–3μM (Slack-Davis *et al.* 2007). Jones *et al.* (2009) determined the effect of PF228 on platelet function and associated signalling pathways. Similar to data obtained from murine platelets lacking FAK, PF228 effectively blocked human platelet spreading on fibrinogen-coated surfaces but did not affect initial adhesion. The morphology of adherent platelets showed the defect in spreading occurred during transition from filopodia to lamellipodia, a process involving cytoskeletal

dynamics. These data promote PF228 as a valuable means for analysis of FAK function in cells.

The aim of this study was to further our understanding of the signalling molecules involved in MSC mechanotransduction during cyclic tensile strain. A 5% strain was applied for a period of 24hr to investigate activation of various proteins implicated in mechanotransduction and to establish signalling responses in both young and aged MSCs. This rate of strain was selected since previous work in the laboratory has indicated that strains lower that 5% elicit differentiation to MSCs on a 2-D substrate whilst higher strains induce apoptosis. Thus a mid-range strain was selected for short term stimulation to observe potential activation of various mechanosensitive proteins. Furthermore, since integrin receptors and FAK are widely recognised as mediators of mechanical signals within cells, inhibition of these proteins was undertaken in this study to assess their role in MSC mechanotransduction during the response to tensile strain. Expression of integrin receptor subunit α2, actin, pFAK, pERK and active caspase-3 were observed following application of tensile strain and exposure to integrin and FAK inhibition, applied singly or in combination.

5. 2 Results

5.2.1 Integrin inhibition does not influence caspase-3 activity in MSCs

In the previous chapter, 10% strain was shown to induce apoptosis in young and aged MSCs. Although integrin inhibition was not shown to cause increased apoptosis when applied in combination with strain, it was not definitively evident that the response was not in part induced by co-application of these factors. It has previously been reported that strain rates lower than 7.5% do not cause apoptosis in MSCs (Kearney *et al.* 2008), but the relationship between strain and integrin inhibition on apoptosis has not yet been investigated whereby the cells may become more vulnerable to the stretch stimulus. Therefore, the effect of integrin inhibition on caspase-3 activity was assessed by addition of RGDS (0.5μM) to the media for 24hr during strain testing. Expression of active caspase-3 was determined using immunocytochemistry and confocal microscopy. Changes in activity were observed as alterations in mean fluorescence intensity values. There was a significant interaction in young MSCs ($F_{1,\ 8} = 5.99$, $p<0.05$, two-way ANOVA) as shown in Figure 5.1, A. However, *Post hoc* analysis revealed no within group differences (static control:119.11±5.33 units, RGDS:129.87±3.47 units, 5%:138.29±9.30 units, 5%+RGDS:120.07±3.66 units, n=3, mean±SEM).

The effect of the integrin inhibitor RGDS on caspase-3 activity was also assessed in aged MSCs to deduce whether aged cells might be more susceptible to strain-induced apoptosis when applied in combination with integrin inhibition. Aged MSCs were not affected by strain or integrin inhibition as a two-way ANOVA showed no significant effects of either factor on active caspase-3 expression (Figure 5.1, A; static control:114.28±4.77 units, RGDS:121.37±4.73 units, 5%:128.96±11.82 units, 5%+RGDS:115.19±6.35 units, n=3, mean±SEM). Representative images (Figure 5.1, B and C) show caspase-3 expression in the various groups. These results indicate that the combination of integrin inhibition and 5% tensile strain do not cause activation of caspase-3 in MSCs.

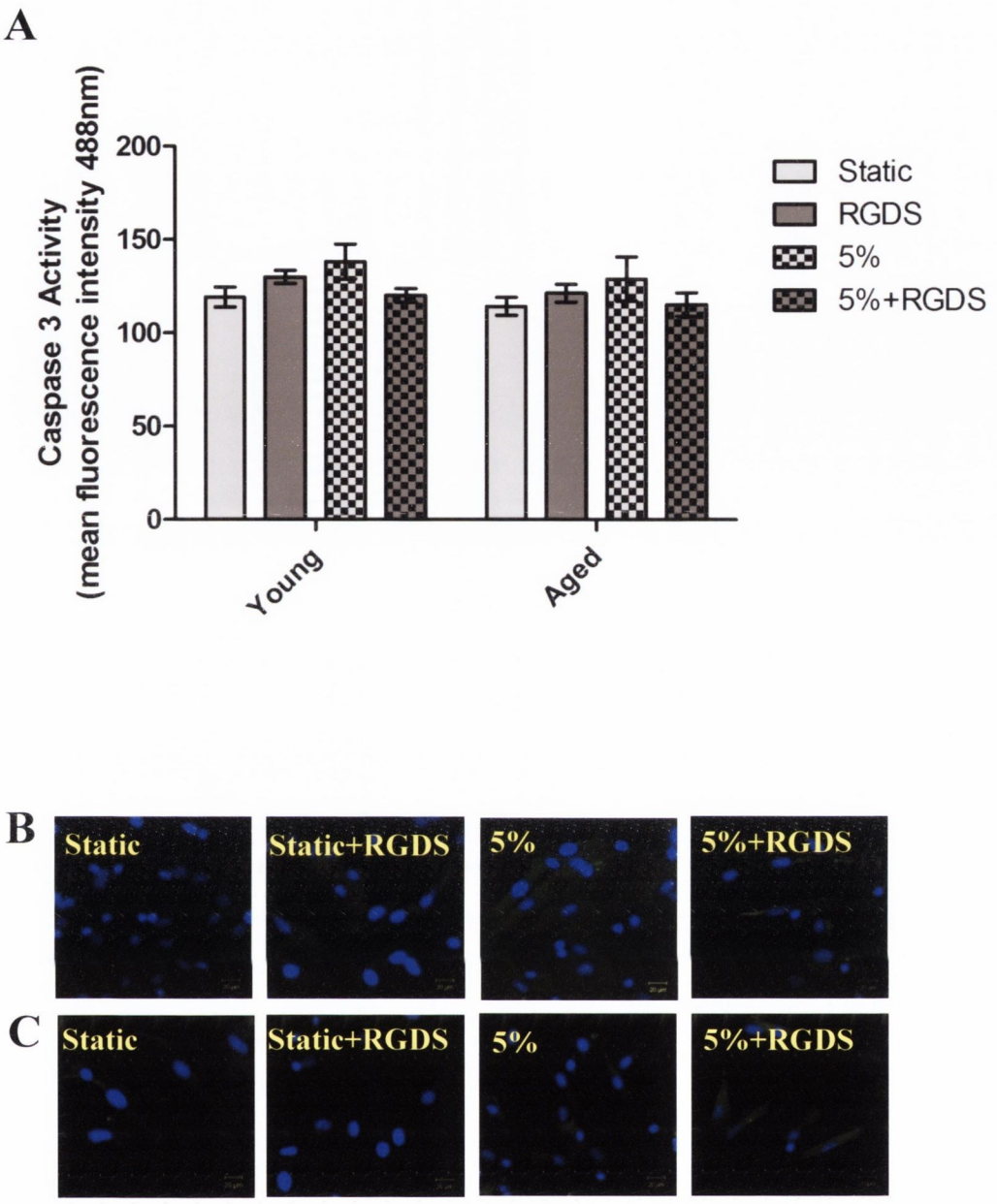


Figure 5.1: Caspase-3 is not activated in MSCs following 5% strain and integrin inhibition for 24hr

MSCs were examined for caspase-3 activation using immunocytochemistry for an anti-active caspase-3 antibody. Changes in expression were quantified by a change in mean fluorescence intensity **A:** 5% strain and integrin inhibition do not affect caspase-3 activity in young and aged MSCs. **B:** Confocal images of casapase-3 expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.2 Cyclic tensile strain increases α2 expression in MSCs and overcomes a reduction in α2 expression induced by FAK inhibition in young cells

The effect of the FAK inhibitor PF228 on MSC signalling was investigated since FAK is a key mediator of integrin signalling and the activity of the two molecules is very closely related. Expression of the integrin subunit α2 was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.2, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 42.62$, $p<0.001$, two-way ANOVA) and drug ($F_{1,12} = 10.16$, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:247.19±5.41 units, 5%:294.91±6.04 units, $p<0.01$, n=4, mean±SEM). Exposure to PF228 (0.05μM) for 24hr caused a significant reduction in α2 expression compared to static untreated controls (static control:247.19±5.41 units, PF228:204.92±4.01 units, $p<0.05$, n=4, mean±SEM). However, application of 5% strain for 24hr in combination with PF228 reversed the decrease in α2 expression observed with FAK inhibition, returning expression to control level (PF228:204.92±4.01 units, 5%+PF228:278.12±16.16 units, $p<0.001$, n=4, mean±SEM).

The effect of the FAK inhibitor (PF228, alone and in combination with the integrin inhibitor RGDS on cell signalling in aged MSCs was also investigated to account for any age-related response to strain that may occur with combined inhibition of two key mechanotransducers.. In aged MSCs (Figure 5.2, A), there was a significant effect of strain ($F_{1,12} = 45.88$, $p<0.001$, two-way ANOVA). Post hoc analysis showed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:225.64 ±2.45 units, 5%:287.74±10.02 units, $p<0.01$, n=4, mean±SEM). Exposure to PF228 (0.05μM) for 24hr did not have any effect when compared to static untreated controls (static control: 225.64 ±2.45 units, PF228:198.74±10.22 units, n=4, mean±SEM). However, application of 5% strain for 24hr in combination with FAK inhibition increased α2 expression compared to the static treated group (PF228: 198.74±10.22 units, 5%+PF228:282.59±15.92 units,

$p<0.001$, n=4, mean±SEM). Representative images (Figure 5.2, B and C) show the changes in α2 expression in the groups exposed to FAK inhibition and 5% strain. Expression appears to be present throughout the cell body as well as localised to the plasma membrane, and there is evidence of intense perinuclear staining in response to tensile strain. These results indicate how tensile strain and inhibition of FAK, a key regulator of integrin-mediated signals, influence integrin expression in young and MSCs. There were no differences observed between age groups.

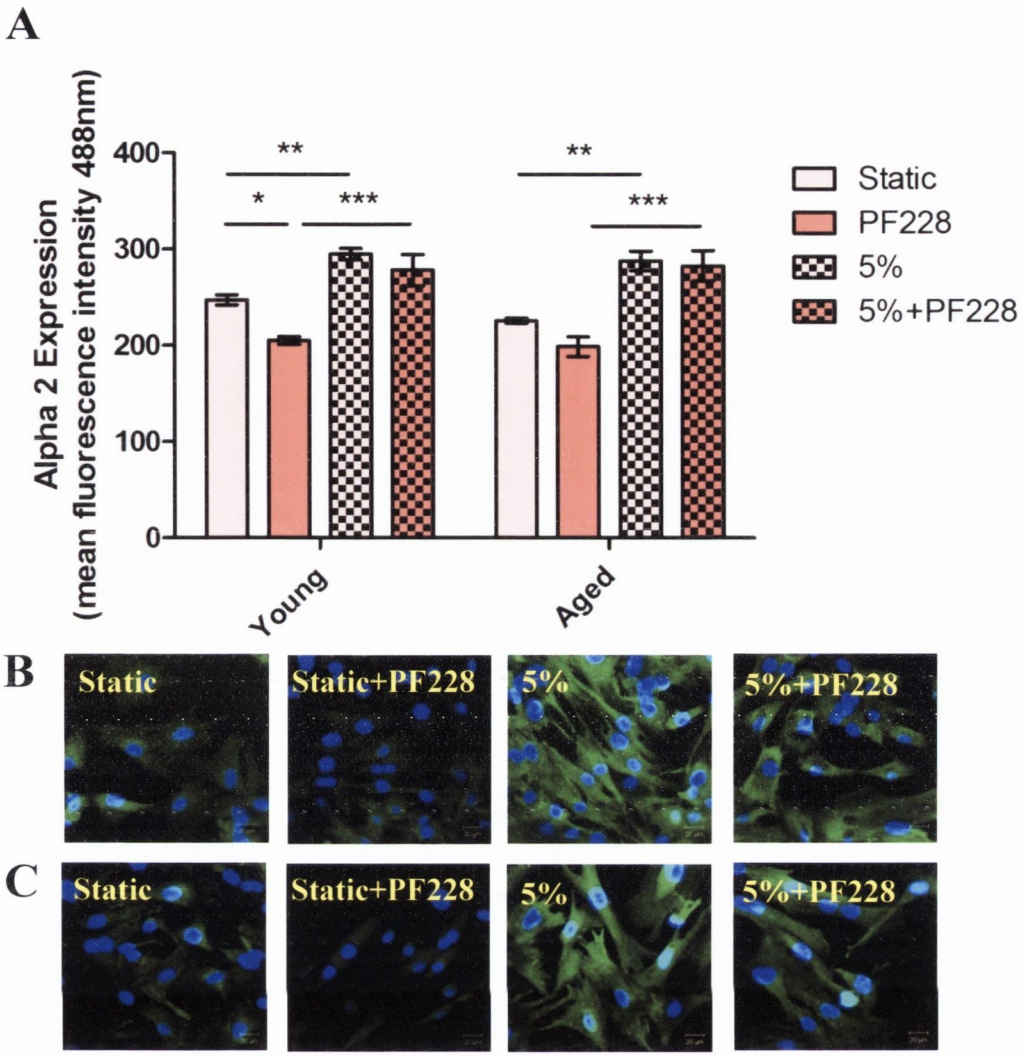


Figure 5.2: Expression of α2 integrin is increased by 5% strain for 24hrs and strain can overcome the reduction in α2 expression evoked by FAK inhibition in young MSCs

MSCs were examined for the expression of α2 by immunocytochemistry using an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 42.62, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 45.88, $p<0.001$, two-way ANOVA) MSCs and drug ($F_{1,12}$ = 10.16, $p<0.01$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in α2 expression following 5% strain for 24hr (**$p<0.01$, n=4 cultures). There was a significant decrease in α2 expression in the young FAK inhibition group compared to untreated static control (*$p<0.05$, n=4 cultures), which was reversed in response to 5% strain (***$p<0.001$, n=4 cultures). **B:** Confocal images of α2 expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.3 Cyclic tensile strain increases α2 expression in MSCs and overcomes a reduction in α2 expression induced by integrin inhibition

The effect of the integrin inhibitor RGDS was assessed alone and in combination with FAK inhibition to address how downregulation of integrin receptor function as well as FAK signalling may impact upon MSC signalling events. Expression of the integrin subunit α2 was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.3, A). In young MSCs, there was a significant effect of strain ($F_{1,12}$ = 48.90, $p<0.001$, two-way ANOVA) and an overall significant interaction ($F_{1,12}$ = 6.03, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:247.19±5.41 units, 5%:294.91±6.04 units, $p<0.05$, n=4, mean±SEM). Exposure to RGDS (0.5μM) for 24hr showed a significant decrease in α2 expression compared to untreated static controls (static control:247.19±5.41 units, RGDS:200.54±4.94 units, $p<0.05$, n=4, mean±SEM) and the application of 5% strain for 24hr in combination with integrin inhibition reversed the decrease in α2 expression observed with integrin inhibition alone, returning expression to control level (RGDS:200.54±4.94 units, 5%+RGDS:299.91±18.77 units, $p<0.001$, n=4, mean±SEM).

In aged MSCs (Figure 5.3, A), there was a significant effect of strain ($F_{1,12}$ = 37.62, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 6.98, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:225.64±2.45 units, 5%:287.74±10.02 units, $p<0.01$, n=4, mean±SEM). Exposure to RGDS (0.5μM) for 24hr showed a significant decrease in α2 expression compared to untreated static controls (static control:225.64±2.45 units, RGDS:179.84±411.73 units, $p<0.05$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with RGDS reversed the decrease in α2 expression observed with integrin inhibition alone, returning expression to control level (RGDS: 179.84±411.73 units,

5%+RGDS:268.59±18.99 units, $p<0.001$, $n=4$, mean±SEM). Representative images (Figure 5.3, B and C) show the changes in α2 expression exposed to RGDS and 5% strain. These results highlight how tensile strain and integrin inhibition influence the expression of α2 in young and aged MSCs. There were no differences observed between age groups.

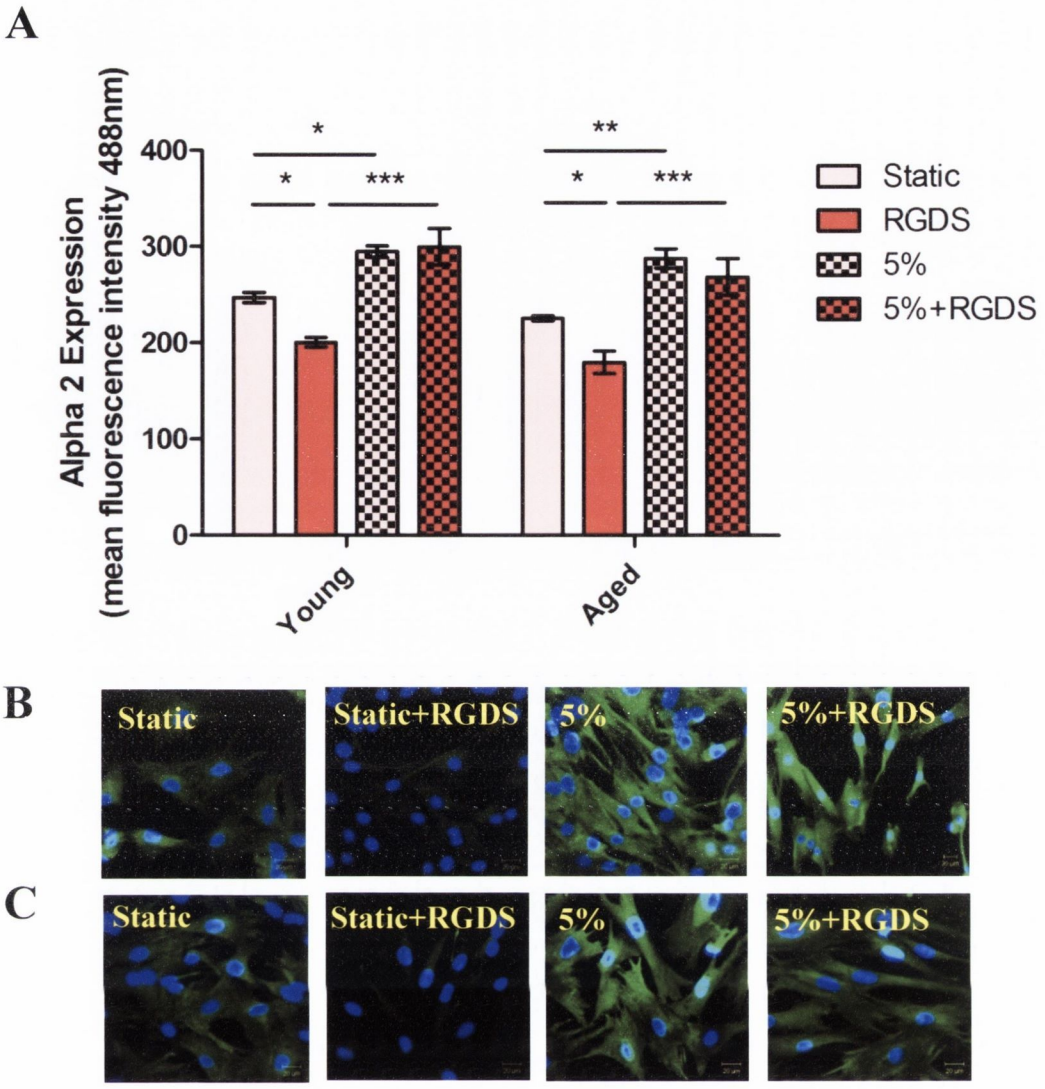


Figure 5.3: Expression of α2 integrin is increased by 5% strain for 24hr and strain can overcome the reduction in α2 expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of α2 by immunocytochemistry using an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 48.90, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 37.62, $p<0.001$, two-way ANOVA) MSCs, drug ($F_{1,12}$ = 6.98, $p<0.05$, two-way ANOVA) in aged MSCs and an overall significant interaction ($F_{1,12}$ = 6.03, $p<0.05$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in α2 expression following 5% strain for 24hr (*$p<0.05$, **$p<0.01$, n=4 cultures). There was a significant decrease in α2 expression in the integrin inhibition group compared to untreated static control (*$p<0.05$, n=4 cultures), which was reversed in MSCs exposed to strain in combination with integrin inhibition (***$p<0.001$, n=4 cultures). **B:** Confocal images of α2 expression in young MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.4 Cyclic tensile strain increases α2 expression in MSCs and overcomes a reduction in α2 expression induced by combined FAK and integrin inhibition

The combined effect of integrin and FAK inhibition on MSC cell signalling was assessed by adding both inhibitors to the media simultaneously during 24hr 5% tensile strain testing. Expression of the integrin subunit α2 was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.4, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 69.96$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 6.80$, $p<0.05$, two-way ANOVA). There was also a significant interaction in this observation ($F_{1,12} = 6.67$, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:247.19±5.41 units, 5%:294.91±6.04 units, $p<0.01$, n=4, mean±SEM). Exposure to PF228 (0.05μM) in combination with RGDS (0.5μM) for 24hr caused a significant reduction in α2 expression compared to static untreated controls (static control:247.19±5.41 units, PF228+RGDS:204.34±4.97 units, $p<0.01$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with FAK and integrin inhibition reversed the decrease in α2 expression observed with FAK and integrin inhibition, returning expression to control level (PF228+RGDS:204.34±4.97 units, 5%+PF228+RGDS:294.70±13.49 units, $p<0.001$, n=4 mean±SEM).

In aged MSCs (Figure 5.4, A), there was a significant effect of strain ($F_{1,12} = 57.51$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 8.56$, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of α2 significantly compared to static controls (static control:225.64±2.45 units, 5%:287.74±10.02 units, $p<0.01$, n=4, mean±SEM). Exposure to PF228 (0.05μM) in combination with RGDS (0.5μM) for 24hr caused a significant reduction in expression compared to static untreated controls (static control:225.64±2.45 units, PF228+RGDS:184.79±8.89 units, $p<0.05$, n=4, mean±SEM). Application of 5%

strain for 24hr in combination with FAK and integrin inhibition reversed the decrease in α2 expression observed with FAK and integrin inhibition, returning expression to control level (PF228+RGDS: 184.79±8.89 units, 5%+PF228+RGDS:271.26±14.08 units, $p<0.001$, $n=4$, mean±SEM). Representative images (Figure 5.4, B and C) show the changes in α2 expression in the groups exposed to FAK and integrin inhibition and 5% strain. These results indicate how tensile strain and combined integrin and FAK inhibition influence integrin expression in MSCs. There were no differences observed between age groups.

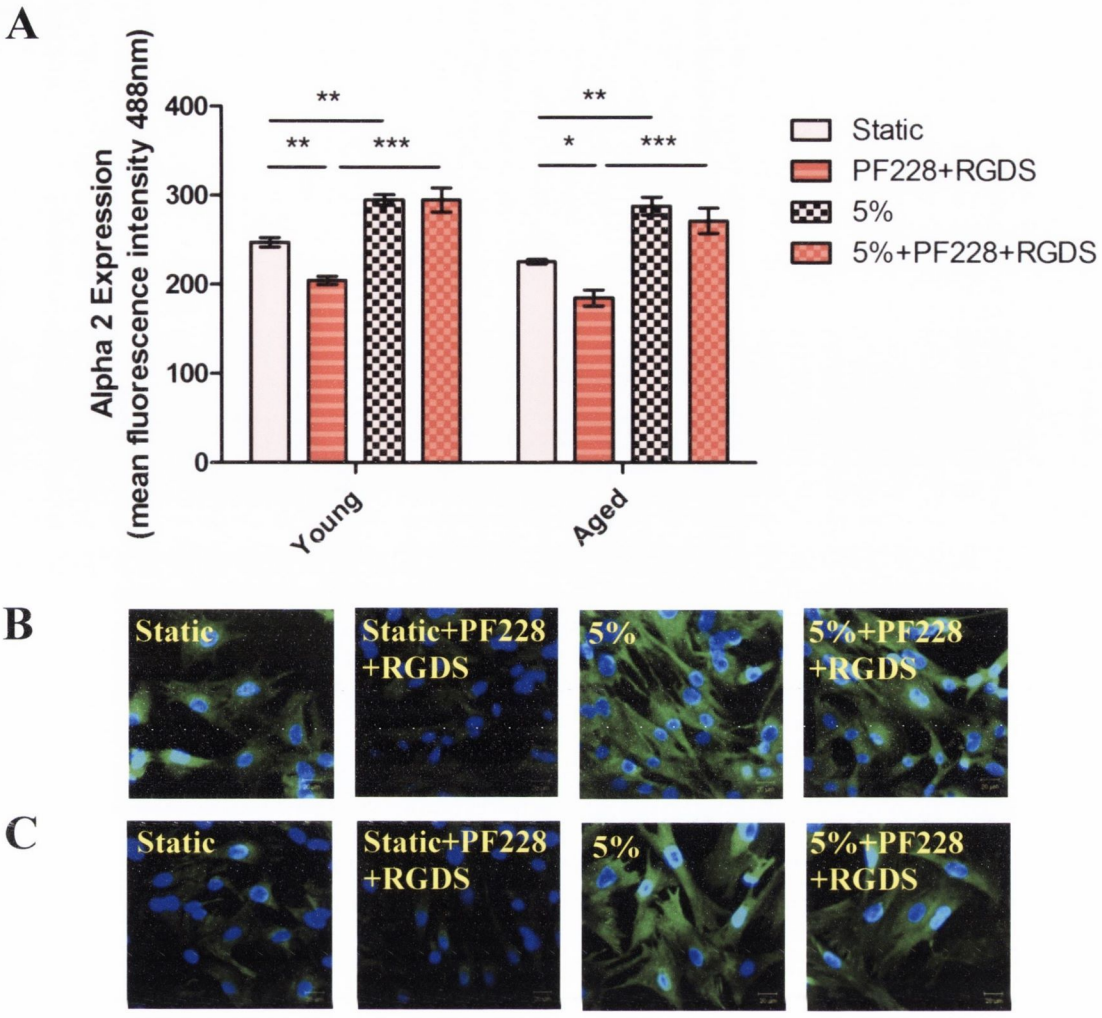


Figure 5.4: Expression of α2 integrin is increased by 5% strain for 24hr and strain can overcome the reduction in α2 expression evoked by combined FAK and integrin inhibition in MSCs

MSCs were examined for the expression of α2 by immunocytochemistry using an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 69.96, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 57.51, $p<0.001$, two-way ANOVA) MSCs and drug combination in both young ($F_{1,12}$ = 6.80, $p<0.05$, two-way ANOVA) and aged ($F_{1,12}$ = 8.56, $p<0.05$, two-way ANOVA) and a significant interaction ($F_{1,12}$ = 6.67, $p<0.05$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in α2 expression following 5% strain for 24hr (**$p<0.01$, n=4 cultures). There was a significant decrease in α2 expression in the FAK and integrin inhibition group compared to untreated static control (*$p<0.05$, **$p<0.01$, n=4 cultures), which was reversed in MSCs exposed to strain in combination with FAK and integrin inhibition (***$p<0.001$, n=4 cultures). **B:** Confocal images of α2 expression in young MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.5 Cyclic tensile strain increases actin expression in MSCs and FAK inhibition causes a reduction in expression under both static and strain conditions

The effect of the FAK inhibitor PF228 on actin expression was investigated since FAK, as a key mediator of integrin signalling, also interacts with actin in the cytoskeleton. Expression of actin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.5, A). In young MSCs, there was a significant effect of strain ($F_{1,12}$ = 66.02, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 51.18, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of actin significantly compared to static controls (static control:395.57±26.25 units, 5%:561.83±22.16 units, $p<0.001$, n=4, mean±SEM). When exposed to PF228 (0.05μM) for 24hr there was a significant decrease in actin expression compared to untreated static controls (static control:395.57±26.25 units, PF228:202.92±22.78 units, $p<0.001$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with FAK inhibition reversed the decrease in actin expression observed with FAK inhibition, returning expression to control level (PF228:202.92±22.78 units, 5%+PF228:418.39±22.53 units, $p<0.001$, n=4, mean±SEM). However, application of strain in combination with FAK inhibition caused a decrease in actin expression compared to strain alone (5%:561.83±22.16 units, 5%+PF228:418.39±22.53 units $p<0.01$, n=4, mean±SEM).

The effect of FAK inhibitor PF228 on actin expression was also investigated in aged MSCs to address differential responses with age. In aged MSCs (Figure 5.5, A), there was a significant effect of strain ($F_{1,12}$ = 35.35, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 31.39, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 5% cyclic tensile strain alone for 24hr exhibit a significant increase in actin expression compared to static controls (static control: 346.85±19.59 units, 5%:455.68±35.23 units, $p<0.05$, n=4, mean±SEM). When exposed to PF228 (0.05μM) for 24hr there was a significant decrease in actin expression compared to untreated static controls (static control:346.85±19.59 units, PF228:160.74±15.68 units, $p<0.001$, n=4,

mean±SEM). Application of 5% strain for 24hr in combination with FAK inhibition reversed the decrease in actin expression observed with FAK inhibition, returning expression to control level (PF228:160.74±15.68 units, 5%+PF228:355.61±27.17 units, $p<0.001$, $n=4$, mean±SEM). However, application of 5% strain for 24hr in combination with FAK inhibition caused a reduction in actin expression compared to strain alone (5%:455.68±35.23 units, 5%+PF228:355.61±27.17 units, $p<0.05$, $n=4$, mean±SEM). Figure 5.5, B and C show representative images of actin expression in young and aged MSCs exposed to both tensile strain and the FAK inhibitor PF228. The number of stress fibres present appears to diminish in response to drug treatment, which may explain the reduction in mean fluorescence intensity. Furthermore, in response to tensile strain it appears that more stress fibres are polymerised within the cells and they also appear to align parallel with the cell's aspect ratio. However, co-application of strain with the FAK inhibitor maintains a decrease in actin expression. The accompanying images show less disctinctive fibres which may signal that fibers are in the process of de-polymerising in response to the drug treatment. These results highlight how actin expression is influenced by both tensile strain and FAK inhibition in MSCs. There were no differences observed between age groups.

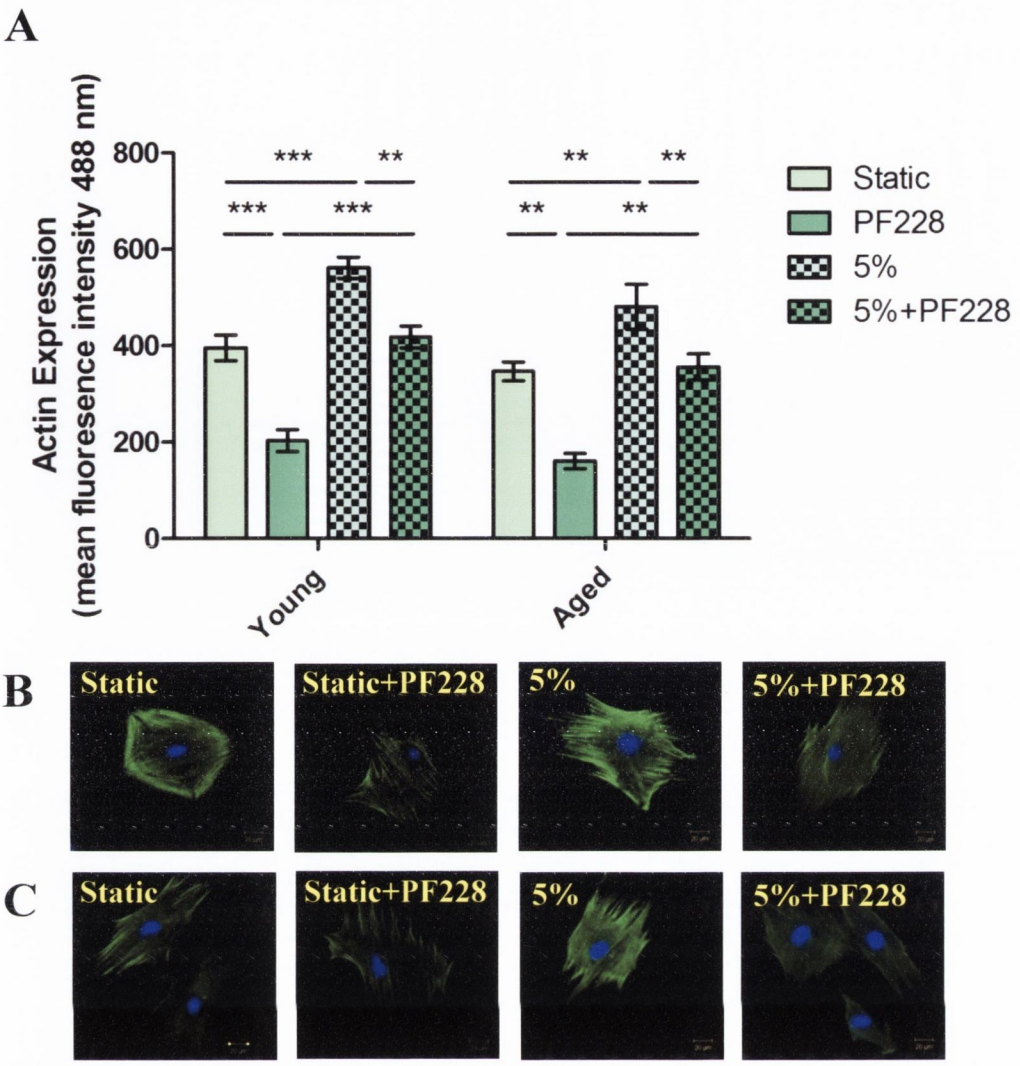


Figure 5.5: Actin expression is increased by 5% strain for 24hr and FAK inhibition reduces expression under static and strain conditions in MSCs

MSCs were examined for the expression of actin by immunocytochemistry using an anti-actin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12} = 66.02$, $p<0.001$, two-way ANOVA) and aged ($F_{1,12} = 35.35$, $p<0.001$, two-way ANOVA) MSCs and drug in young ($F_{1,12} = 51.18$, $p<0.001$, two-way ANOVA) and aged ($F_{1,12} = 31.39$, $p<0.001$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in actin expression with 5% tensile strain for 24hr compared to static control (*$p<0.05$, ***$p<0.001$, n=4 cultures), which was decreased with FAK inhibition (*$p<0.05$, **$p<0.01$, n=4 cultures). There was a significant decrease in actin expression following FAK inhibition (***$p<0.001$, n=4 cultures), which was reversed in MSCs exposed to strain in combination with FAK inhibition (***$p<0.001$, n=4 cultures). **B:** Confocal images of actin expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.6 Cyclic tensile strain increases actin expression in MSCs and overcomes a reduction in actin expression induced by integrin inhibition

The effect of integrin inhibitor RGDS on actin expression was also investigated alone and in combination with FAK inhibition. Expression of actin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.11, A). In young MSCs, there was a significant effect of strain ($F_{1,12}$ = 58.74, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 23.11, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of actin significantly compared to static controls (static control:395.57±26.25 units, 5%:561.83±22.16 units, $p<0.01$, n=4, mean±SEM). When exposed to RGDS (0.5μM) for 24hr there was a significant decrease in actin expression compared to untreated static controls (static control:395.57±26.25 units, RGDS:206.07±26.71 units, $p<0.01$, n=4, mean±SEM), and this was reversed with application of 5% strain in combination with integrin inhibition, returning expression to control level (RGDS:206.07±26.71 units, 5%+RGDS:477.06±36.89 units, $p<0.001$, n=4, mean±SEM).

In aged MSCs (Figure 5.6, A), there was a significant effect of strain ($F_{1,12}$ = 38.72, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 19.01, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 5% cyclic tensile strain alone for 24hr exhibit an increase in actin expression compared to static controls (static control: 346.85±19.59 units, 5%:455.68±35.23 units, $p<0.01$, n=4, mean±SEM). When exposed to RGDS (0.5μM) for 24hr there was a significant decrease in actin expression compared to untreated static controls (static control:346.85±19.59 units, RGDS:221.56±13.49 units, $p<0.01$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with RGDS reversed the decrease in actin expression observed with integrin inhibition, returning expression to control level (RGDS:221.56±13.49 units, 5%+RGDS:388.05±12.28 units, $p<0.001$, n=4, mean±SEM). Figure 5.6, B and C shows representative images of actin expression in young and aged MSCs

exposed to both tensile strain and RGDS. These results highlight how actin expression is upregulated by tensile strain, whilst integrin inhibition downregulates the expression in MSCs but this reduction is reversed with application of 5% tensile strain. There were no differences observed with age.

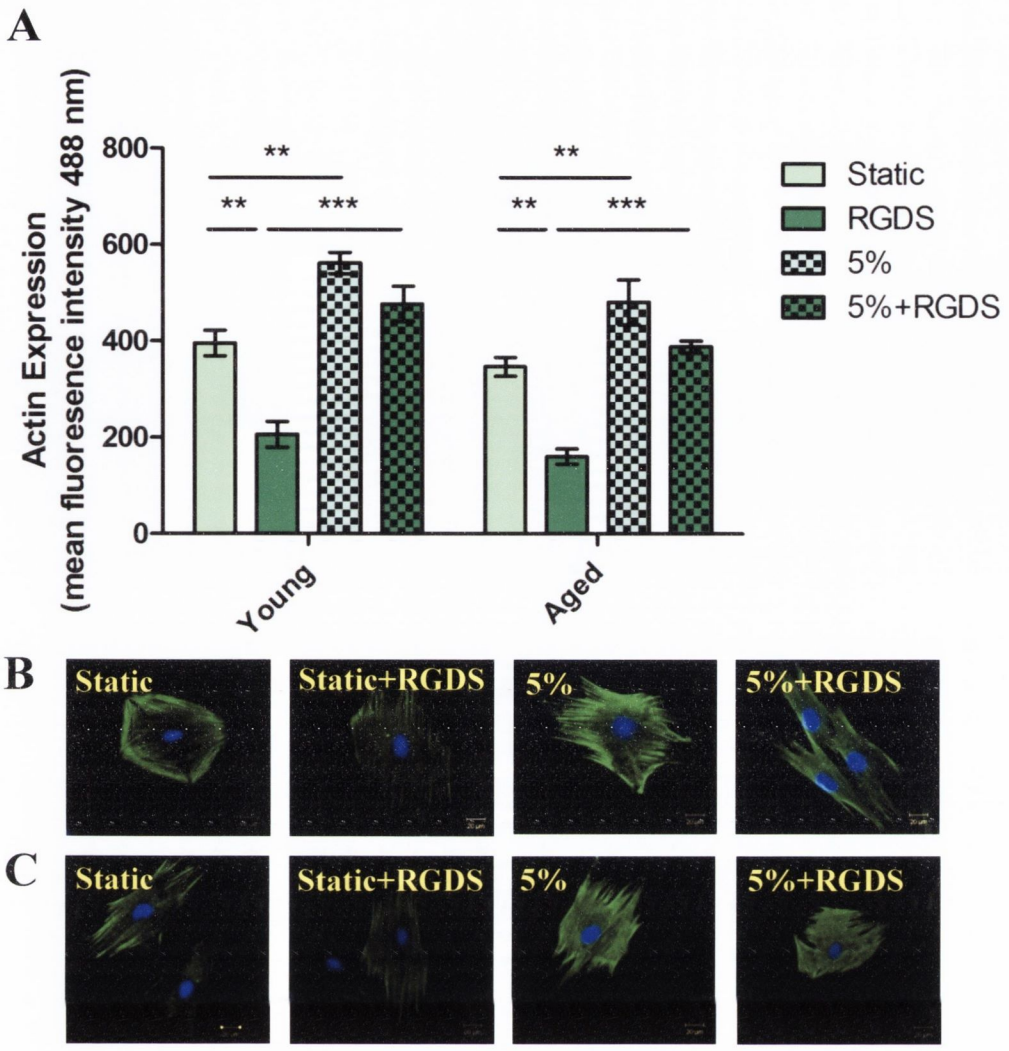


Figure 5.6: Actin expression is increased by 5% strain for 24hr and strain can overcome the reduction in actin expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of actin by immunocytochemistry using an anti-actin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 58.74, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 38.72, $p<0.001$, two-way ANOVA) MSCs and drug in young ($F_{1,12}$ = 23.11, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 19.01, $p<0.001$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in actin expression with 5% tensile strain for 24hr compared to static control (**$p<0.01$, n=4 cultures). Iintegrin inhibition caused a significant decrease in actin expression (**$p<0.01$, n=4 cultures), which was reversed in MSCs exposed to strain in combination with integrin inhibition (***$p<0.001$, n=4 cultures). **B:** Confocal images of actin expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.7 Cyclic tensile strain increases actin expression in MSCs but combined FAK and integrin inhibition cause a reduction in expression under both static and strain conditions

The combined effect of integrin and FAK inhibition on MSC cell signalling was assessed by adding both inhibitors to the media simultaneously during 24hr 5% tensile strain testing. Expression of actin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.7, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 26.65$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 18.52$, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of actin significantly compared to static controls (static control:395.57±26.25 units, 5%:561.83±22.16 units, $p<0.05$, n=4, mean±SEM). When exposed to PF228 (0.05μM) and RGDS (0.5μM) for 24hr there was a significant decrease in actin expression compared to untreated static controls (static control:395.57±26.25 units, PF228+RGDS:227.22±51.11 units, $p<0.05$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with FAK and integrin inhibition reversed the decrease in actin expression observed with combined FAK and integrin inhibition, returning expression to control level (PF228+RGDS:227.22±51.11 units, 5%+PF228+RGDS:425.92±34.70 units, $p<0.01$, n=4, mean±SEM). However, application of strain in combination with FAK and integrin inhibition caused a decrease in actin expression compared to strain alone (5%:561.83±22.16 units, 5%+PF228+RGDS:425.92±34.70 units $p<0.05$, n=4, mean±SEM).

In aged MSCs (Figure 5.7, A), there was a significant effect of strain ($F1,12 = 20.27$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 28.46$, $p<0.001$, two-way ANOVA). Post hoc analysis revealed that aged MSCs exposed to 5% cyclic tensile strain alone for 24hr show a significant increase in actin expression compared to static controls (static control:346.85±19.59 units, 5%:455.68±35.23 units, $p<0.05$, n=4, mean±SEM). When exposed to PF228 (0.05μM) and RGDS (0.5μM) for 24hr showed a significant decrease in actin expression compared to untreated static controls (static control:346.85±19.59 units,

PF228+RGDS:187.54±21.93 units, $p<0.01$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with FAK and integrin inhibition reversed the decrease in actin expression observed with FAK and integrin inhibition, returning expression to control level (PF228+RGDS:187.54±21.93 units, 5%+PF228+RGDS:324.13±29.44 units, $p<0.01$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with FAK and integrin inhibition caused a reduction in actin expression compared to strain alone (5%:455.68±35.23 units, 5%+PF228+RGDS:324.13±29.44 units, $p<0.05$, n=4, mean±SEM). Figure 5.7, B and C show representative images of actin expression in young and aged MSCs exposed to tensile strain, FAK and integrin inhibition. These results highlight how combined FAK and integrin inhibition downregulates the expression of actin in MSCs but this reduction is reversed with application of 5% tensile strain. There were no differences observed between age groups.

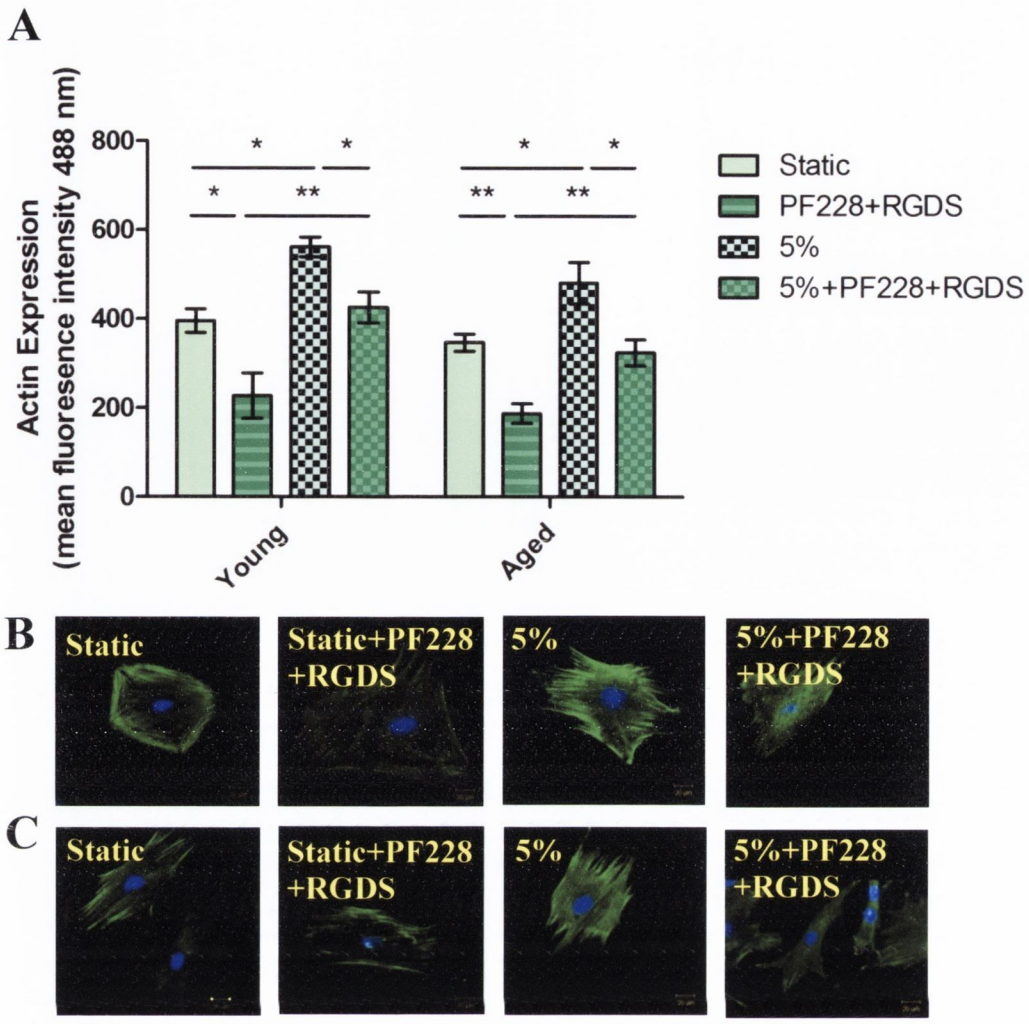


Figure 5.7: Actin expression is increased by 5% strain for 24hr and combined FAK and integrin inhibition reduce expression under static and strain conditions in MSCs

MSCs were examined for the expression of actin by immunocytochemistry using an anti-actin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 26.65, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 20.27, $p<0.001$, two-way ANOVA) MSCs and drug combination in young ($F_{1,12}$ = 18.52, $p<0.01$, two-way ANOVA) and aged ($F_{1,12}$ = 28.46, $p<0.001$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in actin expression with 5% tensile strain for 24hr compared to static control (*$p<0.05$, n=4 cultures), which was decreased with FAK and integrin inhibition (*$p<0.05$, **$p<0.01$, n=4 cultures). There was a significant decrease in actin expression following combined FAK and integrin inhibition for 24hr (*$p<0.05$, **$p<0.01$, n=4 cultures), which was reversed by 5% strain for 24hr (**$p<0.01$, n=4 cultures). **B:** Confocal images of actin expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.8 Cyclic tensile strain increases pFAK expression in MSCs and overcomes a reduction in pFAK expression induced by FAK inhibition

The effect of the FAK inhibitor PF228 on pFAK expression was investigated to assess the efficacy of this inhibitor in downregulating its specific target. Expression of pFAK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.8, A). In young MSCs, there was a significant effect of strain ($F_{1,12}$ = 101.50, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 9.48, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:204.43±20.03 units, 5%:356.88±24.09 units, $p<0.001$, n=4, mean±SEM). Exposure to PF228 (0.05μM) for 24hr caused a significant reduction in pFAK expression compared to static untreated controls (static control:204.43±20.03 units, PF228:117.03±3.50 units, $p<0.05$, n=4, mean±SEM). However, application of 5% strain for 24hr in combination with PF228 reversed the decrease in pFAK expression observed with FAK inhibition, returning expression to control level (PF228:117.03±3.50 units, 5%+PF228:332.01±18.33 units, $p<0.001$, n=4, mean±SEM).

In aged MSCs (Figure 5.8, A), there was a significant effect of strain ($F_{1,12}$ = 91.30, $p<0.001$, two-way ANOVA) and drug ($F_{1,12}$ = 7.16, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:194.03±15.13 units, 5%:337.65±17.77 units, $p<0.001$, n=4, mean±SEM). Exposure to PF228 (0.05μM) for 24hr caused a significant reduction in pFAK expression compared to static untreated controls (static control:194.03±15.13 units, PF228:115.29±4.56 units, $p<0.05$, n=4, mean±SEM). However, application of 5% strain for 24hr in combination with PF228 reversed the decrease in pFAK expression observed with FAK inhibition, returning expression to control level (PF228:115.29±4.56 units, 5%+PF228:319.09±27.51 units, $p<0.001$, n=4, mean±SEM). Representative images (Figure 5.8, B and C) show the changes in pFAK expression in young and aged MSCs exposed to FAK

inhibition and 5% strain. These results indicate how tensile strain and inhibition of FAK, a key regulator of integrin-mediated signals, influence pFAK expression in MSCs.

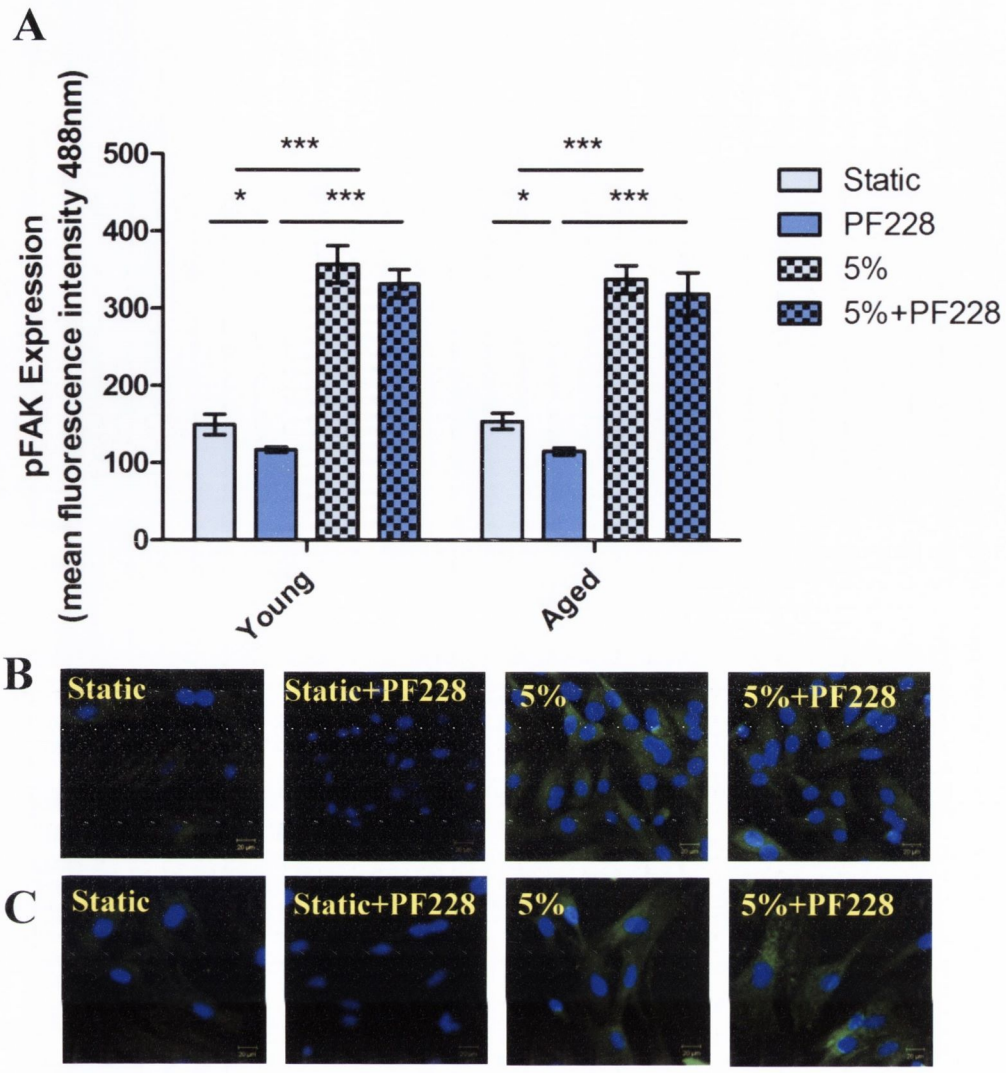


Figure 5.8: Expression of pFAK is increased by 5% strain for 24hr and strain can overcome the reduction in pFAK expression evoked by FAK inhibition in MSCs

MSCs were examined for the expression of pFAK by immunocytochemistry using an anti-pFAK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 101.50, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 91.30, $p<0.001$, two-way ANOVA) MSCs and drug in young ($F_{1,12}$ = 9.48, $p<0.01$, two-way ANOVA) and aged ($F_{1,12}$ = 7.16, $p<0.05$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in pFAK expression following 5% strain for 24hr (***$p<0.001$, n=4 cultures). There was a significant decrease in pFAK expression in the FAK inhibition group compared to untreated static control (*$p<0.05$, n=4 cultures), which was reversed following application of 5% strain (***$p<0.001$, n=4 cultures). **B:** Confocal images of pFAK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.9 Cyclic tensile strain increases pFAK expression in MSCs and overcomes a reduction in pFAK expression induced by integrin inhibition

The effect of the integrin inhibitor RGDS was assessed alone and in combination with FAK inhibition to address how downregulation of integrin receptor function as well as FAK signalling may impact upon MSC signalling events. Expression of the integrin subunit pFAK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.9, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 65.19$, $p<0.001$, two-way ANOVA) and drug ($F_{1,12} = 9.43$, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:204.03±20.03 units, 5%:356.88±24.09 units, $p<0.001$, n=4, mean±SEM). Exposure to RGDS (0.5μM) for 24hr showed a significant decrease in pFAK expression compared to untreated static controls (static control:204.03±20.03 units, RGDS:129.06±9.58 units, $p<0.05$, n=4, mean±SEM) and the application of 5% strain for 24hr in combination with RGDS reversed the decrease in pFAK expression observed with integrin inhibition alone, returning expression to control level (RGDS:129.06±9.58 units, 5%+RGDS:306.72±24.46 units, $p<0.001$, n=4, mean±SEM).

The effect of the integrin inhibitor RGDS was assessed alone and in combination with FAK inhibition to address how downregulation of integrin receptor function as well as FAK signalling may impact upon signalling events in aged MSCs (Figure 5.9, A).There was a significant effect of strain ($F_{1,12} = 112.60$, $p<0.001$, two-way ANOVA) and drug ($F_{1,12} = 10.41$, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:194.03±15.13 units, 5%:337.65±17.77 units, $p<0.001$, n=4, mean±SEM). Exposure to RGDS (0.5μM) for 24hr showed a significant decrease in pFAK expression compared to untreated static controls (static control:194.03±15.13 units, RGDS:127.98±8.27 units, $p<0.05$, n=4, mean±SEM) and the application of

5% strain for 24hr in combination with RGDS reversed the decrease in pFAK expression observed with integrin inhibition alone, returning expression to control level (RGDS:127.9±8.27 units, 5%+RGDS:305.92±17.48 units, $p<0.001$, $n=4$, mean±SEM). Representative images (Figure 5.18, B) show the changes in pFAK expression in young and aged MSCs exposed to RGDS and 5% strain. These results highlight how tensile strain and integrin inhibition influence the expression of pFAK in MSCs.

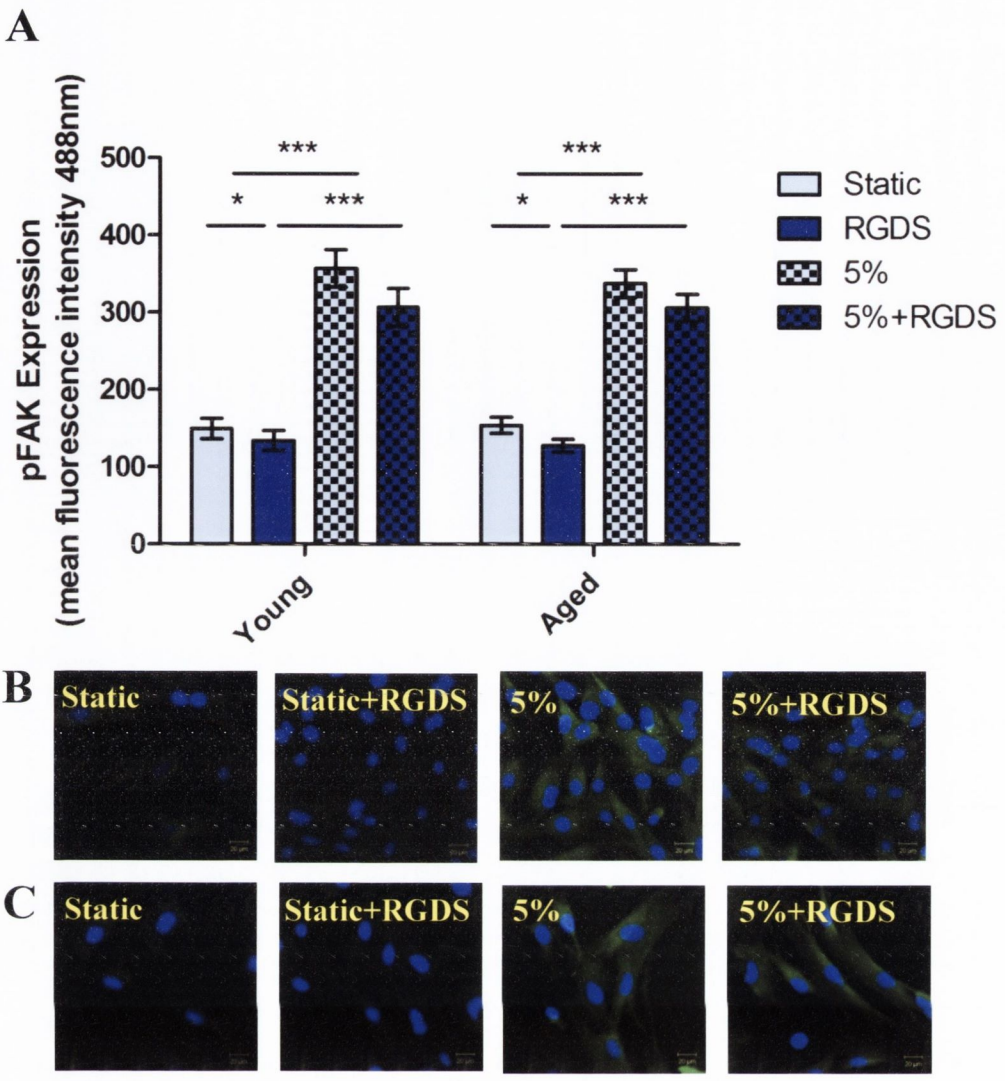


Figure 5.9: Expression of pFAK is increased by 5% strain for 24hr and strain can overcome the reduction in pFAK expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of pFAK by immunocytochemistry using an anti-pFAK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12}$ = 65.19, $p<0.001$, two-way ANOVA) and aged ($F_{1,12}$ = 112.60, $p<0.001$, two-way ANOVA) and drug in young ($F_{1,12}$ = 9.43, $p<0.01$, two-way ANOVA) and aged ($F_{1,12}$ = 10.41, $p<0.01$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in pFAK expression following 5% strain for 24hr (***$p<0.001$, n=4 cultures). There was a significant decrease in pFAK expression in the integrin inhibition group compared to untreated static control (*$p<0.05$, n=4 cultures), which was reversed following application of 5% strain (***$p<0.001$, n=4 cultures). **B:** Confocal images of pFAK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.10 Cyclic tensile strain increases pFAK expression in MSCs and overcomes a reduction in pFAK expression induced by combined FAK and integrin inhibition

The combined effect of integrin and FAK inhibition on MSC cell signalling was assessed by adding both inhibitors to the media simultaneously during 24hr 5% tensile strain testing. Expression of pFAK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.10, A). In young MSCs, there was a significant effect of strain ($F_{1,12} = 97.20$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 6.17$, $p<0.05$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:204.03±20.03 units, 5%:356.88±24.09 units, $p<0.001$, n=4, mean±SEM). Exposure to PF228 (0.05μM) in combination with RGDS (0.5μM) for 24hr caused a significant reduction in pFAK expression compared to static untreated controls (static control:204.43±20.03 units, PF228+RGDS:132.34±10.66 units, $p<0.05$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with PF228 and RGDS reversed the decrease in pFAK expression observed with FAK and integrin inhibition, returning expression to control level (PF228+RGDS:132.34±10.66 units, 5%+PF228+RGDS:338.62±15.12 units, $p<0.001$, n=4, mean±SEM).

In aged MSCs (Figure 5.10, A), there was a significant effect of strain ($F_{1,12} = 68.81$, $p<0.001$, two-way ANOVA) and drug combination ($F_{1,12} = 10.43$, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed that aged MSCs exposed to 5% cyclic tensile strain for 24hr increased the expression of pFAK significantly compared to static controls (static control:194.03±15.13 units, 5%:337.65±17.77 units, $p<0.001$, n=4, mean±SEM). Exposure to PF228 (0.05μM) in combination with RGDS (0.5μM) for 24hr caused a significant reduction in pFAK expression compared to static untreated controls (static control:194.03±15.13 units, PF228+RGDS:124.91±5.61 units, $p<0.05$, n=4, mean±SEM). Application of 5% strain for 24hr in combination with PF228 and RGDS reversed the decrease in pFAK expression observed with FAK and integrin inhibition, returning expression

to control level (PF228+RGDS:124.91±5.61 units, 5%+PF228+RGDS:287.53±28.05 units, $p<0.001$, mean±SEM).Representative images (Figure 5.10, B and C) show the changes in pFAK expression in young and aged MSCs exposed to combined FAK and integrin inhibition and 5% strain. These results indicate how tensile strain and combined integrin and FAK inhibition influence pFAK expression in MSCs. There were no differences observed with age.

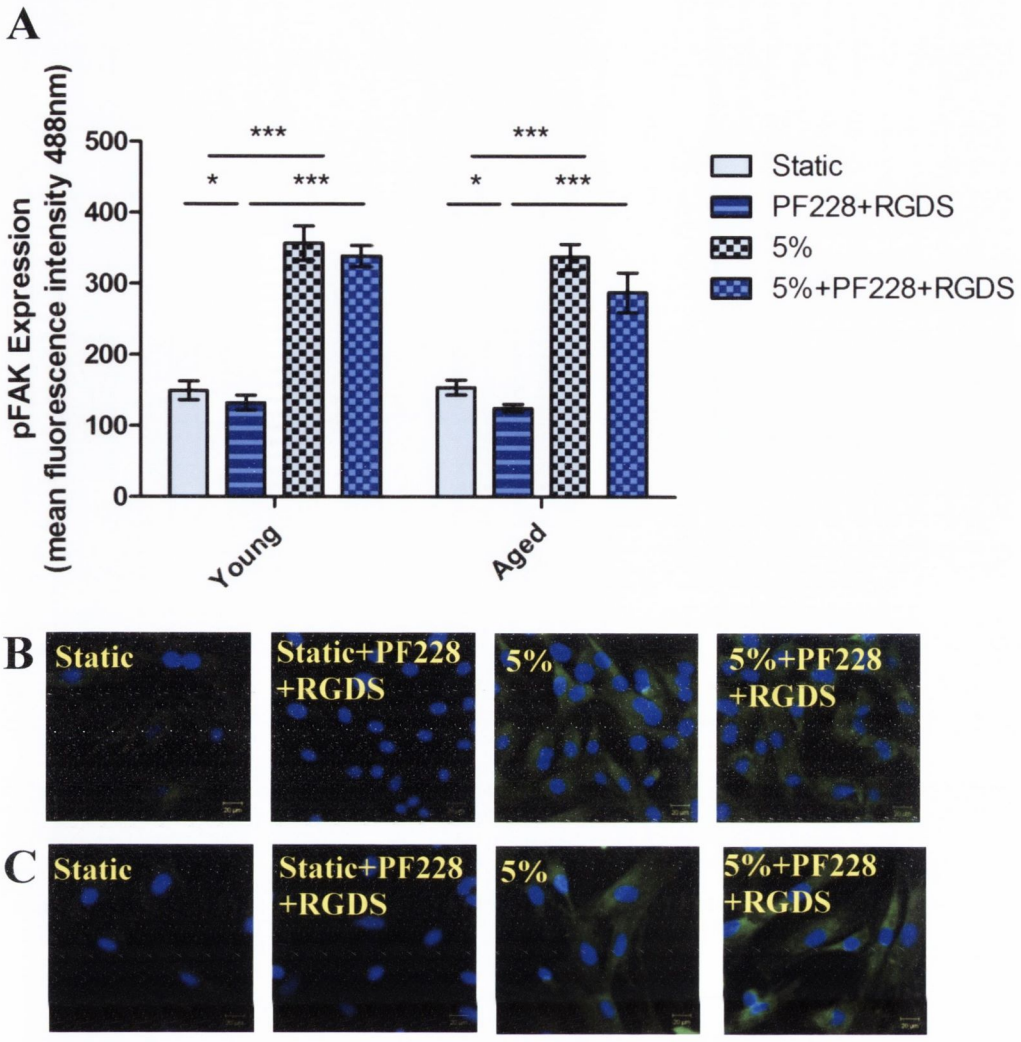


Figure 5.10: Expression of pFAK is increased by 5% strain for 24hr and strain can overcome the reduction in pFAK expression evoked by combined FAK and integrin inhibition in MSCs

MSCs were examined for the expression of pFAK by immunocytochemistry using an anti-pFAK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,12} = 97.20$, $p<0.001$, two-way ANOVA) and aged ($F_{1,12} = 68.81$, $p<0.001$, two-way ANOVA) MSCs and drug combination in young ($F_{1,12} = 6.17$, $p<0.05$, two-way ANOVA) and aged ($F_{1,12} = 10.43$, $p<0.01$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in pFAK expression following 5% strain for 24hr (***$p<0.001$, n=4 cultures). There was a significant decrease in pFAK expression in the FAK and integrin inhibition group compared to untreated static control (*$p<0.05$, n=4 cultures), which was reversed following application of 5% strain (***$p<0.001$, n=4 cultures). **B:** Confocal images of pFAK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.11 Cyclic tensile strain and FAK inhibition do not affect pERK expression in MSCs

The effect of the FAK inhibitor PF228 on pERK expression was investigated in MSCs to assess the effect on downstream targets when the signalling pathway is altered. Expression of pERK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.11, A). In young MSCs, there was no effect of either strain or drug on pERK expression. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and FAK inhibition, applied alone or in combination (static control:224.42±18.92 units, PF228:200.25±34.43 units, 5%:239.18±17.90 units, 5%+PF228:250.23±10.71 units, n=4, mean±SEM).

In aged MSCs (Figure 5.11, A), there was a significant effect of strain ($F_{1,12}$ = 5.83, $p<0.05$, two-way ANOVA). However, *Post hoc* analysis showed no within group differences. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and FAK inhibition, applied alone or in combination (static control:215.56±17.35 units, PF228:208.58±10.76 units, 5%:256.70±16.06 units, 5%+PF228:234.43±9.73 units, n=4, mean±SEM). Representative images (Figure 5.11, B and C) show pERK expression in young and aged MSCs exposed to FAK inhibition and 5% strain. These results indicate how tensile strain and inhibition of FAK, a key regulator of integrin-mediated signals, have no influence on pERK expression in MSCs.

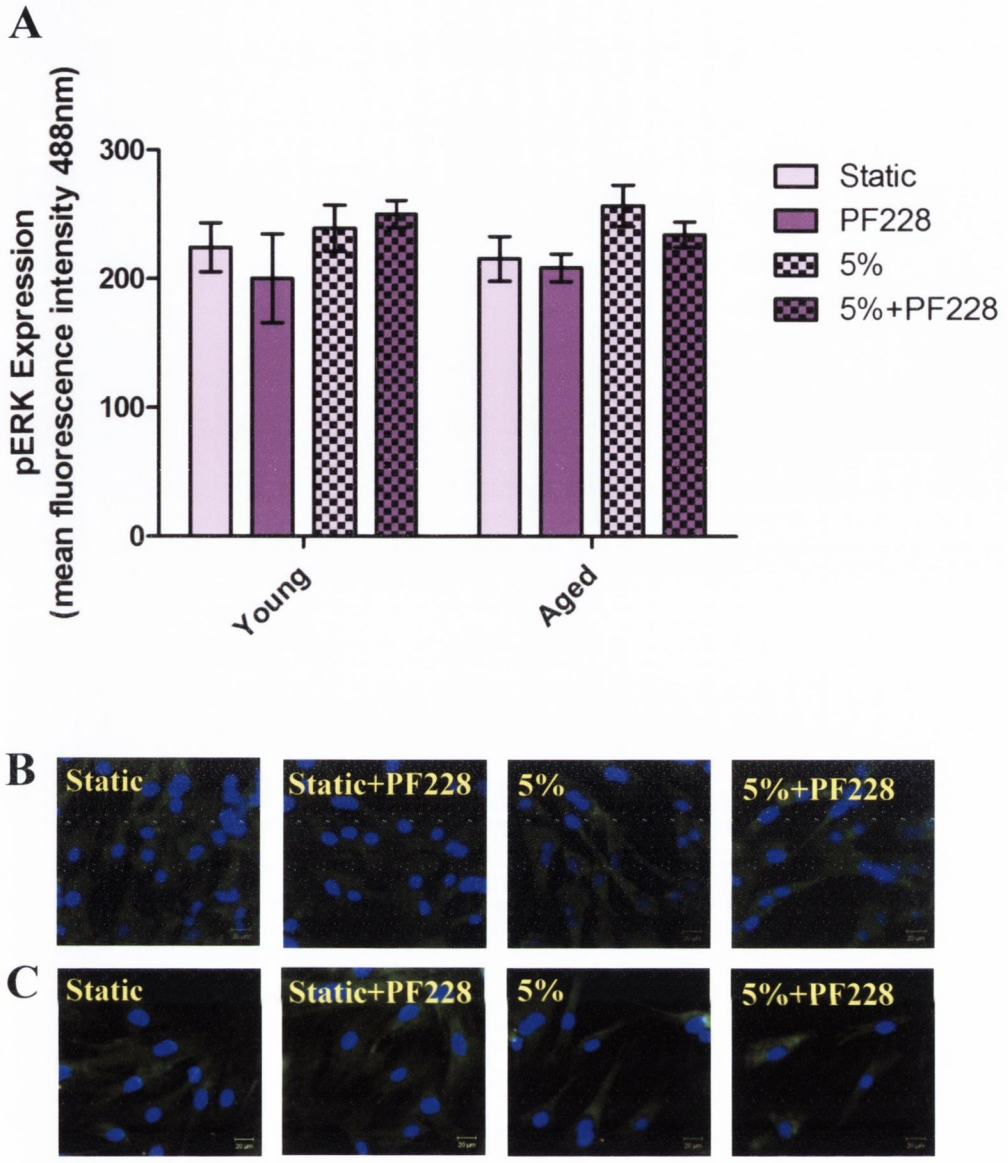


Figure 5.11: Expression of pERK is unaffected by 5% strain and FAK inhibition for 24hr in MSCs

MSCs were examined for the expression of pERK by immunocytochemistry using an anti-pERK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was no effect of either strain or FAK inhibition on pERK expression in young MSCs. There was a significant effect of strain ($F_{1,12} = 5.83$, $p<0.05$, two-way ANOVA) in aged MSCs however, *Post hoc* analysis showed no within group differences. **B:** Confocal images of pERK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.12 Cyclic tensile strain and integrin inhibition do not affect pERK expression in MSCs

The effect of the integrin inhibitor RGDS on pERK expression was investigated in MSCs to assess the effect of altering the signalling pathway on downstream targets. Expression of pERK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.12, A). In young MSCs, there was no effect of either strain or drug on pERK expression. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and integrin inhibition, applied alone or in combination (static control:224.42±18.92 units, RGDS:196.20±17.76 units, 5%:239.18±17.90 units, 5%+RGDS:206.99±18.01 units, n=4, mean±SEM).

There was a significant effect of strain ($F_{1,12} = 7.29$, $p<0.05$, two-way ANOVA) in aged MSCs (Figure 5.12, A) however, *Post hoc* analysis showed no within group differences. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and integrin inhibition, applied alone or in combination (static control:215.56±17.35 units, RGDS:204.71±18.84 units, 5%:256.70±16.06 units, 5%+RGDS:245.36±2.05 units, n=4, mean±SEM). Representative images (Figure 5.12, B and C) show pERK expression in young and aged MSCs exposed to integrin inhibition and 5% strain. These results indicate how tensile strain and integrin inhibition yield no influence on pERK expression in MSCs.

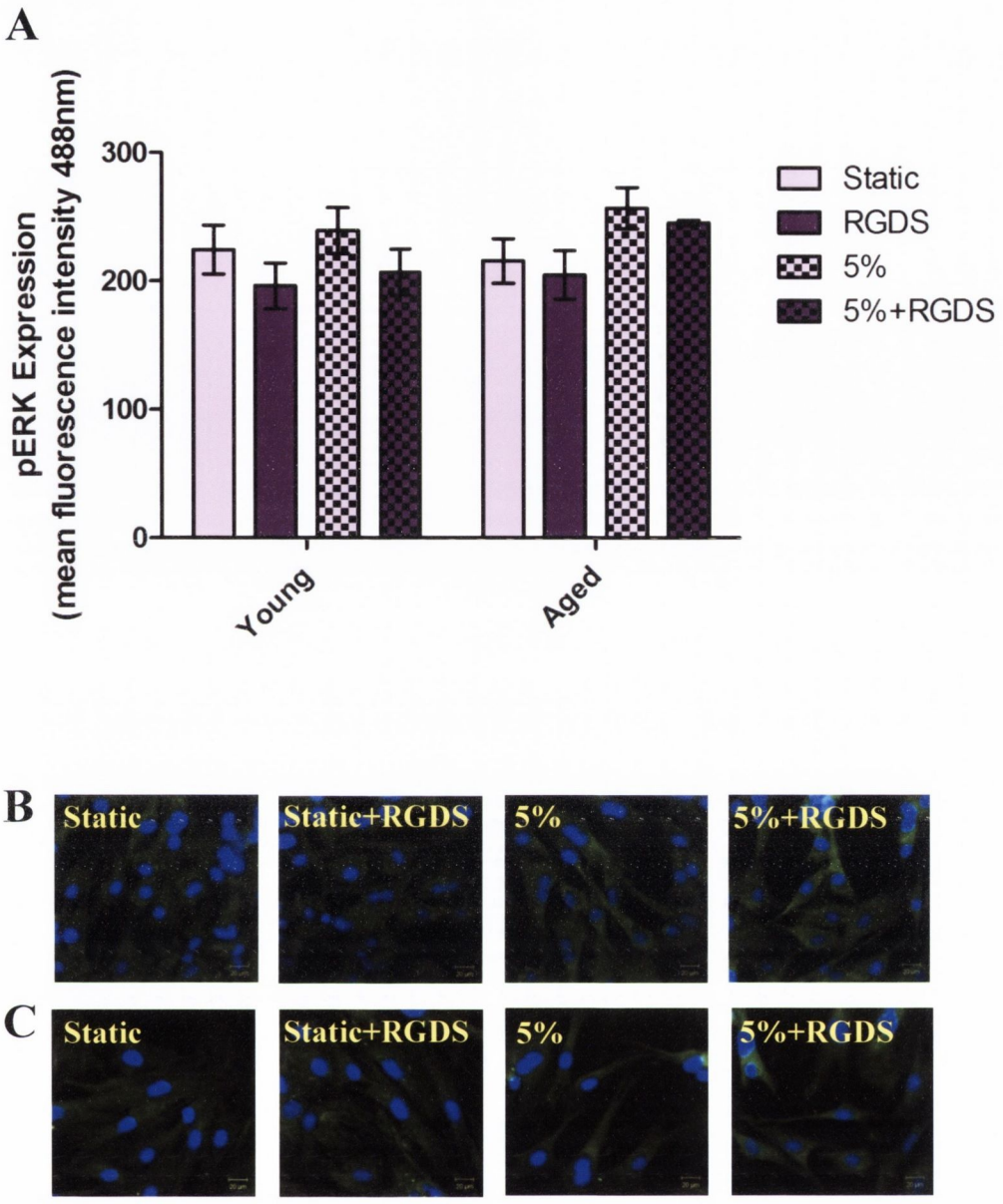


Figure 5.12: Expression of pERK is unaffected by 5% strain and integrin inhibition for 24hr in MSCs

MSCs were examined for the expression of pERK by immunocytochemistry using an anti-pERK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was no effect of either strain or integrin inhibition on pERK expression in young MSCs. There was a significant effect of strain ($F_{1,12} = 7.29$, $p<0.05$, two-way ANOVA) in aged MSCs however, *Post hoc* analysis showed no within group differences. **B:** Confocal images of pERK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.13 Cyclic tensile strain and combined FAK and integrin inhibition do not affect pERK expression in MSCs

The effects of the FAK inhibitor PF228 and the integrin inhibitor RGDS on pERK expression were investigated in MSCs by adding both inhibitors to the media simultaneously during 24hr 5% tensile strain testing. Expression of pERK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 5.13, A). In young MSCs, there was no effect of either strain or drug combination on pERK expression. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and combined FAK and integrin inhibition, applied alone or in combination (static control:224.42±18.92 units, PF228+RGDS:189.41±25.18 units, 5%:239.18±17.90 units, 5%+PF228+RGDS:242.35±19.43 units, n=4, mean±SEM).

There was a significant effect of strain ($F_{1,12} = 9.61$, $p<0.01$, two-way ANOVA) in aged MSCs (Figure 5.12, A) however, *Post hoc* analysis showed no within group differences. No changes in pERK expression were observed following 24hr exposure to 5% cyclic tensile strain and combined FAK and integrin inhibition, applied alone or in combination (static control:215.56±17.35 units, PF228+RGDS:186.15±18.17 units, 5%:256.70±16.06 units, 5%+PF228+RGDS:246.65±9.56 units, n=4, mean±SEM). Representative images (Figure 5.12, B and C) show pERK expression young and aged MSCs exposed to combinedFAK and integrin inhibition and 5% strain. These results indicate how tensile strain and combined FAK and integrin inhibition exert no influence on pERK expression in MSCs.

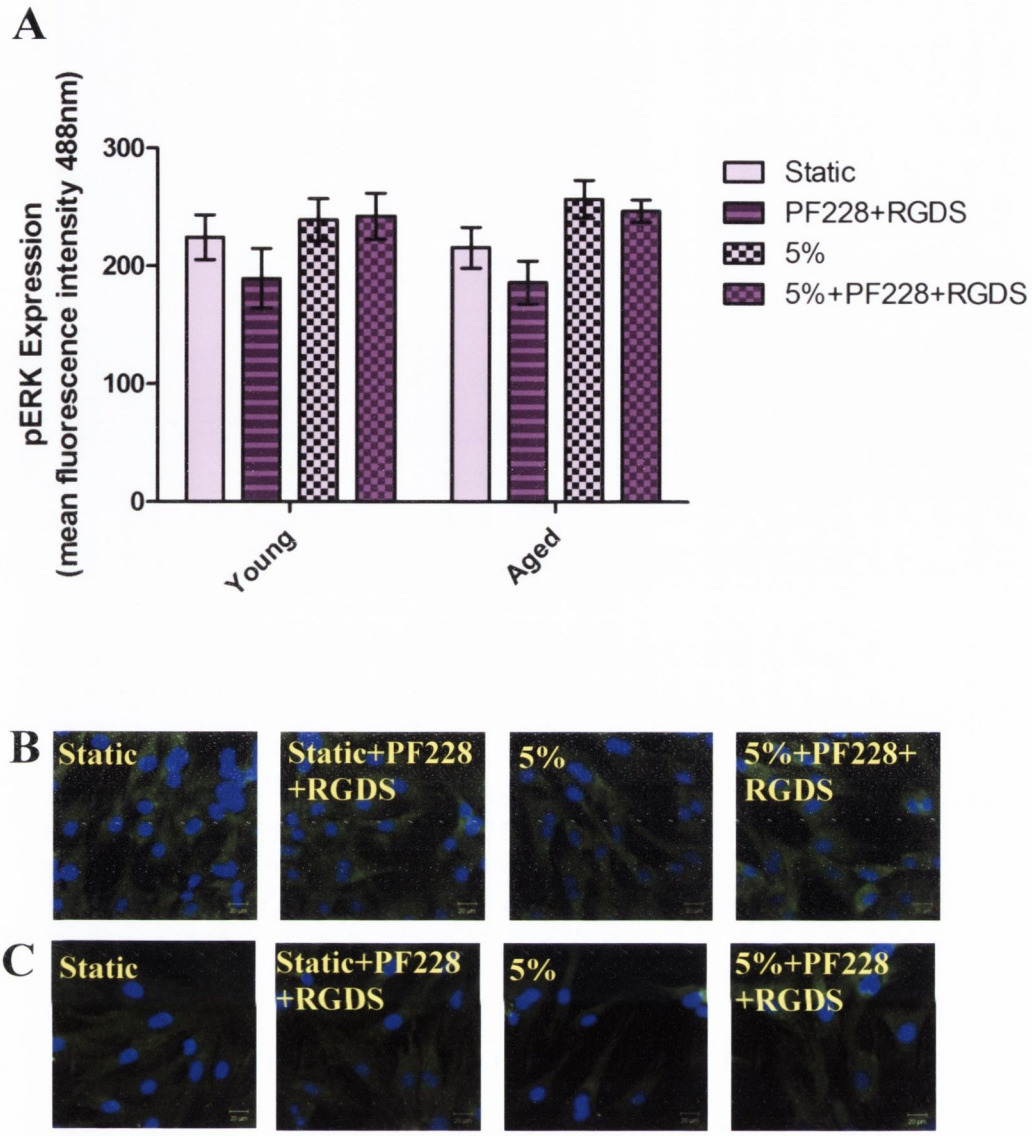


Figure 5.13: Expression of pERK is unaffected by 5% strain and combined FAK and integrin inhibition for 24hr in MSCs

MSCs were examined for the expression of pERK by immunocytochemistry using an anti-pERK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was no effect of either strain or combined FAK and integrin inhibition on pERK expression in young MSCs. There was a significant effect of strain ($F_{1,12} = 9.61$, $p<0.01$, two-way ANOVA) in aged MSCs however, *Post hoc* analysis showed no within group differences. **B:** Confocal images of pERK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.2.14 Expression of integrin subunit α2 is upregulated on a collagen I-coated silicone substrate

It was highlighted in Section 3.23/Figure 3.3 that expression of integrin subunit α2 is reduced in aged MSCs compared to young. These experiments were carried out on glass coverslips 2-3 days following initial cell seeding. Interestingly, it was noted in the current study that expression of α2 was unaltered with age in the static control groups. However, in this study the cells were seeded onto a different substrate, silicone, which was coated with a solution of ECM protein collagen I. Using immunocytochemistry and confocal microscopy, changes in the expression of α2 were indicated by alterations in mean fluorescence intensity values (Figure 5.14). There was a significant increase in α2 expression in young MSCs seeded onto the silicone substrate compared to glass coverslips (Young glass:180.27±8.15 units, Young silicone: 252.90±9.77 units, $p<0.001$, Student's t-test, 8 independent observations, n=3-5, mean±SEM). Similarly, in aged MSCs there was a significant increase in α2 expression in MSCs seeded onto the silicone substrate compared to glass coverslips (Aged glass:141.52±7.91 units, Aged silicone: 230.53±5.95 units, $p<0.001$, Student's t-test, 8 independent observations, n=3-5, mean±SEM). When comparing α2 expression on the different substrates between age groups it was found that there was a significant effect of both substrate ($F_{1,28} = 100.60$, $p<0.001$, two-way ANOVA) and age ($F_{1,28} = 14.38$, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed a significant decrease in α2 expression in aged MSCs seeded to glass coverslips compared to young (Young glass:180.27±8.15 units, Aged glass:141.52±7.91 units, $p<0.01$, 8 independent observations, n=3-5, mean±SEM) but no change was observed in the silicone groups. These results indicate that expression of α2 is upregulated when seeded to a silicone substrate, coated with an adherence promoter. Since α2 naturally binds to collagen in the ECM it is possible that the presence of the collagen I coating functions in upregulating integrin expression in both young and aged MSCs.

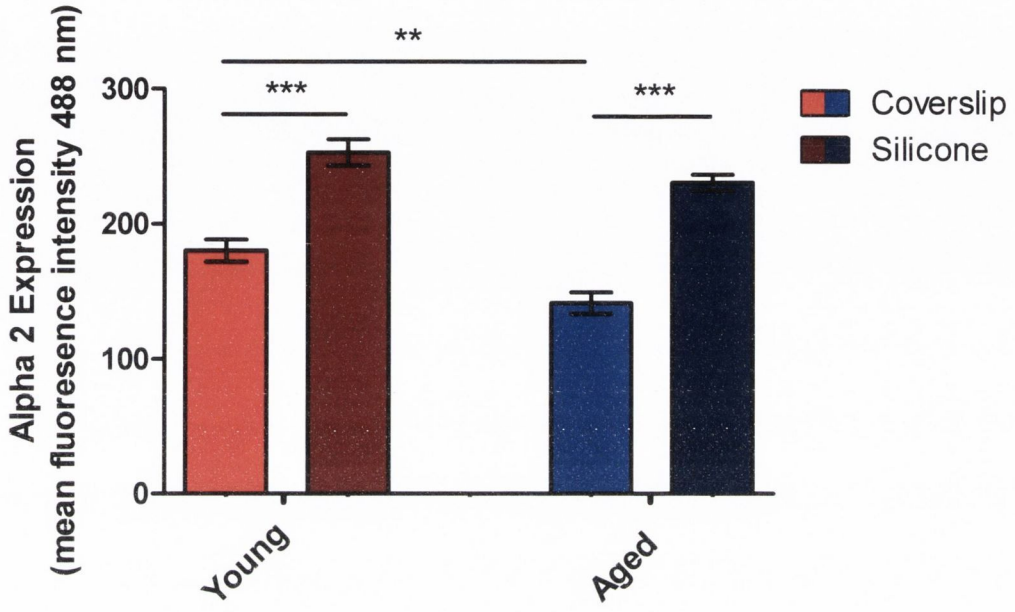


Figure 5.14: Integrin expression is upregulated in MSCs seeded onto a collagen I-coated silicone substrate

MSCs were examined for the expression of α2 by immunocytochemistry using an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. Expression of α2 was significantly increased in MSCs seeded to the coated silicone substrate compared to glass (***$p<0.001$, Student's t-test, 8 independent observations, n=3-5). When comparing expression with age, there was a significant effect of substrate ($F_{1, 28} = 100.60$, $p<0.001$, two-way ANOVA) and age ($F_{1, 28} = 14.38$, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed a significant decrease in α2 expression in MSCs seeded to glass coverslips (**$p<0.01$, 8 independent observations, n=3-5) but there was no change in cells seeded to the silicone substrates. Results are displayed as mean ± SEM. Scale bar is 20μm.

5.3 Discussion

The aim of this study was to assess the effect of a mid-level rate of strain (5%) on mechanotransduction pathways in young and aged MSCs and to evaluate the role of integrin receptors and FAK in such pathways by including inhibitors for these proteins in the media during strain testing. It was first established that 5% cyclic tensile strain applied for 24hr maintained cell viability such that no changes were observed in caspase-3 activity in either young or aged MSCs. Expression of integrin subunit α2, actin and the phosphorylated form of FAK (pFAK) were all observed to increase in response to 5% cyclic tensile strain for 24hr, whilst integrin and FAK inhibition reduced the expression of all three proteins when cells were treated with either inhibitor or a combination of both. The only exception was that FAK inhibition had no effect on α2 expression in aged MSCs. In all cases where inhibitor treatment was applied in combination with tensile strain, expression of α2, actin and pFAK increased in comparison to individual and combined inhibitor treatment in the absence of tensile strain. Thus, 5% cyclic tensile strain effectively reverses the decrease in protein expression induced by integrin and FAK inhibition in both age groups. Actin expression was also decreased in response to FAK inhibition, even in the presence of tensile strain. Furthermore, no changes were observed in phosphorylated-ERK (pERK) expression following application of tensile strain or inhibitor treatment. Finally, it was also observed that expression of α2 is significantly upregulated in MSCs seeded to a collagen I-coated silicone substrate compared to glass coverslips.

Prior to investigating mechanotransduction related protein expression, it was established whether application of 5% cyclic tensile strain induced apoptosis when combined with integrin inhibition. No change in active caspase-3 expression was observed in response to tensile strain for 24hr, in the absence or presence of integrin inhibition, in either young or aged MSCs. These results are in agreement with previous findings reported from this laboratory whereby continuous strain of magnitudes lower than 7.5% failed to initiate apoptosis in young MSCs (Kearney *et al.* 2008). Various studies have demonstrated induction of apoptosis or activation of associated pathways by tensile strain in tendon cells (Arnoczky *et al.* 2006) and vascular smooth muscle cells (Sotoudeh *et al.* 2001) while others have

reported maintenance of cell viability in MSCs (Sumanasinghe *et al.* 2006) and dermal fibroblasts (Nishimura *et al.* 2007). Opposing findings such as those described stem from variability in experimental set up present from study to study. For instance results can differ depending on cell type used, strain magnitude, strain frequency, duration of stimulus and length of experiment. In this study, 5% continuous strain and integrin inhibition for 24hr did not induce activation of an apoptotic signal, therefore these factors were deemed suitable for further experimentation.

Integrin molecules are considered apt candidates in mechanotransduction as they span the cell membrane, connecting both the cytoskeleton and the ECM. Through this connection, they fulfil one of the primary functions of a mechanosensor, which is to link the transcriptional machinery of a cell to its external environment (Ingber 2006, Katsumi *et al.* 2004). Their expression in the cell membrane is also regulated by interactions with various ECM ligands. It has been suggested that integrin binding to a substrate-bound ligand may initiate a series of signalling events that ultimately regulate the expression of integrins themselves (Chen *et al.* 1992). It has been reported that synthesis and transcription of the α2β1 integrin is selectively upregulated when fibroblasts are seeded onto type I collagen gels (Klein *et al.* 1991). Similarly, it has been reported here that expression of the integrin subunit α2 is significantly upregulated in both young and aged MSCs when seeded onto a silicone substrate coated with a collagen I solution. In several biological systems, integrins are altered to a high affinity state in response to mechanical force. Although, the means by which physical stimuli are transduced into biological responses through integrins and then integrated across multiple cell types culminating in a coordinated tissue-level response remains inadequately understood (Leucht *et al.* 2007).

A study by Peake *et al.* (2000) investigated the regulatory pathways involved in the rapid response of the AP-1 transcription factor, c-fos, to mechanical load in human primary osteoblast-like cells and the human MG-63 bone cell line. It was reported that both cell types showed upregulation of c-fos expression on fibronectin and collagen I substrates. The upregulation of c-fos transcription has been identified as an early member of a larger flow of mechanically stimulated

transcriptional responses, which potentially results in load-mediated remodelling of the matrix. Addition of Cytochalasin D and Arg-Gly-Asp (RGD) peptides during loading did not inhibit the response, whereas addition of β1 integrin subunit antibodies inhibited the load response. To further explore the importance of the RGD-binding mechanism in the load response, they used soluble RGD peptides to bind to the available sites on the membrane, before and during loading. Their results suggest that extended blocking of the RGD cell-matrix binding sites is not sufficient to block c-fos gene expression in either cell type investigated. This may indicate that, in the absence of RGD-mediated integrin interactions, RGD-independent but integrin-mediated interactions are enough to activate the mechanical load response in this case. In primary bone cells, their results indicate that β1 integrin-mediated interactions may be essential for the c-fos load response, as treatment with the anti-β1 antibody completely blocks this response. Although mechanotransduction may occur through an RGD-mediated pathway, it may not be critical, as other non-RGD-mediated pathways are available. The β1 integrin subunit link, however, appears to be necessary here.

In the current study, it was observed that integrin inhibition using a soluble RGDS peptide led to the reduced expression of integrin subunit α2 and its downstream targets actin and pFAK. Application of tensile strain in combination with integrin inhibition reversed the reduced expression previously observed indicating the potential for non-RGD-mediated mechanotransduction. In the Peake study actin disruption and RGD-peptide treatment failed to inhibit the loading response under investigation which has also been shown in this study whereby FAK, actin and integrin downregulation by RGDS treatment did not prevent a loading-induced increase in expression. A similar lack of reliance on RGD-mediated mechanotransduction was also observed in rat cardiac fibroblasts where load-induced activation of JNK was not inhibited by RGD peptides, indicating that an RGD-independent integrin interaction was involved. Biaxial 4% static strain was employed to assess integrin- and matrix-dependent activation of ERK2, JNK, and p38. The mechanical stretch rapidly activated ERK2 and JNK, but not p38, when the fibroblasts were permitted to synthesise their own matrix. Adhesion to all matrices was found to be integrin-dependent because it could be blocked by inhibitors of specific integrins. ERK2 activation was blocked with a combination

of anti-α4 and -α5 antibodies and an RGD peptide, but separate antibody or peptide treatment failed to block ERK2 activation. This suggests that integrins, RGD-directed in part, activate ERK2 in response to mechanical stimulation (McKenna *et al.* 1998). However, it is necessary to note that the duration of mechanical stimulation of substrates in this study was 1, 5 or 10min. In the present study, strain and RGDS were applied for a period of 24hr indicating either a lack of RGD-mediated signalling during strain or a robust response of MSCs to tensile strain which surpasses the effects of the RGDS peptide. Furthermore, pERK activation remained unchanged following 24hr of tensile strain. It is possible that earlier time points may highlight an initial increase in pERK expression in response to a mechanical stimulus which stabilises by 24hr or perhaps alternative signalling pathways unaffected by strain are activated at this time maintaining expression at basal levels. Studies have shown a rapid increase in ERK phosphorylation which declines over time. For example, rat vascular smooth muscle cells (VSMCs) were subjected to cyclic tensile strain and it was found that mechanical forces evoked rapid and transient activation of ERK, JNK and p38. ERK activation occurred within 8min of stimulation and persisted for 2hr but then declined (Li *et al.* 1999). Similarly, Reusch *et al.* (1997) reported that application of cyclic tensile strain to VSMCs activated both ERK and JNK, with ERK activation peaking between 10-30min of stimulation.

As a key-mediator of integrin signalling, the role of FAK in mechanotransduction was also under investigation in this study. FAK is a central focal adhesion protein and is known to regulate several cytoskeletal and other focal adhesion proteins, but its role in cell mechanical stability is unknown. The mechanical properties of wild-type, FAK-deficient fibroblasts have been measured by magnetic tweezer, atomic force microscopy and traction microscopy. FAK-deficient cells showed reduced cell stiffness, lower adhesion strength, and increased cytoskeletal dynamics compared to wild-type cells. These observations imply a diminished stability of the cytoskeleton in FAK-deficient cells. The decreased cytoskeletal stability suppresses the formation of ordered stress fibre bundles and reduces cell spreading (Fabry *et al.* 2011). In this study, FAK inhibition caused a reduction in integrin subunit α2, actin and pFAK expression. Application of tensile strain reversed the effect of the inhibitor in all cases; however, FAK inhibition in

combination with tensile strain maintained a reduction in actin expression compared to cells exposed to strain only. These results indicate the role FAK plays in regulating integrin expression via 'inside-out' signalling mechanisms and its regulation of cytoskeletal stability. In a separate study to gain insight into the genetic basis for skeletal mechanotransduction in bone marrow cells, FAK was conditionally inactivated. As a result of FAK deletion, the cellular response to physical stimuli was eliminated. There was no upregulation of skeletogenic genes; Sox9 and Runx2, collagen fibrils were disorganised, and the mechanically-induced osteogenic response was inhibited (Leucht *et al.* 2007). Collectively, these data lend further strength to the role of FAK in mechanotransduction and in the cellular response to mechanical stimuli.

Increased tension within focal adhesions can trigger increased integrin clustering and FAK phosphorylation, which in turn could mediate downstream effects of strain on ERK and JNK (Katsumi *et al.* 2004). There are several probable mechanisms for mechanotransduction within focal adhesions. Force applied to integrins leads to increased clustering, which could be due to increased recruitment of actin and myosin and cytoskeletal assembly (Schoenwaelder & Burridge 1999). Another possibility is that tension from strain alters the conformation of force-sensitive components of focal adhesions to induce novel binding interactions. These conformational changes would lead to activation of tyrosine kinases like FAK to mediate mechanotransduction. Identification of such force-dependent interactions is therefore an important target in mechanobiology (Katsumi *et al.* 2004). In the present study, it was demonstrated that application of 5% strain, in the absence of any additive treatments, was able to upregulate the expression of various mechanosensitive proteins implicated in MSC mechanotransduction. This was also effective in the presence of integrin and FAK inhibition, highlighting the potential in using tensile strain to study MSC mechanobiology. Various mechanisms may be accountable for increased cell surface integrin subunit expression in response to mechanical strain; these include increased recruitment at the surface, increased mRNA and protein synthesis or decreased protein degradation. The effect of cyclic strain on integrin subunit expression was determined in canine anterior cruciate ligament (ACL) and medial collateral ligament (MCL) fibroblasts. In response to 5% cyclic strain, β1 and α5

expression was increased in all fibroblasts with MCL cells exhibiting a higher magnitude of expression. The duration of applied strain from 2 versus 22hr for a period of 3 days had no effect on cell proliferation or integrin expression indicating maintenance of integrin expression changes over time during tensile strain (Hannafin *et al.* 2005).

The study of age-related alterations in cellular mechanotransduction is gaining interest for its potential role in mediating cellular function and dysfunction. There is a possibility that the ability of the cell to sense, process, and respond to mechanical stimuli is altered with aging and such changes may be implicated in the etiology of diseases associated with aging. Due to the complexity of mechanical stimuli, the range of potential mechanotransducers involved and the possible interaction between different elements, the initial phase of the mechanoresponse is highly intricate and quite difficult to reproduce in the laboratory. It is for this reason that very little research has been undertaken in the area of age-related mechanotransduction (Wu *et al.* 2011). In a study to compare the expression, basal activation, and ability of increased intraluminal pressure to activate FAK and focal adhesion-associated proteins in the aorta of adult and aged rats, the loading protocol consisted of applying 200mmHg of pressure to aortic vessels for 15min. Increases were reported in the aortic content of FAK, FAK related non-kinase, Src, RhoA and paxillin in the aged aortae. Increased age significantly altered the basal phosphorylation status of FAK and its downstream effector paxillin. Application of aortic intraluminal pressure amplified the phosphorylation of FAK, Src and paxillin in adult rats while loading failed to induce FAK phosphorylation in the aged rats. In addition, aging did not alter the load-induced regulation of RhoA. These results indicate that FAK and focal adhesion-associated proteins are mechanically regulated and suggest that FAK mechanotransduction is altered with aging (Rice *et al.* 2007). In the current study, the profile of mechanotransduction observed in aged MSCs was almost identical to that of young MSCs, contradicting the limited reports in the literature. However, a different cell type, mechanical stimulus and loading regime were under investigation in this study, highlighting novel observations in MSC mechanostransduction with age.

To summarise, this study has identified the mechanoresponse of several key components implicated in MSC mechanotransduction. These include integrin subunit α2, actin and pFAK, and expression of these mechanosensitive proteins was upregulated in MSCs in response to continuous 5% cyclic tensile strain for a period of 24hr. The significance of customary integrin and FAK signalling was highlighted by the deleterious effect their inhibition held on the expression of integrin subunit α2, actin and pFAK in both young and aged MSCs. Interestingly, there was no additive effect of simultaneous FAK and integrin inhibition but application of tensile strain, even in the presence of inhibitor treatment, reversed the diminished expression induced by FAK and integrin inhibition. Expression of pERK was unaffected by either inhibitor or tensile strain indicating an alternative mechanism of ERK activation. It is also possible that activation may occur in the initial response to strain or perhaps following prolonged periods of tensile strain. Also of extreme consequence is the fact that no differences between age groups were identified in this study. In each observation, aged MSCs responded in a similar manner to young MSCs, indicating the potential for utilising cells isolated from aged donors in tissue engineering studies and applications. This study promotes the benefit of using tensile strain as a means of exploring mechanotransduction pathways in MSCs but additionally its potential as an investigative tool for directed differentiation in tissue engineering applications.

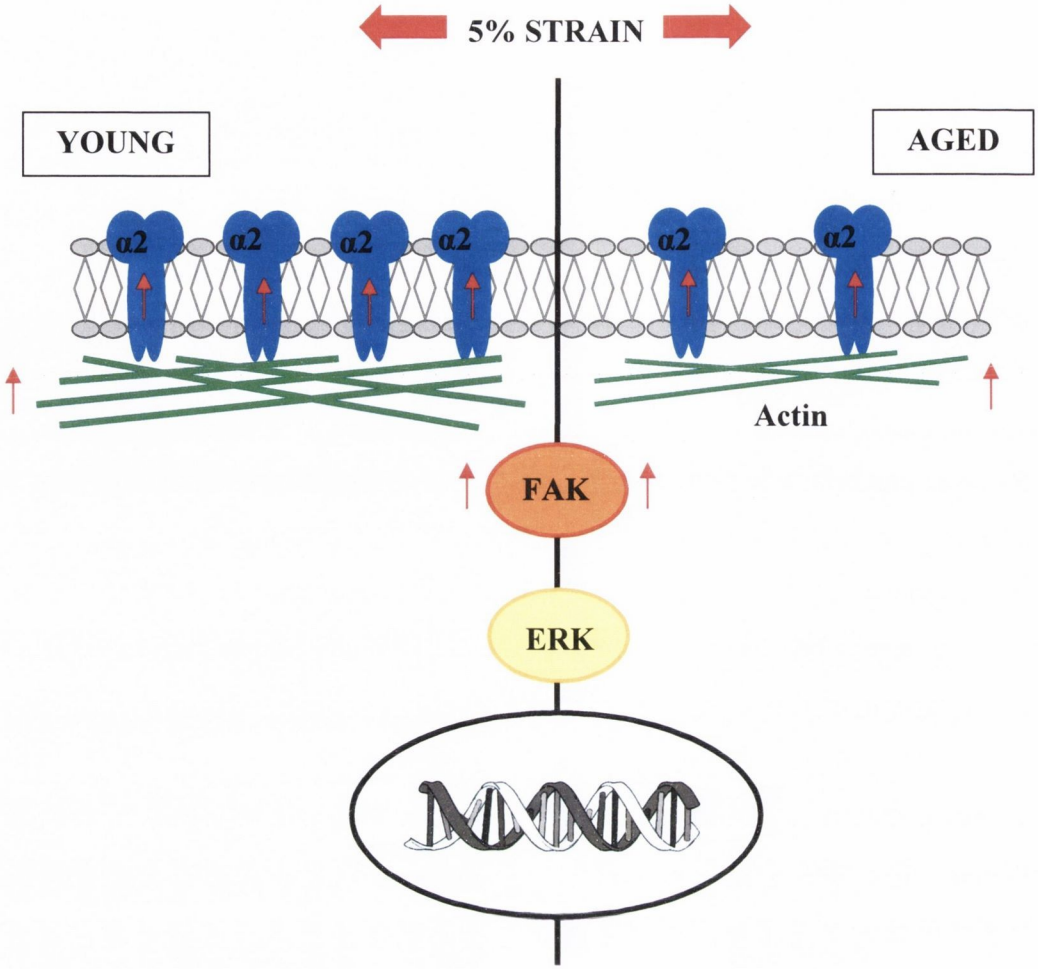


Figure 5.15: Effects of tensile strain on mechanotransduction in young and aged MSCs

Application of 5% cyclic tensile strain for 24hr increases the expression of the mechanosensitive signalling proteins; integrin subunit α2, actin and pFAK but pERK was unaffected (red arrows indicate upregulation induced by tensile strain).

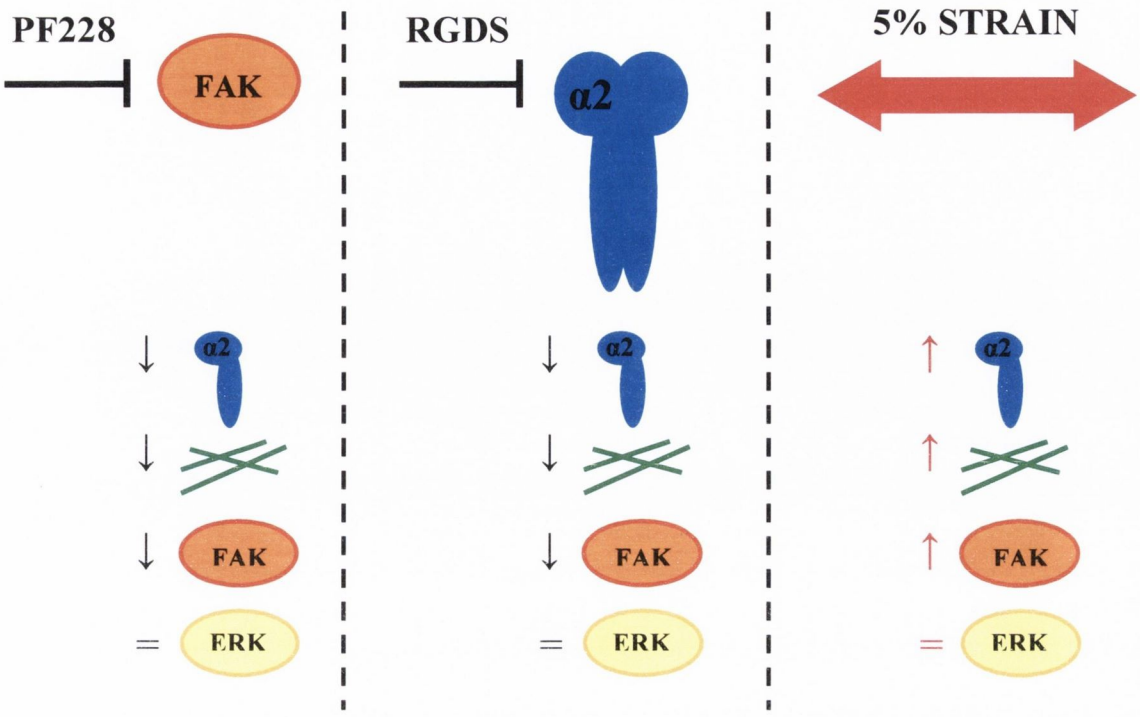


Figure 5.16: Effects of FAK and integrin inhibition on mechanotransduction in young and aged MSCs when applied alone and in combination with tensile strain

Application of the FAK inhibitor PF228 for 24hr decreases the expression of mechanosensitive signalling proteins; integrin subunit α2, actin and pFAK, whilst pERK expression was unaffected in both young and aged MSCs. Similarly, exposure to the integrin inhibitor RGDS for 24hr decreases the expression of the the same proteins, whilst pERK expression was unaffected. However, co-application of 5% cyclic tensile strain and PF228 or RGDS for 24hr generally increased mechanosensor expression, highlighting how tensile strain upregulates this integrin associated pathway in both young and aged MSCs, but ERK is not regulated in the same manner in response to these stimuli.

Chapter 6 Cyclic Tensile Strain-Induced Mesenchymal Stem Cell Differentiation and the Effect of Integrin Inhibition

6.1 Introduction

In recent years, stem cells have shown significant promise as a source of undifferentiated progenitor cells for therapeutic applications in tissue or organ repair. It is well established that the micro-environment of MSCs can have a significant influence on their differentiation and phenotypic expression. In the past, emphasis has been placed on the role of soluble mediators such as growth factors and cytokines on stem cell differentiation, but there is now significant evidence that mechanical signals may also regulate stem cell fate. Using specially designed bioreactors to deliver mechanical signals *in vitro* may provide important information on biochemical signalling pathways for promoting engineered tissue growth. It is hoped that by further understanding the biomechanical and biochemical pathways involved in mechanical signal transduction by stem cells, new insights for the improvement of stem-cell based therapies can be achieved (Estes *et al.* 2004). Mechanical force can be classified into three main categories: tension, compression and shear force. Various studies suggest that mechanical stimuli inherent to the ECM and mechanical forces applied via the ECM are important regulators of MSC differentiation implicating the biophysical connection between the ECM and the cytoskeleton, which is mediated by integrins (Park *et al.* 2004, Simmons *et al.* 2003).

Tensile strain is employed as an investigative tool in tissue engineering and it has been shown to induce osteogenic (Huang *et al.* 2010, Kearney *et al.* 2010, Simmons *et al.* 2003) and chondrogenic differentiation in MSCs (McMahon *et al.* 2007) whilst higher tensile strains can evoke apoptosis (Kearney *et al.* 2010). In order to exploit the differentiation inducing ability of this mechanical stimulus it is imperative to gain insight into the intracellular signalling pathways modulated by tensile strain. In dermal fibroblasts, high frequency strain for 7 days significantly activates p38 MAPK and ERK1/2 (Nishimura *et al.* 2007). Interestingly, Simmons *et al.* (2003) report that application of equibiaxial cyclic strain to hMSCs activates the ERK1/2 and p38 MAPK pathways. Strain-induced mineralisation was principally regulated by ERK1/2 signalling, as calcium deposition was severely reduced following inhibition of ERK1/2. An inhibitory role for p38 signalling was identified in strain-induced osteogenic differentiation,

as inhibition of the p38 pathway resulted in a more mature osteogenic phenotype. Several previous studies support the hypothesis that integrins are signal transducers that regulate *in vitro* differentiation of committed osteoblasts (Chastain *et al.* 2006, Carvalho *et al.* 2003, Schneider *et al.* 2001). In the Schneider study isotype-specific antibodies were used to inhibit the α5 and β1 integrin subunits in osteoblasts which reduced mineralisation by approximately 20% and 45% respectively. Similarly, antibodies blocking αvβ3 and α2β1 reduced mineralisation by 65% and 95%, indicating preferential adhesion and signalling via these integrins may be necessary for osteoblastic differentiation. It has also been established that matrix responsiveness during osteogenesis is due to aggregation of the α2β1 integrin subsequently activating intracellular signalling cascades (Salasznyk *et al.* 2007). Collagen I and α2β1 interaction mediates differentiation and down-regulates TGF-β receptors through activation of FAK and its assorted downstream signals. Attachment of cells to collagen I stimulates tyrosine phosphorylation of FAK and ERK and enhances MAPK activity. These results demonstrate that such signalling pathways may play an important role in the sequential differentiation of osteoblasts during bone formation (Takeuchi *et al.* 1997). In most cells, integrin binding leads to activation of FAK, which distributes integrin signals by forming complexes with SH2 domain-rich signalling proteins. Association of FAK with Src causes activation of ERK (Schaller 2001) and it has been demonstrated that specific ECM proteins induce MSC differentiation via the FAK-ERK pathway (Salasznyk *et al.* 2007). As a member of the MAPK family ERKs are known to play an important role in cell proliferation and differentiation and it has been demonstrated in osteoblasts that ERKs are not only essential for growth and differentiation but are also important for cell adhesion, spreading, migration and integrin expression (Lai *et al.* 2001).

Classically, chondrogenic differentiation of MSCs has been observed in response to compressive loading when cells have been encapsulated within agarose hydrogels (Thorpe *et al.* 2010, Kisiday *et al.* 2009), hyaluronan-gelatin scaffolds (Angele *et al.* 2004) and fibrin gels (Pelaez *et al.* 2009). However, it has been reported previously from this laboratory that mechanical strain can exert regulatory effects on chondrogenic differentiation of MSCs in a collagen I-glycosaminoglycan (GAG) scaffold. It was demonstrated that the rate of GAG

synthesis was increased following application of continuous 10% cyclic tensile strain at 1Hz for a period of 7 days. Analysis of a poroelastic finite element model of the 3-D scaffold computed a maximum fluid flow of 19μm/s and maximum principal strains of 8% under 10% stretch suggesting these magnitudes were adequate for the chondrogenic differentiation process by mechanical stimulation. This study brought new insight into the effects of mechanical loading in a tissue engineered scaffold with a view toward treating damaged or diseased articular cartilage. Additionally, it demonstrated that the application of mechanical loading can overcome reduced cell synthesis, induced by mechanical constraint, implying that *in vitro* conditioning or post-operative joint loading would be beneficial to patients (McMahon *et al.* 2008). Thus, there is potential in mechanically priming MSCs in controlled *in vitro* conditions for both osteogenic and chondrogenic differentiation in the overall goal of treating osteochondral defects.

There is currently a lack of consensus and many conflicting reports regarding the effect of donor age on MSC function. A number of studies have previously shown no age related differences in differentiation using human bone marrow derived MSCs (Scharstuhl *et al.* 2007, Stenderup *et al.* 2001). Although, many studies that demonstrate no change in differentiation have found changes in proliferation, attachment, senescence or self-renewal in the same cells (Mareschi *et al.* 2006, Stenderup *et al.* 2003). Kretlow *et al.* (2008) found that osteogenic and chondrogenic potentials are adversely affected by aging, while adipogenic differentiation potential is maintained in all but the eldest donors. In their study, murine bone marrow derived MSCs maintained their potential for adipogenic differentiation in early aging but showed decreased potential for osteogenic and chondrogenic differentiation, with age thus favouring adipogenesis over osteogenesis and chondrogenesis. Aging has been shown to alter the cellular proteome of MSCs in the presence and absence of chemical stimulation for differentiation. Using a high resolution 2-D electrophoresesis technique several thousand protein spots were detected from MSC lysates. Among the detected proteins, 14 were age-dependently expressed under both normal and osteogenic conditions. From the detected age affected protein spots, more than half were altered following osteogenic stimulation, indicating that a high number of proteins affected by aging of MSCs are also regulated during osteogenic differentiation of

the cells. Functional annotation clustering of the differentially expressed proteins in the aged versus the young cells revealed that alterations seem to be related to antioxidant defence and cytoskeleton organisation as assessed by cytoskeletal protein binding, and actin binding (Kasper *et al.* 2009). Virtually no studies have investigated the effect of tensile strain on the differentiation potential in aged MSCs. In one such study though, stem cells isolated from adipose tissue (ADSCs) of young, adult and old mice were utilised to test the hypothesis that mechanical loading modifies age-related changes in the self-renewal and osteogenic and adipogenic differentiation potential. It was concluded that mechanical stretching counteracts the loss of self-renewal in aging ADSCs by enhancing their proliferation while reducing the heightened adipogenesis of old cells (Huang *et al.* 2010).

The aim of this study was to gain further insight into the effect of tensile strain on MSC differentiation, with a focus on aging and differentiation potential. It was also hoped to identify various signalling molecules involved in MSC mechanotransduction during strain-induced differentiation. A 2.5% strain was applied for a period of 7 days to investigate the differentiation response and activation of various proteins implicated in mechanotransduction in both young and aged MSCs. This rate of tensile strain has previously been shown in the laboratory to promote osteogenic differentiation in young MSCs. Integrin receptors are widely implicated as mediators of mechanical signals during loading-induced differentiation, thus by inhibiting these proteins it was hoped to assess their role in MSC mechanotransduction during strain-induced differentiation. Expression of the bone protein osteocalcin, cartilage protein collagen II, integrin receptor subunit α2, actin, pFAK and pERK were observed in addition to several markers of osteogenic, chondrogenic and adipogenic (see Appendix) differentiation following application of tensile strain and integrin inhibition, applied singly or in combination.

6.2 Results

6.2.1 Cyclic tensile strain increases osteocalcin expression in MSCs, independent of integrin inhibition

Tensile stain is understood to induce osteogenic differentiation in MSCs isolated from young rats (Kearney *et al.* 2010). It remains unclear if MSCs isolated from aged donors differentiate in response to low rates of strain in the same way as has been demonstrated in young MSCs. Cellular differentiation was assessed by seeding MSCs onto collagen I-coated strips of silicone and exposing them to 2.5% cyclic tensile strain for a period of 7 days in the absence of stimulating growth factors. The effect of the integrin inhibitor RGDS on MSC differentiation was also assessed in combination with 2.5% cyclic tensile strain for 7 days. Expression of the bone protein osteocalcin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.1, A). In young MSCs, there was a significant effect of strain ($F_{1,8}$ = 26.34, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of osteocalcin significantly compared to static controls (static control:289.52±14.87 units, 2.5%:436.31±27.37 units, $p<0.05$, n=3, mean±SEM). Exposure to RGDS (0.5μM) alone for 7 days had no effect on osteocalcin expression and a significant increase in osteocalcin expression was still observed following application of 2.5% strain in combination with integrin inhibition for 7 days (RGDS:212.59±15.11 units, 2.5%+RGDS:377.92±50.00 units, $p<0.01$, n=3, mean±SEM).

In aged MSCs (Figure 6.1, A), there was a significant effect of strain ($F_{1,8}$ = 45.00, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed a significant increase in osteocalcin expression following application of 2.5% strain for 7 days, compared to static controls (static control:244.93±20.65 units, 2.5%:331.94±12.16 units, $p<0.05$, n=3, mean±SEM). Exposure to RGDS (0.5μM) alone for 7 days had no effect on osteocalcin expression, however, a significant increase in osteocalcin expression was still observed following application of 2.5% strain in combination with integrin inhibition (RGDS:206.32±6.39 units, 2.5%+RGDS:308.83±13.53

units, $p<0.01$, $n=3$, mean±SEM). Representative images (Figure 6.1, B and C) show the changes in osteocalcin expression in young and aged MSCs exposed to integrin inhibition and 2.5% strain. These results indicate how tensile strain enhances osteocalcin expression and that integrin inhibition has no influence on the strain-induced increase in the expression of the bone protein osteocalcin in MSCs.

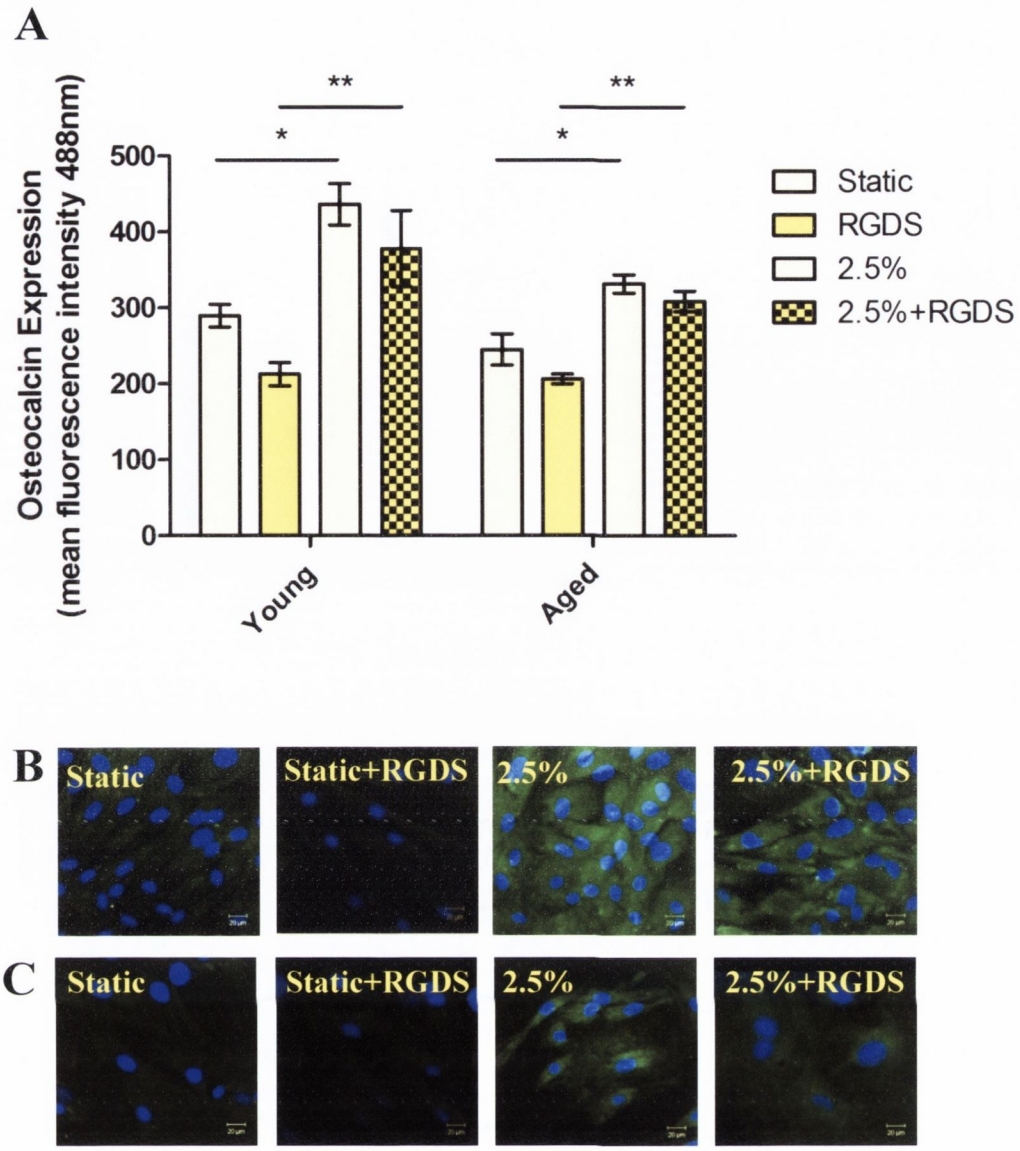


Figure 6.1: Osteocalcin expression is increased by 2.5% strain for 7 days and integrin inhibition has no effect in MSCs

MSCs were examined for the expression of osteocalcin by immunocytochemistry using an anti-osteocalcin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A**: There was a significant effect of strain in young ($F_{1,8}$ = 26.34, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 45.00, $p<0.001$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in osteocalcin expression following application of 2.5% strain for 7 days (*$p<0.05$, n=3 cultures). There was no change in osteocalcin expression by integrin inhibition, but an upregulation in expression following application of 2.5% strain in combination with integrin inhibition (**$p<0.01$, n=3 cultures). **B:** Confocal images of osteocalcin expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.2 Cyclic tensile strain increases osteocalcin expression in MSCs and this is significantly lower in aged MSCs

The pattern of osteocalcin expression in response to tensile strain and integrin inhibition was similar in both young and aged MSCs, however differences in expression were apparent. Figure 6.2, A shows the comparison between young and aged MSCs exposed to strain. There was a significant effect of both strain ($F_{1,8} = 35.39$, $p<0.001$, two-way ANOVA) and age ($F_{1,8} = 14.37$, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed a significant increase in osteocalcin expression in both young (static control:289.52±14.87 units, 2.5%:436.31±27.37 units, $p<0.01$, n=3, mean±SEM) and aged MSCs (static control:244.93±20.65 units, 2.5%:331.94±12.16 units, $p<0.05$, n=3, mean±SEM) exposed to 2.5% strain for 7 days. However, the increase in osteocalcin expression was greater in young MSCs exposed to strain compared to aged (Young 2.5%:436.31±27.37 units, Aged 2.5%:331.94±12.16 units, $p<0.05$, n=3, mean±SEM). Figure 6.2, B shows images of osteocalcin expression in young and aged MSCs. These results highlight the benefit of tensile strain in promoting MSC osteogenesis, which is more pronounced in young MSCs

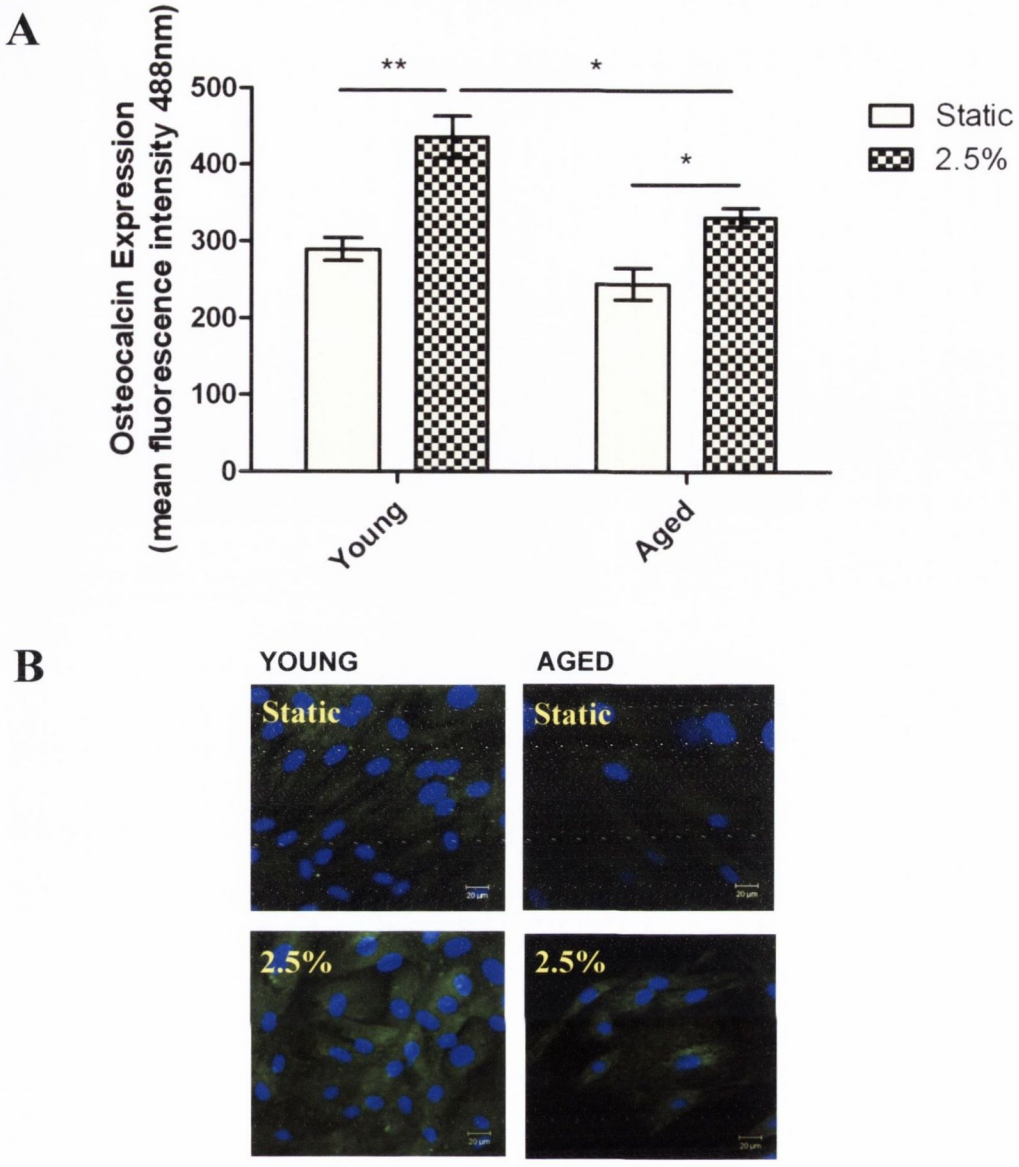


Figure 6.2: Osteocalcin expression is increased in both young and aged MSCs by 2.5% strain for 7 days and this response is significantly lower in aged MSCs

MSCs were examined for the expression of osteocalcin using immunocytochemistry for an anti-osteocalcin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain ($F_{1,8} = 35.39$, $p<0.001$, two-way ANOVA) and age ($F_{1,8} = 14.37$, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed there was a significant increase in osteocalcin expression caused by 2.5% tensile strain for 7 days in both young and aged MSCs (*$p<0.05$, **$p<0.01$, n=3 cultures). The increase was lower in aged MSCs compared to young (*$p<0.05$, n=3 cultures). **B:** Confocal images of osteocalcin expression in young and aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm

6.2.3 Index of osteogenic differentiation in young and aged MSCs: Von Kossa

Von Kossa staining for calcium phosphate deposition is used as a marker of osteogenic differentiation. This staining technique was undertaken to assess the phenotypic differentiation of MSCs along the osteogenic lineage. Five independent observations were made for each treatment group and scored according to an index of differentiation (described in Chapter 2, Section 2.11.2). The index ranges from 0-2 arbitrary units, rising in 0.5 increments, whereby 0 represents no evidence of positive staining, and 2 represents very strong positive staining. Results are graphed as a vertical scatter plot to highlight the distribution of differentiation scores made within each treatment group.

In young MSCs (in Figure 6.3, A) cultured on glass coverslips for 7 days (Glass Control) there was a range of staining intensities observed, however several deeper and more concentrated areas of staining were noted, indicating potential spontaneous osteogenic differentiation. Exposure to integrin inhibition under the same conditions (Glass+RGDS) showed a robust effect of the inhibitory peptide, whereby all observations of positive staining fell on the lower end of the differentiation index. When cells were seeded to silicone sections calcium phosphate deposition was evident in the static group, with levels of positive staining appearing at 1.5 on the differentiation index. Following integrin inhibition with RGDS a lower index of differentiation was evident. Application of 2.5% tensile strain for 7 days resulted in a majority of observations with a higher differentiation index, reaching a score of 2. This high level of staining intensity persisted when strain was applied in combination with integrin inhibition. Figure 6.3, B shows images of calcium phosphate deposition in all treatment groups for young MSCs.

In aged MSCs (Figure 6.3, C) cultured on glass coverslips for 7 days there was a range of staining intensities observed, some with a high index of differentiation indicating potential for spontaneous osteogenic differentiation in aged MSCs also. Exposure to integrin inhibition under the same conditions showed all observations had a low differentiation index. In cells seeded to the silicone substrates, a similar

pattern of calcium phosphate deposition was observed. Positive staining was evident in the static group, with one sample showing a level of positive staining at 1.5 on the differentiation index. Following, integrin inhibition, more samples were observed with a low index of differentiation. Application of tensile strain for 7 days caused a slight increase of observations with a higher differentiation index which was also evident when strain was applied in combination with integrin inhibition. Figure 6.3, D shows images of calcium phosphate deposition in all treatment groups for aged MSCs.

Taken together, these results highlight an upregulation of calcium phosphate deposition in young and aged MSCs exposed to 2.5% cyclic tensile strain for 7 days, compared to static groups, indicating the potential of mechanical strain in inducing differentiation in the absence of growth factor therapy. Interference with mechanotransduction by integrin inhibition causes a reduction in differentiation potential; however this is ineffective when applied in combination with tensile strain. Most importantly, these findings show that although aged MSCs exhibit a lower capacity for differentiation, they retain the ability to respond to low rates of strain in a similar fashion to young MSCs.

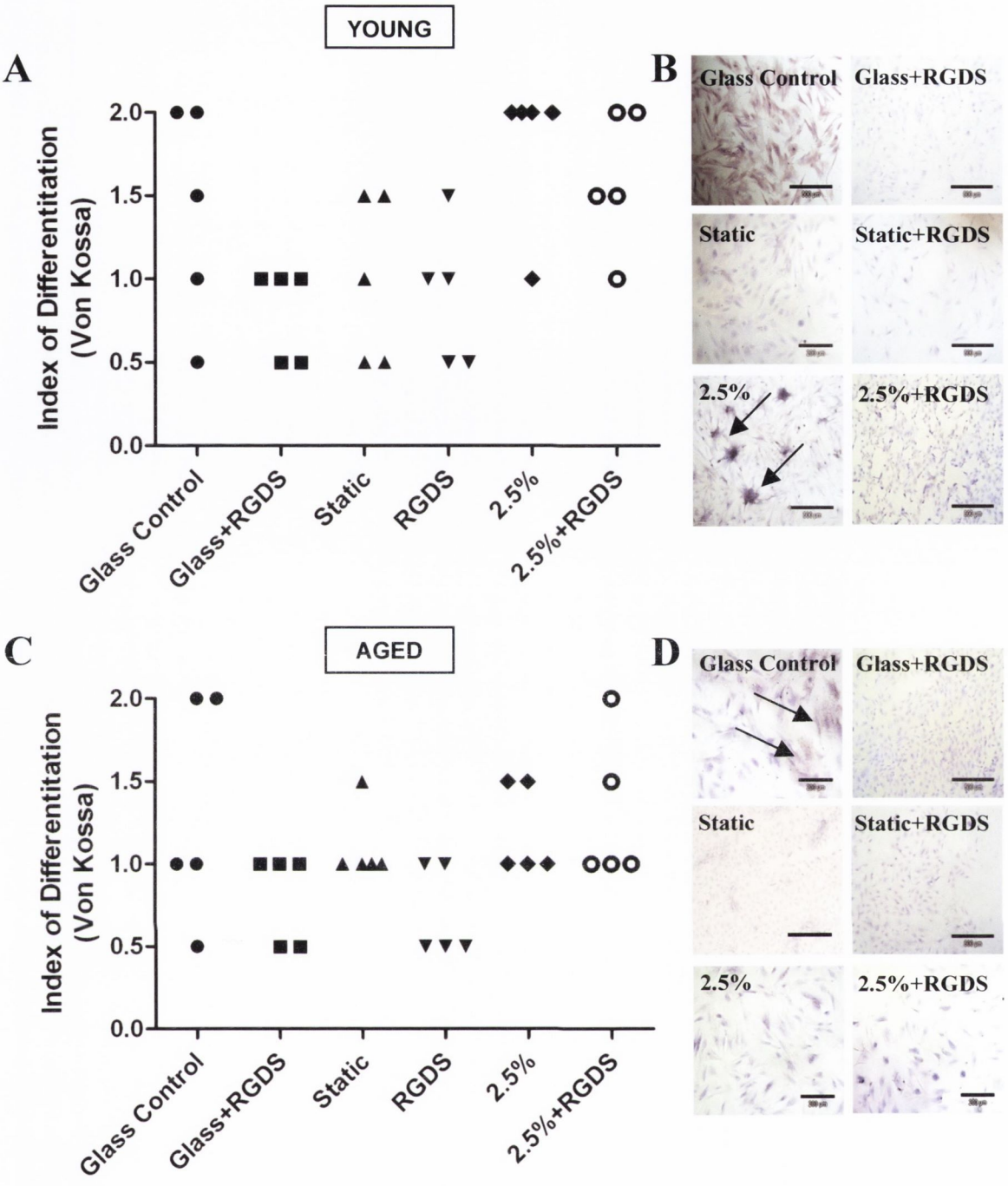


Figure 6.3: Effect of 2.5% strain and integrin inhibition for 7 days on calcium phosphate deposition and spontaneous differentiation on a glass substrate

Calcium phosphate deposition was assessed using the Von Kossa staining technique. **A+C:** On glass coverslips, both age groups show positive staining which was reduced by integrin inhibition. On silicone substrates, there was also a reduction in staining intensity by integrin inhibition, which was reversed by application of 2.5% tensile strain, alone or in combination with integrin inhibition. **B+D:** Images of calcium phosphate deposition in young and aged MSCs (arrows indicate areas of intense staining). Scale bars are 200μm and 500μm.

6.2.4 Cyclic tensile strain increases collagen II expression in MSCs, independent of integrin inhibition in young cells

Tensile strain has also previously been utilised to induce chondrogenic differentiation in MSCs isolated from young rats (McMahon *et al.* 2008) but it remains unclear whether aged cells respond in the same way. Therefore, the effects of tensile strain and the integrin inhibitor RGDS on MSC differentiation were also assessed in young and aged MSCs in terms of chondrogenic differentiation. Expression of the cartilage protein collagen II was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.4, A). In young MSCs, there was a significant effect of strain ($F_{1,8}$ = 26.40, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of collagen II significantly compared to static controls (static control:282.15±19.51 units, 2.5%:360.45±9.56 units, $p<0.01$, n=3, mean±SEM). Exposure to RGDS (0.5μM) alone for 7 days had no effect on collagen II expression, however, a significant increase in collagen II expression was still observed following application of 2.5% strain in combination with integrin inhibition (RGDS:281.55±11.96 units, 2.5%+RGDS:351.25±14.65 units, $p<0.05$, n=3, mean±SEM).

In aged MSCs (Figure 6.4, A), there was a significant effect of strain ($F_{1,8}$ = 17.61, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed a significant increase in collagen II expression following application of 2.5% strain for 7 days, compared to static controls (static control:233.76±13.42 units, 2.5%:348.58±21.68 units, $p<0.01$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days had no effect on collagen II expression (RGDS:263.79±4.37 units, 2.5%+RGDS:308.02±27.70 units, n=3, mean±SEM), whether it was applied alone or in combination with tensile strain. Representative images (Figure 6.4, B and C) show the changes in collagen II expression in young and aged MSCs exposed to integrin inhibition and 2.5% strain. These results indicate how tensile strain upregulates the expression of collagen II and that integrin inhibition exerts no effect on the strain-induced increase in collagen II expression observed in young MSCs. This was the only difference observed between age groups.

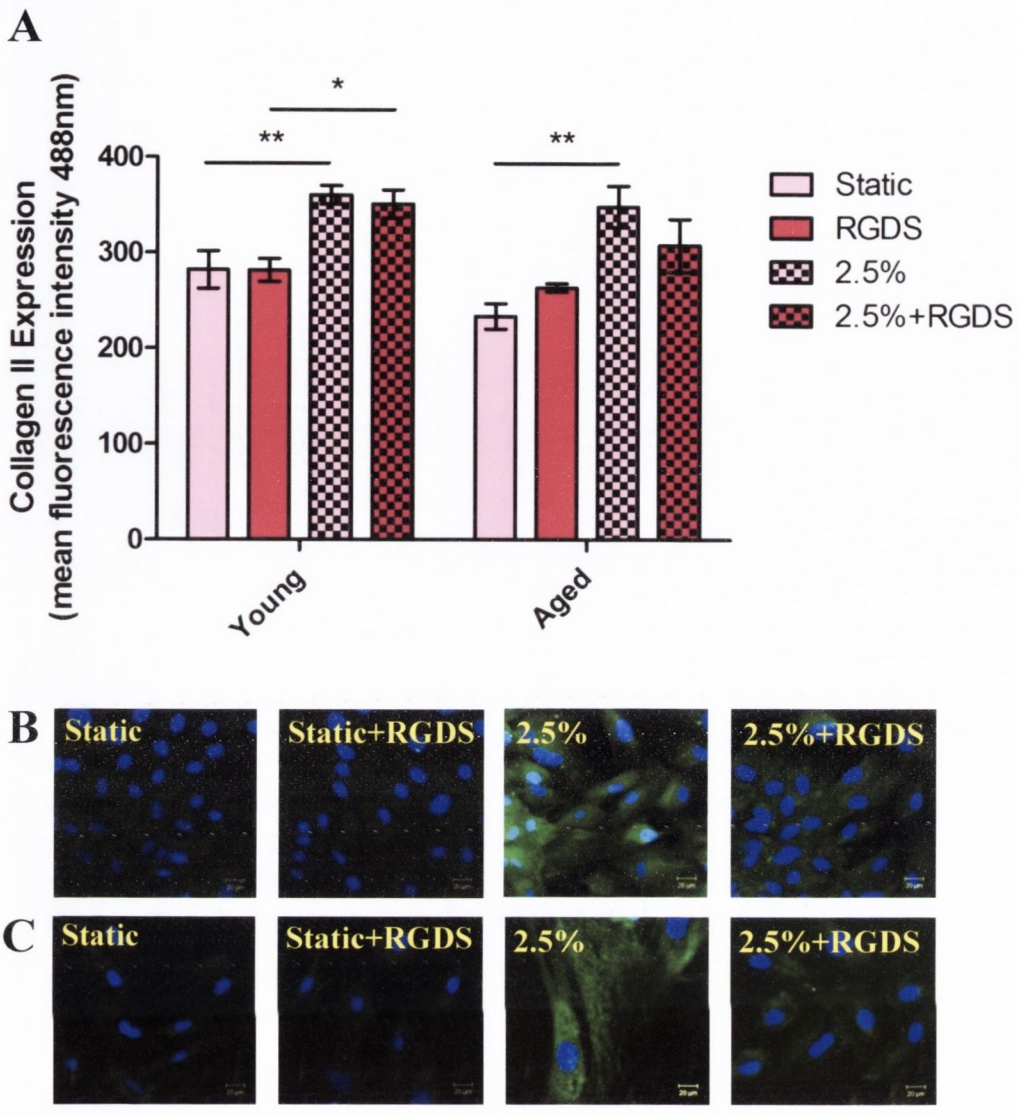


Figure 6.4: Collagen II expression is increased by 2.5% strain for 7 days, independent of integrin inhibition in young MSCs

MSCs were examined for the expression of collagen II by immunocytochemistry using an anti-collagen II antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8}$ = 26.40, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 17.61, $p<0.01$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in collagen II expression following application of 2.5% strain for 7 days (**$p<0.01$, n=3 cultures). There was also an increase in collagen II expression following application of 2.5% strain for 7 days in combination with integrin inhibition compared to the static treated group (*$p<0.05$, n=3 cultures). There was no effect of integrin inhibition alone or in combination with tensile strain in aged MSCs. **B:** Confocal images of collagen II expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.5 Index of chondrogenic differentiation in young and aged MSCs: Safranin-O

Safranin-O staining for proteoglycan deposition is used as a marker of chondrogenic differentiation. This type of staining was employed as an alternative marker to collagen II expression to determine the phenotypic differentiation of MSCs down the chondrogenic lineage. Five independent observations were made for each treatment group and scored according to an index of differentiation (described in Chapter 2, Section 2.11.3). The index ranges from 0-2 arbitrary units, rising in 0.5 increments, whereby 0 represents no evidence of positive staining, and 2 represents very strong positive staining. Results are graphed as a vertical scatter plot to highlight the distribution of differentiation scores made within each treatment group.

In young MSCs (Figure 6.5, A) cultured on glass coverslips for 7 days (Glass Control), most observations were scored at the high end of the index due to the observation of several concentrated areas of staining, indicating potential for spontaneous chondrogenic differentiation. Exposure to integrin inhibition under the same conditions (Glass+RGDS) showed a robust effect of the inhibitory peptide, whereby all observations of positive staining had a low differentiation index. In cells seeded onto silicone substrates, a wide range of staining intensities was evident in the static group. Following integrin inhibition with RGDS, a lower index of differentiation was evident. Application of 2.5% tensile strain for 7 days caused a shift to the top of the differentiation index with almost all observations scoring a 2 on the index. Tensile strain applied in combination with integrin inhibition, resulted in observations with a mid- to high differentiation index. Figure 6.5, B shows images of proteoglycan deposition in all treatment groups for young MSCs.

In aged MSCs (Figure 6.5, C) cultured on glass coverslips for 7 days, most observations were high on the differentiation index indicating potential for spontaneous chondrogenic differentiation in aged MSCs as well. Following integrin inhibition under the same conditions showed all observations of positive staining had a low differentiation index. On the silicone substrate sections, a

similar pattern of proteoglycan deposition was observed. Low levels of positive staining were evident in the static control group, with peak intensities observed at the mid-level of the differentiation index. Integrin inhibition led to more observations with a low index of differentiation compared to the static group. Application of tensile strain for 7 days resulted in a definitive increase of observations with a high differentiation index which was also observed in combination with integrin inhibition. Figure 6.5, D shows images of proteoglycan deposition in all treatment groups for aged MSCs.

Taken together, these results show an upregulation of proteoglycan deposition in young and aged MSCs exposed to 2.5% cyclic tensile strain for 7 days, compared to static groups, indicating the potential of mechanical strain in inducing chondrogenic differentiation in the absence of growth factor treatment. Interference with mechanotransduction by integrin inhibition appears to cause a reduction in differentiation potential; which is ineffective when applied in combination with tensile strain. These results show the ability of aged MSCs to respond to a low rate of strain in a similar manner to young MSCs, however there is also a parallel between osteogenic and chondrogenic differentiation apparent, which is addressed in the next section.

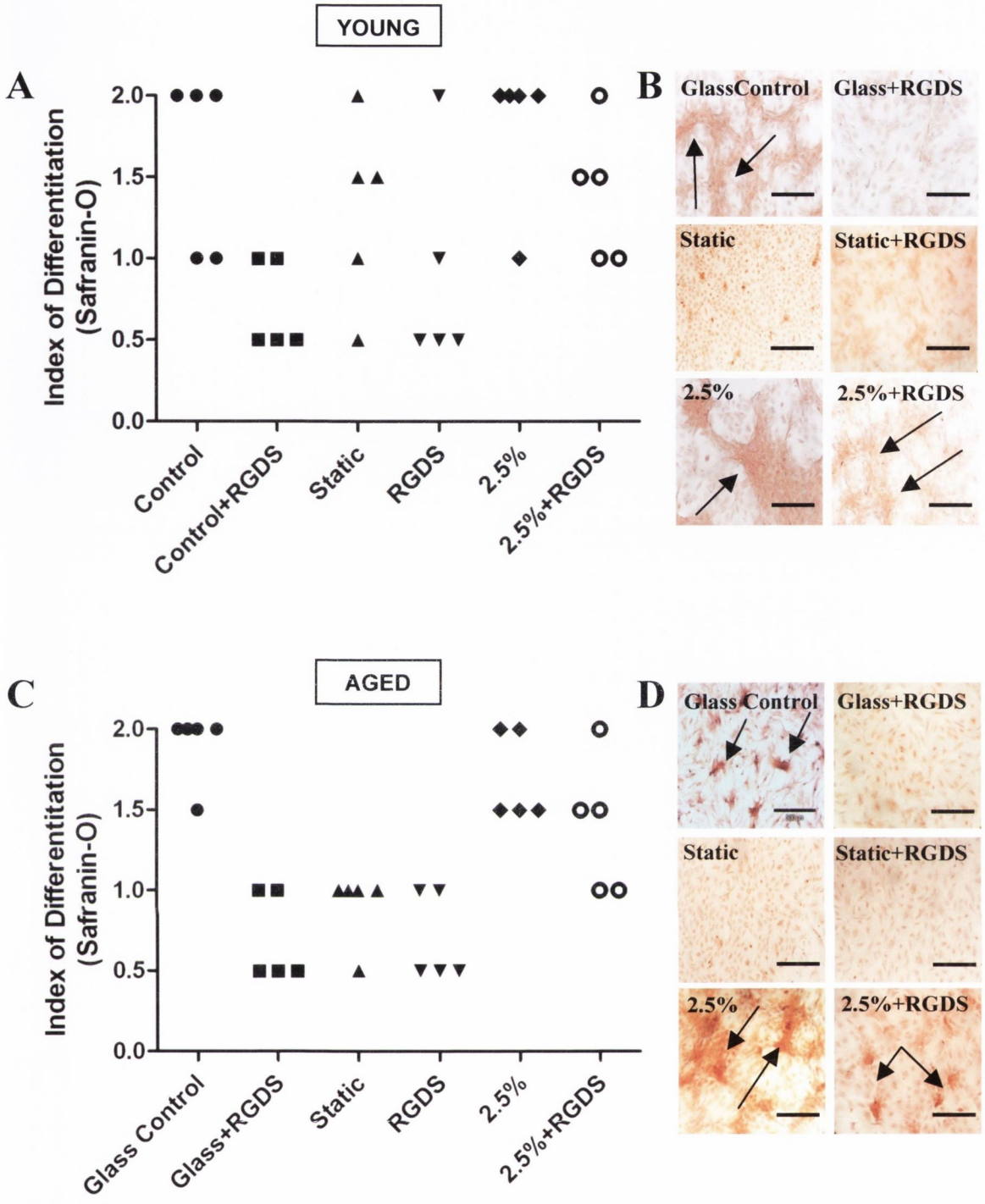


Figure 6.5: Effect of 2.5% strain and integrin inhibition for 7 days on proteoglycan deposition

Proteoglycan deposition was assessed using the Safranin-O staining technique. **A+C:** On glass coverslips, both age groups showed positive staining for proteoglycan deposition, which was reduced by integrin inhibition. On silicone substrates, there was a reduction in proteoglycan deposition by integrin inhibition, which was reversed by application of 2.5% tensile strain, alone or in combination with integrin inhibition. **B+D:** Images of proteoglycan deposition in young and aged MSCs (arrows indicate areas of intense staining). Scale bars are 200μm and 500μm.

6.2.6 Cyclic tensile strain increases expression of osteocalcin and collagen II, which are co-expressed in young and aged MSCs

To determine whether positive staining previously observed for both osteocalcin and collagen II indicated a co-expression of these proteins; sections were co-stained for both proteins simultaneously using different secondary antibodies conjugated to Alexa Fluor 488 and 594.

Osteocalcin was labelled with a secondary antibody conjugated to Alexa Fluor 594 which has a red emission at 594nm. Expression of osteocalcin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.6, A). The results obtained were almost identical to those observed and described previously. In young MSCs, there was a significant effect of strain ($F_{1,21}$ = 276.20, $p<0.001$, two-way ANOVA) and drug ($F_{1,21}$ = 10.82, $p<0.01$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days significantly increased the expression of osteocalcin compared to static controls (static control:259.31±7.90 units, 2.5%:435.92±13.54 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days had no effect on osteocalcin expression, however, a significant increase in osteocalcin expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:228.68±7.15 units, 2.5%+RGDS:398.05±11.62 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). In aged MSCs, there was a significant effect of strain ($F_{1,21}$ = 306.60, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days significantly increased osteocalcin expression compared to static controls (static control:237.34±3.69 units, 2.5%:329.73±7.47 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). Integrin inhibition for 7 days had no effect on osteocalcin expression in aged cells either, and a significant increase in osteocalcin expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:223.68±5.59 units, 2.5%+RGDS:329.29±6.60 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). When comparing the response of young and aged MSCs exposed to tensile strain and integrin inhibition

there was a significant effect of strain ($F_{1,26}$ = 269.90, $p<0.001$, two-way ANOVA), age ($F_{1,26}$ = 61.26, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,26}$ = 26.45, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed a significant reduction in osteocalcin expression in the aged static control group compared to young (Young static:259.31±7.90 units, Aged static:237.34±3.69 units), a novel finding in this study. There was also a significant reduction in osteocalcin expression in aged MSCs exposed to strain only compared to young (Young 2.5%:435.92±13.54 units, Aged 2.5%:329.73±7.47 units) which has been observed and described previously in this chapter. Representative images (Figure 6.6, C) show the changes in osteocalcin expression in the groups exposed to integrin inhibition and 2.5% strain. These results indicate how tensile strain increases expression of the bone protein osteocalcin in MSCs, which is unaffected by integrin inhibition. There was a significantly lower expression of osteocalcin in aged MSCs observed in the static and strained groups compared to young cells, which may imply a reduced potential of aged MSCs toward spontaneous and strain-induced osteogenic differentiation

Collagen II expression was assessed using a secondary antibody conjugated to Alexa Fluor 488 which has a green emission at 488nm and expression was identified using immunocytochemistry and confocal microscopy with changes in mean fluorescence intensity values indicating a change in expression (Figure 6.6, B). The results obtained were also similar to those observed and described previously. In young MSCs, there was a significant effect of strain ($F_{1,21}$ = 65.91, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days significantly increased the expression of collagen II compared to static controls (static control:264.46±9.78 units, 2.5%:357.97±7.05 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days had no effect on collagen II expression, however, a significant increase in expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:255.18±8.77 units, 2.5%+RGDS:330.59±9.60 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). In aged MSCs, there was a significant effect of strain ($F_{1,21}$ = 226.90, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,21}$ = 11.58, $p<0.01$, two-way ANOVA). *Post hoc*

analysis showed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days significantly increased collagen II expression compared to static controls (static control:233.22±5.10 units, 2.5%:357.76±7.20 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). Integrin inhibition for 7 days had no effect but a significant increase in collagen II expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:254.53±6.91 units, 2.5%+RGDS:333.16±7.50 units, $p<0.001$, 5-10 independent observations, n=2-3, mean±SEM). When comparing the response of young and aged MSCs exposed to strain and integrin inhibition there was a significant effect of strain ($F_{1,26} = 158.60$, $p<0.001$, two-way ANOVA). *Post hoc* analysis revealed a significant reduction in collagen II expression in the aged static control group compared to young (Young static:264.46±9.78 units, Aged static:233.22±5.10 units), another novel finding in this study. Representative images (Figure 6.6, C) show collagen II expression in the groups exposed to integrin inhibition and 2.5% strain. These results indicate how aged MSCs may have a reduced potential toward spontatneous chondrogenic differentiation but that tensile strain significantly upregulates the expression of the cartilage protein collagen II in young and aged MSCs, while integrin inhibition is ineffective.

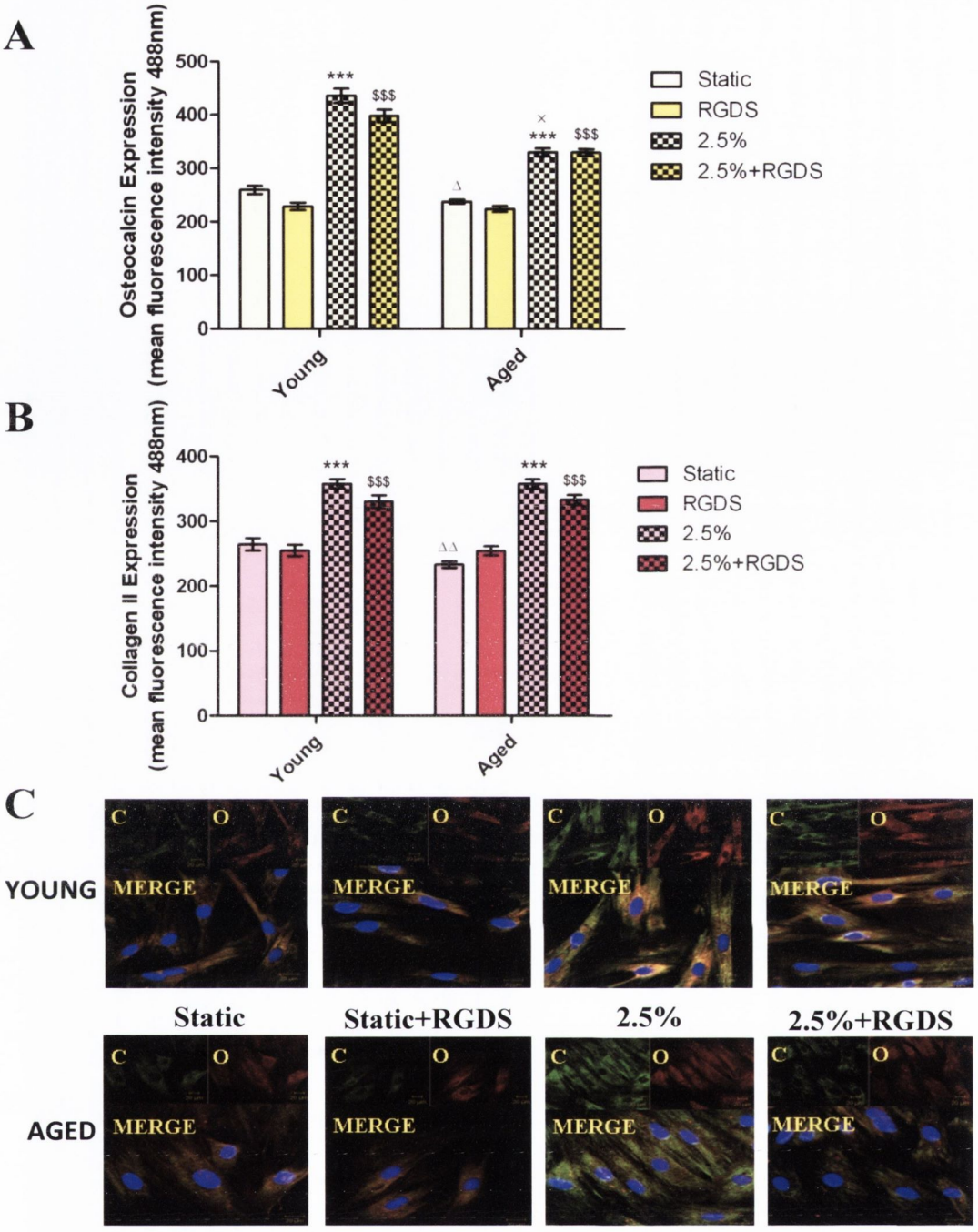


Figure 6.6: Osteocalcin and collagen II expression in MSCs exposed to 2.5% strain and integrin inhibition for 7 days

MSCs were examined for co-expression of osteocalcin and collagen II. **A:** Osteocalcin expression and **B:** Collagen II expression (***$p<0.001$ compares static to strain only in both age groups, $^{\$\$\$}p<0.001$ compares static drug-treated to strained drug-treated, $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$, compares young and aged static and $^{\times}p<0.05$ compares young and aged strain) in young and aged MSCs. **C:** Confocal images of osteocalcin (O-594nm-Red) and collagen II (C-488nm-Green) expression in young and aged MSCs (including merged images). Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.7 Differentiation ratios indicate potential for both osteogenic and chondrogenic differentiation of MSCs

To evaluate whether MSCs exhibit a tendency toward one lineage over another (i.e.: osteogenic versus chondrogenic and vice versa) ratios of differentiation were calculated as a qualitative indication of lineage preference. The mean fluorescence intensity values from both separate and co-staining studies were combined to give an overall mean value of osteogenic or chondrogenic expression for each group. These values were then divided to provide a ratio of differentiation for osteogenesis and chondrogenesis and values >1 indicated preference for a particular lineage (Figure 6.7). In young MSCs osteogenic differentiation appears to be favoured since three out of four groups display a ratio value greater than 1. However, in the static control group, the ratio is equal and it appears in the absence of integrin signalling chondrogenic differentiation is elected, as a ratio value greater than 1 was calculated in the group treated with the integrin inhibitor RGDS. In aged MSCs chondrogenic differentiation appears to be preferred as most of the groups exhibit a ratio value greater than 1. Unlike young MSCs, chondrogenic differentiation is favoured in the static control group, although in the strain group exposed to integrin inhibition the ratio is equal for both lineages. This data suggests a potential tendency toward osteogenic differentiation in young MSCs, especially in response to tensile strain, and a preference for chondrogenic differentiation when integrin activity is compromised.

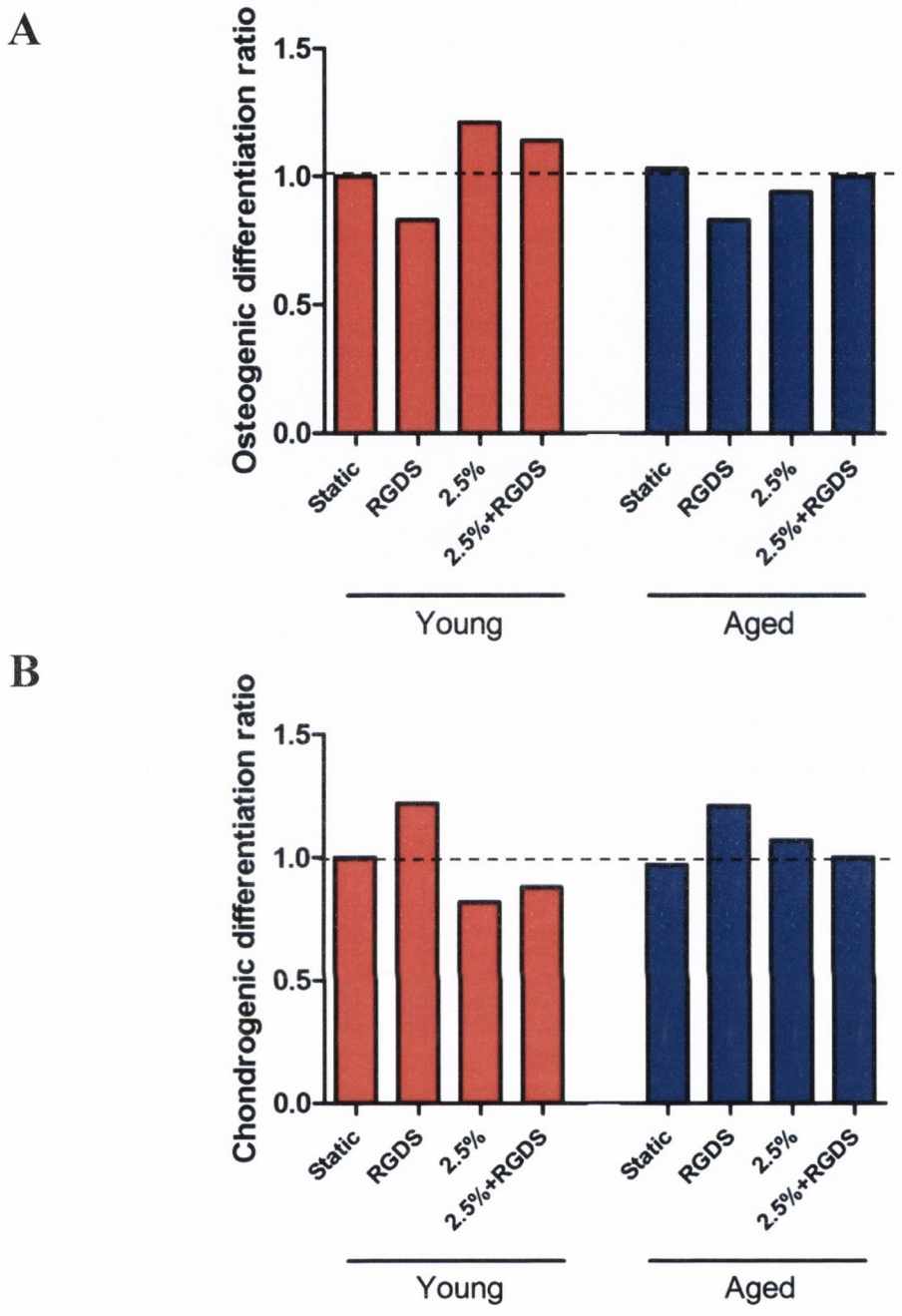


Figure 6.7: Ratio values indicating distribution of osteogenic and chondrogenic differentiation in young and aged MSCs

Differentiation ratios were calculated by combining mean fluorescence intensity values for osteocalcin and collagen II staining and dividing them. Values >1 indicate preference for a particular lineage and this is graded on the histogram. **A:** Young MSCs exposed to tensile strain grade a higher osteogenic response, even in the presence of integrin inhibition. **B:** Young and aged MSCs exposed to integrin inhibition grade a higher chondrogenic response, indicating potential control of osteogenic differentiation through integrin signalling.

6.2.8 Cyclic tensile strain increases α2 expression in MSCs and overcomes a reduction in α2 expression induced by integrin inhibition

The effects of 2.5% cyclic tensile strain and integrin inhibition on MSC mechano-signalling events were also assessed during 7 day differentiation testing. This was to determine the activity of particular signalling molecules during tensile strain and whether any changes correlate with strain-induced differentiation. Expression of the integrin subunit α2 was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.8, A). In young MSCs, there was a significant effect of strain ($F_{1,8}$ = 77.49, $p<0.001$, two-way ANOVA), drug ($F_{1,8}$ = 15.16, $p<0.01$, two-way ANOVA) and a significant interaction ($F_{1,8}$ = 8.73, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of α2 significantly compared to static controls (static control:269.95±5.08 units, 2.5%:337.84±19.66 units, $p<0.01$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in α2 expression compared to untreated static controls (static control:269.95±5.08 units, RGDS:190.47±4.17 units, $p<0.01$, n=3, mean±SEM) and the application of 2.5% strain in combination with integrin inhibition reversed the decrease in α2 expression observed with integrin inhibition alone, returning expression to control level (RGDS:190.47±4.17 units, 2.5%+RGDS:326.93±10.45 units, $p<0.001$, n=3, mean±SEM).

The effects of 2.5% cyclic tensile strain and integrin inhibition on MSC signalling events were also assessed during 7 day differentiation testing in aged MSCs to determine potential changes in signalling patterns with age. In aged MSCs (Figure 6.8, A), there was a significant effect of strain ($F_{1,8}$ = 51.28, $p<0.001$, two-way ANOVA) and drug ($F_{1,8}$ = 5.72, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of α2 significantly compared to static controls (static control:236.34±3.51 units, 2.5%:319.49±18.54 units, $p<0.05$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in α2 expression compared to untreated static controls (static control:236.34±3.51 units, RGDS:170.61±9.17 units, $p<0.05$, n=3, mean±SEM) and the application of 2.5%

strain in combination with RGDS reversed the decrease in α2 expression observed with integrin inhibition alone, returning expression to control level (RGDS:170.61±9.17 units, 2.5%+RGDS:310.67±23.06 units, $p<0.001$, $n=3$, mean±SEM). Representative images (Figure 6.8, B and C) show the changes in α2 expression in MSCs following integrin inhibition and application of 2.5% strain. In response to tensile strain, there is a clear upregulation in the expression of the α2 integrin subunit seen throughout the cell, specifically in the perinuclear region which is indicative of receptor trafficking from the endoplasmic reticulum to the plasma membrane. These results highlight how tensile strain upregulates α2 expression and conversely how integrin inhibition alone reduces expression of α2. However, integrin inhibition has no influence on the strain-induced increase in α2 expression observed in MSCs.

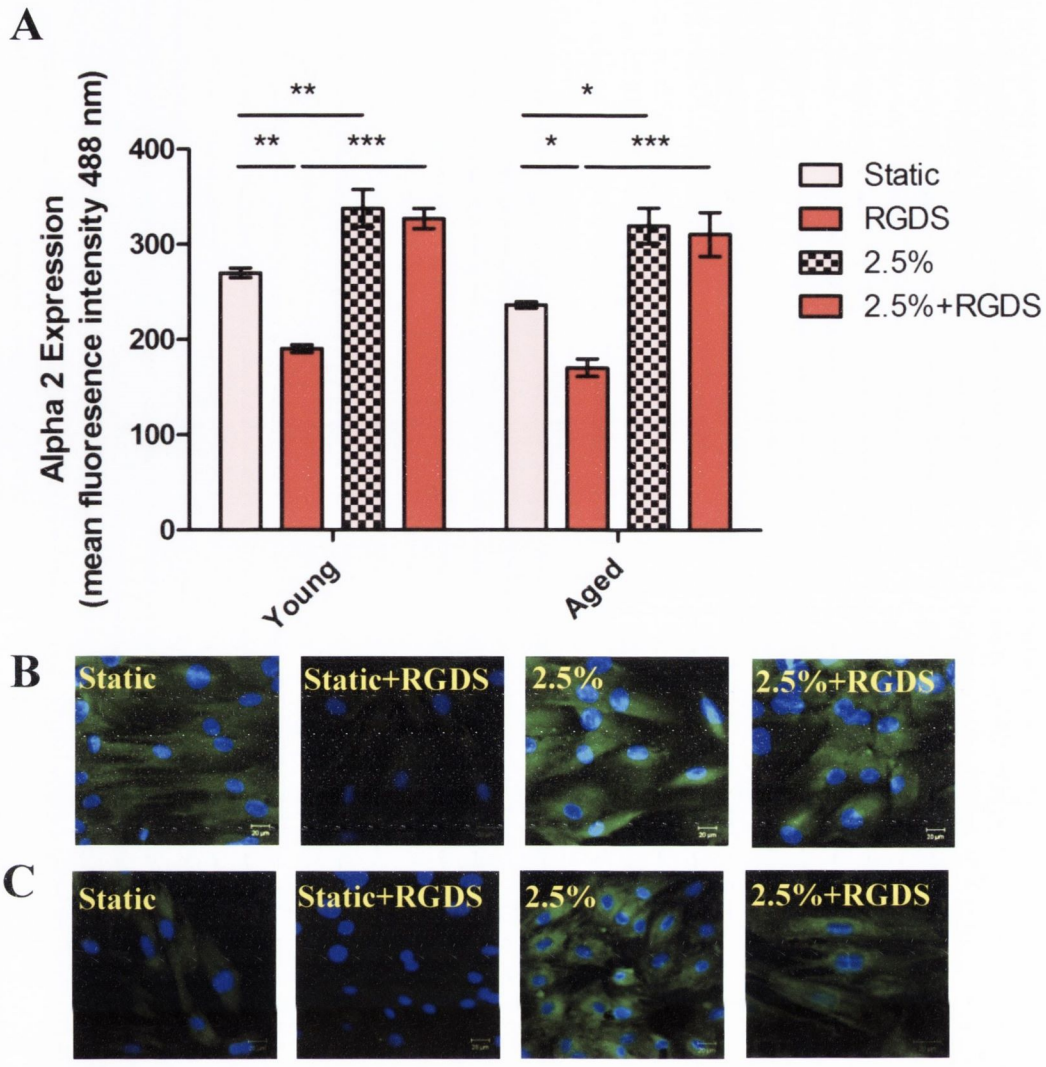


Figure 6.8: Expression of α2 integrin is increased by 2.5% strain for 7 days and strain can overcome the reduction in α2 expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of α2 by immunocytochemistry using an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8}$ = 77.49, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 51.28, $p<0.001$, two-way ANOVA) MSCs, drug in young ($F_{1,8}$ = 15.16, $p<0.01$, two-way ANOVA) and aged ($F_{1,8}$ = 5.72, $p<0.05$, two-way ANOVA) MSCs and a significant interaction ($F_{1,8}$ = 8.73, $p<0.05$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in α2 expression following 2.5% strain for 7 days (*$p<0.05$, **$p<0.01$, n=3 cultures). There was a significant decrease in α2 expression in the integrin inhibition group compared to untreated static control (*$p<0.05$, **,$p<0.01$, n=3 cultures), which was reversed in MSCs exposed to strain in combination with integrin inhibition (***$p<0.001$, n=3 cultures). **B:** Confocal images of α2 expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.9 Expression of α2 is decreased with age and integrin inhibition induces a reduction in α2 expression in both young and aged MSCs under static conditions

The pattern of α2 expression in response to tensile strain and integrin inhibition was similar in both young and aged MSCs, however differences in expression were apparent. Figure 6.9, A shows the comparison between young and aged MSCs exposed to integrin inhibition for 7 days. There was a significant effect of both drug ($F_{1,8}$ = 151.00, $p<0.001$, two-way ANOVA) and age ($F_{1,8}$ = 20.48, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed that integrin inhibition caused a significant decrease in α2 expression in both young (static control:269.95±5.08units, RGDS:190.47±4.17 units, $p<0.001$, n=3, mean±SEM) and aged MSCs (static control:236.34±3.51 units, RGDS:170.61±9.17 units, $p<0.001$, n=3, mean±SEM). However, a significant decrease in α2 expression was also observed in aged MSCs compared to young (Young static control:269.95±5.08 units, Aged static control:236.34±3.51 units, $p<0.01$, n=3, mean±SEM). Figure 6.9, B shows images of α2 expression in young and aged MSCs. This result highlights the age-related difference in integrin expression and the influence of integrin inhibition on α2 expression in young and aged MSCs.

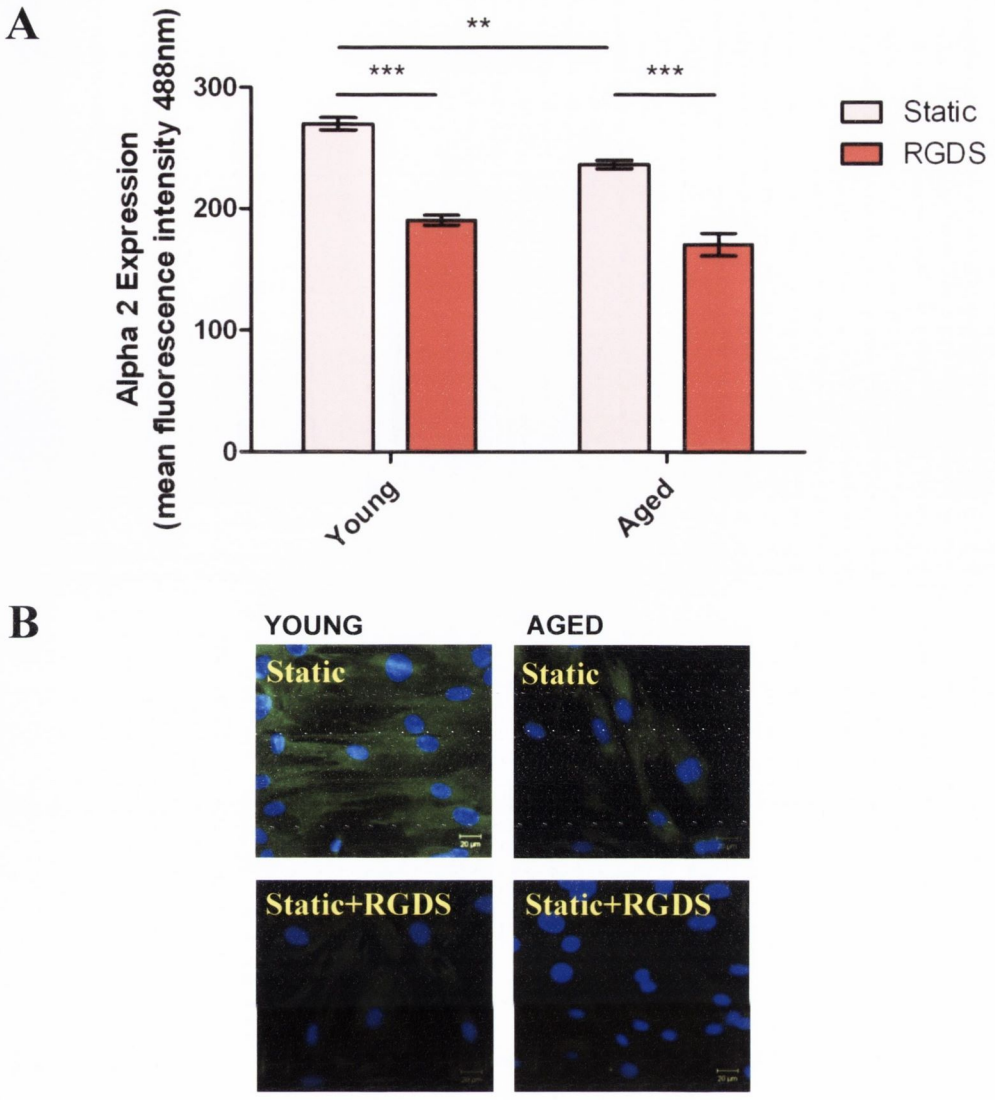


Figure 6.9: Expression of α2 integrin is significantly lower in aged MSCs and integrin inhibition for 7 days reduces α2 expression in both young and aged MSCs under static conditions

MSCs were examined for the expression of α2 using immunocytochemistry for an anti-α2 antibody. Changes in expression were quantified by a change in mean fluorescence intensity **A:** There was a significant effect of drug ($F_{1,8}$ = 151.00, $p<0.001$, two-way ANOVA) and age ($F_{1,8}$ = 20.48, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed there was a significant decrease in α2 expression caused by integrin inhibition (***$p<0.001$, n=3 cultures), which was greater in aged MSCs compared to young (**$p<0.01$, n=3 culture). **B:** Confocal images of α2 expression in young and aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.10 Cyclic tensile strain increases actin expression in MSCs and overcomes a reduction in actin expression induced by integrin inhibition

The effects of 2.5% cyclic tensile strain and integrin inhibition on the integrin signal transducer actin were also assessed during 7 day differentiation testing to evaluate its possible role in mechanotransduction of strain-induced differentiaion. Expression of actin was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.10, A). In young MSCs, there was a significant effect of strain ($F_{1,8} = 59.39$, $p<0.001$, two-way ANOVA), drug ($F_{1,8} = 9.95$, $p<0.05$, two-way ANOVA) and a significant interaction ($F_{1,8} = 13.66$, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of actin significantly compared to static controls (static control:390.76±5.95 units, 2.5%:462.53±28.71 units, $p<0.05$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in actin expression compared to untreated static controls (static control:390.76±5.95 units, RGDS:268.20±7.34 units, $p<0.01$, n=3, mean±SEM) and the application of 2.5% strain in combination with RGDS reversed the decrease in actin expression observed with integrin inhibition alone, returning expression to control level (RGDS:268.20±7.34 units, 2.5%+RGDS:472.20±19.15 units, $p<0.001$, n=3, mean±SEM).

In aged MSCs (Figure 6.10, A), there was a significant effect of strain ($F_{1,8} = 297.60$, $p<0.001$, two-way ANOVA) and drug ($F_{1,8} = 11.57$, $p<0.01$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of actin significantly compared to static controls (static control:296.41±9.66 units, 2.5%:446.27±12.41units, $p<0.001$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in actin expression compared to untreated static controls (static control:296.41±9.66 units, RGDS:242.86±5.31 units, $p<0.01$, n=3, mean±SEM) and the application of 2.5% strain in combination with integrin inhibition reversed the decrease in actin expression observed with integrin inhibition alone, returning expression to control level (RGDS:242.86±5.31 units,

2.5%+RGDS:432.81±10.60 units, $p<0.001$, $n=3$, mean±SEM). Representative images (Figure 6.10, B and C) show the changes in actin expression in young and aged MSCs exposed to RGDS and 2.5% strain. Similar to the previosu study, the accompanying images highlight how drug treatment reduces the number of actin stress fibres present in the cell but application of tensile strain increases fibre formation as well as directing their orientation perpendicular to the direction of stretch and parallel with the cell's aspect ratio. These results indicate the enhancing effect of tensile strain on actin expression in MSCs and the diminishing effect of integrin inhibition but co-application of tensile strain prevents the inhibitor-induced reduction in actin expression.

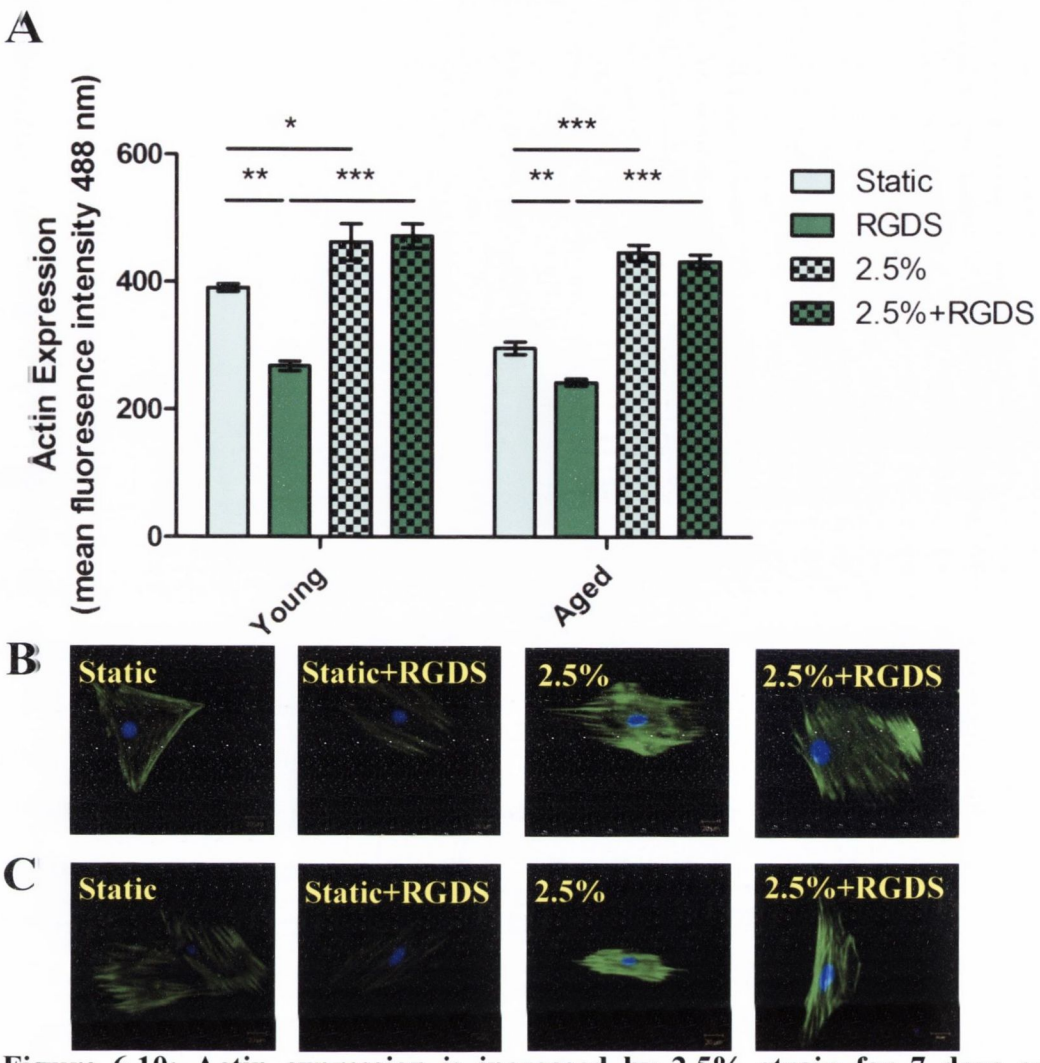


Figure 6.10: Actin expression is increased by 2.5% strain for 7 days and strain can overcome the reduction in actin expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of actin by immunocytochemistry using an anti-actin antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8} = 59.39$, $p<0.001$, two-way ANOVA) and aged ($F_{1,8} = 297.60$, $p<0.001$, two-way ANOVA) MSCs, drug in young ($F_{1,8} = 9.95$, $p<0.05$, two-way ANOVA) and aged ($F_{1,8} = 11.57$, $p<0.01$, two-way ANOVA) MSCs and a significant interaction ($F_{1,8} = 13.66$, $p<0.01$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in actin expression with 2.5% strain for 7 days (*$p<0.05$, ***$p<0.01$, n=3 cultures). There was a significant decrease in actin expression with integrin inhibition (**$p<0.01$, n=3 cultures), which was reversed in MSCs exposed to strain with integrin inhibition (***$p<0.001$, n=3 cultures). **B:** Confocal images of actin expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.11 Actin expression is decreased with age and integrin inhibition induces a reduction in actin expression in both young and aged MSCs under static conditions

The pattern of actin expression in response to tensile strain and integrin inhibition was similar in both young and aged MSCs; however, differences in expression were apparent. Figure 6.11, A shows the comparison between young and aged MSCs exposed to integrin inhibition for 7 days. There was a significant effect of both drug ($F_{1,8} = 147.10$, $p<0.001$, two-way ANOVA), age ($F_{1,8} = 67.93$, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,8} = 22.58$, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed that integrin inhibition caused a significant decrease in actin expression in both young (static control:390.76±5.95 units, RGDS:268.20±7.34 units, $p<0.001$, n=3, mean±SEM) and aged MSCs (static control:296.41±9.66 units, RGDS:242.86±5.31 units, $p<0.01$, n=3, mean±SEM). A significant decrease was also observed in actin expression in aged MSCs compared to young (Young static control:390.76±5.95 units, Aged static control:296.41±9.66 units, $p<0.001$, n=3, mean±SEM). Figure 6.11, B shows images of actin expression in young and aged MSCs. This result highlights the age-related difference and the influence of integrin inhibition on actin expression in young and aged MSCs.

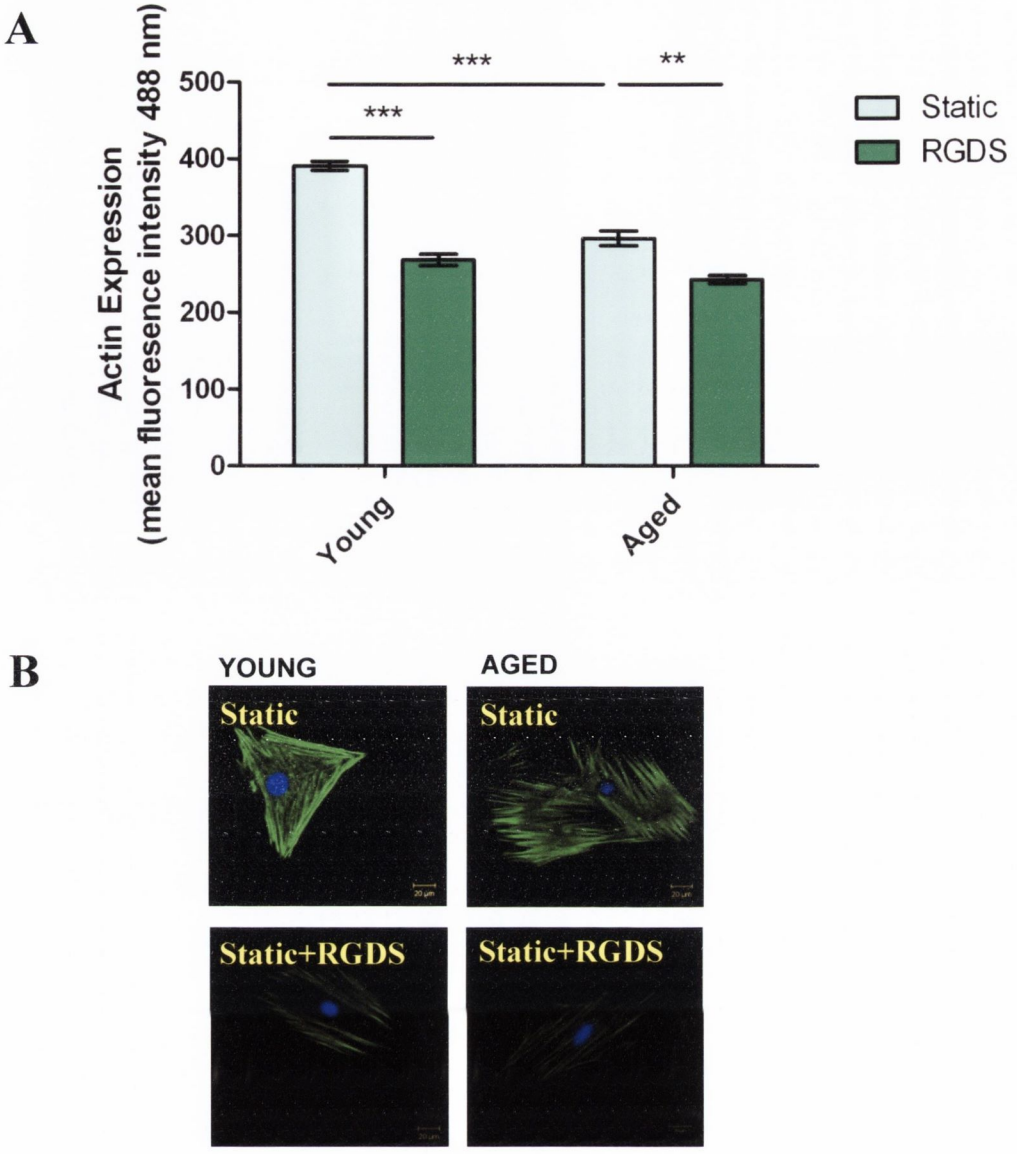


Figure 6.11: Actin expression is significantly lower in aged MSCs and integrin inhibition for 7 days decreases actin expression in both young and aged MSCs under static conditions

MSCs were examined for the expression of actin using immunocytochemistry for an anti-actin antibody. Changes in expression were quantified by a change in mean fluorescence intensity **A:** There was a significant effect of drug ($F_{1,8}$ = 147.10, $p<0.001$, two-way ANOVA), age ($F_{1,8}$ = 67.93, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,8}$ = 22.58, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed there was a significant decrease in actin expression in aged MSCs compared to young (***$p<0.001$, n=3 cultures). There was also a significant reduction in actin expression in both age groups caused by integrin inhibition (**$p<0.01$, ***$p<0.001$, n=3 cultures). **B:** Confocal images of actin expression in young and aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.12 Cyclic tensile strain increases pFAK expression in MSCs and overcomes a reduction in pFAK expression induced by integrin inhibition

FAK is well established as a mediator of integrin signalling and has been implicated in mechanotransduction during differentiation (Shih *et al.* 2011, Leucht *et al.* 2007). Therefore, the effects of 2.5% cyclic tensile strain and integrin inhibition on the activated form of the integrin signal mediator FAK were also assessed during 7 day differentiation testing. Expression of pFAK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.12, A). In young MSCs, there was a significant effect of strain ($F_{1,8}$ = 128.20, $p<0.001$, two-way ANOVA), drug ($F_{1,8}$ = 32.81, $p<0.001$, two-way ANOVA) and a significant interaction ($F_{1,8}$ = 11.18, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of pFAK significantly compared to static controls (static control:218.46±4.49 units, 2.5%:306.12±9.89 units, $p<0.001$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in pFAK expression compared to untreated static controls (static control:218.46±4.49 units, RGDS:118.82±9.51 units, $p<0.001$, n=3, mean±SEM) and the application of 2.5% strain in combination with integrin inhibition reversed the decrease in pFAK expression observed with integrin inhibition alone, returning expression to control level (RGDS:118.82±9.51 units, 2.5%+RGDS:279.92±16.56 units, $p<0.001$, n=3, mean±SEM).

In aged MSCs (Figure 6.12, A), there was a significant effect of strain ($F_{1,8}$ = 82.96, $p<0.001$, two-way ANOVA), drug ($F_{1,8}$ = 17.09, $p<0.01$, two-way ANOVA) and a significant interaction ($F_{1,8}$ = 8.58, $p<0.05$, two-way ANOVA). *Post hoc* analysis revealed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of pFAK significantly compared to static controls (static control:203.02±11.91 units, 2.5%:276.59±14.14 units, $p<0.01$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days showed a significant decrease in pFAK expression compared to untreated static controls (static control:203.02±11.91 units, RGDS:118.93±9.21 units, $p<0.01$, n=3, mean±SEM)

and the application of 2.5% strain in combination with integrin inhibition reversed the decrease in pFAK expression observed with integrin inhibition alone, returning expression to control level (RGDS:118.93±9.21 units, 2.5%+RGDS:262.25±11.84 units, $p<0.001$, $n=3$, mean±SEM). Representative images (Figure 6.12, B and C) show the changes in pFAK expression in young and aged MSCS exposed to integrin inhibition and 2.5% strain. These results indicate the enhancing effect of tensile strain on pFAK expression in MSCs. Integrin inhibition reduces pFAK expression but co-application with tensile strain prevents the inhibitor-induced reduction observed.

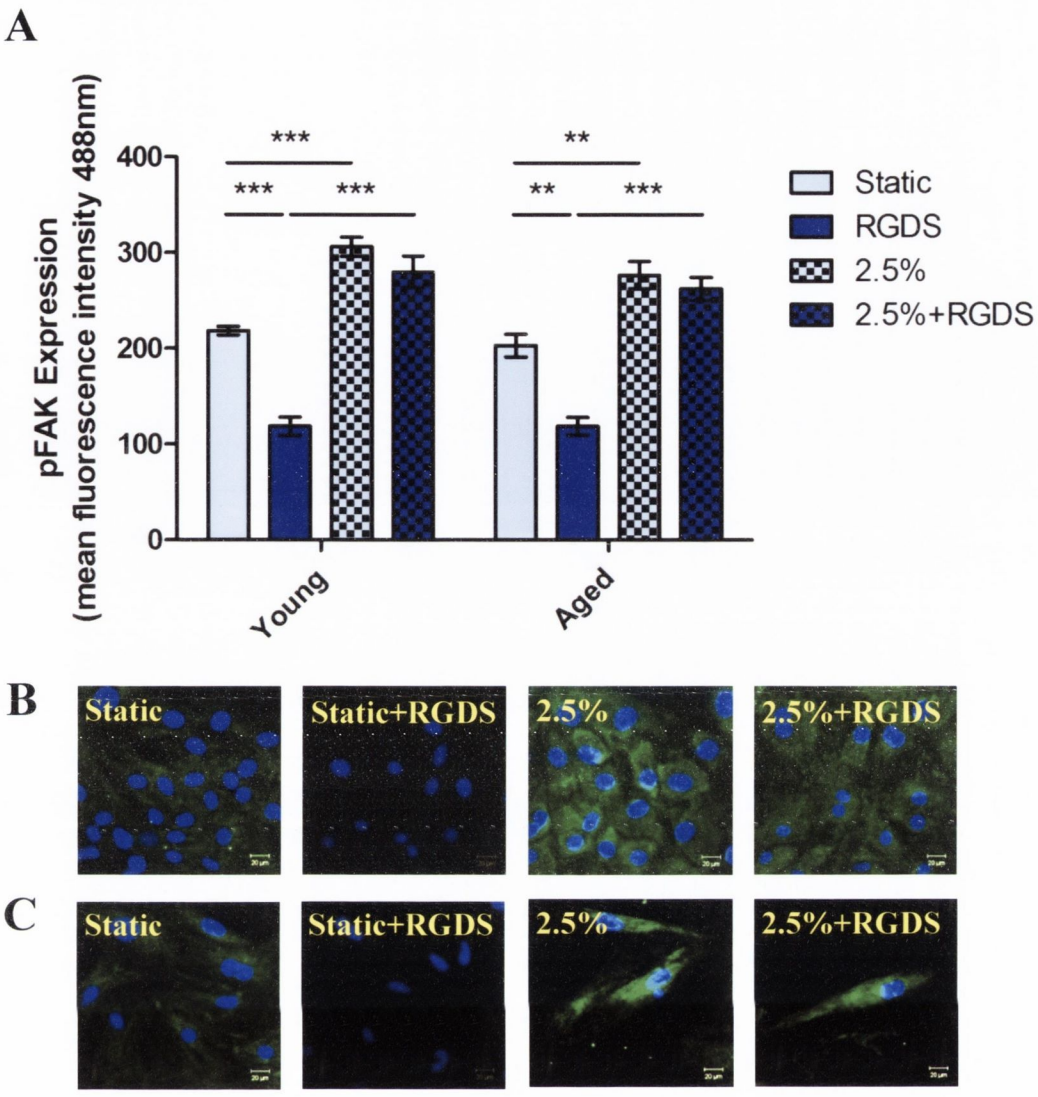


Figure 6.12: Expression of pFAK is increased by 2.5% strain for 7 days and strain can overcome the reduction in pFAK expression evoked by integrin inhibition in MSCs

MSCs were examined for the expression of pFAK by immunocytochemistry using an anti-pFAK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8}$ = 128.20, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 82.96, $p<0.001$, two-way ANOVA), drug in young ($F_{1,8}$ = 32.81, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 17.09, $p<0.01$, two-way ANOVA), and a significant interaction in young ($F_{1,8}$ = 11.18, $p<0.05$, two-way ANOVA) and aged ($F_{1,8}$ = 8.58, $p<0.05$, two-way ANOVA) MSCs. *Post hoc* analysis showed a significant increase in pFAK expression following 2.5% strain for 7 days (**$p<0.01$, ***$p<0.001$, n=3 cultures). There was a significant decrease in pFAK expression in the integrin inhibition group compared to untreated static control (**$p<0.01$, ***$p<0.001$, n=3 cultures), which was reversed in MSCs exposed to strain with integrin inhibition (***$p<0.001$, n=3 cultures). **B:** Confocal images of pFAK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.13 Cyclic tensile strain increases pERK expression in MSCs, independent of integrin inhibition

The effects of tensile strain and the integrin inhibitor RGDS on pERK expression were also investigated, since ERK is often associated with strain-induced MSC differentiation (Shih *et al.* 2011, Kearney *et al.* 2010, Simmons *et al.* 2003). Expression of pERK was determined using immunocytochemistry and confocal microscopy. Changes in expression were observed as alterations in mean fluorescence intensity values (Figure 6.13, A). In young MSCs, there was a significant effect of strain ($F_{1,8}$ = 30.44, $p<0.001$, two-way ANOVA) and drug ($F_{1,8}$ = 10.00, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that young MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of pERK significantly compared to static controls (static control:333.31±7.01 units, 2.5%:426.50±12.30 units, $p<0.01$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days had no effect on pERK expression, however, a significant increase in pERK expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:285.37±25.63 units, 2.5%+RGDS:371.60±14.16 units, $p<0.05$, n=3, mean±SEM).

In aged MSCs (Figure 6.13, A), there was a significant effect of strain ($F_{1,8}$ = 62.49, $p<0.001$, two-way ANOVA). *Post hoc* analysis showed that aged MSCs exposed to 2.5% cyclic tensile strain for 7 days increased the expression of pERK significantly compared to static controls (static control:308.84±11.16 units, 2.5%:379.13±3.49 units, $p<0.001$, n=3, mean±SEM). Exposure to RGDS (0.5μM) for 7 days had no effect on pERK expression, however, a significant increase in pERK expression was observed following application of 2.5% strain in combination with integrin inhibition (RGDS:311.12±5.20 units, 2.5%+RGDS:367.09±9.56 units, $p<0.01$, n=3, mean±SEM). Representative images (Figure 6.13, B and C) show the changes in pERK expression in young and aged MSCs exposed to integrin inhibition and 2.5% strain. These results indicate how tensile strain enhances pERK expression and that integrin inhibiton has no influence on the strain-induced increase in the expression of pERK in MSCs.

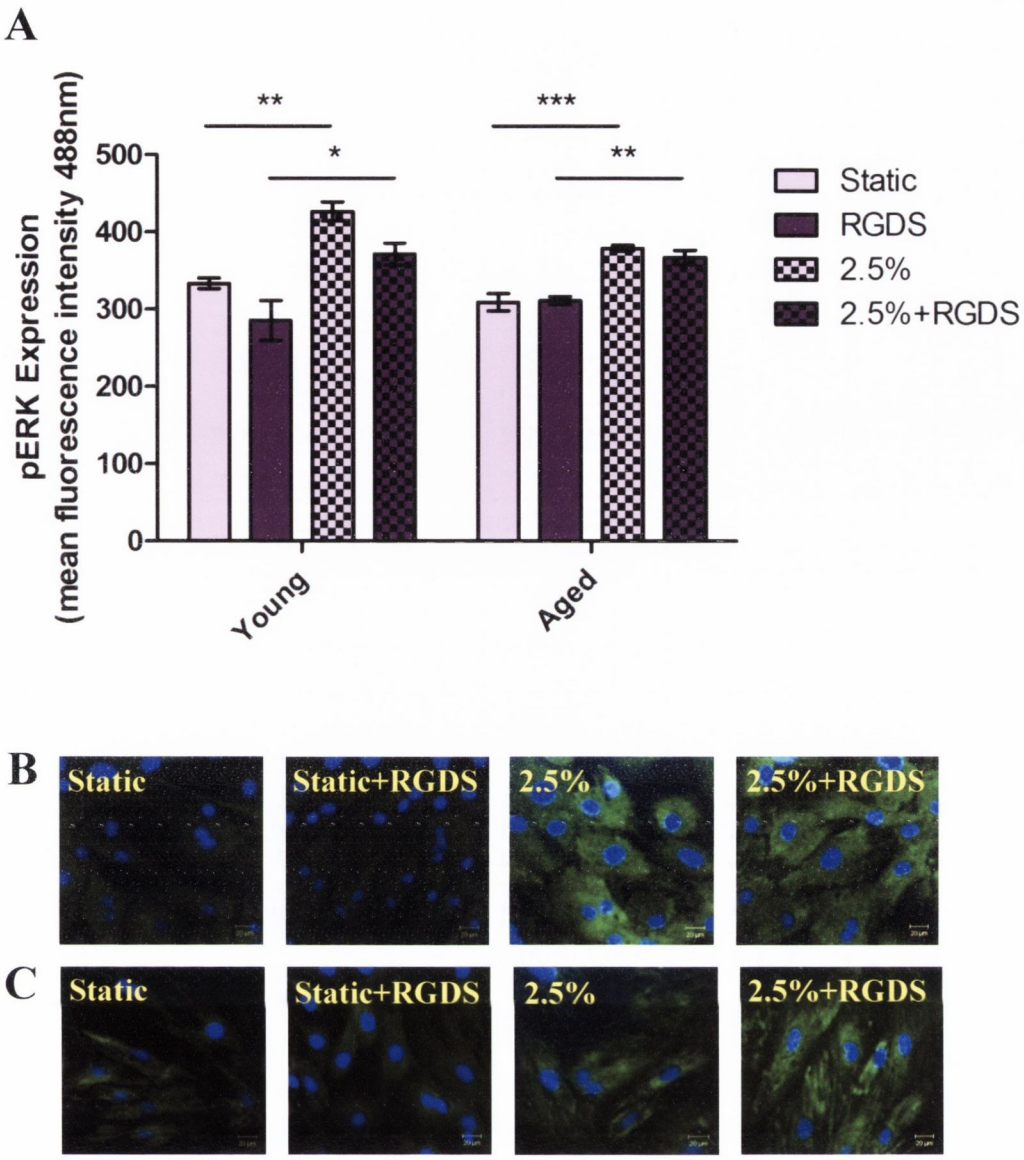


Figure 6.13: Expression of pERK is increased by 2.5% strain for 7 days and integrin inhibition has no effect in MSCs

MSCs were examined for the expression of pERK by immunocytochemistry using an anti-pERK antibody. Changes in expression were quantified by a change in mean fluorescence intensity. **A:** There was a significant effect of strain in young ($F_{1,8}$ = 30.44, $p<0.001$, two-way ANOVA) and aged ($F_{1,8}$ = 62.49, $p<0.001$, two-way ANOVA) MSCs and drug ($F_{1,8}$ = 10.00, $p<0.001$, two-way ANOVA) in young MSCs. *Post hoc* analysis showed a significant increase in pERK expression following 2.5% strain for 7 days (**$p<0.01$, ***$p<0.001$, n=3 cultures). There was a significant increase in pERK expression in the combined strain and integrin inhibition group compared to treated static control (*$p<0.05$, **$p<0.01$, n=3 cultures). **B:** Confocal images of pERK expression in young and **C:** aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.2.14 Cyclic tensile strain increases pERK expression in MSCs and this is significantly lower in aged MSCs

The pattern of pERK expression in response to tensile strain and integrin inhibition was similar in both young and aged MSCs; however, differences in expression were apparent. Figure 6.14, A shows the comparison between young and aged MSCs exposed to strain. There was a significant effect of both strain ($F_{1,8} = 79.28$, $p<0.001$, two-way ANOVA) and age ($F_{1,8} = 15.31$, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed a significant increase increase in pERK expression in both young (static control:333.31±7.01 units, 2.5%:426.50±12.30 units, $p<0.001$, n=3, mean±SEM) and aged MSCs (static control:308.84±11.16 units, 2.5%:379.13±3.49 units, $p<0.01$, n=3, mean±SEM) exposed to 2.5% strain for 7 days. However, the increase in pERK expression was lower in aged MSCs exposed to strain compared to young (Young 2.5%:426.50±12.30 units, Aged 2.5%:379.13±3.49 units, $p<0.05$, n=3, mean±SEM). Figure 6.14, B shows images of pERK expression in young and aged MSCs. These results highlight how tensile strain enhances pERK expression but that the response to strain is more robust in young MSCs compared to aged cells.

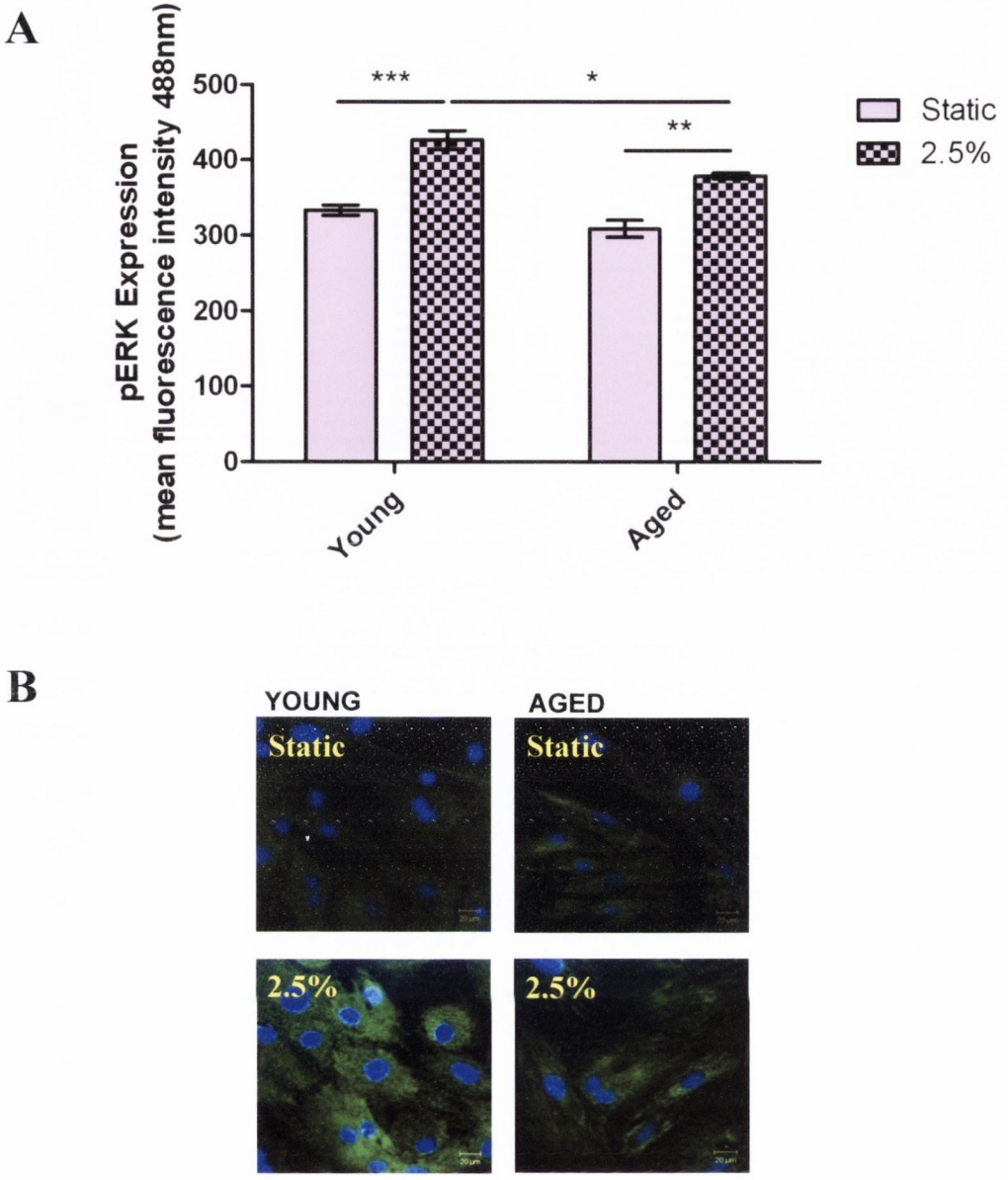


Figure 6.14: Expression of pERK is increased in MSCs by 2.5% strain for 7 days and this response is significantly lower in aged MSCs

MSCs were examined for the expression of pERK using immunocytochemistry for an anti-pERK antibody. Changes in expression were quantified by a change in mean fluorescence intensity **A:** There was a significant effect of strain ($F_{1,8}$ = 79.28, $p<0.001$, two-way ANOVA) and age ($F_{1,8}$ = 15.31, $p<0.01$, two-way ANOVA) in this observation. *Post hoc* analysis showed a significant increase in pERK expression in the strained young and aged groups compared to static controls (**$p<0.01$, ***$p<0.01$, n=3 cultures). The response to tensile strain was significantly lower in aged MSCs compared to young MSCs (*$p<0.05$, n=3 cultures). **B:** Confocal images of pERK expression in young and aged MSCs. Results are displayed as mean ± SEM. Scale bar is 20μm.

6.3 Discussion

6.3.1 Summary of results

The aim of this study was to determine the effect of a low rate of strain (2.5%) on MSC differentiation and mechanotransduction pathways in young and aged MSCs, and to evaluate the role of integrin receptors in such pathways. Expression of both bone-specific osteocalcin and cartilage-specific collagen II were upregulated in response to 2.5% tensile strain for 7 days in young and aged MSCs. The increase in osteocalcin expression in response to strain remained evident following integrin inhibition. Furthermore, the strain-mediated increase in osteocalcin expression was significantly greater in MSCs isolated from young rats compared to MSCs isolated from aged rats. This indicates a decreased differentiation response to strain in aged MSCs. Histological staining to identify calcium deposition by Von Kossa staining followed similar patterns of staining intensity according to a scored index of differentiation, based on the intensity of the staining observed. There was an overall increase in staining intensity in groups exposed to tensile strain and higher scores of osteogenic differentiation were also observed in cells grown in static conditions on glass coverslips compared to silicone substrates. On both glass and silicone substrates, there was a general reduction in staining intensity score following integrin inhibition.

Collagen II expression was also upregulated in response to tensile strain in young and aged MSCs and this increase persisted in young MSCs following integrin inhibition. Histological staining to identify proteoglycan synthesis by Safranin-O staining exhibited comparable patterns of staining intensity according to a scored differentiation index, similar to that employed for osteogenic differentiation. There was a general increase in the intensity of the staining in samples exposed to tensile strain. There was also intense staining observed in cells grown in static conditions on glass coverlsips compared to silicone substrates but comparable to the differentiation index observed in silicone substrates exposed to strain. On both types of substrate, there was a common reduction in staining intensity with integrin inhibition.

To investigate the co-expression of osteocalcin and collagen II, both proteins were stained simultaneously using different fluorescent labels. It was found that expression of both skeletogenic markers was significantly lower in aged MSCs compared to young but that all groups exhibited an upregulation in expression of these proteins when exposed to 2.5% strain, indicating the ability of tensile strain to modulate both markers of differentiation. A reduction in strain-induced osteocalcin expression was observed in aged MSCs compared to young. Differentiation ratios were calculated based on combined immunostaining data which showed an overall preference for osteogenic differentiation but in groups exposed to integrin inhibition the balance was tipped toward chondrogenic differentiation.

In terms of signalling molecules associated with mechanotransduction during differentiation, it was revealed that the integrin subunit α2, actin, pFAK and pERK are all upregulated in response to 2.5% tensile strain for 7 days. Integrin inhibition reduced the expression of all proteins, except pERK, which was unaffected by RGDS treatment. Finally, it was observed in α2 and actin that there was an age-related decrease in expression of α2 and actin. Also, there was a reduction in pERK expression in the response to strain in aged MSCs compared to young.

6.3.2 MSC Differentiation

A role for mechanical stimulation in MSC differentiation has previously been investigated within this laboratory. The cellular mechanisms of mechanotransduction in young MSCs were assessed by straining cells in the presence of the stretch-activated cation channel (SACC) blocker gadolinium chloride ($GdCl_3$), the ERK inhibitor U0126, the p38 inhibitor SB203580 and the PI3-kinase inhibitor LY294002. Following application of tensile strain, the osteogenic markers CBF-α1, collagen I, osteocalcin, and BMP2 were expressed over time. Synthesis of BMP2 by mechanical strain was found to be reduced by the various kinase inhibitors for ERK, p38 and PI3-kinase. It was concluded from that study that mechanical forces play a considerable role in regulating osteogenic

differentiation in young MSCs. Although, those findings suggest that short-term mechanical stimulation induces differentiation toward an osteogenic phenotype, whereas long-term stimulation evokes desensitisation to tensile strain in bone cells (Kearney *et al.* 2010). The current doctoral study has shown a similar upregulation of the osteogenic markers, osteocalcin and calcium phosphate deposition, in response to tensile strain in both young and aged MSCs. However, this response was reduced in aged MSCs, indicating an age-related change in the response to tensile strain. The expression of α2 and actin were found to be reduced with age under static conditions. Although, the expression of these mechanosensors is upregulated in response to strain, indicating there may be an alternative signalling process associated with the observed reduction that doesn't influence differentiation through strain. However, the expression of pERK is reduced in the response of aged MSCs to strain which may implicate the involvement of this signalling molecule in strain-induced osteogenic differentiation, and the reduced expression of this protein with age may explain the reduced differentiation response observed in aged MSCs. Although, the expression of pERK was upregulated in response to strain as well, its activation was not influenced by integrin inhibition which implies that RGD-mediated integrin signalling does not influence ERK activity under static conditions, however the receptors could be involved in mechanoregulation of ERK expression during tensile strain.

Interestingly, upregulation of several chondrogenic markers, collagen II and proteoglycan synthesis were also observed simultaneously to the induction of osteogenic markers in this study. Typically, tension as a form of mechanical stimulation is associated with induction of osteogenic differentiation. This is the first indication of chondrogenic differentiation of MSCs exposed to tensile strain on a 2-D substrate. A review by Kelly & Jacobs (2010) summarise the modest amount of literature pertaining to tensile strain and chondrogenesis. Tensile strain has been shown to increase BMP-2 expression in MSCs embedded in 3-D collagen matrices, suggesting that strain alone can stimulate osteogenic differentiation (Sumanasinghe *et al.* 2006). It has also been demonstrated that tension regulates both fibroblastic and osteogenic associated genes, while compression upregulates chondrogenic gene expression (Haudenschild *et al.*

2009). Compressive strains are generally associated with the induction of chondrogenic differentiation (Thorpe *et al.* 2010, Kisiday *et al.* 2009, Pelaez *et al.* 2009, Campbell *et al.* 2006, Angele *et al.* 2004). However, tensile strain is in fact capable of regulating chondrogenic differentiation and GAG synthesis of MSCs embedded in collagen-GAG scaffolds as described previously from this laboratory (McMahon *et al.* 2008). In another study, chick limb bud MSCs were exposed to a 5% circumferential tensile strain by inserting a balloon catheter into the lumen of a tubular scaffold and inflating the tube (Amos 2008). Results from that study suggested that embryonic MSCs followed known expression patterns of cartilage development and were not undergoing maturation; whereas MSCs at a later stage of development produced a more fibrous tissue and exhibited signs of chondrogenic differentiation in response to tensile strain. Cyclic tensile strain was shown to upregulate the expression of chondrogenic markers in the present study. This has not been previously demonstrated on a 2-D substrate in the absence of chondro-inductive growth factors.

A large number of growth factors and hormones have been implicated as regulators of chondrocyte biology but relatively little is understood about the intracellular signalling pathways concerned. Members of the transforming growth factor-beta (TGF-β) superfamily are a key mediator of MSC chondrogenesis and studies have attempted to define the role of specific TGF-β dependent signalling pathways associated with the regulation of MSC chondrogenesis. Controlled local delivery of TGF-β3 is essential to neocartilage formation by MSCs but further insight is required to prevent the differentiation of chondrogenically induced MSCs adopting a hypertrophic phenotype. (Bian *et al.* 2011, Lee *et al.* 2004). Takeuchi *et al.* (1996) have previously shown that interaction of collagen I with integrin α2β1 causes differentiation and TGF-β receptor down-regulation in osteoblastic cells. In a later study, it was observed that attachment of cells to collagen I stimulated tyrosine phosphorylation of FAK and ERK. Inhibition of tyrosine kinase by Herbimycin A, abolition of focal adhesion by Cytochalasin D or overexpression of antisense FAK mRNA averted ERK/MAPK activation and alkaline phosphatase activity and TGF-β receptor down-regulation was prevented by inactivation of FAK. These results demonstrate that collagen I and α2β1 integrin interaction mediate differentiation and down-regulation of TGF-β

receptors through activation of FAK and its various downstream signals (Takeuchi *et al.* 1997). In the current study, all substrates in the strain experiments consisted of silicone coated with a collagen I solution. Therefore, it is possible that upregulation of osteogenic markers may be due to interaction of integrin α2β1 on MSCs interacting with collagen I on the substrate that the cells were seeded onto. One study has reported an inhibition of chondrogenesis exposed to tensile strain, whereby rat limb bud MSCs were cultured on a silicone membrane and exposed to stepwise stretching following chondrogenic induction. Expression of collagen II and the appearance of chondrogenic nodules decreased following strain but expression of these markers was completely recovered when cell-matrix interaction was inhibited by the Gly-Arg-Gly-Asp-Ser-Pro-Lys peptide or by the application of blocking antibodies for α2, α5 or β1 integrins (Onodera *et al.* 2005). In the present study, integrin inhibition exerted a decrease in the expression of α2, actin and FAK which may explain why those groups showed a slightly higher inclination toward chondrogenesis (Tables 6.1 and 6.2) since TGF-β receptor down-regulation may be averted in this circumstance in addition to disrupted cell-matrix interaction which might normally inhibit chondrogenesis.

However, this does not fully explain simultaneous expression of osteogenic and chondrogenic markers observed in this study. Addition of lectin Dolichos biflorus agglutinin (DBA) to limb bud mesenchymal cells from mouse embryos induces both an osteogenic and chondrogenic response, highlighting the effectiveness of certain stimuli in producing dichotomous results (Talaei-Khozani *et al.* 2011). Alternatively, the occurrence of both osteogenic and chondrogenic differentiation has also been described by Kii *et al.* (2004) who found a novel mechanism for the differentiation of MSCs induced by cadherin-11– and N-cadherin–mediated cell–cell interaction. Both cadherins could directly regulate the osteoblastic and chondrogenic gene expression through specific cell–cell interactions. Friedl *et al.* (2007) found a significant cyclic tensile strain-related stimulation of the major transcription factors known to be crucially involved in both chondrocyte and osteoblast differentiation; Sox9 and Runx2. Accompanying this was a significant increase in mRNAs encoding for the proteoglycans LUM and DCN, which are both swiftly upregulated in early chondrogenic differentiation, and mRNAs for COL1A1, ALPL, SPP1, and SPARC, all markers associated with early

osteoblastic differentiation. These results compellingly illustrate that tensile strain embodies a robust transcriptional stimulus for both osteogenic and chondrogenic differentiation in hMSCs. These mixed findings were attributed to the characteristic donor-related heterogeneity of undifferentiated hMSCs, thus the application of mechanical strain promoted early chondrogenic and osteogenic differentiation *in vitro*.

In the current study, it is possible that at the time point tested, potential for differentiation along either lineage remains possible and mechanical stimulation induces differentiation signals for both osteogenic and chondrogenic differentiation. It is also possible that at a later time point, either lineage may become dominant and override the potential for differentiation along other lineages. For example, prolonged application of strain may continue to down regulate TGF-β receptor expression and therefore diminish a prominent signalling pathway associated with chondrogenesis allowing for osteogenic differentiation to occur. Previous work within our laboratory has found an upregulation in osteogenic marker expression following 3 days of cyclic tensile strain at 2.5% in the absence of osteo-inductive growth factors (Kearney *et al.* 2010). In another study, chondrogenic differentiation has been demonstrated following 7 days of tensile strain at 10% with the addition of chondrogenic growth factors. It is possible that an osteogenic response to tensile strain is elicited at the earlier time point of 3 days, whereas prolonged exposure to strain may encourage an overriding chondrogenic response. Although, the chondrogenic response observed in that study may be fortified by the application of a higher rate of strain and the inclusion of chondro-inductive factors. The bi-lineage potential observed in the current study has serious implications for tissue engineering, such that commitment to a specific lineage has not been observed. This implies that the response of MSCs to tensile strain is not lineage specific and therefore requires some other form of directed differentiation such as specific inductive growth factors to compliment the effects of strain or further investigation into the rate, frequency and duration of strain and how MSCs respond to these signals.

6.3.3 Mechanotransduction during MSC differentiation

A wide variety of signals affect the differentiation of MSCs and it is highly unlikely that a single signalling pathway is responsible for regulating lineage commitment induced by interaction with the ECM. It is more reasonable to acknowledge that a network of signalling pathways is likely at work and it is well established that cell-matrix interactions play a crucial role in the development of the differentiated phenotype (Rowlands *et al.* 2008, Salasznyk *et al.* 2007). Integrin receptors provide the link between the ECM and the cytoskeleton, essential for transduction of mechanical signals. Integrins are not solely responsible for cell adhesion and mechanosensing, they also have a direct role in cell signalling. Each integrin receptor (a combination of an α and β subunit) possesses affinity for a single, or a few specific ligand sequences located on different ECM molecules, for example several integrins bind specifically to the RGD amino acid sequence (Rowlands *et al.* 2008). In a study by Engler *et al.* (2006) it was shown that during *ex vivo* culture of MSCs, lineage commitment could be directed by the matrix elasticity on which the cells were grown. The elastic modulus of collagen I-coated polyacrylamide was tuned to that of brain, muscle and collagenous bone, which the authors demonstrated caused MSCs to differentiate into neurogenic, myogenic, and osteogenic precursors when cultured on gels of varying elasticity. MSCs sense and respond to matrix stiffness by engaging with their pericellular environment through integrin adhesions. The results of their study indicated that MSC differentiation is responsive to both the substrate stiffness and the adhesive ligand presented on the surface of the gel. Specific combinations of substrate stiffness and ECM molecule provide a directed microenvironment for MSC lineage specification into both osteogenic and myogenic lineages. Differentiation of MSCs toward osteogenic precursors was noted predominately on the stiffest substrate gel; the strongest expression of Runx2 (or CBF-α1, an osteoblastic transcription factor) was observed on an 80kPa collagen I-coated gel. This osteogenic response may be due to the fact that this combination of factors best imitates the natural microenvironment of bone. However, it was also reported that substrate stiffness alone is not sufficient to initiate osteogenic differentiation of MSCs, the differentiation process appears to be modulated by the type of ECM molecule present for a given substrate stiffness.

It has also been demonstrated that α2 and collagen interaction is required for activation of CBF-α1 and induction of osteoblast-specific gene expression (Xiao *et al.* 1998). It is possible that in the present study, the collagen I coating on silicone substrates may be promoting the preference of MSC differentiation toward the osteogenic lineage and may explain part of the spontaneous differentiation observed in static control conditions.

Cells sense matrix elasticity via integrin adhesion to ECM molecules and tension through cytoskeletal contractions. Cells on stiff substrates develop permanent anchors through focal adhesions generating high cytoskeletal tension, which is evidenced by increased actin stress fibers and large spread area (Engler *et al.* 2006). In this study, it has been demonstrated that tensile strain significantly upregulates α2 and actin expression, which may represent the initiating steps in a mechanoregulated differentiation pathway. Additionally, integrin inhibition caused significant decreases in α2 and actin expression, and the groups treated with the integrin inhibitor consistently displayed a reduced differentiation response. These results imply that integrin signalling is key to MSC differentiation induced by mechanical stimulation and that tensile strain is a robust promoter of this occurrence. FAK is well suited to integrate these signalling activities and as an intracellular component of the integrin signalling pathway it is widely implicated in mechanotransduction. Leucht *et al.* (2007) found that physical forces triggered the site-specific expression of Sox9 and Runx2, two transcription factors associated with MSC commitment to the skeletogenic lineage. This was followed by conditional inactivation of FAK, which abolished the cellular response to physical stimuli such that bone marrow cells ceased to upregulate skeletogenic genes, collagen fibrils remained disorganised, and the mechanically-induced osteogenic response was inhibited. It was observed in the current study that tensile strain elicited the increased expression of two late stage skeletogenic markers osteocalcin and collagen II, indicating the potential for tensile strain in multiple lineage differentiation. Integrin inhibition also significantly down regulated pFAK expression which may have contributed to the weaker differentiation response exhibited by those groups.

Various cell stimuli, including mechanical stress, converge on the MAPK family members during osteogenic differentiation of hMSCs. Signalling by ERK1/2 MAPKs is crucial for stimulating osteogenic gene expression in hMSCs (Salasznyk *et al.* 2004). A later study by Salasznyk *et al.* (2007) established a link between FAK and ERK during hMSC differentiation. FAK mRNA levels were reduced by half using siRNA treatment which also decreased ECM-mediated phosphorylation of FAK residue Y397 and ERK1/2. FAK inhibition blocked osterix transcriptional activity and the osteogenic differentiation of hMSCs, as evidenced by decreased alkaline phosphatase activity and calcium deposition. These results suggest that FAK signalling plays an important role in regulating ECM-induced osteogenic differentiation of hMSCs. Furthermore, these observations suggest that FAK phosphorylation of ERK1/2 may be one of the mechanisms for regulating Runx2/CBF-α1 in MSCs. In the present study, expression of pFAK was significantly upregulated following application of 2.5% tensile strain for 7 days. This increase could be facilitating the increase in pERK expression and osteogenic differentiation that were also observed following strain. In the previous chapter, it was shown that ERK was not phosphorylated following 5% tensile strain for 24hr. Therefore, a low level of tensile strain may be the optimum stimulus for pERK upregulation or the prolonged length of stimulus delivery may be maintaining the increased expression observed in this study.

It is often suggested that the effects of aging on MSCs lead to a decline in the number and differentiation potential which may contribute to aging and age-related diseases (Zhou *et al.* 2008, Bonab 2006, Fehrer & Lepperdinger 2005, D'Ippolito *et al.* 1999). However, several studies have highlighted a maintenance capacity of MSCs to differentiate with age. For example, Shi *et al.* (2005) demonstrated that adipose-derived MSCs (ADSCs) retain the potential for osteogenic differentiation in aged cells. Similarly, mechanical strain has been shown to counteract the loss of self-renewal in aging murine ADSCs by enhancing their proliferation and simultaneously reducing the heightened adipogenesis of aged cells, while osteogenic potential was not significantly affected by aging. Pre-strain diminished the expression of adipogenic marker genes in adult and old ADSCs and caused an increase in the number of osteogenic colonies with calcium deposition. When subjected to simultaneous mechanical

loading and adipogenic induction, a stronger inhibition of adipogenesis than that caused by pre-strain was observed (Huang *et al.* 2010). In the current study, continuous 2.5% tensile strain for 7 days of aged MSCs was under investigation for the first time. In all experiments in this chapter, differentiation markers and signalling molecules were upregulated in response to strain in both young and aged MSCs, indicating aged MSCs are also mechanoresponsive to an applied stimulus. However, the osteogenic response to strain was reduced in aged MSCs compared to young cells. The only signalling molecule exhibiting an age-associated reduction in response to strain was pERK, which may implicate this protein in strain-induced osteogenic differentiation. All other molecules under investigation, α2, actin and pFAK showed no reduction with strain in aged cells compared to young, which may suggest osteogenic differentiation is not regulated by these particular molecules or that they are in fact associated with strain-induced differentiation but perhaps some other mechanism is mediating the reduced osteogenic response observed in aged MSCs here.

To summarise, this study has identified both an osteogenic and chondrogenic response of young and aged MSCs to the application of 2.5% tensile strain for 7 days (Figure 6.15). In most cases the response of aged MSCs was weaker than that observed for young MSCs, however aged MSCs exposed to strain did show significant increases in markers of differentiation when compared to control groups. These results have positive implications for the potential use of MSCs isolated from aged donors in tissue engineering strategies. Furthermore, this study has illustrated the mechanoresponse of several key signalling molecules associated with MSC differentiation. These include integrin subunit α2, actin, pFAK and pERK and expression of these mechanosensitive proteins was upregulated in MSCs in response to continuous 2.5% cyclic tensile strain for a period of 7 days. The impact of normal integrin signalling was highlighted by the adverse effect integrin inhibition exerted on the expression of integrin subunit α2, actin and pFAK in both young and aged MSCs (Figure 6.16). However, osteocalcin, collagen II and pERK expression were all unaffected by integrin inhibition applied alone, indicating a lack of mediation of RGD-associated integrin signals under static conditions. Although aged cells responded with a similar pattern of expression to young MSCs in most observations, the response of

aged MSCs to strain was significantly lower for osteogenic differentiation and pERK expression, highlighting the necessity for further investigation into the biology of aging in MSCs. This study promotes the benefit of using tensile strain as a means of inducing MSC differentiation and exploring the mechanotransduction pathways involved but further examination is necessary on how to control differentiation along a specific lineage and whether using MSCs isolated from aged donors is truly feasible in the field of regenerative medicine.

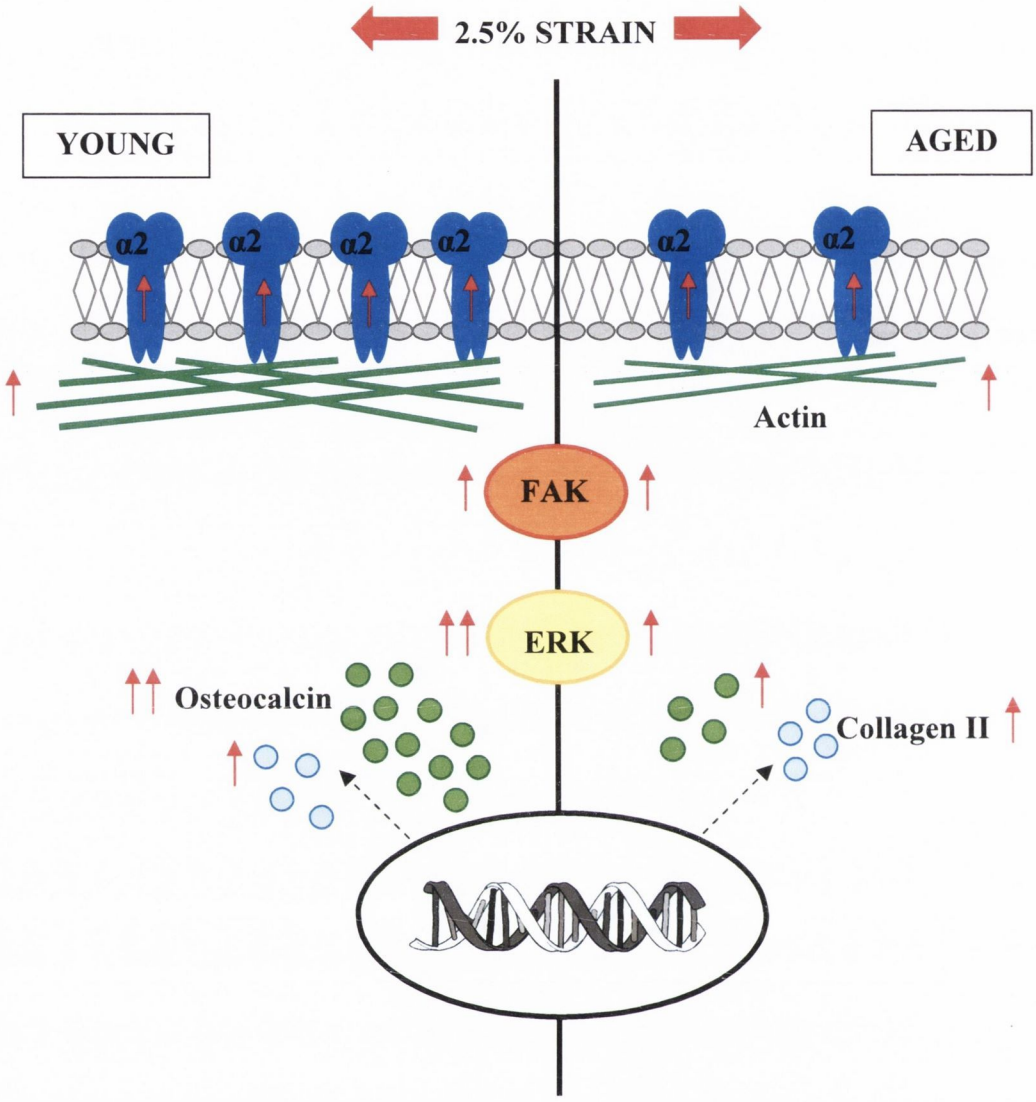


Figure 6.15: Effects of tensile strain on differentiation potential in young and aged MSCs

Application of 2.5% cyclic tensile strain for 7 days increases the expression of osteocalcin and collagen II. The osteogenic response is weaker in cells isolated from aged rats. The increase in differentiation markers was correlated with an upregulation in the mechanosensitive signalling proteins; integrin subunit α2, actin, pFAK and pERK (red arrows indicate upregulation induced by tensile strain).

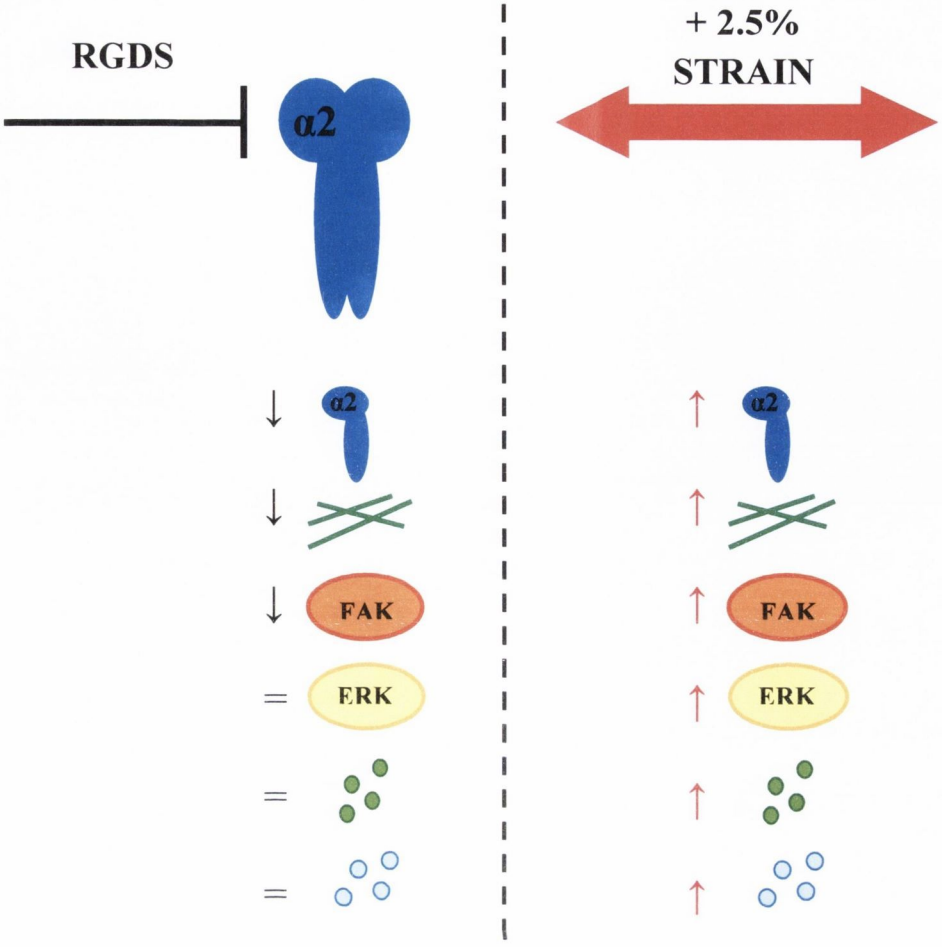


Figure 6.19: Effects of integrin inhibition on differentiation potential in young and aged MSCs when applied alone and in combination with tensile strain

Application of the integrin inhibitor RGDS for 7 days decreases the expression of mechanosensitive signalling proteins; integrin subunit α2, actin and pFAK, whilst pERK expression was unaffected in both young and aged MSCs. Similarly, expression of the differentiation markers osteocalcin and collagen II remained unchanged in response to integrin inhibition following 7 days. However, co-application of 2.5% cyclic tensile strain and RGDS for 7 days increases the expression of osteocalcin, collagen II and all of the signalling molecules under investigation, highlighting how tensile strain upregulates differentiation potential and can overcome the effects of integrin inhibition in both young and aged MSCs.

Chapter 7 Final Discussion

7.1 Discussion

Mesenchymal stem cells are considered a promising progenitor cell source in terms of tissue engineering applications due to their capability to differentiate into several skeletal tissues, their high proliferative capacity and ease of expansion in culture (Caplan 2007, Chamberlain *et al.* 2007, Tuan *et al.* 2002, Pittenger *et al.* 1999). Following isolation, these cells can be manipulated or primed *in vitro* by biochemical and biophysical means for application to sites of injury or disease for the development of new, healthy tissue. Skeletal health is an important factor to consider in the day to day lives of the aging population. Improved therapeutic strategies are necessary for the treatment of osteochondral diseases, which are prevalent in older people (Suri & Walsh 2011, Carrington 2005). Tissue engineering has potential in the field of regenerative medicine for treating osteochondral disease using MSCs. The mode of MSC employment may be by direct implantation or *ex vivo* engineering of a tissue construct, in combination with biocompatible materials or naturally derived biological materials (Caplan, 2007, Neagu *et al.* 2005, Tuan *et al.* 2002). However, the exact mechanism underlying the response of MSCs to stimuli such as mechanical force remains unclear (Nombela-Arrieta *et al.* 2011, Han *et al.* 2004). There is also an obligation to understand how signals are sensed by the cells and transduced to achieve various cellular outcomes in order that optimum conditioning environments can be developed for successful tissue engineering strategies. Additionally, little is known about the response and potential of MSCs isolated from aged donors; how the cells change with age and whether this affects their response to external stimuli (Wagner *et al.* 2009, Zhou *et al.* 2008, Stolzing & Scutt 2006). Ultimately, it is hoped that a broader insight into the biological attributes of MSCs will result in a more rational exploitation of their therapeutic use (Nombela-Arrieta *et al.* 2011). Therefore, the main aims of this thesis were to investigate the effect of aging on MSCs and to further examine their response to cyclic tensile strain. The effects of tensile strain were observed in terms of cell viability, differentiation and signal transduction pathways and whether these are affected by age.

7.1.2 Mesenchymal stem cell aging

In recent years, the phenomenon of aging has gained increasing interest with regard to MSCs and their potential for use in regenerative medicine. There is an accelerating increase in the proportion of aged people worldwide and, coupled with the prevalence of osteochondral disease amongst the aged population, it is critical that therapies improve to allow for successful aging. Chief among potentially improved therapies include the use of MSCs in tissue engineering applications. Unfortunately, insufficient knowledge of the effect of aging on this promising cell source hinders the progression of successful treatment of aged patients. Thus, one of the aims of this study was to examine the effect of age on several cellular characteristics of MSCs. Aging of MSCs is reportedly characterised by a reduction in the number of small colony forming cells, loss of multipotentiality, attainment of a comparatively large and mature appearance, and the propensity of slowly replicating cells (Jiang *et al.* 2008, Stolzing *et al.* 2008, Fehrer *et al.* 2006, Stolzing & Scutt 2006). Alterations related to aging of the cell could have an impact on MSC proliferation and differentiation which are critical in tissue engineering.

The extent to which MSCs are subjected to the causes of aging remains unclear and furthermore, whether age-related changes are caused by intrinsic or extrinsic environmental factors (Zhou *et al.* 2008). It was noted that the aged cells in this study appeared larger in culture as well as sparser in their nature. It was found that the rate of proliferation was decreased in cells isolated from aged rats compared to young, a finding consistent with many other reports. An age-associated decrease in proliferation potential and an acceleration of cellular senescence has been reported in multiple studies, investigating both rat and human bone marrow derived MSCs (Kretlow *et al.* 2008, Zhou *et al.* 2008, , Tokalov *et al.* 2007, Mareschi *et al.* 2006, Stolzing & Scutt 2006, Stenderup *et al.* 2003). It has been suggested that increased doubling time could be due to aging causing an increase in the duration of each phase of the cell cycle. Consistent with decreased proliferation, age-related increases have been found in p53 expression and its targets p21 and BAX, which are known to mediate proliferation, indicating intrinsic alterations in MSCs with aging that may contribute to skeletal, cellular,

and tissue aging (Zhou *et al.* 2008). Wagner *et al.* (2009) investigated the molecular relationship between aging and cellular senescence and found that aging causes gene expression changes in human MSCs that are related to changes observed in replicative senescence of MSCs *in vitro*, indicating that the cells undergo a similar process *in vivo.* In that study, changes in gene expression were analysed on multiple donor ages and it was found that age-related changes in expression occur in several genes. They then analysed the expression of the same genes in a single donor's MSCs exposed to multiple passages and found an overlap in the alteration of gene expression. This finding proposes that the decrease in the proliferative potential of aged MSCs, often associated with cellular senescence, may be a cause of differential gene expression with increasing age.

Another aim of this study was to investigate the mechanosensitivity of MSCs with age. Mechanotransduction describes the means by which mechanical forces are translated into biochemical signals leading to functional responses by the cell. Physical stimuli play a role in regulating cell proliferation, differentiation, and apoptosis by activating various intracellular signal transduction pathways. Integrin mechanoreceptors interact with components of the extracellular matrix and are implicated in mechanotransduction via interaction with actin and focal adhesion kinase activating downstream signalling pathways but it is unknown whether receptor expression is altered with age. A reduction was observed in the expression of the integrin subunit α2 in MSCs isolated from aged rats compared to young. The α2 subunit is a component of the α2β1 integrin which binds to collagen in the ECM (Miller *et al.* 2009, Takada *et al.* 2007, Bosnakovski *et al.* 2006, Eble & Tuckwell 2003). Differential expression of integrins during MSC aging has yet to be investigated but a study by Shakibaei *et al.* (1995) examined the distribution of integrins and collagen types I and II in MSCs of the embryonic mouse limb bud. They discovered an exchange in the expression of the α1- and α3-integrin over time, depending on the type of collagen being synthesised. These findings suggest a possibility that integrins may be differentially expressed over time, such as with increasing age. The migratory capacity in young and aged fibroblasts has been studied and significant differences were revealed in α2β1 integrin function and cytoskeletal organisation, which was correlated with the cells capacity to migrate (Reed *et al.* 2001).

A key aspect of mechanotransduction involves integrin interaction with the cytoskeleton, following binding of an ECM component. Matrix adhesions are mediated through actin-dependent protrusions that bring integrins to the leading edge, at the site of matrix contact, allowing for binding to occur. This is swiftly followed by integrin binding to the actin cytoskeleton, causing additional components to be recruited (Puklin-Faucher & Sheetz 2009). At the cell-matrix interface, focal adhesions link the ECM to the contractile cytoskeleton in response to mechanical force. This linking activates a signalling cascade, including activation of FAK, a mediator of mechanotransduction (Yim *et al.* 2009). Interaction between integrins in the plasma membrane and actin in the cytoskeleton is mediated by mechanical stimulation leading to FAK activation and in some circumstances, downstream activation of transcriptional factors associated with cell differentiation (Salasznyk *et al.* 2007, Ward *et al.* 2007, Brakebusch & Fassler 2003).

This study has demonstrated an age-related reduction in the expression of actin and a reduction in actin filament thickness with age. A disruption in the interaction of myosin and actin is noted in muscle tissue with increased age. The cellular content of proteins depends on the balance between the competing processes of protein synthesis and degradation. With aging, there is evidence to suggest decreased myosin heavy chain synthesis rates and a loss in the regulation of the proteasome, the major protease responsible for degrading myofibrillar proteins. Thus, changes in rates of synthesis or degradation could lead to protein-specific declines in either actin or myosin content (Thompson *et al.* 2006, Ferrignton *et al.* 2005, Toth *et al.* 2005). Smooth muscle cells derived from the bone marrow of adult sheep reportedly display significantly lower expression of an isoform; smooth muscle alpha actin, compared to neonatal cells (Han *et al.* 2010). Furthermore, stimulus-induced actin polymerisation has been shown to be lower in human granulocytes and lymphocytes from aged donors (Rao *et al.* 1992). A link also exists between actin stabilisation and aging through the biomarker SM22, which crosslinks actin filaments *in vitro* and studies in mammalian cells have observed that senescent cells have higher levels of SM22 (Gourlay & Ayscough 2005, Camoretti-Mercado *et al.* 1998). It has been demonstrated that several genes associated with aging are also responsible for

cellular senescence (Wagner *et al.* 2009). There is evidence to suggest that there could be a reduction in the expression of actin with age, which has been verified in this study.

Lipid peroxidation is one of the main products of reactive oxygen species (ROS)-mediated damage to tissue, which causes structural damage to plasma membranes and generation of the secondary product malondialdehayde, a mutagen and carcinogen (Marnett 1999). Free radicals react with membrane fatty acids and phospholipid components to form lipid peroxides and membrane damage is caused by generation of fragmented fatty acyl chains, lipid-lipid cross-links and lipid-protein cross-links. This leads to the induction of irreversible impairment of membrane fluidity, plasticity and biophysical properties thereby causing irreversible damage to the cells integrity and exerting profound effects on membrane-bound protein activity (Montine *et al.* 2002, Pratico 2002). Thus it has been proposed that membrane fluidity can be modulated by peroxidation of membrane phospholipids (Choe *et al.* 1999). It was found that aged MSCs exhibit a greater degree of lipid peroxidation compared to young MSCs in this study. Lipid peroxidation is linked with cellular aging and it has previously been shown to increase with age in several tissue and cell types in the rat, including MSCs (Stolzing *et al.* 2006, Farooqui *et al.* 1987). The increase in peroxidation observed in this study may contribute to an increase in membrane rigidity with age and altered mechanotransduction as a result of this which would be caused by alterations in membrane fluidity associated with lipid peroxidation, which disrupts vital functions necessary for signal transduction or the cells selective permeability to several ions (Rikans & Hornbrook 1997, Chen & Yu 1994). Therefore, it is possible that aged MSCs may respond differently to an applied stimulus such as mechanical strain, which would have implications for their employment in tissue engineering applications. Coupled with the age-related decreases in α2 and actin expression, this could point towards an altered mechanoresponsiveness of MSCs with increasing age. The response of young and aged MSCs to various rates of tensile strain was also under investigation in this study and will be discussed further in the following sections.

7.1.3 Strain-mediated apoptosis in MSCs

Apoptosis is a tightly regulated form of programmed cell death active in development and in the regulation of cells in tissues. It is an active, intrinsically controlled process, either initiated or inhibited by several environmental stimuli, both physiological and pathological (Kerr *et al.* 1972). External stimuli can cause apoptosis in several cell types (Simon *et al.* 2000, Adams & Horton 1998, Li *et al.* 1997, Kajstura *et al.* 1996, Phelouzat *et al.* 1996). Mechanical strain is one such external stimulus capable of inducing apoptosis; cyclic tensile strain has demonstrated this in rat MSCs (Kearney *et al.* 2008), rat spinal cord cells (Uchida et al. 2010) and rat vascular smooth muscle cells (Sedding *et al.* 2008). Cyclic tensile strain is utilised as an investigative tool in tissue engineering studies to mimic *in vivo* conditions and study the effects on various cell types under *in vitro* conditions. However, evidence suggests that the response of the cell is strain magnitude dependent as this type of mechanical stimulus can lead to apoptosis.

One of the main findings of this study was that apoptosis is significantly increased in aged cells exposed to 10% cyclic tensile strain compared to young cells also exposed to strain. To assess age-related alterations in mechanosensitivity of MSCs to strain, cells were exposed to 10% cyclic tensile strain for a period of 3 days. It has previously been demonstrated by Kearney *et al.* (2008) that a minimum of 7.5% magnitude tensile strain or higher induces apoptosis in MSCs isolated from young rats. Results from that study indicated that L-type voltage-activated calcium channels coupled mechanical stress to activation of calpain and JNK, which lead to apoptosis through DNA fragmentation. The aim of the current study was to assess if the same effect observed by Kearney *et al.* (2008) would be attained in MSCs isolated from aged rats or whether their response would be altered due to the age difference and observations of age-related alterations occurring in MSCs; reduced mechanosensory protein expression and increased membrane lipid peroxidation. An investigation into the effect of tensile strain on aged MSC differentiation potential has been undertaken (Huang *et al.* 2010) but to date, no other studies have determined whether there is an apoptotic response in aged MSCs in response to tensile strain. Strain-induced apoptosis was greater in aged MSCs in this study, as evidenced by an increase in caspase-3 activity and

DNA fragmentation. This implies that aged cells are more vulnerable to a high rate of strain and this could be due to changes in the cell occurring with age, affecting the mechanoresponse to strain. The mechanisms of apoptosis in MSCs isolated from aged rats are not widely reported but aging is speculated to make cells more sensitive to apoptotic stimuli (Jiang *et al.* 2008). A striking age-related increase in apoptosis and p53 pathway genes, p21 and BAX, was observed in human MSCs isolated from aged individuals compared to cells isolated from younger individuals (Zhou *et al.* 2008). Stolzing & Scutt (2006) identified an increase in apoptosis in aged MSCs compared to young, coupled with an increase in p53 expression, a tumour suppressor gene which mediates apoptosis. In the present study, apoptosis was associated with an increase in caspase-3 activation, which was significantly greater in aged MSCs compared to cells isolated from young rats. An age-related increase in oxidative stress and associated accumulation of damage has also been suggested (Sethe *et al.* 2006, Pelicci 2004). Such findings suggest that aged MSCs have a higher tendency towards apoptosis in the presence or absence of tensile strain mediated by activity of the p53 pathway and increased levels of oxidative stress. In this study, increased apotosis in aged MSCs may be, in part, associated with oxidative damage caused by increased lipid peroxidation. Other studies have reported similar findings; for example, Beecher *et al.* (2007) demonstrated an apoptotic response in chondrocytes exposed to abnormal cyclic loading, mediated by oxidants. Smooth muscle cells sourced from human, rat and mouse have been exposed to 15% tensile strain, inducing apoptosis via p53 activation. The activity of p38 MAPK and oxidative DNA damage contributed to p53 activation, as well as mitochondrial dysfunction as evidenced by cytochrome c leakage into the cytosol (Mayr *et al.* 2002). In contrast, Sumanasinghe *et al.* (2008) reported overall cell viability in human MSCs following exposure to 10% and 12% cyclic tensile strain for a period of 2 weeks. However, cells were only exposed to the strain for 4hrs per day indicating that strain-mediated apoptosis may also depend on the length of exposure and continuous application of strain as opposed to intermittent loading.

Mechanical stress is thought to elongate the cell membrane resulting in activation of rac-p38 MAPKs and production of ROS. Both signals converge at the level of p53. The p38 pathway directly phosphorylates p53, while ROS production causes

oxidative DNA damage. The p38 MAPKs might indirectly activate p53 by regulating oxidase activation and p53 might be involved in a positive feedback loop by stimulating free radical generation itself. Finally, Bax expression and mitochondrial dysfunction mediate apoptosis following mechanical stress. Thus, the transformation of mechanical force into a biological response might involve receptor-dependent and -independent pathways such as via integrin receptors and oxidative stress (Mayr *et al.* 2002), which has previously been addressed. Integrin receptors have been implicated in maintaining cell survival but the precise molecular mechanisms governing cell survival by integrins are not fully understood. Nonetheless, the PI3-K/Akt and Ras/Raf/MEK/ERK pathways constitute the most well known cell survival-promoting pathways (Vachon 2011, Reddig & Juliano 2005, Marastoni *et al.* 2008, Meredith & Schwartz 1997, Roux & Blenis 2004). Heterotypic and homotypic cell-cell adhesion can also protect from adhesion-dependent apoptosis and evidence suggests that this too is integrin-mediated. Integrin signalling can contribute to tumor development by enabling escape from apoptosis (Bates *et al.* 1995). If integrins play an integral part in cell survival, a reduction in their expression, as has been demonstrated in aged MSCs, could render a cell more vulnerable to stress.

One study has shown that disturbance of α2β1-mediated interaction with collagen I results in MSC cell death. The function of α2β1 in hMSCs was addressed by applying short hairpin RNA (shRNA)-mediated knockdown of the corresponding α-subunits. Deficiencies of the α2 subunit in hMSCs lead to the initiation of cell apoptosis via the Akt pathway. This was the first report of a collagen I-binding integrin transmitting essential signalling for MSCs inhabiting a collagen I-rich cell niche and that interference with the collagen I–integrin interaction resulted in stem cell death (Popove *et al.* 2011). The actin cytoskeleton is also a crucial component in mediating cell responses to both internal and external signals. Central to its function is its dynamic structure and ability to reorganise into distinct structures that are suitable for particular tasks required by the cell. Defects in the capacity to regulate the dynamics of actin have detrimental effects on cell function thus a cell that has lost the ability to signal to and reorganise its actin elicits a signal for cell death. The exact role for actin in the pathway remains unclear but alterations in actin dynamics and reorganisation have been linked to

increasing age (Gourlay & Ayscough 2005). This study has shown a reduction in both α2 and actin expression in aged MSCs which may be contributing to the increased apoptotic response to strain.

Complete loss of cell attachment can cause a form of apoptosis in many cell types, termed anoikis (Stupack 2005). Studies have shown that substrate attachment without integrin engagement resulted in programmed cell death, while integrin-mediated attachment rescued cell survival (Meredith *et al.* 1993). In the present study, RGDS binding peptides were added to the media during 3 day 10% tensile strain testing but they did not induce an additional apoptotic response when applied in combination with strain, nor did they induce such a response when applied in static conditions. In fact, RGDS treatment showed a reduction in DNA fragmentation in aged MSCs exposed to tensile strain. However, anoikis has previously been shown to occur in the presence of immobilised ECM molecules when non-immobilised soluble ligands like RGD peptides were present (Hersel *et al.* 2003, Stupack *et al.* 2001). The potential attenuation in apoptosis observed in this study could be related to an inhibition of apoptosis mediated by RGDS interaction with the substrate surface (Grigoriou *et al.* 2005). Pfaff (1997) reported the presence of cell adhesive RGD sites in several ECM proteins, including collagen. Silicone substrates in this study were coated with a collagen I solution prior to cell seeding. The lack of RGDS-mediated apotosis in this study could in fact be due to upregulation in the activity of RGDS-mediated survival pathways, rescuing the cells from strain-induced apoptosis.

7.1.4 Mechanotransduction in MSCs

Mechanotransduction refers to the sequential process by which cells translate a mechanical signal into a biochemical response via specialised molecules whose biochemical activity is altered upon mechanical distortion, thus converting mechanical energy into biochemical energy. Integrins can trigger signal transduction cascades and induce focal adhesion formation as a result of ECM ligand binding and associated changes in receptor conformation. As the main receptors connecting the cytoskeleton to the ECM, integrins transmit mechanical

stresses across the plasma membrane, developing tractional force in the cytoskeleton (Katsumi *et al.* 2004, Huang & Ingber 1999). This linkage to the actin cytoskeleton is mediated by focal adhesion complex formation by recruiting proteins such as α-actinin, talin, vinculin and other modulators of actin dynamics (Martin *et al.* 2002). Force applied to integrins promotes focal adhesion assembly by activating the small GTPase Rho and its downstream target Rho-associated kinase (ROCK), which promotes actin filament polymerisation, inducing cytoskeletal contraction (Riveline *et al.* 2001). Cell–matrix adhesions are continuously used to sense structural and mechanical features in the surrounding environment. Actin cytoskeletal dynamics can be altered via a Rac1- and RhoA-dependent mechanism and remodel cell mechanosensors in response to incoming signals. Cytoskeletal reorganisation regulated by small GTPases is essential for this adaptation to mechanical force (Asparuhova *et al.* 2009). FAK is a key mediator of integrin signals from the ECM to the cytoskeleton and downstream signalling molecules. Upstream conformational changes in focal adhesions lead to activation of tyrosine kinases such as FAK which mediate mechanotransduction (Salasznyk *et al.* 2007, Riveline *et al.* 2001). It is activated by phosphorylation at specific tyrosine residues, which then stimulate downstream signalling events including activation of the ERK1/2 pathway, leading to a variety of cellular responses (Ward *et al.* 2007).

The response of mesenchymal stem cells to various forms of mechanical stimulation has been investigated in terms of cell viability and differentiation potential. Signalling pathways associated with these cellular outcomes are often addressed in such studies however, one of the aims of this study was to simply investigate the response to tensile strain of several proteins implicated in transducing mechanically-stimulated signals. These molecules include the integrin subunit α2, actin, FAK and ERK. Previous investigations within this laboratory have reported strains greater than 5% as inducing apoptosis in young MSCs on a 2-D substrate and differentiation in 3-D constructs while lower strains induced differentiation on the 2-D substrates. Duration of these experiments also varied between 3-14 days. It was decided for this study to assess signal transduction following 24hr exposure to 5% cyclic tensile strain. It was found that tensile strain significantly upregulated the expression of α2, actin and pFAK after 24hr but

pERK was unaffected. Increased integrin clustering and FAK phosphorylation can be triggered by increased tension within focal adhesions, which in turn can mediate downstream effects of strain on ERK and JNK (Katsumi *et al.* 2004). This was observed in the present study by upregulated α2 and pFAK expression. However, pERK expression remained unchanged by tensile strain which may be a consequence of the 24hr time course. Several other studies have demonstrated a rapid increase in ERK phosphorylation within minutes of exposure to a strain stimulus and which declines over time (Li *et al.* 1999, Reusch *et al.* 1997) so it is possible ERK has been activated but subsequently declined over the course of the 24hr. It has also been demonstrated that force applied to integrins causes increased clustering, which could be due to increased recruitment of actin and myosin and cytoskeletal assembly (Schoenwaelder & Burridge 1999).

In the present study, strain has shown an increase in both α2 integrin and actin expression. Various mechanisms may account for increased cell surface integrin subunit expression in response to mechanical strain, such as increased recruitment at the surface, increased mRNA and protein synthesis or decreased protein degradation. Mechanical stressing of both the α2 and the β1 integrin subunits induces an increased tyrosine phosphorylation of proteins and application of cyclic strain causes tyrosine-phosphorylated proteins to become physically anchored to the cytoskeleton as a result of mechanical integrin loading (Schmidt *et al.* 1998). Integrins sense physical forces that control gene expression through activation of the MAPK pathway. It is suggested that the cytoskeleton plays a significant role in the physical anchoring of activated signalling molecules, enabling the switch of physical forces to biochemical signalling events. It is possible in this study that mechanical strain modulates an increase in integrin receptor expression, which in turn upregulates actin polymerisation to allow for anchorage and necessitates an up-regulation in FAK phosphorylation to sufficiently transmit strain-induced signals.

To determine the impact of integrin- and FAK-mediated signalling in the response to strain, inhibitors for these proteins were included in testing, both alone and in combination.

Integrin receptor function can be inhibited by synthetic integrin binding sequences, RGDS peptides. These sites of integrin adhesion or RGD sites have been identified in many ECM proteins including collagen. Out of the 24 known integrin combinations, roughly half recognise the RGD sequence and bind to the ECM in an RGD dependent way (Pfaff 1997). RGD peptides promote cell adhesion when they are attached to a substrate, and inhibit cell adhesion when they are available to cells in solution, reducing adhesion and potentially leading to apoptosis (Grzesik & Robey 1994). Integrin-mediated cell attachment can regulate processes such as cell migration, growth, differentiation, and apoptosis. Exploitation of RGD peptides can further our knowledge of integrin functions in various biological systems (Ruoslahti 1996). Application of RGDS, or integrin inhibition, for 24hr in static conditions resulted in a reduced expression of α2, actin and pFAK in nearly all groups, whilst pERK expression remained unchanged. The expression of α2 was not significantly reduced in aged MSCs by integrin inhibition which could be due to the time point tested, thus further incubation with RGDS may have led to a significant decrease in expression. The reduction in α2 expression occurs as a direct result of RGDS treatment, which may then subsequently cause the downregulation of actin and pFAK due to the inability to mediate contact with the ECM to trigger intracellular signal transduction. In fact, specific blocking antibodies to α2 have been demonstrated to inhibit FAK activation (Sawhney *et al.* 2006). There was no effect of RGDS on ERK expression which indicates an integrin-independent mechanism of ERK regulation under static conditions.

Following application of tensile strain in combination with integrin inhibition, the decreases observed by RGDS treatment alone were abolished and α2, actin and pFAK expression levels were restored to a level comparable to control conditions. Actin disruption and RGD-peptide treatment fail to inhibit the loading response in osteoblasts (Peake *et al.* 2001) which has also been demonstrated in this study whereby actin, FAK and integrin downregulation by RGDS treatment did not prevent a loading-induced increase in expression. A similar lack of reliance on RGD-mediated mechanotransduction has been reported in rat cardiac fibroblasts, where load-induced activation of JNK was not inhibited by RGD peptides (McKenna *et al.* 1998), and in bovine aortic endothelial cells where pretreatment

with RGD peptides to block RGD-dependent integrin signalling failed to attenuate strain-induced increases in the ECM-degrading enzyme matrix metalloproteinase (MMP)-2 expression (von Offenberg Sweeney *et al.* 2004). Combined application of tensile strain and integrin inhibition failed to have an effect on pERK expression in this study, indicating two possibilities; ERK is rapidly increased early on during strain stimulation and declines over time or its activation is possibly mediated in an integrin-independent manner.

Integrins also play a role in the dynamic turnover and remodelling of focal adhesion complexes by activating FAK, a central focal adhesion protein that regulates several cytoskeletal and other focal adhesion proteins. The dominant mode of FAK regulation is by integrin-dependent adhesion to the ECM and it is a crucial component of the integrin-signalling pathway (Schaller 2001). The actin cytoskeleton is also necessary for FAK tyrosine phosphorylation, as experiments have shown that Cytochalasin D eradicates tyrosine phosphorylation of FAK in response to many stimuli, including integrin-dependent cell adhesion (Schlaepfer *et al.* 1998) and FAK inhibition is associated with a loss of mesenchymal stem cell motility (Zachary 1997). The novel small molecule inhibitor PF228 blocks the catalytic activity of recombinant FAK protein or endogenous FAK expressed in multiple cell lines. Treatment of cells with PF228 blocks FAK phosphorylation on tyrosine residue Tyr397 and there is an associated decrease in cell migration and restrained adhesion turnover, but little effect on cell growth or apoptosis in culture. This molecule exhibits *in vitro* selectivity towards FAK and inhibits activation of FAK in various cell lines (Slack-Davis *et al.* 2007). Jones *et al.* (2009) found that PF228 effectively blocks human platelet spreading on fibrinogen-coated surfaces but does not affect initial adhesion, which is similar to reports on murine platelets lacking FAK. In this study, PF228 was shown to decrease expression of α2, actin and pFAK with no effects observed on pERK. Given FAK's close involvement with integrins and the cytoskeleton it is unsurprising that inhibition of this protein elicits the decreases observed. However, the mechanism by which this is achieved for α2 and actin is unclear, for instance, whether the down regulation of α2 is caused by disrupted actin dynamics induced by the inhibition of FAK activation or whether the inhibition of FAK directly causes the decrease in receptor activation and subsequently the decrease

in actin expression as a result of this. These signalling molecules are intricately linked in their functions, it would be favourable to determine the order of governing signals in which these mechanosensory proteins are activated and how inhibition of the various components affect the pathway.

Tensile strain applied in combination with FAK inhibition, eliminated the decreases in α2, actin and pFAK expression observed following FAK inhibition and protein levels were increased to a level similar to control conditions. Combined application of tensile strain and FAK inhibition did not have an effect on pERK expression suggesting that potential activation of ERK has subsequently declined by 24hr or its activation is possibly mediated in a FAK-independent manner here. In a myoblast cell line transfected with a shortened mutant of FAK, known as FAK-related non-kinase (FRNK), which acts as a dominant negative inhibitor of FAK and over-expression of FRNK significantly reduces the stretch-induced proliferation of C2C12 cells (Kumar *et al.* 2004). In Caco-2 cells, a cell line selected for enterocytic differentiation, 10% strain was applied by a vacuum manifold regulated by computer-controlled solenoid valves to assess mitogenic effects of cyclic strain. Blockade of FAK and ERK activation further blocks downstream effects of strain on the cells. FAK397 transfection prevents strain-induced activation of co-transfected ERK2 and JNK1. Thus, strain activation of ERK and JNK kinases might be at least in part FAK related in Caco-2 cells (Li *et al.* 2001). The effects of FAK inhibition in these studies highlight an inability of strain to overcome the inhibition. In this study actin expression was decreased in strained cells in the presence of FAK inhibition. However, for other proteins (α2, pFAK) strain eliminated the effect of FAK inhibition and restored mechanosensory protein expression. It may be due to a difference in experimental procedure and duration that explain this result compared to what has been reported in the literature. It was also noted that FAK and integrin inhibition did not produce an additive effect when applied in combination. Individual inhibition of both proteins exhibited similar responses within the cell; it is possible that when added in combination, the effects induced by either inhibitor do not allow for an additional downregulation as this has already been achieved by one of the inhibitors at least or a contribution of both.

It should also be noted in this study that contradictory to earlier reports, no age-related decline in α2 expression was observed when cells were in static conditions. Integrin expression in the cell membrane is regulated by interactions with various ECM ligands. It is understood that integrin binding to a substrate-bound ligand can initiate a series of signalling events that regulate the expression of integrins themselves (Chen *et al.* 1992). In fact, the synthesis and transcription of α2β1 is selectively upregulated when fibroblasts are seeded onto type I collagen gels (Klein *et al.* 1991). In this study, MSCs were seeded onto silicone sections coated with a collagen I solution for tensile testing and α2 expression was upregulated compared to expression in cells seeded to a glass substrate. Furthermore, integrin expression was upregulated in aged MSCs to a similar level as young cells on the collagen I-coated substrate.

7.1.5 Strain-mediated differentiation in MSCs

Tissue engineering involves the use of living cells to develop biological substitutes for implantation into the body or to enhance the remodelling of tissue in an alternative active manner. The immediate goal is to repair, replace, improve or maintain the function of a specific tissue or organ with a further view to develop an implantable system or *in vivo* remodelling of tissue. Successful applications require a thorough understanding of how cells respond to their environment in normal tissues and in engineered devices prior to and following implantation. Mesenchymal stem cells represent an ideal cell source for use in tissue engineering applications due to their ease of expansion in culture and immunomodulatory properties (Butler *et al.* 2000, Nerem and Sambanis 1995). Many of the tissues and organs requiring a tissue engineered intervention (e.g.: bone and cartilage) have important biomechanical functions. In fact, the biomechanical properties of these tissues are critical to their proper function *in vivo* and increasing evidence suggests that mechanical stress may significantly increase the biosynthetic activity of cells in a bioartificial environment (Butler *et al.* 2000). One of the aims of this thesis was to evaluate the effect of strain as a tool to promote MSC differentiation, to address potential age-related changes in the response to strain and investigate the mechanotransduction pathway involved.

Young and aged MSCs exhibited differentiation along both osteogenic and chondrogenic lineages in response to 2.5% strain for 7 days, indicating the potential of strain-mediated differentiation but a lack of specificity toward a single phenotype. There is an abundance of evidence in the literature describing mechanically stimulated differentiation in young MSCs but few observations of aged MSCs undergoing strain-induced differentiation have been reported. Other studies have reported similar dichotomous results in response to certain stimuli though (Talaei-Khozani *et al.* 2011, Kii *et al.* 2004). Cyclic tensile strain stimulation has even been reported to upregulate the major transcription factors known to be crucially involved in both chondrocyte and osteoblast differentiation; Sox9 and Runx2 (Friedl *et al.* (2007). It is possible that after 7 days of strain stimulation there is potential for differentiation along either lineage which induces differentiation signals for both osteogenic and chondrogenic differentiation. Increasing the testing time could possibly lead to either lineage gaining dominance and cells adopting a single phenotype. The potential for MSCs to differentiate along two lineages which has been observed in this study has serious implications for tissue engineering, such that commitment to a specific lineage has not been observed. This implies that the response of MSCs to the tensile strain stimulus employed here is not lineage specific and therefore requires further induction such as defined growth factors to compliment the effects of strain or further investigate the response of MSCs to various rates, frequencies and durations of strain.

Cyclic tensile strain has been demonstrated as having an effect on the secretion of osteogenic differentiation markers in human MSCs. Application of an 8% strain significantly increases secretion of alkaline phosphatase and osteocalcin and expression of collagen 1 and core binding factor alpha-1 (Cbfα-1) compared with no stimulation (Jagodzinski *et al.* 2004). Expression of genes involved in cell proliferation, osteoblastic differentiation and matrix production by cyclic uniaxial mechanical strain were investigated in a human osteoblastic precursor cell line in 3-D collagen I matrices by analysing the mRNA of histone H4, Cbfα-1, alkaline phosphatase, osteopontin, osteocalcin, and collagen I. Cyclic stretching of cell seeded matrices increases cell proliferation and modestly increases the expression of most of the investigated genes compared to unstrained controls. Mechanical

loading promotes the differentiation of osteoblastic precursor cells in a collagen I matrix suggesting that application of a mechanical stimulus may have a beneficial effect on *in vitro* tissue formation (Ignatius *et al.* 2005). It was previously reported from this laboratory that application of a 2.5% strain induces osteogenic differentiation of young MSCs. Following exposure to tensile strain, the osteogenic markers Cbfα-1, collagen I, osteocalcin and bone morphogenetic protein (BMP) 2 are temporally expressed. The strain-induced synthesis of BMP2 is mediated by activation of the kinases ERK, p38 and PI3 kinase (Kearney *et al.* 2010). In this study, 2.5% strain was applied for 7 days to young and aged MSCs. Expression of the bone protein osteocalcin was significantly upregulated by tensile strain in both age groups but the aged response was lesser compared to young MSCs. The increase in osteocalcin expression was correlated with an increase in calcium phosphate deposition.

It is understood that mechanical strain and ECM proteins play pivotal roles in osteogenic differentiation of MSCs. Huang *et al.* (2009) applied 3% cyclic mechanical stretching to human MSCs which activated the phosphorylation of FAK, upregulated the transcription and phosphorylation of Cbfa-1, leading to increased alkaline phosphatase activity and mineralised matrix deposition. In this study, there was a strain-induced increase in α2, actin, pFAK and pERK after 7 days. Several of the signalling cascades that have been implicated in strain-induced differentiation are known to interact with integrin receptors in the plasma membrane. This study has shown a reduction in the expression of α2 in the aged MSC membrane which would lead to the presumption that the response to strain may be reduced in aged MSCs. However, Meyers *et al.* (2004) studied the effect of modelled microgravity on integrin expression and function in human MSCs. They found that exposure to modelled microgravity reduces the expression of collagen I while simultaneously increasing expression of the collagen I-specific integrin receptor α2β1. Despite the upregulation in integrin expression, autophosphorylation of FAK and downstream MAPK signalling were reduced as evidenced by a reduction in ERK activation. Together, these findings suggest that modelled microgravity decreases integrin/MAPK signalling, which is likely to contribute to the observed reduction in osteogenic differentiation. It was also observed in the current study that the response to strain was reduced for pERK

and osteocalcin expression in aged MSCs. Interruption of collagen–integrin interaction by the addition of an Asp-Gly-Glu-Ala (DGEA) peptide inhibits the expression of osteoblastic phenotypes of bone marrow fibroblastic cells. Furthermore, an anti-α2 integrin antibody, which blocks the binding of integrin with collagen, suppresses the expression of osteoblastic phenotypes, implying that collagen-α2β1 integrin interaction is an important signal for the osteoblastic differentiation of bone marrow cells (Mizuno *et al.* 2000). Disruption of integrin activity using an RGDS peptide failed to have an inhibitory effect on osteogenic differentiation in this study, however the Mizuno study also found that RGDS did not suppress the osteoblastic differentiation induced by collagen I. These findings point toward a possible RGD-independent signalling pathway for osteogenic differentiation.

Strain-induced mineralisation is reported to be mediated by ERK1/2 signalling, as inhibition of ERK1/2 attenuates calcium deposition by 55% in human MSCs following cyclic tensile strain (Simmons *et al.* 2003). There was a strain-associated increase in ERK phosphorylation in this study which may be mediating the increase observed in osteocalcin expression. Additionally, the decreased osteogenic response to strain in aged MSCs was also observed for pERK expression. The osteogenic response to mechanical stress is reported as being blunted with aging. It has been postulated that this decline in responsiveness is related to a decline in certain hormones or growth factors that may interact with mechanical signals to change the sensitivity of bone cells to strain (Kohrt 2001). In a study investigating the effect of mechanical loading on collagen-induced arthritis it was found that arthritis suppressed bone formation induced by loading (Kameyama 2004). Although young animals were used in this study, arthritis is a disease prevalant amongst the aged population so these results could theoretically be viewed as a diseased age-related response to loading. Sun *et al.* (2001) have investigated whether aging negatively affects MSC replication and osteogenesis. A cell-free ECM was prepared from cultured femoral marrow cells from 3- or 18-month old C57BL/6 mice and it was found that aging negatively affects the formation of an ECM that maintains MSC function, and aged MSCs can be revitalised by culture on a young-ECM. This result provides potential for use of aged MSCs in tissue engineering.

The response to mechanical loading has also been rigorously tested for chondrogenic differentiation. The effect of cyclic tensile strain on the chondrogenic differentiation of MSCs in a 3-D collagen I-glycosaminoglycan (GAG) scaffold was assessed previously in this laboratory using a multi-station bioreactor. The rate of GAG synthesis was increased following application of continuous 10% cyclic tensile loading for 7 days, suggesting this magnitude of strain was sufficient to mechanoregulate the chondrogenic differentiation process (McMahon *et al.* 2008). Several studies have also adopted the use of cyclic and dynamic compression of different magnitudes to induce chondrogenesis in undifferentiated MSCs and demonstrated cell viability following loading regimes as well as GAG accumulation and proteoglycan deposition (Jung *et al.* 2009, Kisiday *et al.* 2009, Pelaez 2008, Mouw *et al.* 2007, Huang *et al.* 2004). An upregulation in collagen II expression and proteoglycan synthesis were observed in this study following application of 2.5% tensile strain to both young and aged MSCs for 7 days. Multiple intracellular signalling cascades have been shown to be activated during loading leading to expression of cartilage specific genes. Such signalling cascades include the MAPKs; p38, ERK1 and JNK (Mouw *et al.* 2007). This study has shown a strain-induced upregulation in the activity of α2, actin, pFAK and pERK which may be contributing to the chondrogenic response observed and simultaneous integrin inhibition during strain testing did not exert any significant effects. However, rat embryonic limb bud cells have previously been seeded to a silicone membrane and exposed to stepwise-increased stretching, which exerts shear stress on the cells. This type of stimulation was shown to down-regulate chondrogenic markers collagen II and nodule formation. This downregulation was completely recovered when cell-ECM attachment was inhibited by Gly-Arg-Gly-Asp-Ser-Pro-Lys peptide or by blocking antibodies for α2, α5 or β1 integrins. It was concluded that shearing stress generated by stepwise stretching inhibits chondrogenesis through integrins (Onodera *et al.* 2005). When examining lineage preference by differentiation ratios, it was observed in this study that groups exposed to integrin inhibition, or the RGDS peptide, exhibited a higher preference for the chondrogenic lineage. Although, integrin inhibition did not exert any significant effects on the expression of chondrogenic markers.

7.2 Study Limitations

There is currently significant interest in MSCs and their ability to differentiate into the various skeletal tissues. Adult MSCs are generally regarded as an autologous source of reparative cells and it has been established that these cells can be isolated from several organs in the body besides bone marrow; including adipose tissue (Huang *et al.* 2010), synovial fluid (Jones *et al.* 2004), dental pulp (Huang *et al.* 2009), umbilical cord blood (Lee *et al.* 2003) and amniotic fluid (Tsai *et al.* 2007). Thus, MSCs are resident in many tissues and organs but Medici *et al.* (2010) recently reported that endothelial cells treated with BMP4 or TGF-β2 regressed to a multipotent type cell which displayed characteristics of MSCs, and could be differentiated into several endodermal cell types. With a wealth of isolation sources and rapidly increasing interest in this cell type it is necessary to address several drawbacks associated with MSCs, to which care should be taken to avoid for tissue engineering and clinical applications. For instance, the extent to which true MSCs can be used in tissue repair may have a bearing on many clinical outcomes such that variability between cell source and purity of isolations must be deduced. High variability amongst preparations has been reported (Solchaga *et al.* 1999), thus although analysis demonstrated that homogenous populations of young and aged MSCs were isolated by immunoreactivity against CD90 in this study, it is acknowledged that variability may exist between preparations and have some bearing on the results obtained.

To investigate the expression and stimulus-induced changes of various proteins in this study, the primary technique employed was staining by immunocytochemistry. This was followed by visual acquisition using a confocal microscope and analysis of images for quantification using image examiner software. Wherever possible, alternative methods of protein or molecule detection were utilised, such as western blotting and PCR. However, the nature of the experiments undertaken generated low n numbers (due to the variety of factors to be tested) and insufficient cell numbers per silicone substrate to satisfy the protein or mRNA requirements necessary for these techniques. Therefore, great care was taken using a single primary technique to ensure standards were maintained equally throughout this study. Staining protocols and confocal settings were

optimised prior to data acquisition and analysis and any variable parameters were kept constant in the numerous experimental settings to rule out potential variation between groups.

The integrin inhibitor RGDS was used in various parts of this study to evaluate the function of these receptors in the response to tensile strain. Although this inhibitor has been established as a blocker of integrin activity, it binds specifically to the tri-amino acid motif RGD on integrins which express it. There are up to 24 known integrins in mammals, approximately half of which contain an RGD binding motif. Using the RGDS peptide ensures a blockade of activity in several integrins at least present in the MSC plasma membrane. It remains unclear which particular integrins are associated with the various responses to tensile strain. Of interest in this study was the integrin $\alpha 2\beta 1$, which was affected by exposure to the RGDS peptide. However, a more specific method of identifying its role in mechanotransduction may be achieved using an siRNA approach for example. This would ensure the efficient silencing of $\alpha 2$ in cells allowing for the mechanoresponse to be examined in its absence.

7.3 Conclusion

This thesis has shown that mesenchymal stem cells can be successfully isolated from both young and aged rats and manipulated *in vitro* along the osteogenic and chondrogenic lineages. Age-related alterations occurring in MSCs have also been observed in this study, including a reduction in the expression of mechanosensitive proteins that are involved in mechanotransduction and increased membrane lipid peroxidation which may alter mechanosesnitivity with age. These age-related alterations in the cell could explain the altered response of aged MSCs to high rates of strain. However, when exposed to low strain rates young and aged MSCs exhibit a similar response. Investigation of signalling cascades activated in response to a mechanical stimulus may highlight the reason for such observations (results summarised in Figure 7.1). With a greater understanding of the mechanisms involved in mechanostranduction and how it may be affected by age, the true potential of MSCs isolated from aged patients

can be determined. It can then be concluded whether these cells are suitable for use in tissue engineering based therapies for treating osteochondral disease.

7.4 Future work

To date, this research has generated several encouraging findings related to the use of MSCs isolated from aged donors in tissue engineering strategies. However, a lot of questions remain to be answered. Age-related changes occurring in MSCs that have been identified require validation and quantification by alternative experimental methods. Where possible it would be ideal to identify the expression levels of the various mechanosensory proteins and differentiation markers investigated by quantifiable measures such as western blotting or PCR. Oxidative stress was investigated using an assay to detect lipid peroxidation in cell samples. Further observations to deduce the effects of oxidative stress on the plasma membrane of aged cells and whether these correlate with alterations in mechanosensitivity with age would be beneficial. This could be achieved using atomic force microscopy to map the response of the cell membrane to applied force and whether it becomes stiffer with age. Considering the integral role played by integrin receptors, cytoskeletal actin and FAK in mechanotransduction it would be of great interest in the future to induce integrin upregulation or downregulation via pharmacological or siRNA techniques to assess how increased expression or total inhibition of these receptors affects the mechanoresponse of young and aged MSCs. It would determine whether differentiation or cell viability are affected by receptor activity and would also elucidate how pivotal their role in mechanotransduction may be. Finally, it is hoped that in the future direct investigation into the effect of cyclic mechanical strain on MSCs can be undertaken using cells isolated from human donors since the aim of tissue engineering is to treat human injury and disease therefore human-derived cells are the most ideal candidates to utilise in this research area.

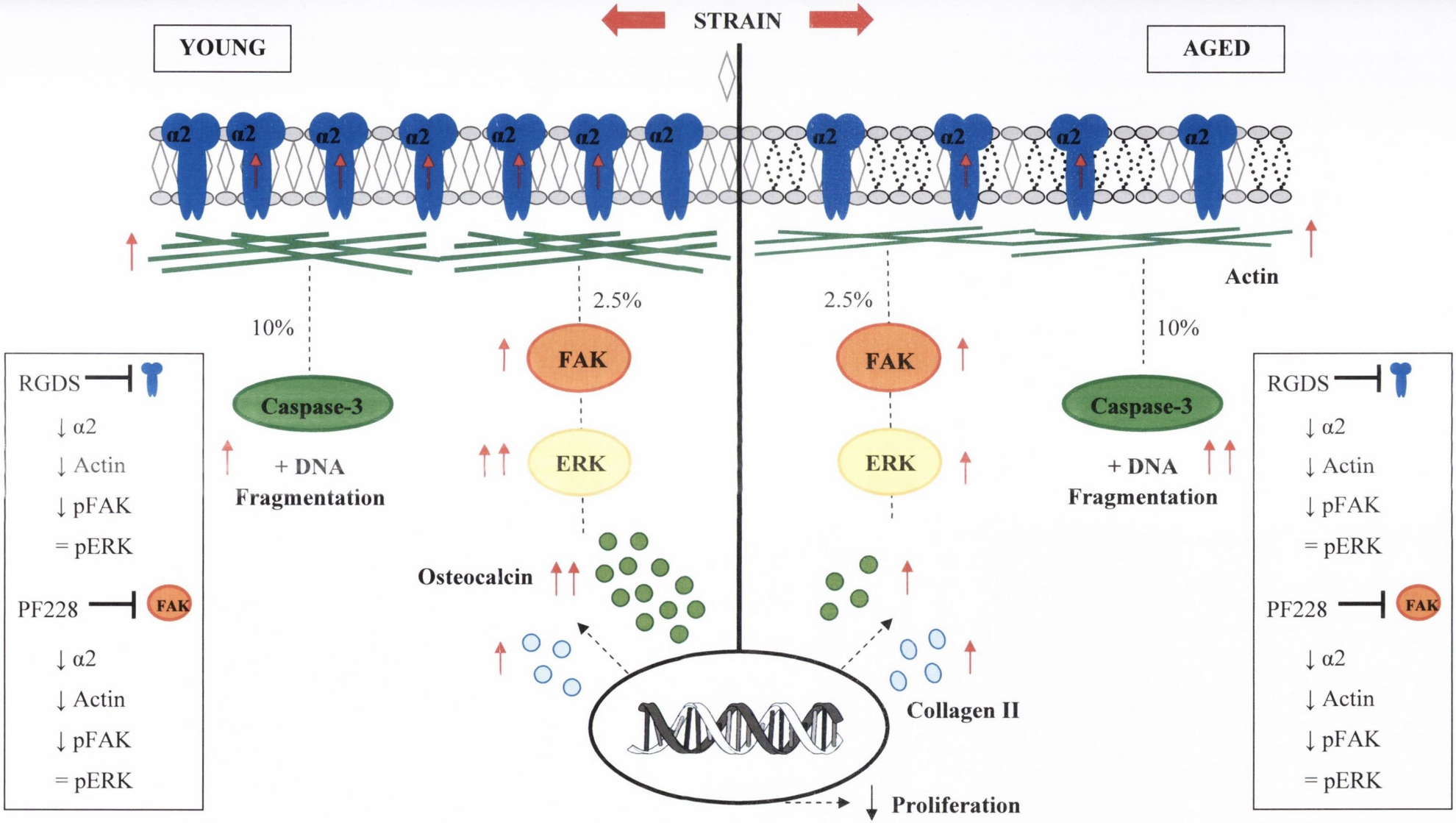


Figure 7.1: Effects of tensile strain on MSC differentiation and viability (red arrows - strain-induced changes in expression)

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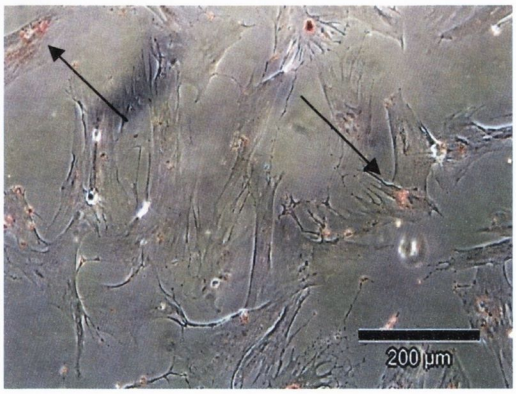
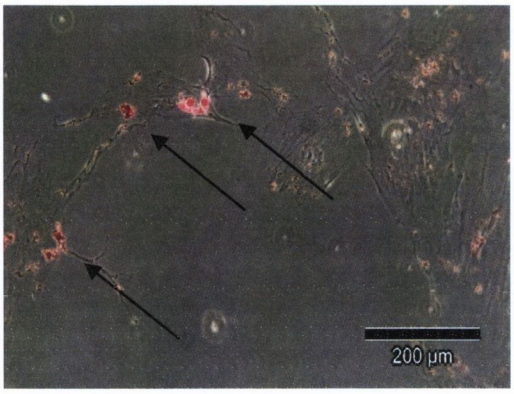
Appendix I

YOUNG

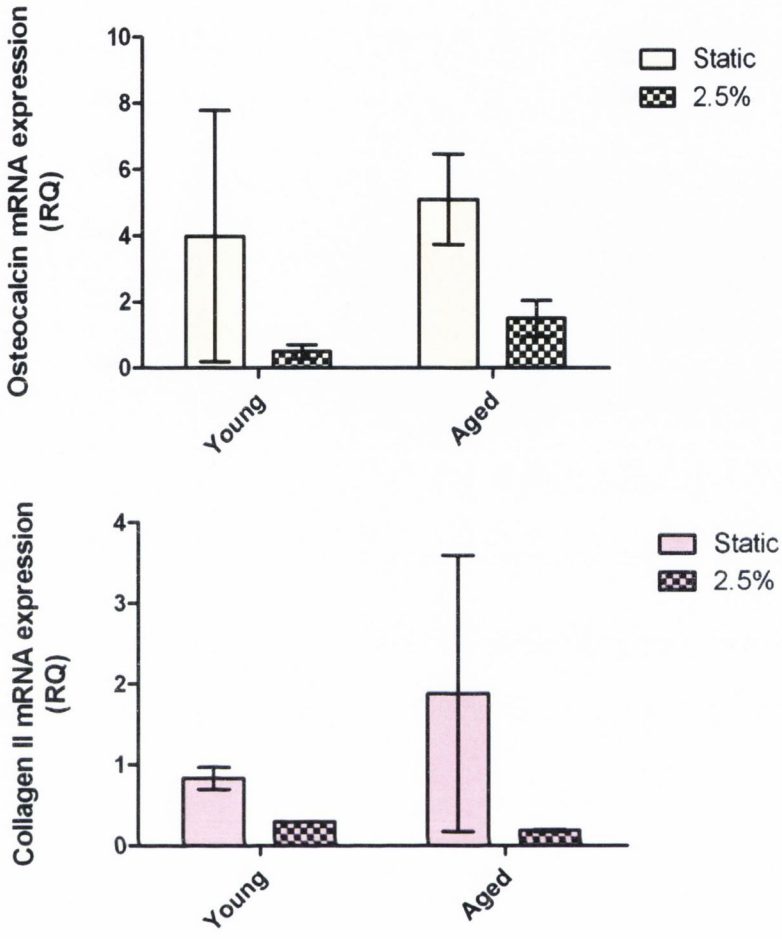


AGED



Adipogenic differentiation of young and aged MSCs was assessed in cells cultured on glass coverslips for 7 days. MSCs were stained with Oil-Red O and it was found that there were very low levels of adipogenic differentiation present, which was more evident in cells isolated from aged rats. This indicates that MSCs do not undergo spontaneous adipogenic differentiation following 7 days but that aged cells exhibit a higher propensity toward adipogenesis compared to young MSCs.

Appendix II



Osteogenic and chondrogenic differentiation of young and aged MSCs were also assessed using RT-PCR. These results have been omitted from Chapter 6 since low sample numbers were available (n=2-4), extracted mRNA levels were low (~3-10μg) and there was great variability observed within several groups. Due to these limiting factors it was concluded that the results obtained were not a true representation of gene expression in MSCs.