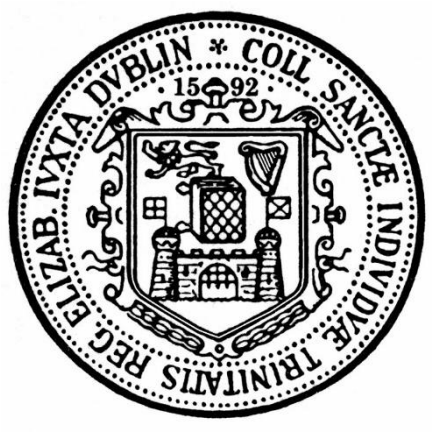

Elena Stavenschi, B.Eng.

Biophysical Regulation of Stem Cell Osteogenic Lineage Commitment: A Role for the Cytoskeleton

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

Doctor of Philosophy

Supervisor: Prof. David A. Hoey

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Declaration

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Elena Stavenschi

Summary

Osteoporosis is a debilitating disease characterised by weakened bone architecture due to increased resorption activity of osteoclasts and diminished bone deposition by mesenchymal stem cell (MSC) derived osteoblasts. Current osteoporosis treatments target activity of osteoclasts but fail to promote bone formation. Bone formation is modulated by biophysical cues and MSCs play an important role in this process by undergoing osteogenic lineage commitment to sustain loading induced adaptation. However, if and how mechanical cues developed during locomotion in the bone marrow niche directly affect the ability of MSCs to commit towards the osteogenic lineage is unknown. Understanding how biophysical cues can modulate MSC osteogenic lineage commitment will enable the identification of novel anabolic therapeutic targets to treat bone disorders such as osteoporosis.

The overall objective of this thesis is to investigate if and how changes in the marrow mechanical environment can directly regulate the osteogenic lineage commitment of MSCs. Specifically, to investigate the effect of bone marrow associated physiologically relevant 1) oscillatory fluid flow induced shear stress and 2) pressure mechanical cues on the ability of MSCs to undergo osteogenic lineage commitment. Furthermore, the mechanisms by which MSCs responds to the pressure biophysical cues will be explored, focusing on 3) cytoskeletal elements and 4) the cytoskeletal extension, the primary cilium.

Initially, a systematic approach was employed to determine whether bone marrow physiologically relevant fluid flow induced shear stress can directly affect the ability of MSCs to undergo osteogenic lineage commitment. MSCs were exposed to oscillatory fluid flow (OFF) of 1Pa, 2Pa and 5Pa shear stress magnitudes at human locomotion associated frequencies of 0.5Hz, 1Hz and 2 Hz for 1hr, 2hrs and 4hrs of stimulation. In this study it was found that OFF elicits a positive osteogenic response in MSCs in a shear stress magnitude, frequency, and duration dependent manner that is gene specific. Based on the mRNA expression of osteogenic markers *Cox2*, *Runx2* and *Opn* after short-term fluid flow stimulation, it was identified that a regime of 2Pa shear stress magnitude and 2Hz frequency induces the most robust and reliable upregulation in osteogenic gene expression. Furthermore, long-term mechanical stimulation utilising this regime, elicits a significant increase in collagen and mineral deposition demonstrating that mechanical stimuli predicted within the marrow is sufficient to directly promote osteogenesis.

In the next chapter, the effect of physiologically relevant pressure cues was explored since fluid flow within the marrow is dependent on pressurisation effects and little is known about its involvement in MSC osteogenesis. A systematic approach was employed whereby MSCs were exposed to cyclic hydrostatic pressures (CHP) of 10kPa, 100kPa and 300kPa magnitudes at frequencies of 0.5Hz, 1Hz and 2Hz for 1hr, 2hrs and 4hrs of stimulation. CHP was found to elicit a positive but variable early osteogenic response in MSCs in a magnitude and frequency dependent manner that is gene specific. *COX2* expression elicited magnitude dependent effects which were not present for *RUNX2* and *OPN* mRNA expression. However, the most robust pro-osteogenic response was found at the highest magnitude (300kPa) and frequency regimes (2Hz). Interestingly, long-term mechanical stimulation utilising 2Hz frequency elicited a magnitude dependent release of adenosine triphosphate (ATP) however, all magnitudes promoted similar levels of collagen synthesis and significant mineral deposition,

demonstrating that lineage commitment is CHP magnitude independent. Therefore, this study demonstrates that physiological levels of pressures, as low as 10kPa, within the bone can drive MSC osteogenic lineage commitment.

In the last two studies, the involvement of the cytoskeleton in transduction of pressure cues into an osteogenic response were explored. Initially, it was demonstrated that CHP is a potent mediator of cytoskeletal reorganisation and increases in early osteogenic responses in MSCs. In particular, the intermediate filament (IF) network associated cytoskeletal element was shown to undergo breakdown and reorganisation with a recoiling effect towards the perinuclear region. Furthermore, this IF remodelling was found to be paramount for loading induced MSC osteogenesis, revealing a novel mechanism of MSC mechanotransduction. In addition, it was identified that chemical disruption of intermediate filaments with Withaferin A induces a similar mechanism of IF breakdown and remodelling and subsequent increase in osteogenic gene expression in MSCs, exhibiting a potential mechanotherapeutic effect to enhance MSC osteogenesis. This study, therefore, highlights a novel mechanotransduction mechanism of pressure-induced MSC osteogenesis involving the understudied cytoskeletal structure, the intermediate filament, and demonstrates a potential new therapy to enhance bone formation in bone loss diseases such as osteoporosis.

Lastly, the role of cytoskeletal extension, the primary cilium was examined in relation to MSC associated pressure mechanotransduction. CHP stimulation of MSCs elicits a rapid release of ATP into the extracellular space, a reorganization of the intermediate filament network with concentration in the perinuclear region and an inhibition of proliferation. Furthermore, CHP results in a modulation of the primary cilium length. Importantly, the integrity of the primary cilium was found to be paramount for pressure induced ATP release and inhibition of proliferation, demonstrating a novel role for the cilium in MSC pressure mechanotransduction. Moreover, primary cilium expression was determined to play a pivotal role in pressure induced remodelling of intermediate filament network, which indicates that the cilium may be upstream of this IF mechanism demonstrated previously. Overall, this study highlights a novel pressure induced mechanotransduction mechanism pertaining to the expression of the primary cilium, which mediates purinergic signalling, anti-proliferative and cytoskeletal remodelling effects in response to pro-osteogenic pressure stimuli.

To conclude, in this thesis it was demonstrated that MSCs are mechanosensitive to physiological bone marrow associated mechanical cues such as fluid shear and pressure and they can have a direct effect on MSC osteogenic lineage commitment. Furthermore, the transduction of pressure mechanical cues was demonstrated to occur via novel mechanisms pertaining to the cytoskeletal associated intermediate filaments and cytoskeletal extension, the primary cilium. Moreover, MSC osteogenesis can be modulated by pharmacologically targeting the disassembly of the intermediate filament network using Withaferin A. These studies provide a framework for bioreactor based orthopaedic strategies and mechanobiology studies to identify biological targets for bone anabolism and how alterations to such mechanisms may lead to debilitating diseases such as osteoporosis.

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scientific mind but also, he reached out on a personal level, in times when I needed support or a blunt feedback.

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Nomenclature

%	Percentage
ΔP	Delta Pressure
μ	Viscosity
μg	micrograms
μPa	micro Pascals
ρ	Density
18s	18s Ribosomal RNA
2D	Two-dimensional cell culture/Monolayer cell culture
3D	Three-dimensional cell culture
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BMP (2,7)	Bone Morphogenetic Protein Isoform 2 and 7
BMSC	Bone marrow derived stem cells
C3H10 T1/2	Embryonic fibroblast cell line developed from C3H mouse
Ca2+	Calcium ions
cAMP	Cyclic Adenosine Monophosphate
cDNA	Circular deoxyribonucleic acid
CFU-F	Colony Forming Units Fibroblasts
CHP	Cyclic hydrostatic pressure
Col1α1	Collagen 1 alpha1
Cox2	Cyclooxygenase 2
Cx43	Connexin 43
Dh	Hydraulic diameter
Dlx5	Distal-Less Homeobox 5
DMEM	Dulbecco's Modified Eagle Medium
DMP1	Dentin Matrix Protein 1
ECM	Extracellular Matrix
EGF1	Epidermal Growth Factor 1
EGR1	Early Growth Response 1
ERK 1/2	Extracellular Signal Regulated Kinase

FAK	Focal Adhesion Kinase
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H2O	Water
hBMSC	Human bone marrow derived stem cells
HP	hydrostatic pressure
Hr(s)	hour(s)
Hz	Hertz
IER3	Immediate Early Response 3
IGFBP1	Insulin Like Growth Factor Binding Protein 1
IMP	Intramedullary pressure
ITGB1	Integrin Subunit Beta 1
kg	Kilogram
kPa	Kilo Pascals
L	Length
LABVIEW	Laboratory Virtual Instrument Engineering Workbench
LCS	Lacunar canalicular system
Le	Entrance length
m	Meters
MC3T3	Mouse preosteoblast cell line
min	Minutes
ml	Millilitres
MLOY-4	Murine Osteocyte-like Cell Line
mM	Millimolar
MMP 1&3	Matrix Metalloproteinases
MPa	Mega Pascals
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
Msx2	Msh Homeobox 2
n	Sample number
NFATC1	Nuclear Factor of Activated T Cells
nM	Nanomolar

NO	Nitric Oxide
^{o}C	degrees Celsius
Ocn	Osteocalcin
OCRL	Inositol Polyphosphate-5-Phosphatase
OPG	Osteoprotegerin
Opn	Osteopontin
Osx	Osterix
P/S	Penicillin streptomycin
Pa	Pascal
pERK1/2	Phosphorylated Extracellular Signal Regulated Kinase 1/2
PGF2	Prostaglandin F2
PGH2	Prostaglandin H2
PGI2	Prostaglandin I2
PKC	Protein Kinase C
PTH	Parathyroid Hormone
Q	Flow rate
qPCR	Quantitative polymerase chain reaction
RANKL	Receptor Activator of Nuclear Factor Kappa-B ligand
Re	Reynolds number
RNA/rna	Ribonucleic acid
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase
RPM	Rates per minute
Runx2	Runt related transcription factor 2
s	Second
Sost	Sclerostin
TGFβ1	Transforming Growth Factor β1
TRPV4	Transient Receptor Potential Cation Channel Subfamily V Member 4
u	Velocity
v/v	volume/volume
VEGFA	Vascular Endothelial Growth Factor A
w	Width
WNT	Wingless-Type

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Chapter 1.
Introduction

1.1 Bone associated diseases and current treatments

Osteoporosis is a disease characterised by weakening of the bone due to loss of bone mass and disruption of bone architecture resulting in an increased risk of fragility fracture. It is predicted that 1 in 3 women and 1 in 5 men will suffer of an osteoporotic related bone fracture worldwide leading to a detrimental effect on quality of life. Osteoporosis related fragility fractures have become a global economic health burden and worryingly, the incidence of these fractures are expected to rise due to an ageing population (Svedbom et al., 2013, Feng and McDonald, 2011). One of the characteristics of osteoporosis presentation is weakened microarchitecture of the bone due to accelerated activity of bone resorbing cells, osteoclasts, which overpowers the compensatory bone formation (Yu and Wang, 2016). Therefore, current treatments target the function and survival of bone resorbing osteoclasts to prevent further loss of skeletal mass but fail to restore bone mass and architecture (Canalis, 2018). However, current antiresorptive therapies have been associated with severe side effects including increased risk of heart disease, cancer and jaw osteonecrosis among others (Salari Sharif et al., 2011, Mupparapu and Akintoye, 2017, Zhou et al., 2014). Therefore, a shift in treatment strategy is emerging through targeting the cellular component responsible for bone anabolism such as the osteocytes, osteoblasts and osteoprogenitors/skeletal stem cells. Parathyroid hormone (PTH) is one of the first bone anabolic agents prescribed for osteoporosis treatment targeting osteoblast activity and is administered either as hormonal treatment (Teriparatide) or as PTH agonist (Abaloparatide). Both treatments display good clinical indications of lower fracture risk however this effect is lost if PTH treatment is discontinued, hence it requires concurrent administration with anti-resorptive agents (Black et al., 2005). Moreover, its use is limited to 2 years due to safety concerns of osteosarcoma development (Canalis, 2018). The most recent class of anabolic treatment for osteoporosis is Romosuzumab, a monoclonal antibody that targets

the osteocyte derived protein, sclerostin, an antagonist of Wnt pathway and thereby modulating bone formation whilst inhibiting resorption. Clinical trials to date demonstrated that Romosuzumab exhibited an increase in bone formation and decreased the risk of fracture, however it is yet to gain approval for standard clinical use by Food and Drug Administration due to reported adverse cardiovascular effects (Taylor, 2017, Langdahl et al., 2017, Canalis, 2018). One obvious target that has yet to be explored in relation to its involvement in disease progression and treatment, are mesenchymal stem cells (MSCs), which reside in the bone marrow and play a pivotal role in bone anabolism (Chen et al., 2016a, Corral et al., 1998). Osteoporosis also presents with a decrease in both MSC population and their ability to undergo osteogenic lineage commitment. This subsequently has a deleterious effect on sustaining bone anabolism and thereby leading to increased risk of fracture (Tewari et al., 2015). Hence, MSCs represent a potential therapeutic avenue to target bone formation which is yet to be fully explored.

1.2 Stem cell mechanobiology

Mesenchymal Stem Cells (MSCs) are multipotent cells that are capable of differentiation into multiple lineages of the musculoskeletal system. Although, the definition of a stem cell has become a scientific debate, it is undeniable that stem cells within the bone and marrow niche play a vital role in bone regeneration and modelling by being a source of osteoprogenitors (Robling et al., 2006, Robey, 2017, Caplan, 2017, Bianco et al., 2013). The debated MSC terminology arises from its initial labelling as derived from the mesenchyme- a type of embryonic tissue characterized by loosely associated cells that lack polarity and are surrounded by a large ECM with differential potential towards mesoderm associated lineages, whereas adult bone marrow stem cells are defined as being of post-natal origin and have a more limited differential capacity geared towards musculoskeletal tissues only (Caplan, 2017, Robey, 2017). Furthermore, some argue that bone marrow stem cells are pericytes since they are found to reside near perivascular niches within bone and share surface markers similar to adventitial reticular cells (Jones and McGonagle, 2008). In this thesis, the MSC terminology will be used for both human bone marrow stem cells and mesenchymal stem cells, however the author acknowledges the origin and differences between these cell types. The contribution of MSCs in maintaining bone integrity has yet to be fully elucidated, in particular how adaptation to physiological mechanical cues can dictate the differential potential of MSCs.

Bone integrity is known to be modulated by physical loads from observations of in vivo human models of bone loss due to lack of mechanical loading in astronauts and bedridden patients as well as increase in bone mass in athletes with sustained physical exercise. Bone mechanoadaptation has been attributed to the mechanosensory abilities of the osteocytes, which have been extensively studied to date (Schaffler et al., 2014, Klein-Nulend et al., 2013, Iolascon et al., 2013, Bonewald, 2011). However, recent evidence has shown that mechanical cues also engage MSCs in loading induced bone anabolism independent of osteocyte signalling using models of fluorescent tagged MSCs transplant in ulnar loading, ex vivo model of vertebral bone explants exposed to vibrational stimulation and osteocyte ablation (Robling et al., 2006, Chen et al., 2016a, Curtis et al., 2018, Kwon et al., 2012). Although these observations are indirect, it highlights that MSCs are mechanosensitive to the surrounding micromechanical environment and their osteogenic potential may be regulated by the mechanical cues

developed during bone loading. Therefore, deciphering how loading cues at the tissue scale are translated to MSCs and subsequently elicit an osteogenic response will potentially open novel avenues to enhance bone formation leading to new treatments for bone loss diseases such as osteoporosis by targeting these mechanisms, mimicking the beneficial effect of loading. However, how the bone marrow mechanical environment under physiological loading affects MSC osteogenesis is yet to be fully explored.

Loading-induced bone formation is a tightly regulated process that requires recruitment and differentiation of mesenchymal stem cells that reside predominantly within bone sinusoids, marrow and the periosteum (Bianco et al., 2013, Chen and Jacobs, 2013). During locomotion, the skeletal system including the marrow, sustains a complex dynamic loading environment where pressure gradients, developed because of bone deformation, lead to pressure-induced fluid flow and shear. In addition, due to loading and unloading of bones, these stimuli are dynamic and follow an oscillatory pattern. The synergistic action of loading induced pressure and fluid flow modulate mechanical and chemical signalling, thus enabling bone adaptation (Ren et al., 2015). To understand how these mechanical cues, translate into a biological response, and specifically their independent effects on stem cell induced osteogenesis, in vitro mechanobiology studies employing bioreactor systems to mimic the bone mechanical microenvironment on cellular scale are employed. Such studies to date demonstrated that foremost, MSCs are sensitive to mechanical stimuli and secondly, they respond to such stimuli with a characteristic osteogenic fingerprint. Fluid flow is the most studied mechanical stimulus in bone physiology displaying osteogenic effects in MSCs, although with incongruent results (Li et al., 2004, Yourek et al., 2010, Ceccarelli G, 2013, Govey et al., 2013, Becquart et al., 2016). The effect of pressure on osteogenic differentiation of MSCs, although understudied, has also presented mixed results such as promoting osteogenesis and/or chondrogenesis and activation of characteristic pathways of these specific lineages (Zhao et al., 2016, Nessler et al., 2016, Klein-Nulend et al., 1997b, Zhao et al., 2015, Liu et al., 2009, Hess et al., 2010). From a review of the literature, it has been noted that a common factor in these studies which may have lead in the disparity of the current observations is the depiction of mechanical environment of the bone. Due to limited knowledge of the bone marrow mechanical environment, bone mechanobiology studies to date have employed pressures (< 100 kPa) and shear stresses (0-3 Pa) that are predicted to occur within the lacunar canalicular system (McCoy and O'Brien, 2010b,

Nessler et al., 2016, Weinbaum et al., 1994). Recent computational studies identified that under certain physiological loads, cells within the marrow may experience shear stresses up to three times higher than previously described (0-8 Pa) and significantly lower pressures (~40 kPa) during bone loading and muscle activation (Kumar et al., 1979, Coughlin and Niebur, 2012a, Metzger et al., 2015a). Therefore, studies to date describing loading induced responses in MSCs may over or under represent physiological conditions depending on the type of physical cue. Hence, there is a need to develop physiologically mimetic in vitro models of loading-induced marrow mechanics to better determine the role of physical stimuli in driving the lineage commitment of MSCs. These models can be utilised to delineate mechanisms by which cells sense and translate micromechanical cues into an osteogenic response, therefore identifying potential therapeutic targets to drive bone anabolic responses. Moreover, this knowledge can be utilised to advance current tissue engineering strategies for orthopaedic applications.

1.3 Stem cell mechanotransduction

Bone adaptation to the mechanical environment through the translation of mechanical cues to organ, tissue and cell scale via a biological feedback system is a seminal hypothesis introduced by Frost in 1987 as the mechanostat theory (Frost, 1987). The mechanostat hypothesis, highlights the presence of biological thresholds and mechanisms that control bone mechanoadaptation whereby nowadays they are referred to as mechanobiology and mechanotransduction mechanisms (Yavropoulou and Yovos, 2016, Frost, 2003). This theory represents a vast biologic macrocosm as several mechanotransduction mechanisms to date have been discovered and many have yet to be identified as hierarchical distribution of biological signalling is complex with both local and systemic interactions (Frost, 2003).

To date, loading-induced osteogenic responses have been attributed to many cellular mechanisms pertaining to both transmission of extracellular cues from the pericellular environment to the internal relay of these signals to the cellular effectors. Integrins are surface-adhesion receptors that form focal adhesion processes providing anchorage and means of communication from the extracellular matrix (ECM) to the internal cell cytoskeleton allowing for bidirectional transmission of forces across plasma membrane (Harburger and Calderwood, 2009). Furthermore, ECM type and stiffness can dictate the expression and the density of particular integrins and thereby modulating MSC differential potential (Docheva et al., 2007). For example, collagen I preferential expression of $\alpha 2\beta 1$ integrin and increase in ECM rigidity activates focal adhesion kinase (FAK), a known mechanotransducer of matrix stiffness in addition to downstream expression of ROCK and ERK1/2 all of which contribute to MSC osteogenesis (Shih et al., 2011). Integrin connection to the cytoskeleton can also be modulated biochemically via activation of cell surface receptors such as mechanosensitive ion channels thereby affecting cell cycle progression, motility and differentiation (Ingber, 1997, Yavropoulou and Yovos, 2016, Pchelintseva and Djamgoz, 2018). Ultimately, the mechanotransduction of extracellular cues impacts the internal cell architecture which in turn is known to modulate the stem cell differential potential.

The cell cytoskeleton represents an internal framework of tension bearing actin and intermediate filaments and compression resisting microtubules. These three elements are always in equilibrium and can regulate cell shape and internal architecture.

This adaptive mechanism allows fine tuning of a cell's ability to intercept and relay signalling cues according to its function and surrounding environment (Ng et al., 2017). For example, osteocytes as terminally differentiated cells are embedded within bone and communicate with nearby osteocytes via long dendritic processes to sense distribution of the mechanical strains throughout the entire bone (Schaffler et al., 2014). These processes are actin rich projections with intermediate filaments and microtubules reaching only to the proximal ends of the processes (Tanaka-Kamioka et al., 1998). In contrast, osteoblasts in 3D culture also develop processes however their stability is dependent on microtubule presence rather than actin (Murshid et al., 2007). Changes in the intracellular architecture have also been observed during osteogenic lineage commitment. MSC osteogenic differentiation has been attributed mainly to reorganisation of actin cytoskeleton, both via biochemical induction and mechanical modulation whilst the microtubules were identified to mainly regulate actin dynamics (Rubin and Sen, 2017, Arnsdorf et al., 2009, Rodriguez et al., 2004). Moreover, cytoskeletal adaptation in MSCs has been explored only in response to fluid flow and strain mechanical stimulation whilst the effect of pressure on the cytoskeleton and the subsequent downstream osteogenic effects in MSCs are unknown (Mathieu and Loboa, 2012). Furthermore, the intermediate filaments are the least studied cytoskeletal component in MSCs and bone cell associated osteogenesis with only two studies to date showing that presence of intermediate filament associated protein vimentin acting as a brake during osteoblastogenesis (Lian et al., 2012, Lian et al., 2009). Therefore, it is paramount to understand the contribution of each cytoskeletal element to loading induced osteogenesis.

An intriguing apical extension of the cytoskeleton is a microtubule-based organelle known as the primary cilium. Initially thought to be a vestigial part of a cell, now it is known to be involved in extracellular-intracellular communication of both biochemical and mechanical cues (Spasic and Jacobs, 2017). In bone it has been shown to play a critical function in skeletogenesis and loading induced adaptation (Chen et al., 2016a, Moore et al., 2018a, Moore et al., 2018b). In particular, loading induced bone formation promotes osteogenic lineage commitment of MSCs and homing of progenitors to the bone surface. However, inhibition of primary cilia expression in MSCs impairs loading induced osteogenesis resulting in stunted loading induced bone formation (Chen et al., 2016a). In vitro studies demonstrated that primary cilia are involved in fluid flow

mechanotransduction whereby its presence regulates intracellular calcium signalling and downstream osteogenic gene expression in all bone cells including MSCs (Spasic and Jacobs, 2017, Hoey et al., 2012). To date, primary cilia mediated pressure mechanotransduction has been reported only in chondrocytes and trabecular meshwork cells. In chondrocytes, primary cilia were found to not be required for compression induced ATP release but necessary for subsequent ATP reception for activation in intracellular calcium signalling and downstream chondrogenesis (Wann et al., 2012). In trabecular meshwork cells, primary cilia mechanotransduction was related to how the eye senses pressure changes via a mechanism involving ciliary mechanosensory channels transient receptor potential vanilloid 4 (TRPV4) and OCRL (Inositol Polyphosphate-5-Phosphatase) (Luo et al., 2014). However, the involvement of primary cilia in mediating pressure mechanotransduction in MSCs is unknown. Understanding how physiologically bone associated mechanical cues directs MSC osteogenic lineage commitment and the mechanotransduction mechanisms that facilitate this process will aid in promoting platforms for bioreactor based orthopaedic strategies. Moreover, this will facilitate the development of mechanobiology models to identify how alterations in loading induced MSC mechanotransduction can lead to diseased states such as osteoporosis and thereby discover novel therapeutic targets.

1.4 Thesis Objective

The primary objective of this thesis is to investigate if and how changes in the marrow mechanical environment can directly regulate osteogenic lineage commitment of mesenchymal stem cells. Specifically, the effect of loading induced physiologically relevant changes in marrow fluid shear and pressure on MSC osteogenesis will be determined. Furthermore, the mechanisms by which MSCs responds to these biophysical cues will be explored, focusing on cytoskeletal elements and the cytoskeletal extension, the primary cilium. To achieve this, I propose the following:

I. Determine whether the mechanical environment of the bone marrow niche can modulate the osteogenic lineage commitment of MSCs.
    a. Investigate the effect of physiologically relevant oscillatory fluid flow induced shear stress on MSC osteogenic lineage commitment.
    b. Investigate the effect of physiologically relevant cyclic hydrostatic pressure on MSC osteogenic lineage commitment

II. Determine the mechanotransduction mechanisms of pressure induced MSC osteogenesis
    a. Investigate the role of the cytoskeletal elements in MSC pressure mechanotransduction
    b. Investigate the role of the primary cilium in MSC pressure induced mechanotransduction

Chapter 2.
Literature Review

2.1 Introduction

Determining how physiological biophysical cues within the marrow micromechanical environment can provide spatial and temporal information to trigger an osteogenic response is a complex question but can provide impactful knowledge, especially in the regenerative medicine field. To achieve the objectives set, the structure, function and mechanical environment of bone and bone marrow niche will be examined. Afterwards, an in-depth review of mechanobiology studies exploring the effect of fluid flow and pressure on bone associated cells including mesenchymal stem cells in relation to eliciting an osteogenic response will be performed, with a particular focus on how these regimes relate to bone and marrow physiology. Furthermore, the mechanotransduction mechanisms pertaining to loading induced osteogenesis will be explored in relation to the involvement of cell cytoskeleton and primary cilia. Lastly, current trends in bioreactor systems for orthopaedic based research will be discussed highlighting design factors for development of custom bioreactors that can mimic bone and marrow mechanical niche.

2.2 Skeletal physiology

The skeletal system contains 206 bones, each with unique physical characteristics to serve its function from organ protection to locomotion, containing a biological ecosystem with immunomodulatory, regenerative and endocrine capabilities (Clarke, 2008). The regenerative properties are related to the presence of hematopoietic and skeletal progenitors within the bone and marrow space and are based on a regulatory system between the skeleton and surrounding tissues where mechanical forces play a central role in this process. Therefore, the bone is considered to be a living tissue due to its adaptative nature to suit the body's intrinsic biological systematic and mechanical needs.

2.2.1 Bone structure and mechanical environment

Bone has a complex architectural arrangement at tissue, cell, matrix and molecular level. Based on tissue scale organisation 80% of bone is cortical and 20% is trabecular (spongy/cancellous) (Eriksen et al., 1994). Cortical bone is compact and encapsulates most of the marrow space whereas the trabecular bone follows a honeycomb pattern which transitions at the metaphysis in the axial long bones. The bone on the outer surface, except at the joint area, is surrounded by a lining sheath of matrix and bone lining/osteoblast cells known as periosteum whereas on the inside is lined by endosteum. The fundamental functional unit of cortical bone is called an osteon. The interconnecting osteons form the Harversian system which is composed of lacunae, where terminally bone committed osteocyte cells reside. The lacunae are interconnected via canaliculi through which osteocyte processes form a signalling network (Figure 2-1) (Clarke, 2008). The bone matrix onto which cellular components reside is composed of organic matter synthesised by bone depositing osteoblast cells such as collagen and other bone related proteins, inorganic matter- hydroxyapatite, and water (Ren et al., 2015). Water based interstitial fluid represents an efficient medium by which biochemical signalling, nutrient and waste exchange occurs, and provision of biophysical cues translated during bone loading (Qin et al., 2003, Cowin, 2002).

Interstitial fluid movement is thought to be one of the main mechanisms which enables loading induced bone adaptation (Qin and Hu, 2014, Qin et al., 2002, McGarry et al., 2005). During loading, the bone undergoes deformation at levels of up to 3000 microstrain at tissue scale (4000-40000 μm at cell scale), which causes pressurisation of

interstitial fluid forcing it to flow within the Haversian system towards the periosteum and at the unloading phase would flow in the opposite direction towards endosteum, in an oscillatory pattern (Ren et al., 2015). Mathematical, computational and imaging modalities to date predict that the fluid flow induces drag effects within the lacunar-canalicular system (LCS) exceeding 5 Pa. Within lacunae, where osteocyte cell processes reside, shear stresses can reach up to 12 Pa magnitudes based on recent computational models (Verbruggen et al., 2014, Price et al., 2011, Weinbaum et al., 1994).

Very little is known about the pressure magnitudes that are elicited during bone physiological loading. Zhang et al. (1998) predicted that a walking physiological cyclic stress of 0–18 MPa at 1Hz would elicit hydrostatic pressures within LCS up to 300 kPa which is approximately 12% of the applied axial stress and 40 times higher than that in the vasculature (Ren et al., 2015, Zhang et al., 1998). These pressures are thought to be sufficient to modulate osteocyte mechanobiological responses (Scheiner et al., 2016). Ex vivo compression of porcine femur at loads of 1-2 kN, 1Hz was found to elicit pressures up to 1.5 kPa in the marrow at the femoral neck (Metzger et al., 2015b). However, pressure gradients elicited during loading regimes that are anabolic such as jogging and jumping in humans have yet to be defined.

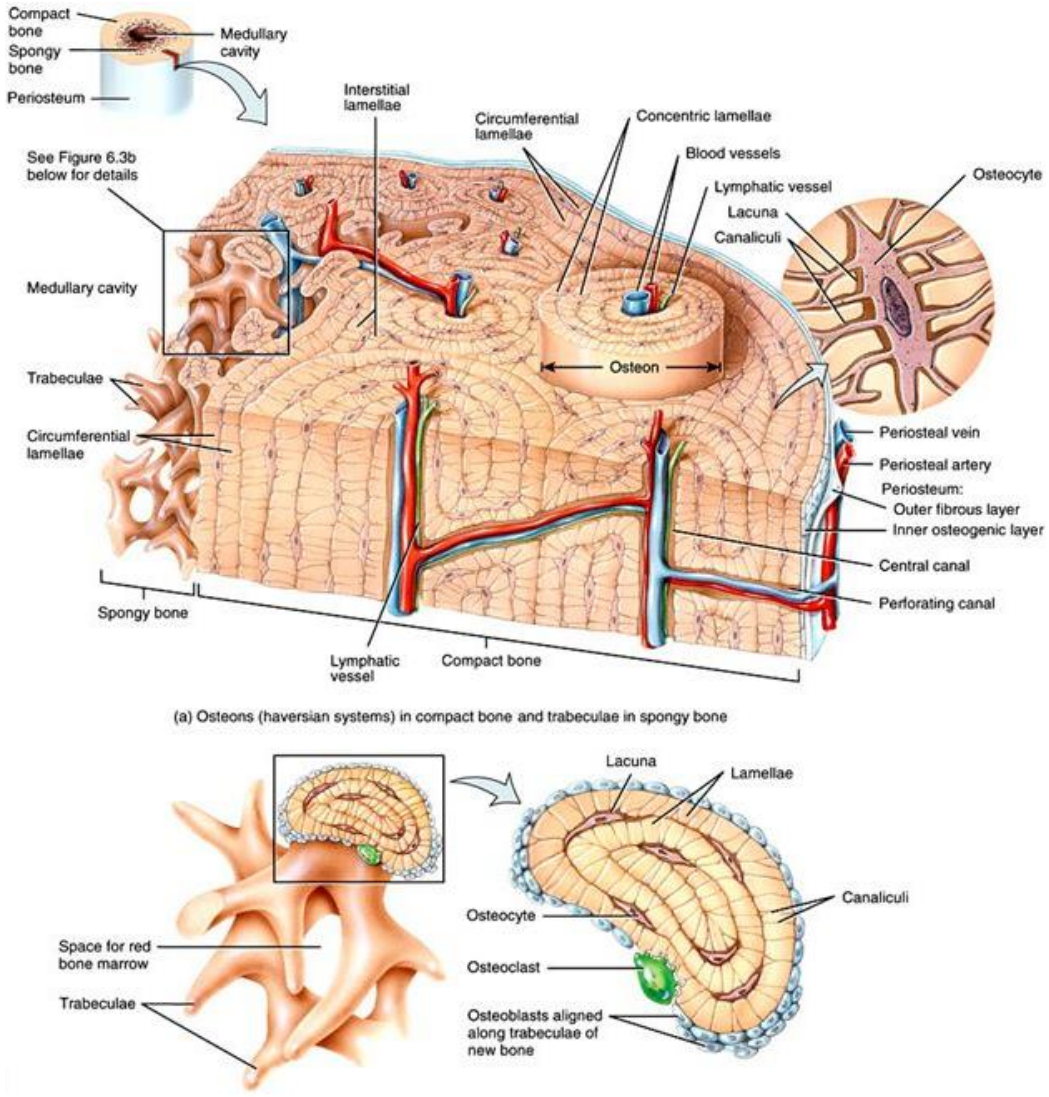


Figure 2-1 Structural organisation of bone adapted from (Tortora and Derrickson, 2014).

2.2.2 Marrow structure and mechanical environment

Bone marrow is a classified as an organ whose functions are related to the maintenance of bone homeostasis, modelling, remodelling, tissue repair and blood production. Found within the lumen of bones, it represents 5% of a human adult's body mass (Standring, 2016, Travlos, 2006). The marrow is a heterogenous tissue colloquially characterised as red and yellow based on its cellular and fat content. Red marrow is the main constituent at birth and it is converted into yellow marrow throughout and individual's life. The marrow distribution within the axial load bearing bones is dependent on the architectural fenestration of the vasculature such as red marrow located within epiphyses interspersed through the trabecular bone; and yellow marrow located

mainly in the diaphysis (Moulopoulos and Koutoulidis, 2015). The stroma represents the marrow's connective tissue and it houses cells that are not directly involved in haematopoiesis: osteoblast, osteoclast, skeletal stem cells, reticular cells, endothelial cells, macrophages and adipocytes (Figure 2-2). The marrow is surrounded by the inner surface of the bone lumen and the outer surface of the cancellous bone spicules which are covered by an endosteal lining consisting of a single layer of flat "bone-lining cells" supported by a thin layer of reticular connective tissue (Travlos, 2006). This provides a unique interface for signalling transfer of both biochemical and biophysical stimuli.

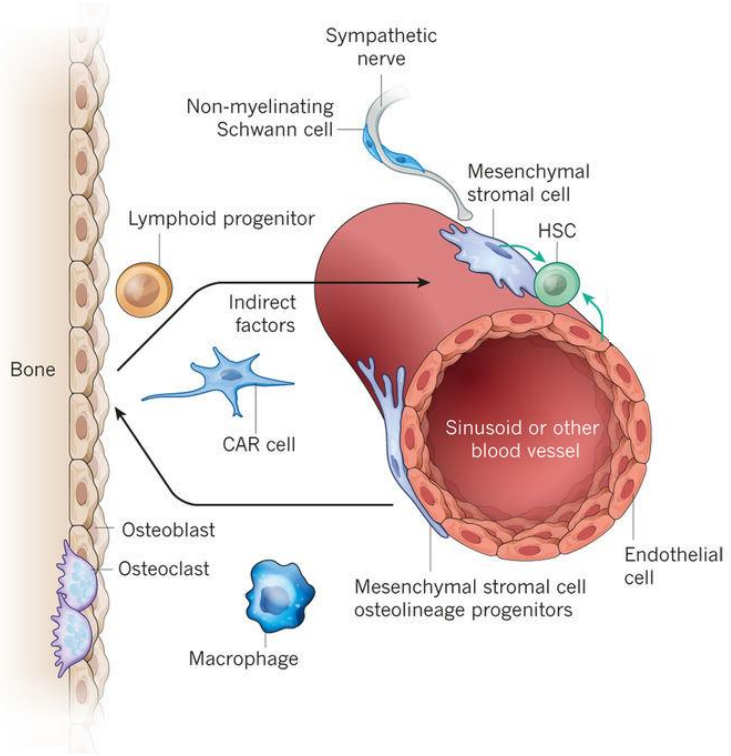


Figure 2-2 Schematic of cellular content of bone marrow depicted by hematopoietic lineage (lymphoid, macrophage, osteoclasts, stem cells), bone lineage (osteoprogenitors/stem cells, osteoblasts) and others. Image adapted from (Morrison and Scadden, 2014).

Recent evidence of loading induced osteogenic lineage commitment of stem cells highlights the involvement of whole bone and marrow biophysical cues in this process (Chen et al., 2016a). However, due to limited access to the tissue and the difficulty to preserve the structure/biophysical properties ex vivo the mechanical characteristics of

the marrow are yet to be fully characterised. The marrow is a viscous material with solid characteristics at room temperature yet and fluid-like behaviour at body temperature (Metzger et al., 2015a, Metzger et al., 2014). The viscosity of the marrow can vary depending on the anatomical location thereby affecting the mechanical interaction with the endosteal surface (Dickerson et al., 2008, Gurkan and Akkus, 2008a). Loading causes oscillations in the marrow pressure within the medullary cavity providing hydraulic strengthening to the bone (Kumar et al., 1979). Moreover, this effect is not exclusive to bone loading, but also to muscle contraction independent of loading which is facilitated by the interlinked network of vasculature interfaced with the bone and marrow. The vasculature provides a baseline intramedullary pressure (IMP) roughly ¼ of systolic pressure (~4kPa), yet with loading, venous occlusion and bone deformation causes pressurisation of marrow and interstitial fluid within bone which is pulsatile in nature and dependent on the rate of loading (Downey et al., 1988, Bryant, 1983, Kumar et al., 1979, Hillsley and Frangos, 1994). Ex vivo experiments on intact sheep tibiae and human femora recorded pressures less than 50 kPa within the marrow niche (Bryant, 1983, Downey et al., 1988). In a rat model, femoral muscle contractions incited by electrical stimulation displayed a linear positive relationship between muscle activation and intramedullary pressure with 4-8 fold increases compared to baseline resting IMP (Kumar et al., 1979). It is noteworthy that the pressure magnitude range within marrow is an order of 100 to 1000-fold less than that of experienced in cartilage. Therefore, the contribution of physiological pressure to MSC osteogenic lineage commitment is unclear and yet to be fully explored (Fahy et al., 2018, Anderson et al., 2008).

Loading induced pressurisation of the marrow plays another critical role in bone mechanics such as shearing effects at the marrow endosteum interface within the intramedullary cavity and trabeculae. These effects were computationally predicted to be of magnitude greater than 0.5 Pa in 75% of the bone marrow (Coughlin and Niebur, 2012b). Moreover, shear stress at the trabecular interface was found to be dependent on the viscosity of the marrow and the density/tortuosity of the trabecular architecture with magnitudes exceeding 5 Pa for higher viscosity range (Figure 2-3) (Metzger et al., 2015a). The importance of the magnitude range predicted for both pressure and shear stress within the marrow niche with physiological loading with respect to MSC osteogenic lineage commitment will be further discussed in the Skeletal Mechanobiology section.

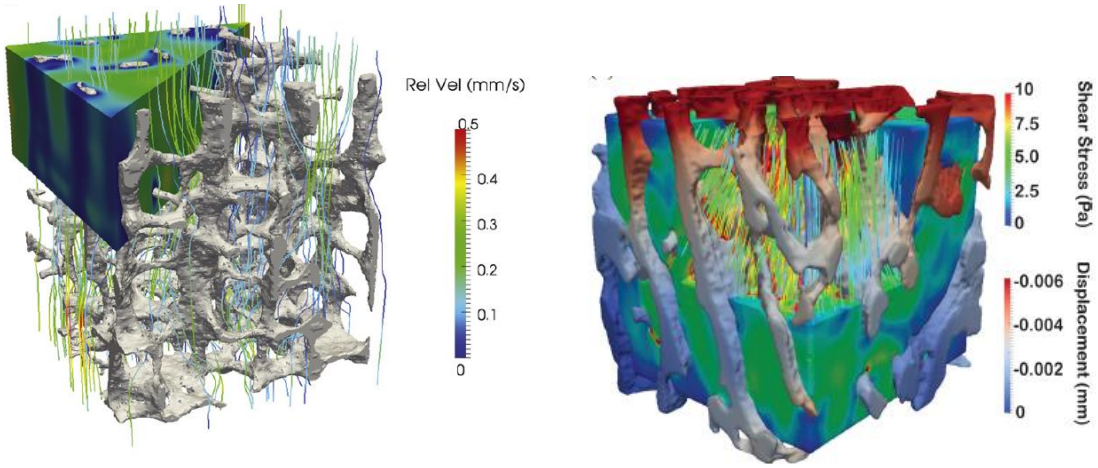


Figure 2-3 A) Computational model of a reconstructed trabecular bone sample showing the streamlines and velocity contours of marrow flow relative to the bone with vibrational stimulation (Coughlin and Niebur, 2012a) and B) distribution of flow induced shear stress of the marrow due to compression (Metzger et al., 2015a).

2.3 Cellular content within skeletal system

Bone metabolism is a finely orchestrated and rigorously controlled interaction of cellular components that facilitate mechanical sensing, bone deposition and resorption. The cellular component of bone is generally classified as following: stem cells, the progenitor pool; bone depositing cells (bone lining and osteoblasts) and bone sensing osteocyte cells in addition to hematopoietic derived bone resorbing osteoclast cells (Mizuno et al., 2018). Bone remodelling is essential for fracture healing, calcium homeostasis and skeleton adaptation to mechanical use. The remodelling mechanism is defined by the coupled interaction between osteocytes, osteoblasts and osteoclasts. Bone modelling, which is also involved in the remodelling process, can also be viewed as an anabolic event in which stem cells and other progenitors undergo osteogenic lineage commitment to support bone synthesis in response to biophysical skeletal demands (Florencio-Silva et al., 2015). In the sections below, the anatomical function of skeletal stem cells and bone committed cells in relation to osteogenesis will be discussed.

2.3.1 Bone and marrow derived stem cells

In 1970, Friedenstein *et al.* reported the isolation of a rare mononuclear cell population from the bone marrow with plastic adherence characteristic described as Colony Forming Units-Fibroblast (CFU-F) that differentiated into cartilage and bone (Friedenstein et al., 1970). These cells were later demonstrated to be multipotent with ability to undergo differentiation towards the mesengenic lineage in vitro (bone, cartilage, fat, tendon, ligament, muscle etc.) and therefore named mesenchymal stem cells (Figure 2-4) (Caplan, 1994, Bianco et al., 2013, Bianco and Robey, 2015). Today, the historical context of MSC definition is debated as mesenchyme represents the mesodermal layer during embryogenesis which leads to development of mesodermal tissues which also includes the musculoskeletal system, whereas post-natal progenitors within skeletal system are not of embryonic origin and have a more limited differential capacity (Robey, 2017, Caplan, 2017, Caplan, 1994). Current guidelines defined by Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed a minimal criterion to define human MSCs: plastic adherence (CFU-F); must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules; MSCs must differentiate to osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006). However,

these guidelines may change as new knowledge unfolds. Due to historical usage, the MSC acronym has been proposed to be kept, however its contextual meaning requires a more appropriate representation of its role in vivo (Caplan, 2017). In this thesis, MSCs will be referred to both human bone marrow derived stem cells and mesenchymal stem cells of embryonic origin although the author acknowledges the differences in between these two cell types. The cell model/type used will be explicitly described in the methods section in all the results chapter.

The current concept of a MSC is thought to be a cell type that is capable of generating a complete heterotopic bone or bone marrow organ in vivo including a compartment of perivascular cells with similar properties and phenotype as the original cells (Jones and McGonagle, 2008). Immunofluorescence studies using MSC markers looking at the MSC distribution within the marrow determined that MSCs are morphologically similar to adventitial reticular cells (ARCs) and are located near hematopoietic niches such as adjacent to vessel walls, on the endosteal surfaces of trabecular bone, within the interfibrillar spaces, or perivascular niches (da Silva Meirelles et al., 2009, Jones and McGonagle, 2008, Tuli et al., 2003). Therefore, the MSCs are arguably thought to be a 'bona fide specialised pericyte of bone marrow' located near vascular sinusoids distributed throughout the post-natal skeleton (Jones and McGonagle, 2008, Robey, 2017). However, stem cells with multilineage potentiality have also been extracted from avascular tissue such as cartilage which may indicate tissue specific progenitors (Barbero et al., 2003).

MSCs play a significant role in maintaining the progenitor pool since 95% of bone cellular make up is represented by non-proliferative bone sensing osteocytes and mineral synthesising cells with limited proliferative capacity such as osteoblasts and bone lining cells (Frost, 1960, Tewari et al., 2015, Park et al., 2012). The homing mechanism of MSCs to sustain bone integrity is yet to be fully elucidated, however current paradigms relate such events to both biochemical paracrine signalling from bone committed cells and direct mechanical stimulation (Duncan and Turner, 1995, Curtis et al., 2018). The number of MSCs undergoing osteogenic lineage commitment is related to the number of secretory osteoblasts entering active synthesis (Bruder et al., 1994). Hence, the location of MSCs and the associated homing mechanism is vital for bone integrity and is yet to be fully elucidated.

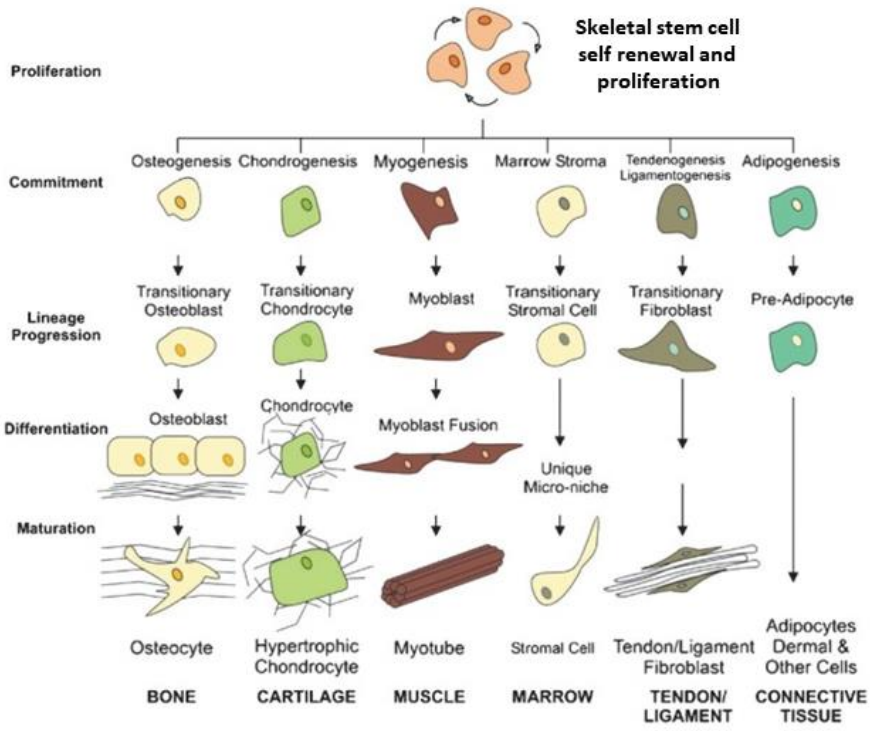


Figure 2-4 Mesengenic process of skeletal stem cells adapted from (Firth and Yuan, 2012)

2.3.2 Bone lineage committed cells

Osteoblasts are MSC derived cells specialised in bone synthesis located along the bone surface and make up 4-6% of total resident bone cells (Capulli et al., 2014). Osteogenic differentiation of MSCs towards an osteoblast, also known as osteoblastogenesis, requires expression of specific genes related to Bone Morphogenetic (BMPs) and Wingless (Wnt) pathways (Grigoriadis et al., 1988). Expression of Runt-related transcription factor 2 (*Runx2*), Distal-less homeobox 5 (*Dlx5*) and osterix are crucial factors required for osteoblastogenesis. *Runx2* is also known as the master osteogenic transcription factor instigating downstream signalling of osteoblast related genes necessary for bone matrix synthesis (Fakhry et al., 2013). The transition from MSC/osteoprogenitor down the osteogenic lineage is described by several stages relating to its function such as pre-osteoblast, transitory osteoblast, secretory osteoblast, osteocytic osteoblast and osteocyte being the terminal form of this lineage. Quiescent osteoblasts known as bone lining cells are also present on bone surfaces functioning as a source of osteoblast pool in addition to modulating osteoclast activity (Mosley, 2000, Andersen et al., 2009, Matic et al., 2016).

Osteoblast function to synthesise bone matrix occurs by secretion of collagen proteins (collagen type I) and other non-collagen proteins such as osteocalcin, osteonectin, bone sialoprotein, osteopontin as well as proteoglycans (decorin and biglycan). Thereafter, mineralisation of the organic matrix occurs through integration/deposition of calcium ions via interaction of osteoblast synthesised enzymes such as ALP among others (Glimcher, 1998, Anderson, 2003). During this process, the osteoblast become trapped by the mineralised osteoid and can progress towards becoming an osteocyte or bone lining cells or undergo apoptosis (Parfitt, 1990, Jilka et al., 1998).

Osteocyte cells compromise 90-95% of bone cellular component and can have a lifespan of up to 25 years (Datta et al., 2008). As osteocytes are derived through terminal differentiation of osteoblasts, the osteoblast associated markers become downregulated and osteocyte specific markers such as dentine matrix protein 1 (DMP1) and sclerostin (SOST) are highly expressed (Bonewald, 2011, Ubaidus et al., 2009, Mikuni-Takagaki et al., 1995). The osteocytes are located within the lacunae with their processes protruding through canaliculi forming an integrated cellular nexus enabling to sense changes in the biochemical and micromechanical environment of the bone (Davis and Praveen, 2006, Ren et al., 2015). This is evident through its wide surface coverage which is 400-fold higher than that of Harversian and Volkmann system and 100-fold higher than that of trabecular bone surface (Mullender et al., 1996, Johnson, 1966). This also allows for communication with nearby cells such as osteoblasts and bone lining cells on the bone surface (Florencio-Silva et al., 2015).

2.4 Skeletal mechanobiology

Frost, in his Utah's paradigm for bone physiology, mentions a mechanical feedback loop that consists of a mechanism that translates bone macroscale mechanical loads to the tissue and cells thereby eliciting a mechanobiological response. The Utah Paradigm (Figure 2-5) proposed by Frost summarises elegantly this feedback system relating bone physiology to 4 conditions (Jee, 2000, Frost, 2003):

1) Skeletal health and disease are determined by an intrinsic mechanism that operates on effector cells and nonmechanical agents (referred to as biochemical/hormone factors)
2) Mechanical loading represents the guiding system that directs spatial and time dependent effects on the intrinsic mechanism in 1)
3) After birth, neuromotor physiology and anatomy dominate control of those biologic mechanisms
4) Most nonmechanical factors can modulate but not mimic the mechanical control.

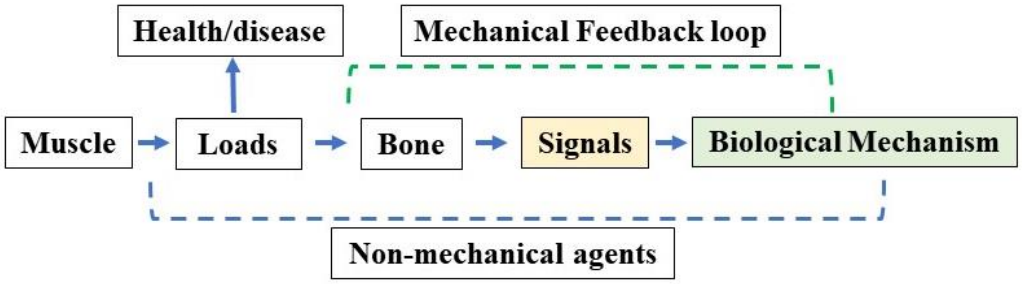


Figure 2-5. Frost's Utah Paradigm of bone physiology describing how mechanical factors dominate control of the biologic mechanisms that control changes in postnatal bone and mass. Adapted from Jee et al. (2000).

The focus of this thesis is to determine on a cellular level, what physiological thresholds incite bone marrow derived stem cells to guide them towards the bone lineage (mechanobiology) and the subsequent mechanism that facilitates this process (mechanotransduction). These two definitions will be briefly discussed below in relation to bone physiological fluid flow and pressure cues with a particular interest to MSCs. Moreover, the effects will be related specific to monolayer (2D) studies as 3D environmental interactions are more complex and have been extensively reviewed in (McCoy and O'Brien, 2010b).

2.4.1 Bone mechanobiology

As osteocytes are embedded in the lacunar-canalicular system, this provides a hierarchical and unique architectural layout with complex physical-biological interactions such as topographical cues from the surrounding matrix, matrix strain impacting cell deformation through adherence coupling mechanisms, build-up of pressure gradients and fluid flow transients due to translation of locomotion associated inertial effects among others (Ren et al., 2015). To date fluid flow has been postulated to be the most influential stimulus in bone adaptation. These observations are based on in vitro studies demonstrating that fluid flow induced shear stress elicits a stronger osteogenic response compared to direct mechanical strain in osteocytes (You et al., 2000, Owan et al., 1997a, McGarry et al., 2005, Verbruggen et al., 2014). The distribution of strain field due to flow induced drag forces or substrate/ECM strain is different, therefore the mechanotransduction mechanism involved in the activation of associated biological response may not be the same (Mullender et al., 2004, McGarry et al., 2005).

Fluid flow has been shown to modulate osteocyte activity through the following mechanisms: release of key signalling molecules such as Ca2+ (Lu et al., 2012, Jing et al., 2013), adenosine triphosphate (ATP) (Genetos et al., 2007), cyclin adenosine monophosphate (cAMP) (Kwon et al., 2010b) nitric oxide (Klein-Nulend et al., 1995a) and PGE2 (Kamel et al., 2010, Klein-Nulend et al., 1997a); release of RANKL cytokine and OPG ligand which in turn regulates osteoclast activity (Li et al., 2013, Li et al., 2012); bone anabolism through the inhibition of sclerostin/SOST as well as promoting dendrite formation and gap junction communication (Li et al., 2013, Kamioka et al., 2001, Zhang et al., 2006, Yellowley et al., 2000). Although only one study to date has investigated the effect of pressure on osteocytes, it reported similar effects to fluid flow such as increases in intracellular calcium activity, RANKL/OPG and *Cox2* mRNA expression (Liu, 2010). Current experimental models summarised with respect to fluid flow and pressure induced mechanoresponses in osteocyte are detailed in Table 1 and Table 3.

Osteoblasts are initially surrounded by soft osteoid matrix in regions with bigger porosities than LCS therefore they are postulated to experience a vastly different mechanical environment compared to osteocytes (McGarry et al., 2005, Bonewald and Johnson, 2008). Identifying the unique osteoblast mechanical environment is difficult due to remodelling kinetics of the ECM and surrounding architecture (Wittkowske et al.,

2016). Therefore, current experimental knowledge is based on osteocyte associated mechanical stimuli and magnitudes (Tables 2,3). Stimulation of osteoblasts with fluid flow induced shear stress demonstrated release of second messengers such as calcium ion flux (You et al., 2001, Hung et al., 1995, Batra et al., 2005), inositol triphosphate (Reich and Frangos, 1991), nitric oxide (Johnson et al., 1996), ATP (Genetos et al., 2007) and cAMP (Ogasawara et al., 2001). Moreover, fluid flow was shown to induce expression of osteoblast associated genes *COX2* and c-Fos (Pavalko et al., 1998, Shivaram et al., 2010, Ogasawara et al., 2001), *OPN* (Owan et al., 1997a, You et al., 2001, Batra et al., 2005), *RUNX2* and ALP (Yu et al., 2014, Mai et al., 2013b, Kapur et al., 2003) in addition to genes regulating matrix synthesis such as collagen type I (Mai et al., 2013a, Mai et al., 2013b, Myers et al., 2007). Long term stimulation has also promoted matrix deposition and mineralisation (Delaine-Smith et al., 2014, Mai et al., 2013a, Mai et al., 2013b).

Table 1. Summary of current experimental evidence regarding fluid flow induced osteogenic mechanoresponses in osteocytes

Magnitude	Frequency	Cell type	Biological effect	Reference
0.5 Pa	PFF, 5 Hz	chicken calvarial osteocytes	Increase in NO, leading to fluid flow induced PGE2 release	(Klein-Nulend et al., 1995a)
0.7 Pa	PFF, 5 Hz	mouse osteocytes	Increase in PGE2, PGI2 and PGF2α release; upregulation of PGH2 mRNA	(Klein-Nulend et al., 1997a)
0.7 Pa	PFF, 5 Hz	chicken calvarial osteocytes	Fluid flow induced PGE2 release dependent on Ca2+ intracellular signaling and actin integrity	(Ajubi et al., 1999)
0.4-1.62 Pa	steady	MLO-Y4	Increase in E11 mRNA expression, marker of osteocyte differentiation	(Zhang et al., 2006)
0.8 and 1.1 Pa	steady, OFF 0.5 Hz	MLO-Y4	Increase in stress fiber formation and dendrite processes, upregulation of *Cox2* mRNA and *Opn* protein release	(Ponik et al., 2007)
2 Pa	OFF, 1 Hz	MLO-Y4	Upregulation in Ca2+, PGE2 and ATP release dependent on the activation of hemichannels via PKC mechanism	(Genetos et al., 2007)
0.2-3.2 Pa	PFF, 0.5 Hz	MLO-Y4	β-catenin nuclear translocation, biphasic *Cox2* expression and PGE2 release	(Kamel et al., 2010)
0.5-5 Pa	OFF, 0.5,1 and 2 Hz	MLO-Y4	Magnitude dependent *Cox2* mRNA expression, OPG/RANKL decrease at higher shear stress magnitude, oscillations and duration of stimulation	(Li et al., 2012)
2 Pa	steady, OFF 1 Hz	MLO-Y4	Kinetics of intracellular Ca2+ transients are dependent on the dynamic nature of fluid flow	(Lu et al., 2012)
1.6-3.2 Pa	steady	MLO-Y4	Upregulation in Cx43 an inhibition of apoptosis; upregulation in OPG, decrease in RANKL and SOST	(Li et al., 2013)
0.05-0.8 Pa	steady	Ocy454	Decrease in SOST/sclerostin and RANKL, upregulation in DMP1	(Spatz et al., 2015)

OFF-oscillatory fluid flow

Table 2. Summary of current experimental evidence regarding fluid flow induced osteogenesis in osteoblasts

Magnitude	Frequency	Cell type	Biological effect	Reference
0.1-2.4 Pa	steady	rat osteoblasts	Upregulation in the intracellular IP3 levels and PGE2 release	(Reich and Frangos, 1991)
0.6 Pa	steady	rat osteoblasts	Upregulation in NO release	(Johnson et al., 1996)
0.7 Pa	PFF, 5Hz	mouse osteoblasts	Upregulation in NO and PGE2 release	(Klein-Nulend et al., 1997a)
Undetermined	n/a	MC3T3-E1	Upregulation in *Opn* mRNA expression independent of strain magnitude	(Owan et al., 1997b)
1.2 Pa	steady	MC3T3-E1	Upregulation in *Cox2* and c-Fos mediated by actin remodelling	(Pavalko et al., 1998)
0.4-1.2 Pa	PFF, 3-9 Hz	mouse osteoblasts	Magnitude dependent NO and PGE2 release	(Bakker et al., 2001)
2 Pa	OFF, 1Hz	MC3T3-E1	Upregulation Ca2+ and *Opn* mRNA expression	(You et al., 2001)
1-63 μPa	orbital motion	human osteoblasts	Increase in mRNA for ALP, fibronectin and fibronectin receptor; PGE2 and TGFβ1 release; cell proliferation	(Liegibel et al., 2004)
0.8/1 Pa	PFF, 0.5Hz	MG-63	Upregulation in Col1, MMP1 and MMP3 mRNA dependent on integrity of microtubule network	(Myers et al., 2007)
0.051 Pa	OFF	MLO-A5	Collagen synthesis and mineral deposition	(Delaine-Smith and Reilly, 2012, Delaine-Smith et al., 2014)
1.2 Pa	steady	MC3T3-E1	Upregulation in mRNA expression for ALP, *Runx2*, *Cox2*, Bmp2; Increased ALP activity, collagen synthesis and mineralisation	(Mai et al., 2013a, Mai et al., 2013b)
0.5 Pa	steady	rat osteoblasts	Upregulation in Col1 mRNA; Increase in fibronectin, collagen synthesis and cell proliferation, decrease in ALP	(Xing et al., 2014)
1.5–412 μPa	steady	MC3T3-E1	Upregulation in mRNA expression for ALP, OCN, Col1, *Runx2* and increase in cell proliferation	(Yu et al., 2014)
Undetermined	250 revolutions per minute	normal human osteoblasts	Increase in proliferation and mitochondrial metabolism, ALP activity and OCN synthesis; decrease in RANKL/OPG	(Aisha et al., 2015)

Table 3. Summary of current experimental evidence regarding pressure induced osteogenic effects in osteoblasts, osteocytes and MSCs

Pressure stimulus	Frequency	Cell type	Biological effect	Reference
50-200kPa	Static	Osteoblast-like MC3T3-E1 cells	Inhibition of osteoblast proliferation, promotion of osteoclast production	(Imamura et al., 1990)
100-200 kPa	Constant	Osteoblast-like MC3T3-E1 cells	Inhibition of proliferation and increase in PGE2 secretion	(Ozawa et al., 1990)
13 kPa	0.3Hz, 24 hrs	Mouse metatarsal rudiments	Increase in *in situ* mineral metabolism	(Burger et al., 1992)
13 kPa	0.3Hz, 24 hrs	Chicken calvariae osteocytes and periosteal fibroblasts	Increase in PGE2 release	(Klein-Nulend et al., 1995b)
13 kPa	0.3 Hz	Neonatal calvaria cells	Stimulation of osteoblastic activity defined by actin remodelling and ALP activity	(Roelofsen et al., 1995)
17.2-69 kPa	1 Hz, 10 cycles	Rat neonatal calvarial bone cells	Increased proliferation and cytosolic calcium concentration	(Brighton et al., 1996)
50-90 kPa	0.1 and 1 Hz, 20 min	Rat osteoblast-like cells 17/2.8	1 Hz increased cell saturation; decrease in ALP activity at 1Hz	(Vergne et al., 1996)
10-40 kPa	0.25 Hz or 1 Hz, 1 hr daily for 5 days	Osteoblasts from neonatal rat calvariae	Decrease in proliferation at 1Hz after 5 days of pressure; increase in ALP synthesis	(Nagatomi et al., 2001)
10–40 kPa	1 Hz, 1 hr daily	Osteoblasts from neonatal rat calvariae	Increase in collagen and calcium deposition	(Nagatomi et al., 2003)
3 MPa	0.33 Hz (triangular wave form), 1 hr/day	Primary osteoblasts from trabecular bone cores taken from epiphyses of metacarpal bone of 3-4-month-old calves	Increased osteoblast function only when osteocytes are present, increased osteocyte viability	(Takai et al., 2004)

Table 3. Summary of current experimental evidence regarding pressure induced osteogenic effects in osteobaslsts, osteocytes and MSCs (continuation)

Pressure stimulus	Frequency	Cell type	Biological effect	Reference
10-16 kPa	1 Hz	Rat bone marrow progenitor cells	Enhanced proliferation	(Maul et al., 2007)
0-68 kPa	0.5 Hz, triangular wave, 1hr	Osteoblast-like MC3T3-E1 cells	Increase in ATP release and *Cox2* mRNA expression, actin and microtubule remodelling	(Gardinier et al., 2009)
10-36 kPa	0.25 Hz, sinusoidal wave, 1hr/day	Rat bone marrow stromal cells from tibiae and femurs	Increase in osteogenic transcription factors Runx2, Dlx5, Msx2, Osterix; Increase in pERK1/2 activity and ALP synthesis	(Liu et al., 2009)
Negative pressure of 50 kPa at frequency of twice per day	30 min, Twice/day	hMSC	Low-intensity intermittent negative pressure inhibited the proliferation of cells but induced osteogenic differentiation.	(Yang et al., 2009)
68 kPa	0.5 Hz, triangular wave, 1-2 hrs	MLO-Y4 osteocyte like cells	Decrease in apoptosis; increase in intracellular activity, RANKL/OPG and *Cox2* mRNA activity	(Liu, 2010)
1-11 kPa	Intermittent 30 min stimulation, 7 hr break	Human bone marrow derived cells from hip	Increase in proliferation, collagen I and ALP synthesis	(Rottmar et al., 2011)
0-279 kPa	0.0001-2 Hz	Whole femurs of chick foetuses	Increased volume of diaphyseal collar, positive relation between frequency of stimulation and bone volume deposition	(Henstock et al., 2013)
10-100 kPa	2 Hz	hMSC	No changes in mRNA expression related to osteogenesis; no biochemical induction used	(Becquart et al., 2016)
10 kPa	static	Human bone marrow UE7T-13 cells	Increase in proliferation; mRNA upregulation of *BMP2*, *RUNX2*, *OSX*, ALP, *Col1α1*; Collagen synthesis and mineralisation	(Sugimoto et al., 2017)

2.4.2 Stem cell mechanobiology

Current paradigms postulate that both direct and indirect physicochemical cues within the stem cell niche allows for MSC self-renewal and differentiation to meet the circumstantial needs of the bone to maintain homeostasis (Lee et al., 2011, Ohlstein et al., 2004). One of the early studies looking at the kinetics of bone resorption and bone formation using an inducible osteoblast ablation model noted that the mice developed a reversible form of osteopenia, although the osteoclast function was not affected (Corral et al., 1998). In vitro and in vivo studies employing osteocyte depletion or transgenic preclinical models have demonstrated that loading induced osteogenesis can occur independently of osteocyte activity and relies on activity of osteoblasts, osteoprogenitors and their precursor, the MSCs (David et al., 2007, Turner et al., 1998, Tewari et al., 2015, Curtis et al., 2018, Kwon et al., 2012, Moore et al., 2018b). In a unique approach, Chen et al. (2016) transplanted fluorescent tagged MSCs to enable lineage tracing of these cells in a model of ulnar loading. The authors detected cells derived from fluorescent MSCs embedded within the bone matrix and near active bone forming surfaces, demonstrating that mechanical cues home the MSCs to the bone surface and promote osteogenic lineage commitment (Chen et al., 2016a). Although, the mechanism by which MSCs contribute to bone anabolism is yet to be fully elucidated, in vitro studies have highlighted that both indirect paracrine signalling and direct biophysical stimulation are at play (Wittkowske et al., 2016, Ren et al., 2015, Chen and Jacobs, 2013).

The involvement of paracrine signalling in MSC osteogenesis was demonstrated utilising in vitro models whereby the secretome of mechanically stimulated mature bone cells was collected and used to treat MSCs. Fluid flow stimulated osteocytes were shown to release factors that enhanced MSC recruitment, proliferation and differentiation over factors secreted under static conditions (Brady et al., 2015a, Hoey et al., 2011, Klein-Nulend et al., 2013). Interestingly, the fluid flow stimulated osteoblast secretome was found to either have no effect or inhibit MSC proliferation (Brady et al., 2015a). The paracrine signalling homing mechanism of MSC and involvement in bone anabolism requires further exploration. Moreover, how the osteocyte secretome changes in response to various physiological mechanical cues and subsequent effects of MSC osteogenesis is yet to be determined.

The biophysical environment of MSCs and how it changes during locomotion is of ongoing exploration. Current mathematical and theoretical models postulate that locomotion induced pressure gradients and associated fluid flow within marrow and the LCS network may be driving forces in MSC osteogenesis (Ren et al., 2015, Metzger et al., 2015a, Gurkan and Akkus, 2008b). In vitro studies to date observed that fluid flow promotes osteogenic effects in MSCs such as increase in intracellular calcium and ATP signalling (Li et al., 2004, Riddle et al., 2006), expression of osteoblastic markers (Kreke et al., 2005, Kreke et al., 2008), changes in DNA methylation of osteoblastic genes (Arnsdorf et al., 2010) and subsequent activation of bone associated signalling pathways (Table 4) (Iolascon et al., 2013, Chen and Jacobs, 2013). This behaviour is in response to fluid shear magnitudes based on seminal research by Weinbaum et al. (1994) describing that osteocytes within LCS network sustain shear stresses up to 3 Pa during bone bending. Therefore, how MSCs behave in response to magnitudes higher than 3 Pa, which have recently been predicted to occur within the marrow space using computational modelling, is yet to be determined (Metzger et al., 2015a, Verbruggen et al., 2014, Price et al., 2011). Moreover, the variation in fluid flow protocols has highlighted the inconsistent and temporal effects in relation to osteogenic markers and commitment which have been attributed to chemotransport and biochemical induction effects (Riddle et al., 2008, Hoey et al., 2012, Li et al., 2004, Delaine-Smith and Reilly, 2012). For example, steady and pulsatile flow profiles were found to provide the greatest stimulatory effect compared to oscillatory fluid flow based on percentage osteoblast cells responding to fluid flow stimulation and the magnitude of the response. This effect has been attributed to the net fluid mass transport, however due to repetitive bone loading/unloading motion, oscillatory fluid flow is considered to be most closely related to human kinematics (Jacobs et al., 1998). Therefore, a unified and standardised approach investigating the effect of physiologically relevant fluid flow parameters on MSC osteogenic responses and commitment is necessary.

Loading induced fluid flow streams within the bone are elicited by strain induced pressure transients. However, the effect of pressure on stem cell osteogenesis has been explored to a lesser degree when compared to fluid flow. Currently, most of the pressure magnitudes utilised in in vitro studies are representative of in vivo conditions at rest periods which are less than 10 kPa or a quarter of systolic pressure (Table 3) (Gurkan and Akkus, 2008b). During locomotion, the pressures within bone and marrow are

known to be of transient nature and oscillate depending on the muscle contraction and impact stresses during locomotion reaching up to 300 kPa in magnitude (Ren et al., 2015, Downey et al., 1988). Current evidence of pressure induced osteogenesis suggests that low physiological pressures (<100 kPa) promote both early osteogenic responses described as upregulation in osteogenic factors (*Runx2*, *Dlx5*, *Msx2*, *Osterix*, *BMP2*, ALP) and osteogenic lineage commitment represented by collagen and mineral deposition (Table 3) (Liu et al., 2009, Sugimoto et al., 2017). However, a recent report suggests that pressure does not promote an osteogenic phenotype (Becquart et al., 2016). The disparity in the results may be attributed to the variation in pressure stimulation protocols. Moreover, it is unknown what optimum pressure conditions are most favourable to trigger MSC osteogenesis given the limited exploration of the effects of pressure magnitudes and frequencies associated with human locomotion. Similar to fluid flow, a systematic and standardised approach is required to fully elucidate the involvement of pressure in MSC osteogenesis and determine its influence in this response compared to fluid flow.

Table 4. Summary of current experimental evidence regarding direct fluid flow induced osteogenesis in MSCs

Magnitude	Frequency	Cell type	Biological effect	Reference
2 Pa	OFF 1Hz	hMSC	Increase Ca2+ mobilisation, proliferation, *OPN* and OCN mRNA levels; No change in Col1 and *Runx2* mRNA levels. Decrease in ALP activity	(Li et al., 2004)
0.16 Pa	1.67 mHz OFF/ steady	rBMSC	Increase in *OPN* (protein and mRNA), and BSP mRNA; decrease in PGE2 release after flow stimulation	(Kreke et al., 2005)
0.5, 1 and 2 Pa	OFF 1Hz	hMSC	Flow rate dependent ATP release via vesicular mechanism; prerequisite for intracellular Ca2+ flux, activation of calcineurin, NFATC1 nuclear translocation and proliferation; Increase pERK1/2	(Riddle et al., 2006, Riddle et al., 2007)
0.5-2 Pa	OFF 1Hz	hMSC	Fluid flow induced proliferation dependent on flow rate associated effects rather shear stress magnitude; ATP release associated mechanism	riddle 2008 (Riddle et al., 2008)
0.23 Pa	1.67 mHz OFF/ steady	rBMSC	Increase in pERK and p38 for steady flow; PGE2 release-OFF; upregulation in mRNA Col1α1, *OPN*, BSP, and OCN long term with either flow regime	(Kreke et al., 2008)
1 Pa	OFF 1Hz	C3H10 T1/2	Increase in *Runx2*, PPARγ and SOX9 mRNA expression; RhoA and ROCKII mediated fluid flow induced *Runx2* expression	(Arnsdorf et al., 2009)
0.23 /0.43 Pa	15, 44 and 74 mHz PFF	rBMSC	Increase in mRNA levels: *Cox2*, COL1α1, *OPN*, BSP, VEGFA, TGFβ1, BMP2 and BMP7 and OC; *OPN* and OC secretion	(Sharp et al., 2009)
1.2 Pa	steady	hMSC	Flow induced mRNA expression: increase in ALP, decrease in Col1, no change in *Runx2*. Protein expression: upregulation pERK1/2 and p38, Cx43.	(Grellier et al., 2009)
1 Pa	OFF 1Hz	C3H10 T1/2	Fluid flow induced epigenetic changes: *OPN* increase in mRNA expression and decrease DNA methylation	(Arnsdorf et al., 2010)
0.4, 1.5 and 2.2 Pa	steady	hMSC	Fluid flow upregulated ALP activity and BMP2 and mRNA expression and BSP independent of biochemical induction	(Yourek et al., 2010)
Undetermined	OFF, 45 cycles/min	hES-MP	Long term collagen and mineral deposition in biochemical induction medium is not affected by fluid flow	(Delaine-Smith and Reilly, 2012)
1 and 2 Pa	OFF 1Hz	hMSC	Fluid flow induced upregulation in *COX2*, *RUNX2*, *OPN*, BMP2 mRNA expression and proliferation; primary cilia mediated fluid flow induced osteogenesis	(Hoey et al., 2012)
0.7 Pa (0.1-4.2 Pa)	2.5 Hz	hMSC	Fluid flow induced upregulation in *COX2*, IER3, EGR1, IGF1, IGFBP1, ITGB1, VEGFA and FGF2 mRNA; upregulation in nitric oxide, pERK1/2	(Becquart et al., 2016)

2.5 Stem cell mechanotransduction mechanisms

Translation of mechanical cues into a biochemical response is enabled by the presence of mechanosensors and the subsequent coupling of the intrinsic effector mechanisms. Mechanotransduction mechanisms are related to the conformational changes in the cellular structures such as stretch-activated ion channels, integrin complexes, cell-cell adhesions among others to allow for activation of downstream signalling (Klein-Nulend et al., 2012). To date, several mechanosensors and associated mechanotransduction mechanisms have been discovered in relation to osteogenesis in bone cells, although not all of them have been confirmed to be related to MSC osteogenesis (MacQueen et al., 2013). They are largely categorised into mechanosensitive ion channels, cell-matrix adhesion proteins (integrins), cell-cell membrane proteins (connexins), membrane structures (lipid rafts, glycocalyx), cell cytoskeleton including primary cilia, and mechanically activated intracellular signalling systems (GTPases and G-protein coupled receptors, MAPK, β-catenin, Calcium signalling, Akt, FAK, purinergic signalling among others) (Thompson et al., 2012, Rubin et al., 2006).

One of the early responses of MSCs to fluid flow which were found to be paramount for osteogenic differentiation were identified as purinergic (adenosine triphosphate/ATP) and second messenger signalling as well as intracellular calcium fluxes (Li et al., 2004, Riddle et al., 2006, Riddle et al., 2007). Release of ATP in response to fluid flow stimulation was found to occur via a vesicular mechanism. This purinergic signalling has been shown to be a pre-requisite for calcium signalling, activation of calcineurin, nuclear translocation of NFATC1 and proliferation, although the channels involved in this process are yet to be identified (Riddle et al., 2007). Fluid flow induced calcium signalling and subsequent osteogenic response was found to be dependent on the presence of mechanosensitive channels TRPV4 and TRPM7 (Corrigan et al., 2018, Wen et al., 2012, Liu et al., 2015b). Moreover, TRPM7 was also found to be critical for pressure induced calcium signalling and upregulation of osteogenic markers *Runx2*, ALP, and NFATC1 highlighting that various mechanical stimuli may activate a similar mechanotransduction mechanism (Xiao et al., 2015). Similarly, mechanosensitive channel Piezo1 was also recognised to be paramount for pressure induced osteogenesis and lineage commitment of MSCs (Sugimoto et al., 2017).

Cell-matrix and cell-cell interactions have also been investigated as potential mechanotransduction mechanisms involved in MSC osteogenesis. The cell interacts with the underlying extracellular matrix (ECM) via focal adhesion attachments which are complex dynamic structures that link integrins and scaffold proteins to the cytoskeleton (Baumann, 2018, Ivanovska et al., 2015). Fluid flow induced ERK1/2 and focal adhesion kinase signalling, which are osteogenic associated pathways, were found to be dependent on the presence of β1 integrin (Liu et al., 2014). The expression of integrins are specific to the type of ECM protein and are postulated to trigger potentially different mechanotransduction mechanisms (Xiao and Quarles, 2015, Fraioli et al., 2015, Malmstrom et al., 2011, Treiser et al., 2010). Cell-cell interaction such as gap junctions which are known mechanosensors involved in osteocyte communication in bone were found to be expressed in MSCs as well. However, their involvement in loading induced MSC osteogenesis are yet to be explored (Batra et al., 2012a, Batra et al., 2012b).

MSC fate is known to be coupled with re-arrangement of the cell cytoskeleton architecture which also provides a unique platform for transduction of mechanical cues (Ingber, 1997, Matthews et al., 2006). The cytoskeletal extension, the primary cilium, has also proven to be a pivotal mechanotransducer in MSC osteogenesis and bone anabolism (Chen et al., 2016a, Corrigan et al., 2018, Hoey et al., 2012). Therefore, these two cellular components will be reviewed in greater detail.

2.5.1 Cytoskeleton

The cytoskeleton, a principle component of the internal cell structure, defines the cell shape, stiffness, ability to move, signal transduction and communication (Ingber, 1997, Treiser et al., 2010). It is composed of three structural proteins: tension bearing actin and intermediate filaments and compression bearing microtubule filaments (Figure 2-6). All three elements are arranged into distinct networks which in turn compartmentalizes the intracellular components. The polymerisation and depolymerisation of these networks generate direct forces directing cell shape and kinetics and are regulated by regulatory proteins. Internal and external cues (biochemical and mechanical) can affect the activity of regulatory proteins and thereby regulating organisation of the cytoskeletal network (Fletcher and Mullins, 2010).

Actin is a dynamic cytoskeletal component which responds to changes in extracellular cues such as cell shape, motility and cell-cell communication. Assembly of

actin stress fibre bundles in adherent cells is enabled by its attachment to integrins via adaptor proteins (Naumanen et al., 2008). Cytoskeletal contractility, which is generated by the isomeric tension in the actin network, has been shown to regulates MSC cell fate. Chemical disruption of structural actin (F-actin) was found to favour adipogenesis versus osteogenesis (Kilian et al., 2010). Interestingly, depolymerisation of actin enables trafficking of the soluble form of actin (G-actin) to the nucleus which leads to nuclear transport of Yes-associated protein (YAP) and thereby allowing transcription of *Runx2* (Sen et al., 2015). This highlights the regulatory complexity of actin involved in MSC osteogenesis related to both structural reinforcement and intracellular trafficking. Mechanical stimulation such as fluid flow and pressure has been shown to instigate actin remodelling in all bone cells including MSCs depicted by the stress fibre formation. The integrity and remodelling of the actin network was found to be paramount for fluid flow induced osteogenesis. Small GTPase Rhoa A and its effector protein ROCKII were found to be involved in this process (Arnsdorf et al., 2009). A similar actin remodelling mechanism via RhoA activity was found to be elicited by pressure in hMSCs (Zhao et al., 2015). Overall, mechanically induced osteogenesis in MSCs to date has been attributed mainly to changes in actin dynamics although its interplay with the other two filaments and their contribution to this process has not fully been explored.

Intermediate filaments (IF) are the least studied cytoskeletal element within the musculoskeletal system although it carries functions that go beyond a strictly mechanical role such as adhesion, migration and invasion (Ivaska et al., 2007, Robert et al., 2016). IF proteins are classified into subgroups based on their structure and homology with vimentin being the most widely distributed found in fibroblasts, leukocytes, endothelial cells and MSCs among others (Block et al., 2015). Furthermore, IFs are the least stiff component of the three cytoskeletal structures which allow for fast assembly/disassembly kinetics during cell remodelling and cellular transport. This is exemplified by the IF precursors, known as squiggles and particles, that move at the cell periphery and then join together to create longer filaments that incorporate into the larger network. The reversible action during breakdown of IF into squiggles and particles at the cell edge during lamellipodia formation allows for cell movement (Helfand et al., 2011). Spatial regulation of IF is targeted to different subcellular locations via cytoskeletal linker proteins to both integrin ECM interaction but also the other cytoskeletal elements such as actin and microtubules, extensively described in (Robert et al., 2016, Jiu et al.,

2015). Unlike microtubules and actin filaments, intermediate filaments are not polarized and cannot support directional movement of molecular motors and thus rely on the instigating dynamics of actin (Fletcher and Mullins, 2010).

The involvement of IF in MSC osteogenesis is understudied. Downregulation of IFs associated protein vimentin was found to promote osteogenic differentiation of MSCs and osteoblasts via regulation of transcription factor Atf4 (Lian et al., 2009). Moreover, administration of biochemical factors that disrupt IF demonstrated improved bone quantity and quality in ovariectomised mice (Khedgikar et al., 2013). In a model of high magnitude pressure induced chondrogenesis of MSCs, the IF were found to undergo structural reorganisation suggesting that this understudied cytoskeletal component can be mechanically modulated (Steward et al., 2013).Whether, IF network undergoes changes in response to pro-osteogenic mechanical cues and is involved in loading induced MSC osteogenesis is yet to be explored.

Microtubules are the stiffest cytoskeletal components, given their duty to withstand compressive forces in addition to constant remodelling to sustain cellular activities such as mitosis, motility, intracellular transport, and maintenance of cell shape (Alfaro-Aco and Petry, 2015). The microtubule remodelling mechanism depends on two states: stably growing and rapidly shrinking. This enables rapid reorganisation as it is dynamic and not dependent on regulatory proteins (Holy and Leibler, 1994). Microtubules tend to grow from the microtubule organising center (MTOC), which is also known as centrosome, to the plasma membrane providing basic organisation of the cytoplasm including positioning of organelles (Nature Education, 2014). The role of microtubules in loading induced MSC osteogenesis is yet to be fully explored. Microtubules were found to not be directly involved in biochemical induced osteogenesis of MSCs, but were regulating actin dynamics and thereby cell shape (Rodriguez et al., 2004). In contrast, microtubule mechanotransduction mechanisms were found to be important in bone committed cells. Biochemical disruption of microtubules and not actin abolished fluid flow induced expression of collagen I, MMP 1 and MMP 3 in osteoblasts. Moreover, pressure stimulation has been shown to cause dispersion of microtubules beyond perinuclear regions in osteoblasts, although its biological significance it is yet to be determined (Gardinier et al., 2009). Microtubules in osteocytes exposed to fluid flow were shown to undergo remodelling within the cytoplasm but also aggregate around the primary cilium, as possible reinforcement

mechanism to fluid drag or to modulate the cilium mechanosensation abilities (Espinha et al., 2014b). The primary cilium is composed of nine doublet microtubulues which have been post-translationally modified by acetylation and project into the extracellular space as an antennae filled with receptors, giving it a mechanosensing and transducer capabilities (Figure 2-7) (Ishikawa and Marshall, 2014, Spasic and Jacobs, 2017). In stem cells, the primary cilium has been shown to be implicated in loading induced osteogenesis and bone anabolism which will be discussed further in the section below.

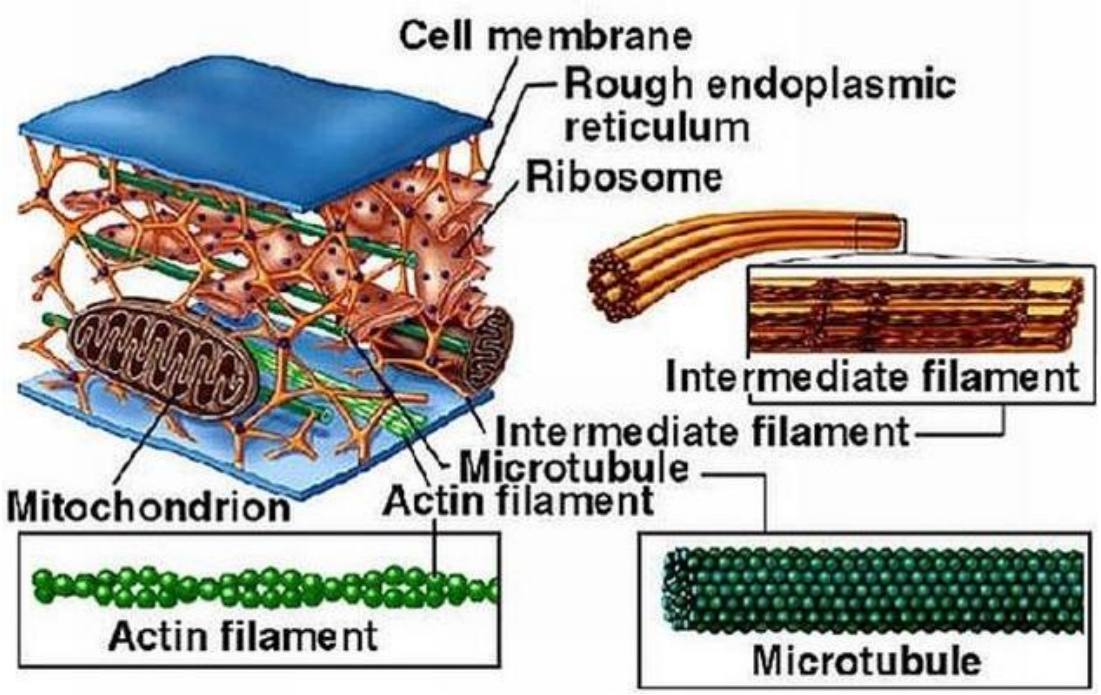


Figure 2-6. Cartoon of cytoskeletal components depicted by actin, microtubule and intermediate filaments (ScienceAid.net).

2.6 Primary cilia- the cytoskeletal antennae

The primary cilium is a small cellular organelle extending apically in the extracellular milieu and ideally positioned to respond to mechanical and biochemical stimuli (Christensen et al., 2012, Prasad et al., 2014, Satir and Christensen, 2007, Singla and Reiter, 2006, Upadhyay et al., 2014). Cilium axoneme assembly relies on a process known as intraflagellar transport (IFT) and is organised into a ring of nine doublet microtubules which extend from the basal body, the microtubule organisational centre (Figure 2-7) (Praetorius and Spring, 2005, Kobayashi and Dynlacht, 2011, Sengupta and Barr, 2014, Reiter et al., 2012). Research into this unique organelle was spurred by discovery of genetic disorders that are associated with dysregulation of primary cilium formation such as polycystic kidney diseases, Bardet Biedl Syndrome, polydactyly among others collectively known as ciliopathies (Reiter and Leroux, 2017). Once thought to be a vestigial organelle, now it is proven to be a complex signalling reactor with many functions yet to be identified.

In the musculoskeletal system, impaired primary cilia formation is associated with serious skeletal defects observed in some ciliopathies such as Jeune Syndrome (JATD), Oro-facial-digital syndrome (ORS) or Ellis van Creveld syndrome (EVC) (Haycraft and Serra, 2008, Serra, 2008, Fliegauf et al., 2007, Ruiz-Perez et al., 2007, Huber and Cormier-Daire, 2012, Pacheco et al., 2012, Koyama et al., 2007). Primary cilia functions are broad and complex due to its unique architecture of a microdomain that concentrates receptors, ion channels and downstream signalling effectors and is involved in embryonic development, cell proliferation and differentiation (Hsiao et al., 2012, Nachury, 2014, Christensen et al., 2012). In adult bone, the primary cilium has been identified as an important component in dictating bone adaptation to loading, in particular modulating osteogenic lineage commitment of osteoprogenitors and MSCs (Spasic and Jacobs, 2017). In a mouse model of conditional knock out of primary cilia in osteoblasts and osteocytes by deleting ciliary protein Kif3a under Colα1(I)2.3 promoter demonstrated that loading induced bone formation was significantly decreased (Temiyasathit et al., 2012). In addition, using a bone implant model in the same transgenic mouse, an impaired healing process around the implant was observed. This was attributed due to the inability of osteoblasts to proliferate, deposit calcium and lay down collagen fibres (Leucht et al., 2013). Kif3a ablation under a osteocalcin promoter which is expressed at later stages of osteoblast differentiation, exhibited decreased

proliferation rate and expression of osteoblast differentiation markers (Qiu et al., 2012). Impairment of primary cilium formation in periosteal osteochondroprogenitor cells (Prx1CreER-GFP; Ift88fl/fl) was found to disrupt endochondral and intramembranous ossification in addition to abrogation of fluid flow induced osteogenic mRNA expression (Moore et al., 2018b, Moore et al., 2018a). These models demonstrate that primary cilium in osteocyte, osteoblasts and periosteal osteochondroprogenitor cells plays a pivotal role in skeletal development and loading induced bone mechanoadaptation.

The role of MSCs in loading induced bone anabolic response was found to be equally important as elegantly demonstrated by Chen et al. (2016). In a unique approach, bone marrow was transplanted from a transgenic mouse containing GFP tagged cells with Kif3a floxed allele to a lethality irradiated mouse. This approach exhibited a dual function: firstly, it enabled tracing of MSCs osteogenic lineage commitment due to mechanical loading. Secondly, by inducing conditional knock out of the floxed Kif3a allele to impair ciliogenesis only in the transplanted cells including MSCs, it provided further evidence that loading induced osteogenic lineage commitment of MSCs is dependent on the primary cilium. This methodology showcased a pivotal role of cilia in loading induced bone anabolic response. However, it is currently unclear whether this is due to paracrine signalling from osteocytes or direct mechanosensing/mechanotransduction pertaining to this specialised organelle in MSCs (Brady et al., 2015a, Hoey et al., 2011, Birmingham et al., 2012). Therefore, in vitro models of loading induced osteogenesis in MSCs can further explore and decouple these effects.

In vitro, the MSC primary cilium has an incidence of 80-90% with a length ranging 0.4-9 μm (Brown et al., 2014, Hoey et al., 2012). Abrogation of primary cilium using siRNA technology against ciliary protein Intraflagellar Transport 88, demonstrated that fluid flow induced early osteogenic responses in MSCs was inhibited (Hoey et al., 2012). Interestingly, transmembrane ion channel transient receptor potential cation channel subfamily V member 4 (TRPV4) was found to localize to the primary cilium in MSCs and similarly implicated in fluid flow induced calcium signalling and early osteogenic gene expression highlighting a ciliary associated mechanotransduction pathway (Corrigan et al., 2018). Biochemically induced osteogenic differentiation of MSCs was equally found to require an intact functioning primary cilium (Tummala et al., 2010). Moreover, this unique organelle has also been highlighted in its involvement

in paracrine signalling from fluid flow stimulated osteocytes (Hoey et al., 2011). Since the primary cilium was found to be paramount for short term fluid flow induced osteogenic response, it is unknown whether it has a similar functional role in pressure induced osteogenesis.

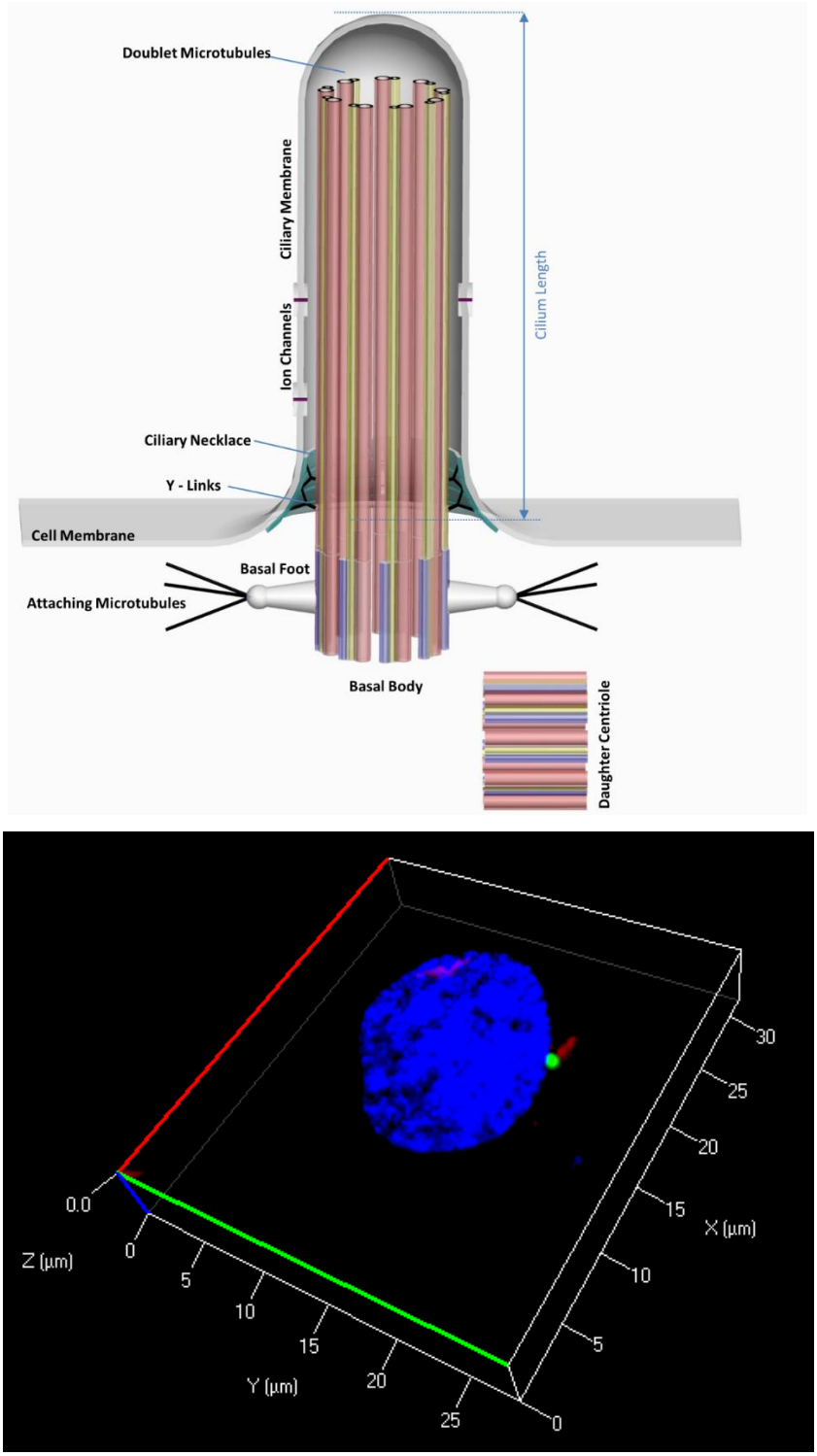


Figure 2-7. Schematic of primary cilia structure adapted from (Eichholz and Hoey, 2017) and 3D reconstruction of a confocal image representing primary cilia stained with acetylated α tubulin and Alexa 564 fluorochrome (red) and centrosome stained by pericentrin and Alexa488 fluorochrome (green) on a human bone marrow stem cell. Nucleus is blue by DAPI (4′,6-diamidino-2-phenylindole).

2.7 Bioreactor systems for orthopaedic research

Bioreactor is a mechanical system that simulates the environmental conditions at various scales and thereby it facilitates the identification of the mechanisms of cellular mechanotransduction, biochemical signalling and tissue morphogenesis. Its application is vast in both basic research and tissue regenerative medicine. Due to complexity of bone anatomy from biochemical signalling, complex biological matrix architecture and its interface with cellular components, in addition to mechanical modulation of bone integrity, it provides a design challenge interfacing several disciplines.

The key variables reported to influence loading induced osteogenic response in bone cells (osteocyte, osteoblasts and stem cells) are: nature of the stimulus (pressure, fluid shear, strain, voltage, microgravity, magnetic fields etc.), chemotransport, O_2 tension, loading time, loading frequency, spatial organisation (2D/3D), substrate matrix, biochemical factors and exposure time; cell source and its culture method (passage) (Donahue et al., 2001, Riddle et al., 2008, Yeatts et al., 2013, Yeatts and Fisher, 2011, McCoy and O'Brien, 2010b, Xie et al., 2016, Chen et al., 2016b, Brady et al., 2015a, Jacobs et al., 1998). Numerous custom designs of *in vitro* flow bioreactors and custom protocols are reported in reported in literature, sometimes with contradictory effects (Yeatts et al., 2013, Potier et al., 2010, McCoy and O'Brien, 2010b). The disparity in observations can be related to the difference in the setup, mechanical and biological designs, as well as lack of standards relating what would be the optimum physiological relevant stimulus for eliciting a biomimetic osteogenic response with inference to a bone physiological response. In this thesis, fluid flow and pressure bioreactors and design features necessary to emulate bone physiological loading conditions on a cellular level will be considered and presented in Chapters 3 and 4. Therefore, current trends in bioreactors for orthopaedic research will be briefly discussed below.

2.7.1 Fluid flow bioreactors

To date, several mechanical designs have been described in literature with the aim to mimic the bone physiological fluid flow: perfusion systems, parallel plate flow chamber (PPFC) and rocking platform (**Figure 2-8**) (Yeatts et al., 2013, Yeatts and Fisher, 2011, Gaspar et al., 2012). These systems, although operate on different physical principles, aim to mimic the fluid drag and inertial effects, to some degree similar to what would be elicited within lacunar-canalicular system. The biophysical nature of

interstitial fluid interaction between bone composite inorganic/organic matrix, cellular component and architectural arrangement of the LCS/bone marrow is a complex physics problem. Therefore, a simplification of the system is the use of Newtonian fluid physics to define a shear stress field within the laminar range (Re<100). To achieve this, parallel plate configuration systems are the most widely used whereby a pressure gradient, either gravity fed or active pumping, forces fluid flow within the chamber created by the system with maximum shear stress achieved at the plate surface (Jacobs et al., 1998, Nauman et al., 1999). The parallel plate design is advantageous compared to other systems (rocking platform and cone and plate bioreactor) due to simplicity of equipment, homogenous shear stress field, ease of medium sampling/exchange, configuration for optical visualisation (microscopy) and small volumetric fluid requirement. In addition, the system can be used for custom flow profiles and shear gradients (Brown, 2000). A disadvantage of this system is difficulty to perform large scale studies due to system constrains (used of multiple PPFCs, pumping system) therefore rocking platform may be an alternative. The rocking platform system imparts fluid shear stress due to translation of inertial effects of see-saw motion to the fluid within the culture plates. The fluid shear within the plate can tailored by adjusting the fluid height, rocking angle and frequency of rocking motion, however the shear stresses generated are limited to >0.5Pa with time dependent heterogenous distribution (Tucker et al., 2014, Corrigan et al., 2018). Cone and plate bioreactor is a simple system that operates on tangential flow whereby a rotation imposed about a cone axis oriented perpendicular to the surface of a flat plate. This system can produce both homogenous and heterogenous shear stresses in laminar and turbulent range by adjusting the height of the cone from plate surface, radius of the cone and rotational speed (Brown, 2000). Although this fluid flow system is usually used for monolayer cell culture of vascular cells, as the flow profiles can be adjusted to be atherogenic or atherosclerotic, modification of this system has been used as a perfusion system for scaffold culture for orthopaedic research (Teixeira et al., 2014, Spruell and Baker, 2013).

Both PPFC, rocking platform and cone and plate bioreactors are considered 2D bioreactors as it caters only for mechanical stimulation of monolayers cultures. However, both PPFC and cone and plate bioreactors have been reconfigured for fluid flow stimulation of cells culture on scaffolds, also known as 3D culture (McCoy and O'Brien, 2010b). These bioreactors have the added complexity of spatial organisation and

substrate-ECM cues. Although 3D culture can be considered more biologically and anatomically mimetic, it also gives rise to heterogenous fluid shear fields and cellular organisation, which makes the biological output difficult to interpret.

Rise in the use of computational fluid dynamics (CFD) modelling has enabled to graphically map the distribution of shear stress, velocity fields as well as interaction of cells with fluid flow allowing for a greater depth in design refinement of bioreactor spatial organisation (Metzger et al., 2015a, Vaughan et al., 2015, Verbruggen et al., 2014, Vaughan et al., 2013). This is particularly important in determining the areas within the chamber that do not abide by laminar flow (inertial forces<drag forces) such as in the use of gaskets with geometrical features to create the microchannel interfacing with cells (Anderson and Knothe Tate, 2007).

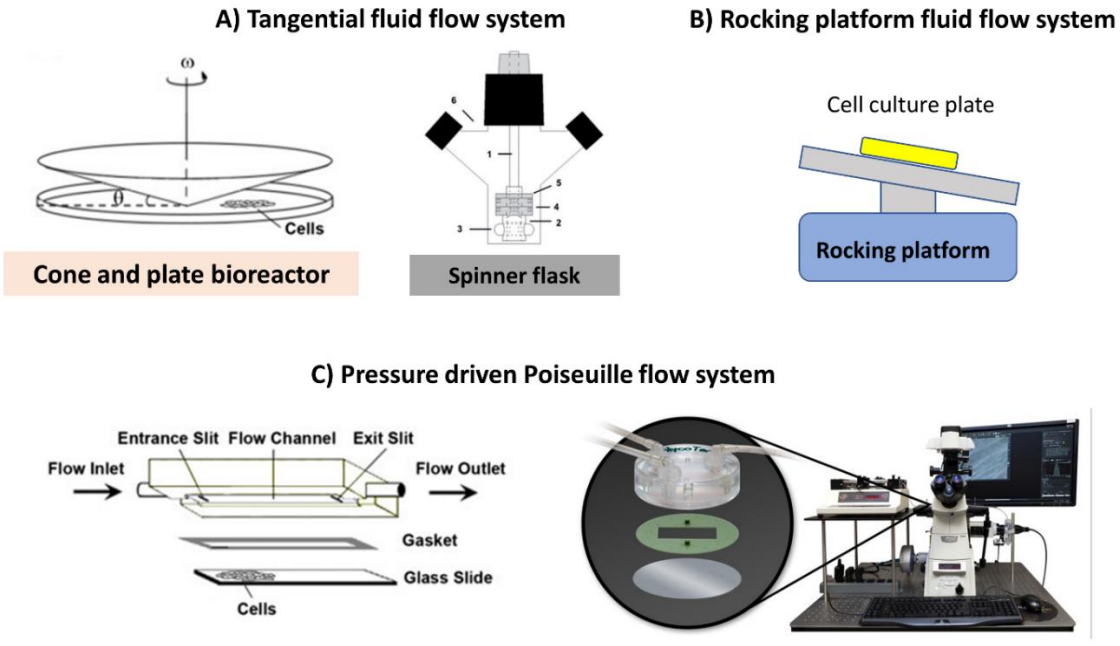


Figure 2-8 Examples of fluid flow bioreactors that operate on a) tangential fluid flow such as plate bioreactors (Hsieh et al., 2014) and spinner flask (Teixeira et al., 2014)b) see-saw rocking motion producing heterogenous flow (Tucker et al., 2014) and c) pressure driven fluid flow bioreactors such as parallel plate flow chamber for larger scale studies (left) (Hsieh et al., 2014) and adaptation of the bioreactor for imaging on microscopes (left) (GlycoTech Corporation) (Palange et al., 2012).

2.7.2 Pressure bioreactors

Few pressure bioreactor designs for bone research have been reported to date and all of them rely on the physics of fluid compressibility (**Figure 2-9**). The pressurisation of the fluid matter is the main feature design that dictates the bioreactor type: gas or liquid (Hess et al., 2010, Nagatomi et al., 2003, Gardinier et al., 2009, Gardinier et al., 2014, Becquart et al., 2016). The gas type bioreactors are simple to use since standard cell culture incubators can be employed as the chamber system, maximising output by following standard operating cell culture protocols. However, it has two limitations: from the engineering aspect, the pressure range may be limited by the inherent safety features of bioreactors which can be overcome by custom chamber designs; from a biological perspective, pressurisation of a nitrogen gas mixture containing the standard 3-5% CO_2 may enhances gas dissolution in medium and thereby affecting the pH balance in the medium, hence inclusion of buffering agents and temporal monitoring may be required (Roelofsen et al., 1995, Klein-Nulend et al., 1997b, Zhao et al., 2015, Reinwald et al., 2015). Fluid based pressurisation systems employing either water or oil-based medium may offer advantages over gas pressure systems in several ways. Fluid based systems allow for a greater range of pressure gradients to occur without causing large volumetric fluctuations due to incompressibility of the fluid. The system can be easily reconfigured from a fluid flow bioreactor by reusing the same perfusion/pump system, although considerations towards tubing material and O-ring/sealing method of the chamber should be taken account of to prevent loss of pressure and dampening effects. Moreover, the effect of pressure and fluid flow can be studied simultaneously effectively mimicking the pressure gradients and inertial effects that occur in bone within the LCS system during bone loading.

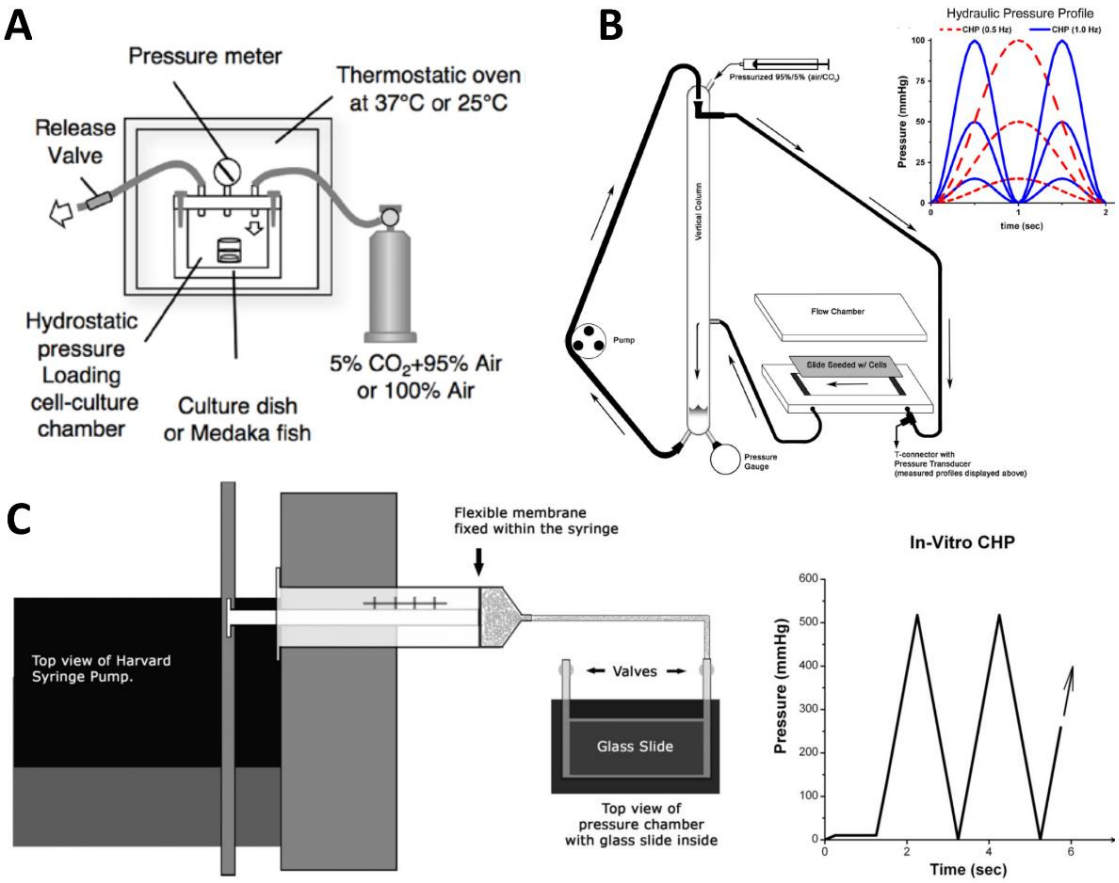


Figure 2-9 Examples of pressure bioreactor systems that operate on gas compression (Sugimoto et al., 2017) (a) and water pressurisation (Gardinier et al., 2009) (c) that can be adapted for fluid flow and pressure stimulation (b) (Gardinier et al., 2014)

2.8 Summary

Bone is a mechanically regulated tissue, whereby translation of biophysical cues from macroscale environment to the microscale cellular milieu modulates tissue homeostasis. Fluid flow and pressure stimuli are thought to be the dominant biophysical cues to instigate an osteogenic biological response and mostly studied in bone committed cells (summarised in Tables 1-4). The effect of physiologically relevant fluid flow and pressure cues within the marrow milieu during loading on MSC osteogenesis is yet to be fully explored. Moreover, the nature of mechanical regimes in terms of magnitude, frequency and duration of stimulation that is most suitable to promote MSC osteogenic lineage commitment has not been identified. Therefore, first two thesis objectives will determine what physiological bone related fluid flow and pressure stimuli promote MSCs osteogenesis.

Whilst several mechanisms of fluid flow induced osteogenesis have been described to date, only one mechanotransduction mechanism of pressure induced osteogenesis has been reported (Sugimoto et al., 2017). Therefore, this requires investigation with particular interest to the cell cytoskeleton, given the observation of cytoskeletal changes in bone cell to pressure stimuli (Gardinier et al., 2009). In addition, the primary cilium, as a cytoskeletal extension, warrants investigation of possible involvement in pressure mechanotransduction, since it has been shown to be paramount for loading induced osteogenesis (Chen et al., 2016a, Hoey et al., 2012). Hence, the last two objectives of the thesis are to determine the role of cytoskeleton and primary cilia in osteogenesis related pressure induced mechanotransduction.

Chapter 3.
Oscillatory fluid flow induces the osteogenic lineage commitment of Mesenchymal Stem Cells: The effect of shear stress magnitude, frequency and duration

3.1 Introduction

Bone structure adapts to meet the daily mechanical demands imposed during locomotion (Robling et al., 2006, Hu et al., 2012, Kwon et al., 2010a). This mechanically driven process dictates synthesis of new bone by osteoblasts derived from mesenchymal stem cells (MSCs). Due to the limited lifespan and non-proliferative nature of osteoblasts *in vivo*, MSCs play a critical role in replenishing the osteoblast population by undergoing osteogenic lineage commitment (Ren et al., 2015, Chen et al., 2016a, Tewari et al., 2015). Despite this important role for MSCs in bone adaptation, it is not fully understood how loading of bone at the macro scale regulates MSC differentiation within the niche. Two mechanisms have emerged whereby loading induced-MSC lineage commitment may be driven indirectly through paracrine signalling from mature bone cells or alternatively, MSCs may sense the applied mechanical stimulus directly (Riddle and Donahue, 2009, Govey et al., 2013, Hoey et al., 2011, Schaffler et al., 2014, Brady et al., 2015b). Although the response of bone cells such as osteocytes to a range of mechanical stimuli predicted within the lacunar-canalicular system (LCS) is known, how MSCs respond to the dynamic mechanical environment of the niche, or whether this mechanical stimulation is sufficient to directly drive osteogenic lineage commitment remains poorly understood.

During daily ambulation, the skeletal system including the marrow experiences complex mechanical stimuli such as pressure and fluid flow-induced shear (Ren et al.,

2015). Bone marrow experiences fluctuations in intramedullary pressure of up to 30kPa, which is dependent on loading, blood flow and muscle contraction that is oscillatory in nature (Gurkan and Akkus, 2008b). The pressure gradients that develop within the marrow cavity with loading, force the marrow to flow imparting a shear stress to resident cells, including MSCs. Until recently, the MSC micromechanical environment was poorly understood, therefore MSCs were subjected to shear stresses known to occur within the LCS of bone such as 0.1-3Pa (Delaine-smith et al., 2012, McCoy and O'Brien, 2010a, Weinbaum et al., 1994, Verbruggen et al., 2014, Espinha et al., 2014a). However, recent work using fluid-structure interaction models of reconstructed trabecular bone and bone marrow has demonstrated that the shear stresses imparted to MSCs are dependent on the deformation rate, bone porosity and bone marrow properties and can result in shear stresses exceeding 5 Pa (Metzger et al., 2015a, Coughlin and Niebur, 2012a). This therefore indicates that *in vitro* experimental studies to date have utilised shear magnitudes that represent the lower spectrum of the range that MSCs can experience *in vivo* with loading.

Direct mechanical stimulation of MSCs in vitro can play a positive role in directing cell lineage commitment. In particular, a number of studies have explored the effect of fluid shear on osteogenic gene expression employing variable biophysical regimes. Cyclooxygenase-2 (*Cox2*), a necessary enzyme involved in bone anabolism, is upregulated in as little as 30 minutes following either steady, pulsatile or oscillatory fluid flow stimulation at shear stress magnitudes of 0.1-1 Pa (Becquart et al., 2016, Zhang et al., 2002, Glossop and Cartmell, 2009). Similarly, fluid flow-induced upregulation of the osteoblastic master transcription factor, *Runx2*, occurs in response to both steady and oscillatory flow; however, this was in the presence of osteogenic supplements (Hoey et al., 2012, Kim et al., 2014, Li et al., 2004, Grellier et al., 2009). MSCs exposed to steady flow of less than 0.3 Pa magnitude demonstrated enhanced expression of the bone matrix proteins such as *Opn*, *Ocln*, *BSP* and *Col1α1* when cultured for up to 21 days (Kreke et al., 2008). However, employing a pulsatile or oscillatory flow results in an upregulation of the gene expression of these bone matrix proteins within an hour of stimulation (Sharp et al., 2009, James G. McGarry, 2005). Furthermore, due to dynamic nature of locomotion, OFF is considered more physiologically mimetic compared to steady and pulsatile regimes, which have predominately been used in bioreactor based tissue engineering strategies (Jacobs et al., 1998). Loading-induced bone formation *in vivo* has

a magnitude-frequency interdependence such as the coupling of high magnitude-low frequency stimulation and vice versa (Turner et al., 1994, Nagaraja and Jo, 2014). Interestingly, most OFF studies utilize frequency of 1Hz in order to mimic the ambulatory pace, however the effect of a range frequencies related to human motion has not been investigated (Danion et al., 2003, Arnsdorf et al., 2010, Riddle et al., 2008, Riddle et al., 2007, Riddle et al., 2006). Given the variable MSC response to fluid flow, a systematic study that investigates the effect of the magnitude, frequency and duration of physiologically relevant OFF on osteogenic responses is required.

It is currently unclear whether fluid flow induced shear predicted to occur within the marrow during daily ambulation is sufficient to directly drive osteogenic lineage commitment of MSCs. Therefore, the objective of this study is to conduct a systematic analysis of oscillatory fluid shear stress magnitude, frequency and duration on early osteogenic responses and to determine whether these mechanical stimuli are sufficient to drive osteogenic lineage commitment of mesenchymal stem cells in the long term. Elucidating how marrow mechanical environment affects osteogenic responses in MSCs will enable the development of advanced strategies for orthopaedic bioreactor-based tissue engineering and will provide a platform to delineate the mechanisms of loading-induced MSC osteogenic differentiation and bone formation.

3.2 Materials and methods

3.2.1 Cell culture

All materials were purchased from Sigma unless otherwise stated. A murine mesenchymal stem cell line (C3H10T1/2) was cultured on fibronectin (10 µg/ml) coated glass slides in low glucose DMEM supplemented with 10% foetal bovine serum (FBS: Biosera) and 1% penicillin-streptomycin (P/S) unless otherwise stated. For short term fluid flow stimulation, cells were cultured for 24hrs under standard conditions followed by 48hrs of serum starvation (0.5% FBS) supplemented with 0.3 nM dexamethasone, 0.025 mM L-ascorbic acid and 10 mM β-glycerolphosphate. These concentrations represent minimal levels for the support of osteogenesis, thereby allowing greater scope to investigate the effect of a biophysical versus a biochemical stimulus. Regarding long-term fluid flow stimulation, the cells were cultured in similar conditions, except supplemented with 2% FBS and 2%P/S. To demonstrate that these minimal osteogenic supplements do not induce osteogenic differentiation of MSCs alone, for long term mechanical stimulation, an additional 'Control' group consisting of standard growth media with no additional osteogenic supplements was included.

3.2.2 Parallel plate flow chamber design

The design of the channel within the parallel plate flow chamber (PPFC) primarily focused on producing uniform laminar flow with small pressure drops to avoid a variable mechanical environment and subsequent variable cellular response. To achieve this, the design parameters such as size of inlet/outlet reservoirs, entrance and exit slits from the channel, dimensions of flow channel and method for maintaining a constant channel height were considered (Fig.1A,B). The channel dimensions such as the length and width were dictated by the glass slides used to culture cells (38mmx75mm). This is to ensure sufficient cell numbers for mRNA isolation. The inlet/outlet reservoir diameters were chosen to be similar to the tubing diameter as to minimise pressure losses (Chung et al., 2003). The slit dimension for the entrance and exit of the channel were based on maintaining the flow rate entering the inlet as to avoid transverse pressure gradients and minimise entrance effects (Nauman et al., 1999). Entrance effects are associated with the development of the velocity field of the flow independent of the axial position along the length and is dependent on cross sectional geometry of the channel, inlet profile and Reynolds number (Chung et al., 2003) (L_e,

Eq.1). Therefore, the fluid flow characteristics such as entrance length, hydraulic resistance (R_h, Eq.2) and pressure drop across the chamber (ΔP, Eq.3) are investigated for various channel heights using the equations below:

(Eq.1) $L_e = 0.05 Re D_h$; (Eq. 2) $R_h = 12\mu l/h^3 w$; (Eq.3) $\Delta P = Q R_h$; (Eq.4) $Re = \frac{\rho u D_h}{\mu}$;

where Re is Reynold number; D_h - hydraulic diameter, l - length of channel, h - chamber height, w - width of channel, Q - flow rate and u - flow velocity. The channel height is chosen based on a compromise between small entrance length and pressure drop across the channel.

Computational fluid dynamics (CFD) was utilised to validate the theoretical predictions (Anderson and Knothe Tate, 2007, Chung et al., 2003, Lu et al., 2014). CFD modelling was employed using StarCCM+ software (CD-adapco^{TM}) to validate the chosen height of the microchannel. To simplify the simulations of the oscillatory fluid flow, it was assumed to be composed of two quasi-steady state flows with forward and reverse direction for the period of oscillations, for which Hagen-Poiseuille steady flow conditions holds true. As detailed by Doorly and Sherwin (2009) in Geometry and Flow (Doorly and Sherwin, 2009), if the reduced velocity of the flow regime is larger than the ratio of length/height of the chamber, the flow regime can be assumed to be quasi steady for the period of oscillation. The reduced velocity (U reduced) is a non-dimensional number which describes the ratio of distance travelled (chamber length) by the mean flow to the pipe diameter and it is a function of Reynolds number and Womersley number. The Womersley number describes the laminar boundary layer growth over pulse period T, hence relates viscous effect to the frequency of oscillations. The reduced velocity has been calculated for each flow regime and was found to be much higher than the length to height ratio (Table 5), hence the quasi steady state flow assumption holds true.

Table 5. Reduced velocity (U reduced) of all flow oscillatory fluid flow regimes

Magnitude	Re	**U reduced**			Length/height
		0.5 Hz	1Hz	2Hz	
1 Pa	64	489.2	978.4	1956.8	230
2 Pa	128	978.4	1956.8	3913.5	
5 Pa	321	2445.9	4891.9	9783.8	

For steady, fully developed laminar flow between 2 infinite parallel plates the magnitude of wall shear stress can be expressed as a function of flow rate, viscosity and chamber dimensions (Eq.5) (Huesa et al., 2010):

$$\text{(Eq. 5)}\ \tau=\frac{6Q\mu}{bh^2}.$$

To simulate fluid flow within the channel, the medium was assumed to be a Newtonian incompressible fluid with a viscosity of 0.0006814 kgm/s (μ H_2O) and a density (ρ H_2O, 37^oC) of 993.33 kg/m^3 (Kestin et al., 1978). The fluid continuum was modelled as steady, segregated laminar 3D flow using Navier Stokes solver. Constant and uniform distribution of the parabolic velocity profile at the inlet is assumed. No slip condition was set for the chamber walls and static gauge pressure boundary defined for the outlet. The meshing of the discretised flow continuum was optimised to a 3 million polyhedral cell mesh with refinement at the channel boundaries. The pressure and fluid velocities were calculated at each iteration until a convergence of 1E-6 was achieved for continuity and momentum in x, y and z direction for the Reynolds Average Navier Stokes equations.

3.2.3 Fluid flow mechanical stimulation

OFF was achieved by applying an oscillatory pressure driven flow via a syringe pump (Alladin 1660) to the custom-made PPFC (Figure 3-2 A). The volumetric rate of flow (Q) necessary for a given shear stress was calculated using equation (Eq.5) and validated and adjusted for any pump losses based on the flow output. To systematically delineate the effect of fluid shear stress magnitude, frequency and duration on early osteogenic response of MSCs, a series of fluid flow regimes were employed and are presented in Table 6. The OFF parameters were grouped such as to examine the independent effect of peak shear stress, frequency and duration. In addition, frequency and flow duration were coupled such as comparisons of flow regimes with constant number of loading cycles can be made i.e 0.5Hz, 4hrs against 1Hz,2hrs and 2Hz,1hr. These flow regimes were modelled on a previous systematic investigation of fluid flow on osteocytes (Li et al., 2012). Control slides, which were not subjected to fluid flow, were assembled in the chambers to reproduce handling effects for a matched duration of mechanical stimulation. To determine whether oscillatory fluid shear could induce osteogenic lineage commitment and based on our results from the short-term systematic analysis, MSCs were subjected to two separate flow regimes (FR) over a long-term

duration as described in Figure 3-1. FR1 consisted of a fluid shear of magnitude 1Pa and frequency 1Hz (most common flow regime for bone cells) while FR2 consisted of a fluid shear of magnitude 2Pa and frequency 2Hz. For both long-term FRs, MSCs were exposed to fluid shear on Days 1, 2, 4, and 5 for 4 hours a day followed by an additional 14 days of static culture.

Table 6. Experimental conditions for short-term fluid flow stimulation

	Frequency (Hz) and [total oscillations]		
Shear stress (Pa) & (flow rate)	0.5 Hz	1 Hz	2 Hz
1 Pa (44.9 ml/min)	2 hrs [3600 oscillations] and 4 hrs [7200 oscillations]	2 hrs [7200 oscillations]	1 hr [7200 oscillations] and 2 hrs [14400 oscillations]
2 Pa (89.8 ml/min)			
5 Pa (224.5 ml/min)			

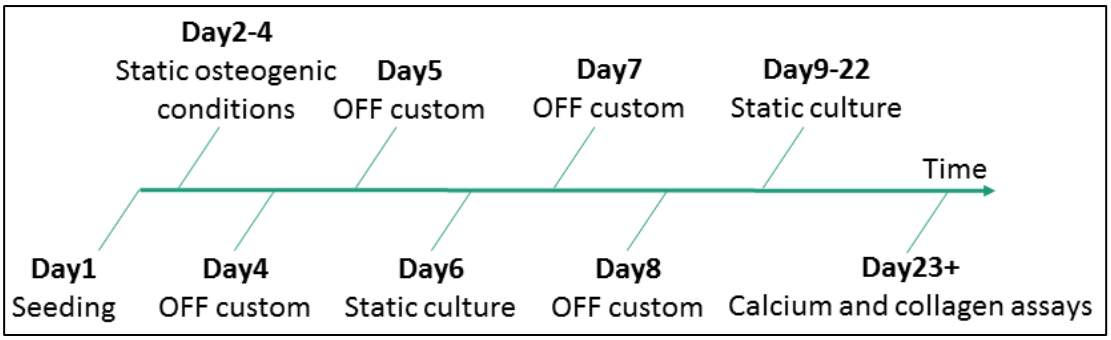


Figure 3-1 Long term oscillatory fluid flow protocol

3.2.4 Quantitative real-time PCR

Immediately post-flow, cells were lysed using TRI Reagent® (Sigma Aldrich) and mRNA was isolated according to the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Life Technologies). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Select Mastermix with ROX passive dye (ThermoFisher 4472903). The expression of *18S*, *Cox2*, *Runx2* and osteopontin (*Opn*) was quantified using primers (Sigma) detailed in Table 7. The amplification was performed with an ABI7500 Fast Real Time qPCR machine. Each sample was normalised to reference gene *18S* and static control using relative quantification method.

Table 7. Mouse primers and experimental conditions used for qPCR analysis

Gene	Tm (°C)	Primer concentration	Sequence
18S	60	400 nM	5'-GTAACCCGTTGAACCCCATT-3'
			5'-CCATCCAATCGGTAGTAGCG-3'
Cox2	60	400 nM	5'-ACTCATAGGAGAGACTATCAAG-3'
			5'-GAGTGTGTTGAATTCAGAGG-3'
Runx2	60	300 nM	5'-ACAAGGACAGAGTCAGATTAC-3'
			5'-CAGTGTCATCATCTGAAATACG-3'
Opn	60	400 nM	5'-GGATGAATCTGACGAATCTC-3'
			5'-GCATCAGGATACTGTTCATC-3'

3.2.5 Osteogenic assays

Cells were fixed in formalin for 10 minutes. For collagen staining, cells were incubated in 0.1% PicroSirius Red solution for 1 hour at room temperature. After washing twice in 0.5% acetic acid and water, samples were mounted with DPX mounting medium. Calcium staining was performed using 2% Alizarin Red solution. The bound dye for both calcium and collagen was observed under light microscopy. The bound Alizarin Red was used to quantify the calcium content by extraction using 10%v/v acetic acid and measuring the absorbance at 405nm.

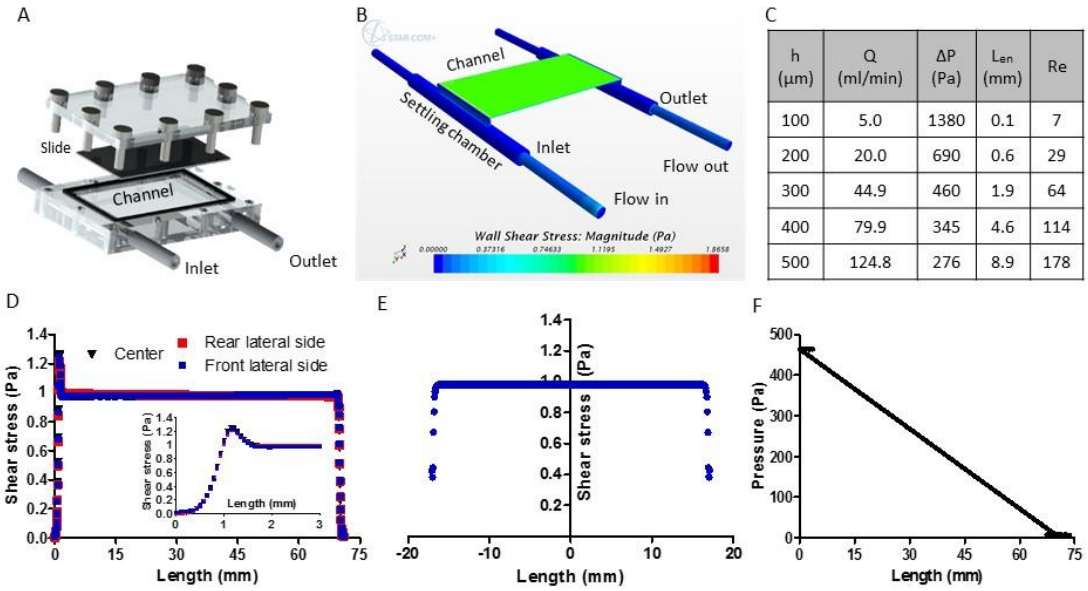
3.2.6 Statistical analysis

All data presented as mean±SEM. For qRT-PCR analysis, each condition was compared to matched static control using a two-tailed student's t-test with Welch's correction. Flow to no flow significance is denoted as *$p<0.05$, **$p<0.01$, ***$p<0.005$. One-way ANOVA with Tukey post hoc test was used to compare the effect of magnitude, frequency and duration and significant differences are indicated as # $p<0.05$, ## $p<0.01$, ### $p<0.005$ except for the 2-hour group, where a two-way ANOVA analysis was employed with Bonferroni post hoc test. For calcium quantification, a one-way ANOVA with Dunnett's post-test was performed.

3.3 Results

3.3.1 Parallel plate fluid flow bioreactor design and validation

The chamber design was optimised to minimise pressure changes and the entrance length while achieving a homogenous fluid flow-induced shear stress. This was achieved by varying the height of the channel to determine the theoretical changes in the entrance length (L_e), pressure drop across the chamber (ΔP) for a 1Pa shear stress magnitude (Figure 3-2 C). Increasing the channel height yielded a proportional increase in entrance length and flow rate whilst decreasing the pressure drop. The flow was laminar for all flow rates as indicated by $Re<1400$ (Vaughan et al., 2013). Therefore, the channel height of 300 µm was chosen as it produced a small entrance length (1.9mm) with small pressure changes across the channel (460Pa) while maintaining a laminar flow profile. To validate the chosen height of the chamber, CFD was used to simulate 1Pa continuous fluid flow within the chamber. Extrapolated data from computational validation confirms that homogeneity of shear stress distribution across the length (Figure 3-2 B, D) and width (Figure 3-2 B, E) of the channel as well as the presence of entrance effects which leads to a 20% increase in shear stress magnitude at the inlet. The entrance length predicted for 1Pa shear stress is 1.7mm, smaller than theoretical prediction by 0.2mm and confirming that 95% of slide sustains homogenous shear. The pressure drop matches the theoretical prediction (Figure 3-2 F).



h (μm)	Q (ml/min)	ΔP (Pa)	L_{en} (mm)	Re
100	5.0	1380	0.1	7
200	20.0	690	0.6	29
300	44.9	460	1.9	64
400	79.9	345	4.6	114
500	124.8	276	8.9	178

Figure 3-2. Development and validation of the parallel plate flow chamber bioreactor design (A) Parallel plate flow chamber design (B) Graphical representation of shear stress distribution in different compartments of the chamber (C) Evaluation of height (h) on entrance length (Le) effects, flow rate (Q), pressure change across the channel (ΔP) and Reynolds number (Re) for a commercial glass slide of 38mmx75mm size. Computational validation of optimised chamber geometry (300μm height) was executed for 1 Pa wall shear stress (WSS) under continuous flow. Extrapolated data from computational validation shows distribution of shear stress across the length of the chamber (D) and at the centre and lateral sides (E); pressure drop across the length of the chamber (F).

3.3.2 Effect of OFF magnitude on early osteogenic gene expression in MSCs

Overall, MSCs stimulated with OFF display positive but variable responses as determined by the upregulation of osteogenic genes *Cox2*, *Runx2* and *Opn* when compared to the static condition. *Cox2* mRNA expression is upregulated in response to fluid shear in a magnitude dependent manner (Figure 3-3 A). This is clearly evident at the 1hr (2Hz) time point, as only 2Pa and 5Pa elicited a significant increase ($p<0.05$) and the increase in response to 5Pa was significantly greater than at 2Pa ($p<0.05$). At 2hrs (1Hz), the magnitude effect is also present as seen by significant increase at 2Pa versus 1Pa (<0.05) whereas at 2hrs (2Hz), the 5Pa response plateaus and mirrors that seen in response to 2Pa. Interestingly, at 4hrs (0.5Hz), a greater and similar response is seen in the 1Pa and 5Pa groups compared to 2Pa ($p<0.05$) although all of them were significant compared to no flow control ($p<0.05$). This may be attributed to a synergistic interplay between frequency and magnitude as 2Hz, 2Pa stimulation exhibits similar upregulation over time. OFF elicited a variable yet overall positive osteogenic response in terms of *Runx2* and *OPN* mRNA expression (Figure 3-3 B, C), although there is no clear effect of OFF magnitude in these genes.

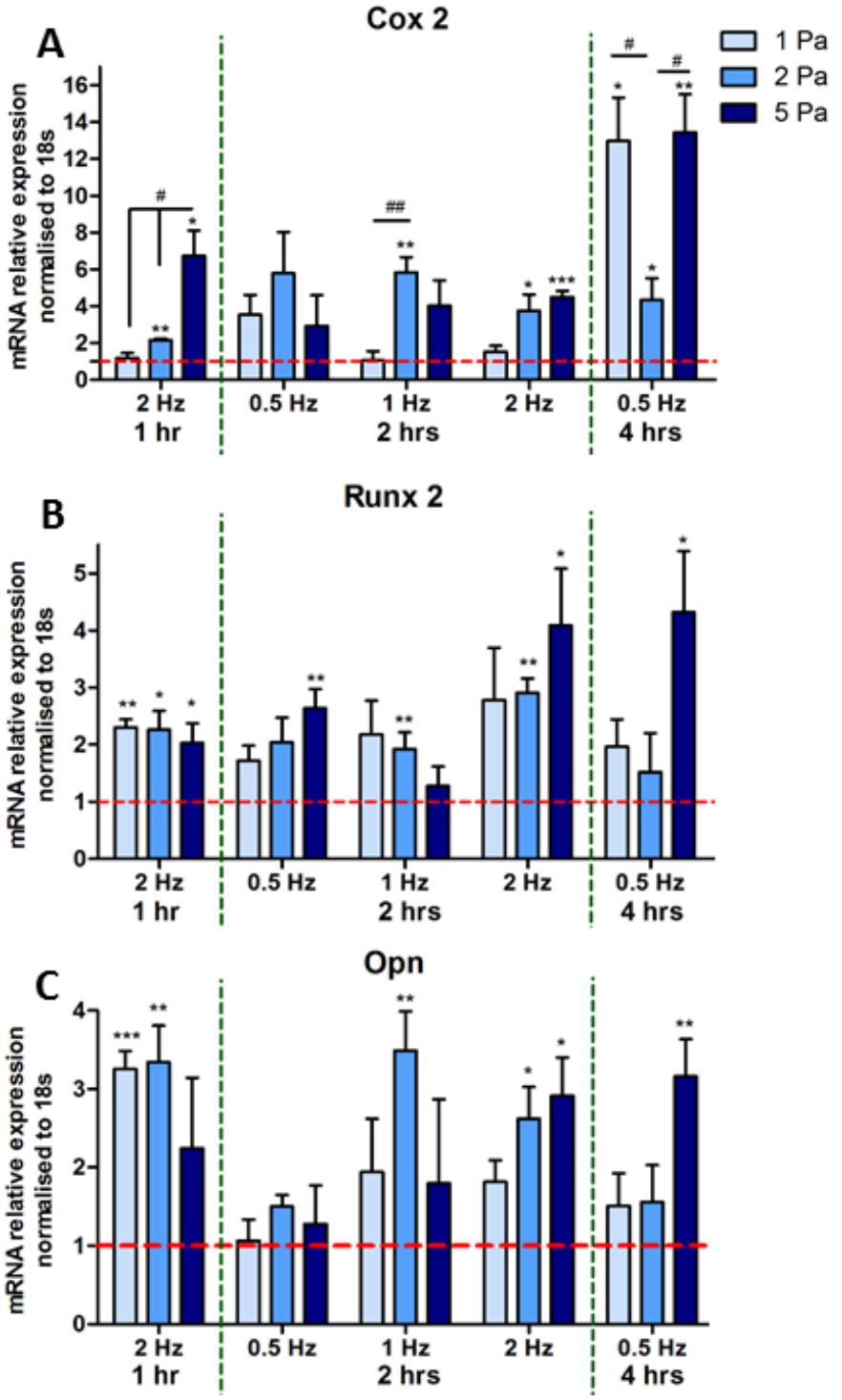


Figure 3-3. Effect of fluid flow magnitude on early mRNA expression of osteogenic genes *Cox2*(A), *Runx2* (B) and *Opn* (C). Data presented is normalised to each time control static culture (1 hour, 2 hours and 4 hours) and grouped by the timing of the stimulus, in ascending order of magnitude for each frequency. Statistical significance comparing flow versus no flow for individual flow experiment is denoted by *p<0.05, **p<0.01, ***p<0.005; whereas ###, p<0.005 denotes significance for the effect of magnitude between different flow conditions. N=3-4 for each flow condition.

3.3.3 Effect of OFF frequency on early osteogenic gene expression in MSCs

To identify whether OFF frequency affects early osteogenic mRNA expression, the 2hr flow group was analysed for 0.5Hz, 1Hz and 2Hz frequencies. OFF-induced *Cox2* mRNA expression displays similar levels of upregulation at all magnitudes irrespective of frequencies, although a higher and more consistent expression is achieved at shear stress magnitudes above 2Pa and at frequency of 2Hz (Figure 3-4 A). Except for the 1Pa group, all genes demonstrate a non-significant trend of a frequency effect at the highest shear stress magnitude of 5 Pa (Figure 3-4 B, C). This is most notable for the *Runx2* mRNA expression which is significantly higher at 2Hz versus 1Hz ($p<0.05$).

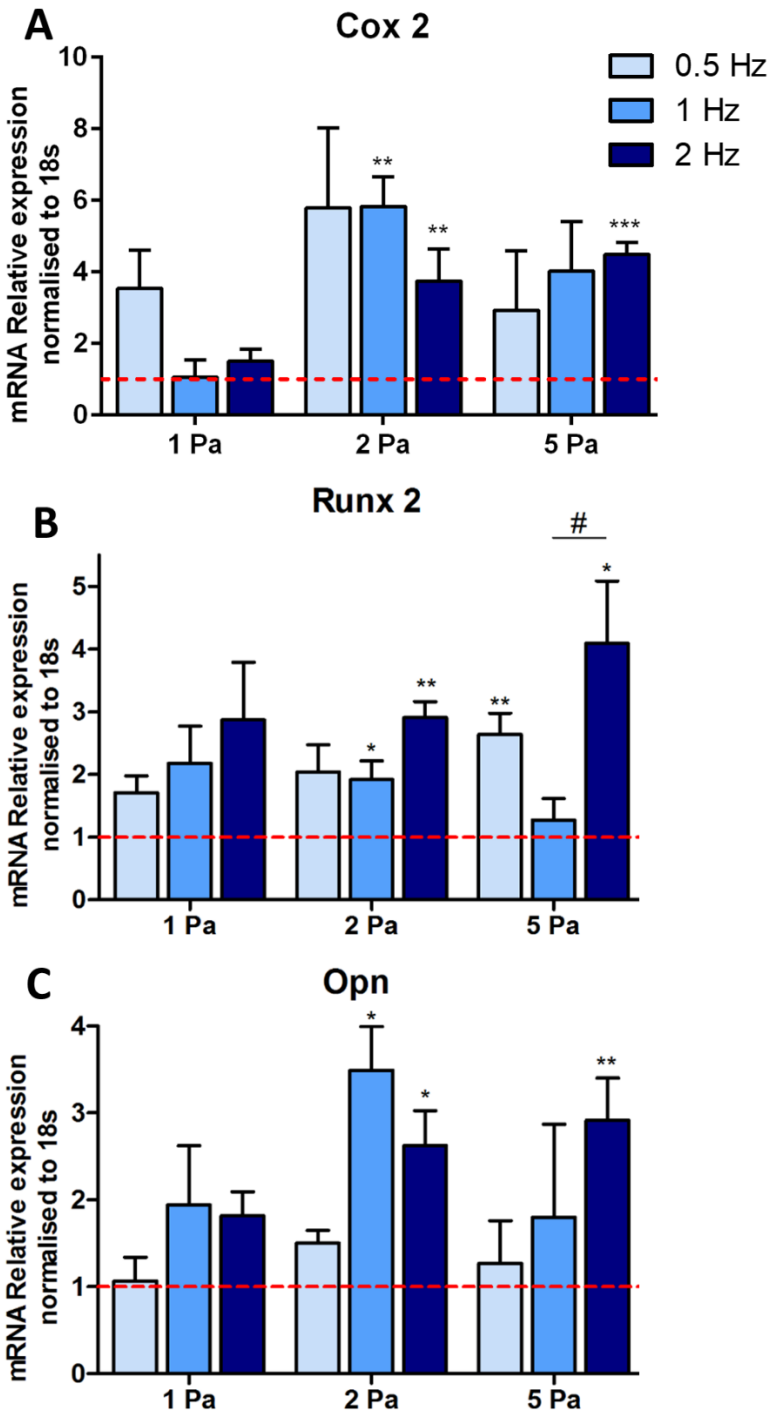


Figure 3-4. Effect of fluid flow frequency on early mRNA expression of osteogenic genes *Cox2* (A), *Runx2* (B) and *Opn* (C). Data presented is normalised to time control static culture (2 hours) and grouped in ascending order for each magnitude of fluid flow. Statistical significance comparing flow versus no flow for individual flow experiment is denoted by *p<0.05, **p<0.01; whereas ###, p<0.01 denotes significance for the effect of magnitude between different flow conditions. N=3-4 for each flow condition.

3.3.4 Effect of OFF duration on early osteogenic gene expression in MSCs

To identify the effect of duration on early osteogenic expression, 1hr and 2hrs as well as 2hr and 4hrs of stimulation were compared while other parameters were held constant. For illustrative purposes, 2hrs (1Hz) group was included as it is most popularly used fluid flow mechanical regime. *Cox2* mRNA expression elicited a higher response when stimulated for longer durations at 0.5Hz than 1Hz and 2Hz (Figure 3-5 A). Despite similar magnitude stimuli, *Cox2* expression at the later time is significantly higher in particular 0.5Hz 1Pa and 5Pa magnitudes ($p<0.05$). *Runx2* mRNA expression displayed a similar yet less pronounced increase with longer duration at 0.5Hz, with significance achieved for 5Pa (Figure 3-5 B). *Opn* mRNA expression displays similar levels of upregulation with flow irrespective of duration at 2Hz (Figure 3-5 C). For the 0.5Hz group, similar expression was elicited at all magnitudes irrespective of duration of stimulation except 5Pa, where longer duration yields a significant higher response ($p<0.05$). However, when comparing the 1hr, 2Hz with 2hrs,1Hz and 4hr, 0.5Hz groups, all of which experience the same shear stress magnitude and number of oscillating cycles (7,200), there is a clear abrogated response with the longer duration for 1Pa and 2 Pa suggesting that *Opn* expression occurs rapidly and diminishes with time. Therefore, duration of mechanical stimulation plays a role in the level of upregulation of osteogenic markers although this effect is gene dependent.

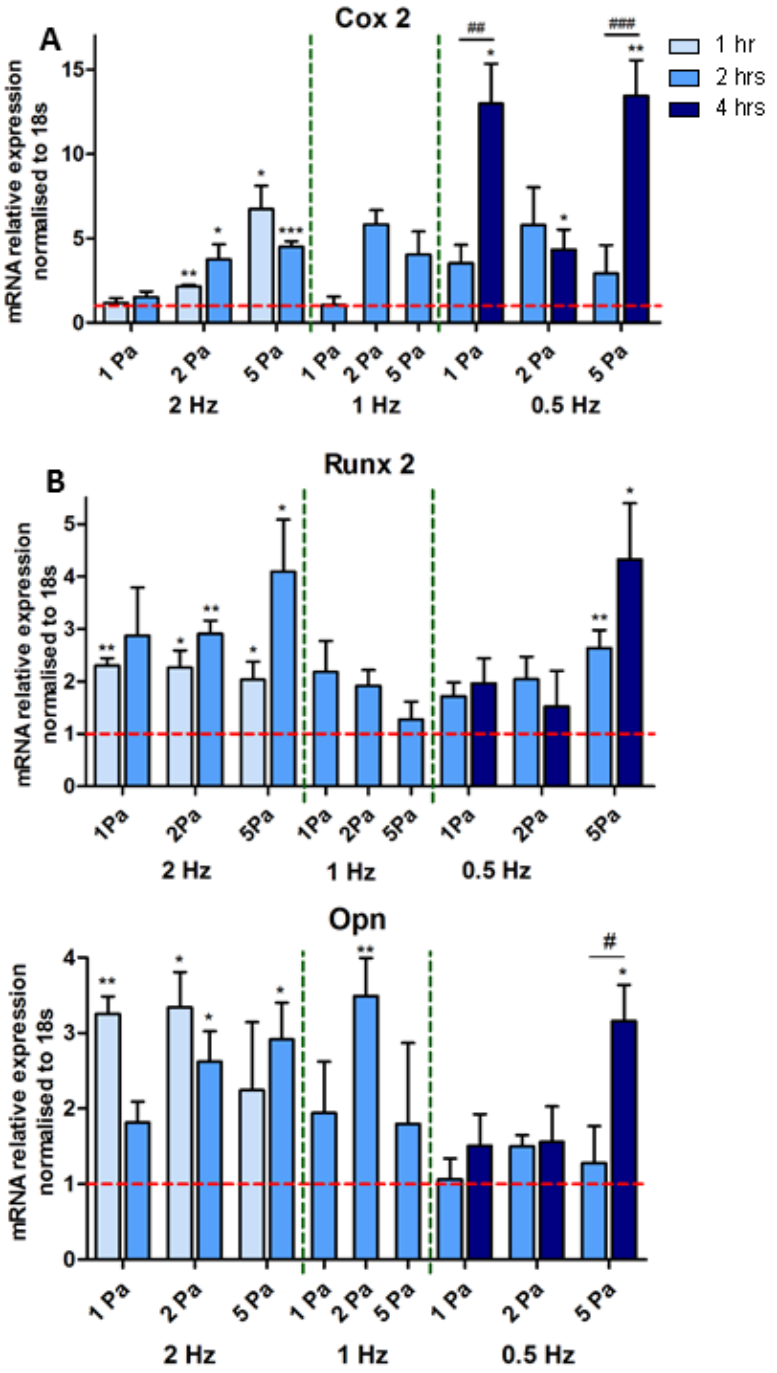


Figure 3-5. Effect of duration of fluid flow mechanical stimulation on early mRNA expression of osteogenic genes *Cox2* (A), Runx (B) and *Opn* (C). Data presented is normalised to each time control static culture (1 hour,2 hours and 4 hours) and grouped in ascending order for the duration of the mechanical stimulation for each magnitude at 2Hz and 0.5 Hz frequencies. Statistical significance comparing flow versus no flow for individual flow experiment is denoted by *p<0.05, **p<0.01; whereas #, p<0.05 denotes significance for the effect of duration of mechanical stimulation between different flow conditions. N=3-4 for each flow condition.

3.3.5 Effect of long-term OFF on the osteogenic lineage commitment of MSCs

Based on the systematic analysis performed above, a flow regime consisting of 2Pa, 2Hz, and 2hr fluid flow duration was chosen based on the robust and reliable upregulation of all osteogenic markers. Therefore, this flow regime was applied twice a day, for a total of 4 days followed by 2 weeks of static culture. A second flow regime of 1Pa, 1Hz, 2hr was also applied for comparison, as this is a common regime found within the literature for short-term studies. After a relative short-term OFF effect on a long-term static culture, the addition of minimal osteogenic supplements alone resulted in an increase in collagen production that is indicative of osteoblastogenesis (Figure 3-6 A, B). The degree of collagen staining intensified with mechanical stimulation with no clear differences between the two fluid flow regimes (Figure 3-6 C, D). Regarding mineralisation, addition of minimal osteogenic supplements in static culture did not alter calcium deposition (Figure 3-7 A, B, E). Increased staining for mineral was observed with OFF although no difference between the two regimes is evident visually (Figure 3-7 C, D). However, fluid flow stimulates synthesis of mineral in a shear stress and frequency dependent manner. Specifically, 2Pa, 2Hz reaches a significant two-fold increase in mineral content compared to static control while 1Pa, 1Hz (FR1) increases mineralisation although not to a significant degree (Figure 3-7 F). Therefore, physiologically relevant OFF predicted to occur within the marrow cavity is sufficient to directly drive osteogenic lineage commitment although high magnitudes and frequencies are required.

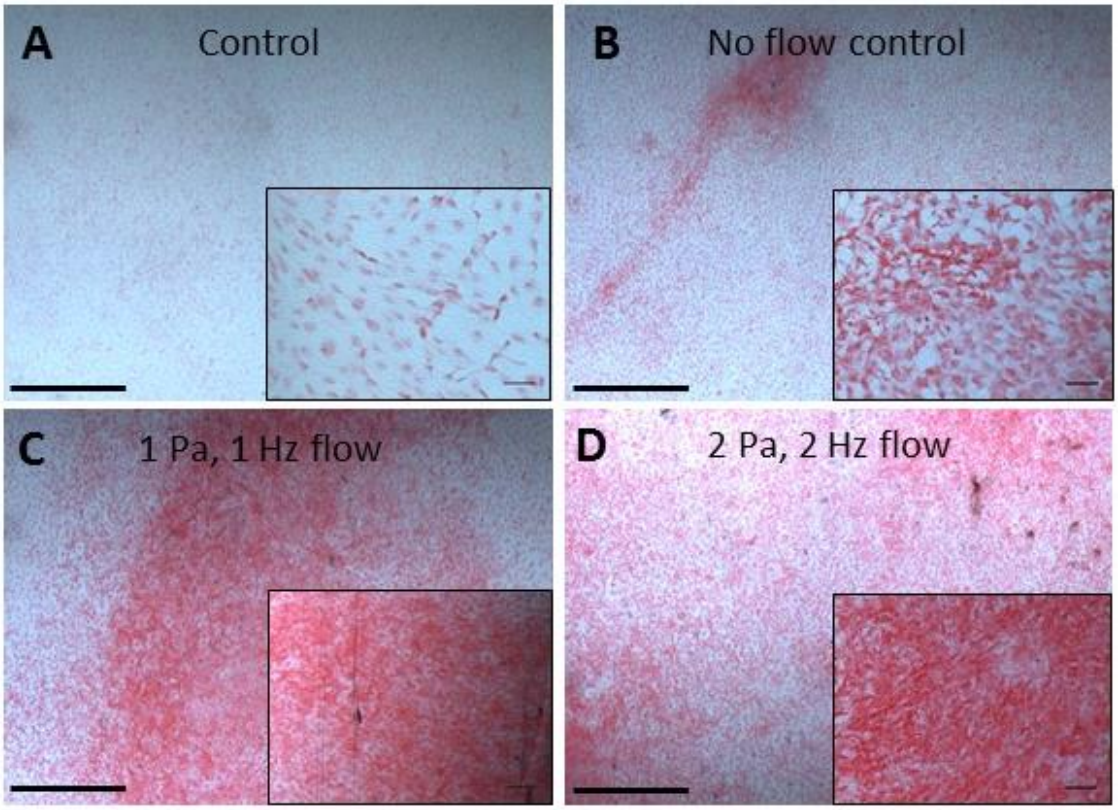


Figure 3-6. Fluid flow stimulation induces collagen expression in MSCs after 2 weeks of culture post mechanical stimulation. Collagen staining depicted by PicroSirius Red for negative control (C-), no flow (NF), 1Pa flow (F1) and 2Pa flow (F2). Scale bar 100μm for corner pictures and 0.5mm for large pictures.

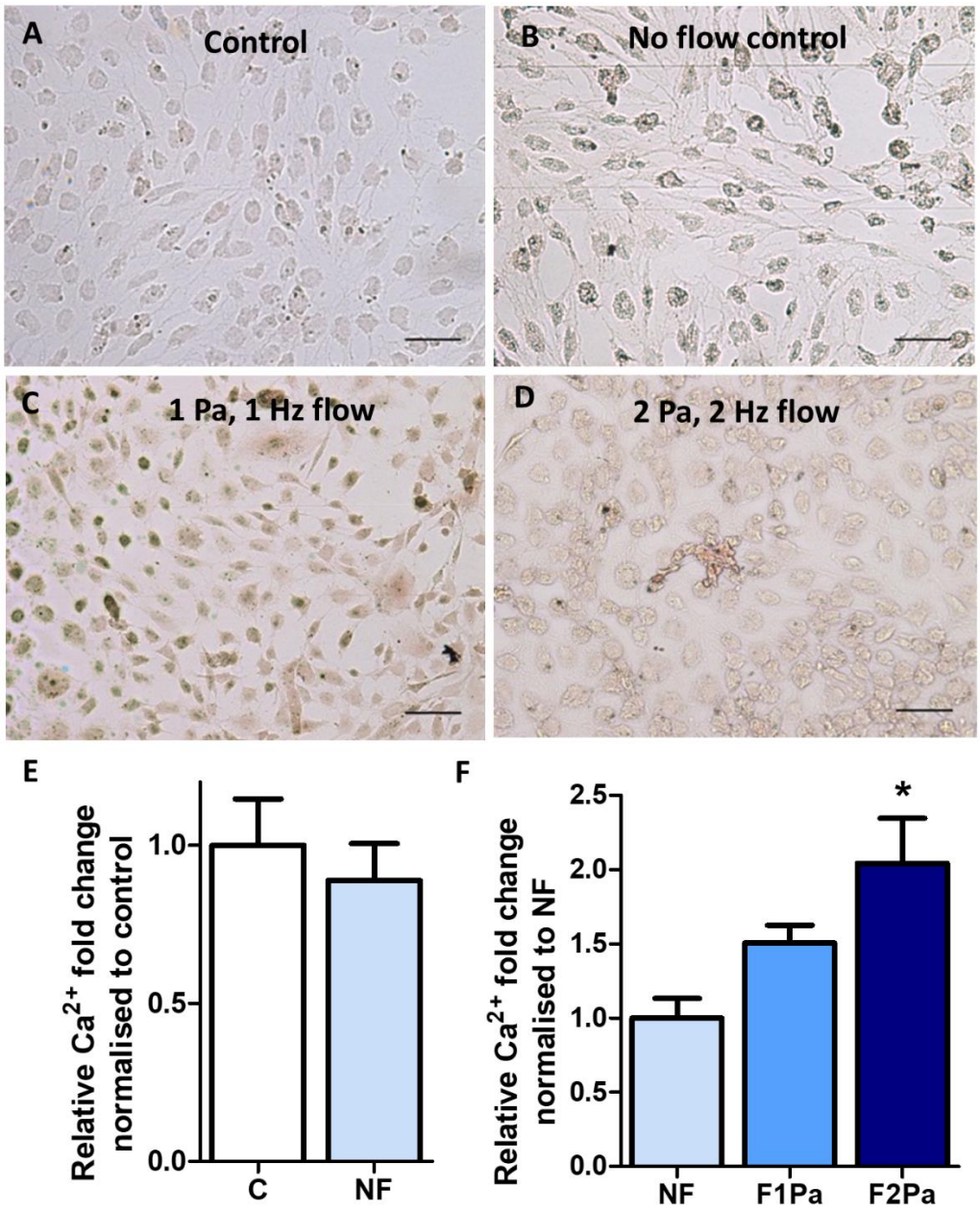


Figure 3-7. Fluid flow stimulation induces calcium expression in MSCs after 2 weeks of culture post mechanical stimulation. Mineralisation depicted by Alizarin Red staining for negative control (A); no flow (B); 1Pa, 1Hz flow (C) and 2Pa, 2Hz flow (D). Calcium quantification from Alizarin Red staining comparing the effect of osteogenic supplements (E) and mechanical stimulation on MSC osteogenic differentiation (F). To compare the effect of osteogenic supplements a student's t test with Welch correction was employed and for the effect of mechanical stimulation a one-way ANOVA analysis with Dunnett's post hoc test was executed; *$p<0.05$. Scale bar 100 μm. Sample number n=3.

3.4 Discussion

As it is unclear whether the mechanical environment of the bone marrow can directly drive the osteogenic lineage commitment of mesenchymal stem cells, a systematic *in vitro* analysis was performed on the effect of OFF induced shear stress magnitude, frequency and duration on MSC osteogenic responses. Through the utilisation of custom-built PPFCs, it was demonstrated that OFF elicits a positive osteogenic response in MSCs in a shear stress magnitude, frequency, and duration dependent manner that is gene specific. Furthermore, it was identified that a regime of 2Pa, 2Hz, which is predicted to occur *in vivo*, induces the most robust and reliable upregulation in osteogenic gene expression. Furthermore, long-term mechanical stimulation utilising this flow regime, elicits a significant increase in collagen and mineral deposition compared to static control demonstrating that mechanical stimuli within the marrow is sufficient to directly drive osteogenic lineage commitment. Furthermore, this flow regime elicited a more robust response when compared to the more commonly utilised flow regime of 1Pa, 1Hz, which did not significantly drive MSC osteogenesis. Therefore, our findings highlight that bone marrow biophysical cues can directly drive MSC osteogenesis, although magnitudes and frequencies representing high intensity exercise are required.

The response of MSCs to OFF-induced shear stress is dependent on magnitude, frequency and duration. Here it is demonstrated for the first time that fluid flow induced *Cox2* mRNA expression is increased with elevation in shear stress magnitude in MSCs. Interestingly, this dose dependent response is not evident with *Opn* or *Runx2* expression, which may suggest alternate mechanotransduction pathways are utilised to activate these unique genes. In this study it is shown that mechanics plays a vital role in MSC early osteogenic responses, especially as cells may experience shear stress magnitudes as high as 8Pa during loading (Metzger et al., 2015a, Coughlin and Niebur, 2012a). A positive effect of magnitude on early osteogenic responses was previously demonstrated in osteocytes, whereby increases in shear stress elicited a corresponding increase in *Cox2* mRNA expression (Li et al., 2012), further validating that this gene is indeed sensitive to magnitude. Upregulation of this *Cox2* is necessary for differentiation as MSCs isolated from *Cox2*$^{-/-}$ mice have inhibited osteoblastogenesis (Zhang et al., 2002). Interestingly, expression of *Runx2* and *Opn* exhibited a weaker dependency to shear stress magnitude. In previous studies, *Runx2* and *Opn* were found to exhibit variable responses to similar

and different fluid flow mechanical regimes depending whether osteogenic supplements were present (Li et al., 2004, Kreke et al., 2008, Franceschi and Xiao, 2003). In addition, differential effects of fluid flow on gene expression has been noted in the osteocyte, such as fluid flow associated decrease in RANKL/OPG expression did not change with increase of magnitude above 2Pa (Li et al., 2012). Therefore, these results demonstrate the importance of shear stress magnitude in driving osteogenic gene expression in MSCs.

Early fluid flow-induced osteogenic responses in MSCs demonstrated a weak dependency on oscillating frequency although subtle positive effects were evident at high shear stress magnitudes. Very few studies to date have investigated the effect of loading frequency on bone cells although *in vivo* it is known to play an important role in bone anabolic responses (Turner et al., 1994, Nagaraja and Jo, 2014). It was found that increasing the frequency of fluid flow oscillations while maintaining shear stress magnitude did not increase *Cox2* mRNA expression. In osteocytes similar findings were reported for *Cox2* (Li et al., 2012). *Runx2* and *Opn* mRNA expression is also not dependent on fluid flow frequency, although an apparent increase with frequency is noticed at magnitudes above 1Pa. To date, the effect of OFF frequency has not been studied, with the majority of studies utilising 1Hz (Li et al., 2004, Riddle et al., 2007, Jacobs et al., 1998, Danion et al., 2003). Interestingly, an *in vitro* study investigating the effect of the frequency of dynamic compression of osteoblasts identified that 2Hz was the optimum for loading-induced mineralisation (Tanaka and Tachibana, 2015), which is in agreement with our finding. Frequency of 2Hz is related to a more intense motion such as jogging compared to 1 Hz for walking. In addition, if coupled with higher shear stress magnitudes generated from the impact due to locomotion, it may generate an optimum mechanical state for osteogenesis (Nagaraja and Jo, 2014). Hence, frequencies above normal walking motion can be considered pro-osteogenic if coupled with higher range of shear stress magnitudes.

Fluid flow stimulation affects osteogenic lineage commitment depending on the intensity of mechanical regime. Short-term fluid flow stimulation identified that 2Pa, 2Hz fluid flow model provides a more robust and reliable osteogenic gene response. Employing this regime for long-term stimulation demonstrated enhanced osteogenic differentiation based on collagen and calcium deposition. Given the fact than 1Pa, 1Hz flow regime is the most widely used, here it is shown that this mechanical regime does stimulate osteoblastogenesis based on collagen staining but was not sufficient to induce

significant mineralisation. This demonstrates that current flow models for studying MSC mechanobiology under represent the biophysical stimulus required to drive osteogenic lineage commitment.

This is the first study to demonstrate that OFF induces osteogenic lineage commitment of MSCs *in vitro* in a 2D culture model. However, a limitation of this study is the use of C3H10T1/2 cell line, which is capable of differentiation into osteoblasts and is responsive to mechanical stimuli but is limited in the ability to deposit mineral (Zhao et al., 2009, Hu et al., 2013). In this study, increases in shear stress magnitude was achieved by a proportional increase in flow rate, therefore mass transport effects may be associated with higher magnitudes. Further investigations are required to delineate the individual roles of chemotransport and shear stress magnitude on inducing MSC osteogenic lineage commitment.

3.5 Conclusion

In this study, it is demonstrated that OFF-induced MSC lineage commitment is dependent on shear stress magnitude and frequency. The systematic approach taken enabled the identification of the differential effects of the fluid flow characteristics on early osteogenic responses and lineage commitment. This will provide new insight for development of optimised strategies for orthopaedic tissue engineering and novel platforms for mechanotransduction studies.

Chapter 4.
Physiological cyclic hydrostatic pressure induces osteogenic lineage commitment of Human Bone Marrow Skeletal Stem Cells: A Systematic Study

4.1 Introduction

Bone is exposed to constant cyclic loading which is necessary to maintain tissue integrity (Robling et al., 2006, Hu et al., 2012, Kwon et al., 2010a, Lee et al., 2014). This effect is mediated in part, by bone marrow derived stem cells (MSC), which undergo osteogenic lineage commitment in response to loading to replenish the population of bone synthesising cells (Ren et al., 2015, Chen et al., 2016a). Recently, it has been shown that bone marrow stem cells undergo osteogenesis in response to mechanical cues independent of osteocyte signalling, suggesting that the marrow micromechanical environment may directly influence MSC osteogenesis (Curtis et al., 2018, Chen et al., 2016a, Kwon et al., 2012). Given the complex mechanical environment of the bone, it is unclear how the translation of macro-scale mechanical cues to the marrow niche directly regulates stem cell differentiation. Fluid flow is recognized as the most potent biophysical stimulus contributing to bone anabolic responses (Hillsley and Frangos, 1994, Qin et al., 2003, Stavenschi et al., 2017, Govey et al., 2013). However, the effect of pressure transients, which are necessary to drive the loading-induced fluid flow, on bone marrow stem cell osteogenesis is poorly understood. Decoupling the effect of pressure transients from fluid shear on stem cell osteogenesis would identify the driving physical forces in loading induced bone formation, focusing efforts to utilise this information to enhance MSCs osteogenesis and bone repair.

The pressurisation of bone intraosseous fluid plays a critical role in bone mechanics as it provides hydraulic strengthening, as well as forcing the interstitial fluid and marrow, to flow through the lacunar-canalicular system (LCS) and within the medullary cavity, respectively (Metzger et al., 2015a, Ren et al., 2015). This, in turn, imparts fluid shear stress stimulation to the resident bone cells, in addition to enhancing mass transport and paracrine signalling (Ren et al., 2015, Ochoa et al., 1991, Brady et al., 2015a, Hoey et al., 2011, Hoey et al., 2012). Under static conditions, the pressure generated within the marrow medullary cavity, known as intramedullary pressure (IMP), is approximately 4kPa and related to the systemic blood pressure. However, fluctuations in IMP, which are pulsatile by nature, were found to be dependent on muscular contraction, the rate of loading and anatomic location, with magnitudes quantified up to 50 kPa (Downey et al., 1988, Bryant, 1983, Kumar et al., 1979, Hillsley and Frangos, 1994). In addition, stem cells resident within Haversian channels and perivascular space may be exposed to pressures up to 300 kPa generated with loading within the LCS (Ren et al., 2015, Qin et al., 2003, Riddle and Donahue, 2009). Pressure transients are paramount for the loading-induced bone anabolic response, yet, it is not fully understood whether pressure plays a direct role in MSC osteogenesis independent of the secondary fluid shear. This is of significant importance given that *in vitro* fluid flow bioreactors rely on pressure gradients to elicit dynamic flow, and these can vary depending on the inertial effects of fluid flow and geometry of the channels (Stavenschi et al., 2017, Chung et al., 2003, Gardinier et al., 2009, Jacobs et al., 1998).

One of the earliest studies quantifying the effect of pressure on ossifying long bones and calvaria rudiments demonstrated that cyclic hydrostatic pressure enhanced mineral deposition, whereas, continuous hydrostatic pressure had catabolic consequences (Burger et al., 1992). Osteoblasts exposed to cyclic hydrostatic pressure (CHP, 10-40 kPa at 0.3-1 Hz) during short and long-term mechanical stimulation exhibited temporal increases in bone associated markers, as well as terminal osteoblastogenesis (Klein-Nulend et al., 1997b, Nagatomi et al., 2003, Roelofsen et al., 1995, Gardinier et al., 2009). Interestingly, osteoblasts treated with 68 kPa, 0.5 Hz CHP for an hour elicited an increase in the expression of the bone associated marker, Cyclo-oxygenase 2 (*Cox2*), whereas treatment of human bone marrow derived stem cells with a similar magnitude, but at 2Hz frequency, did not mimic this response (Becquart et al., 2016, Gardinier et al., 2009). In contrast, short-term administration of 10-36 kPa of hydrostatic or cyclic

pressure (0.25Hz) was sufficient to elicit an early osteogenic response in the expression of Runt-related transcription factor 2 (*Runx2*), Osterix (*Osx*), Distal-Less Homeobox 5 (*Dlx5*), Msh Homeobox 2 (*Msx2*), Bone Morphogenetic Protein 2 (*BMP2*) and Alkaline Phosphatase (ALP) in bone marrow derived stem cells. (Sugimoto et al., 2017, Liu et al., 2009). Furthermore, 21-day treatment of 10 kPa hydrostatic pressure (HP) per day in osteogenic biochemical induction medium was sufficient to induce osteogenic lineage commitment of human MSCs (hMSCs), whereas 90 kPa HP per day for 2 weeks after biochemical induction did not alter mineral deposition (Sugimoto et al., 2017, Zhao et al., 2015). Therefore, given the variable response to pressure in bone cells and limited analysis in human osteoprogenitors, a systematic study that investigates the effect of physiologically relevant pressure on osteogenic responses in human bone marrow derived stem cells is required.

Hence, the objective of this study is to conduct a systematic analysis of cyclic hydrostatic pressure magnitude, frequency and duration on early osteogenic responses, and to determine whether these mechanical stimuli are sufficient to drive osteogenic lineage commitment of bone marrow derived stem cells in the long term. Understanding how bone micromechanical cues modulate osteogenic hMSCs potential, independent of fluid flow, may open new avenues for orthopaedic regenerative medicine strategies in addition to providing novel platforms to characterise loading induced stem cell osteogenesis.

4.2 Materials and methods

4.2.1 Cell culture

All materials were purchased from Sigma unless otherwise stated. Human bone marrow-derived stem cells (hMSCs) isolated from bone marrow aspirates (Lonza) were used in this study as it represents the best cellular in vitro model for translation research. The MSCs multipotency was characterised by trilineage differentiation (Figure 4-1). Unless otherwise stated, hMSCs were cultured on fibronectin (10 µg/ml, Corning) coated glass slides in low glucose DMEM supplemented with 10% foetal bovine serum (FBS, Biosera) and 1% penicillin-streptomycin (P/S). For short-term mechanical stimulation, cells were cultured for 24hrs under standard conditions followed by 48hrs of serum starvation (0.5% FBS) supplemented with 10nM dexamethasone, 0.025 mM L-ascorbic acid and 10 mM β-glycerol phosphate. These concentrations represent minimal levels for the support of osteogenesis, thereby allowing greater scope to investigate the effect of a biophysical versus a biochemical stimulus (Stavenschi et al., 2017). Regarding long-term cyclic hydrostatic pressure stimulation, the cells were cultured in similar conditions, except supplemented with 2% FBS and 2% P/S.

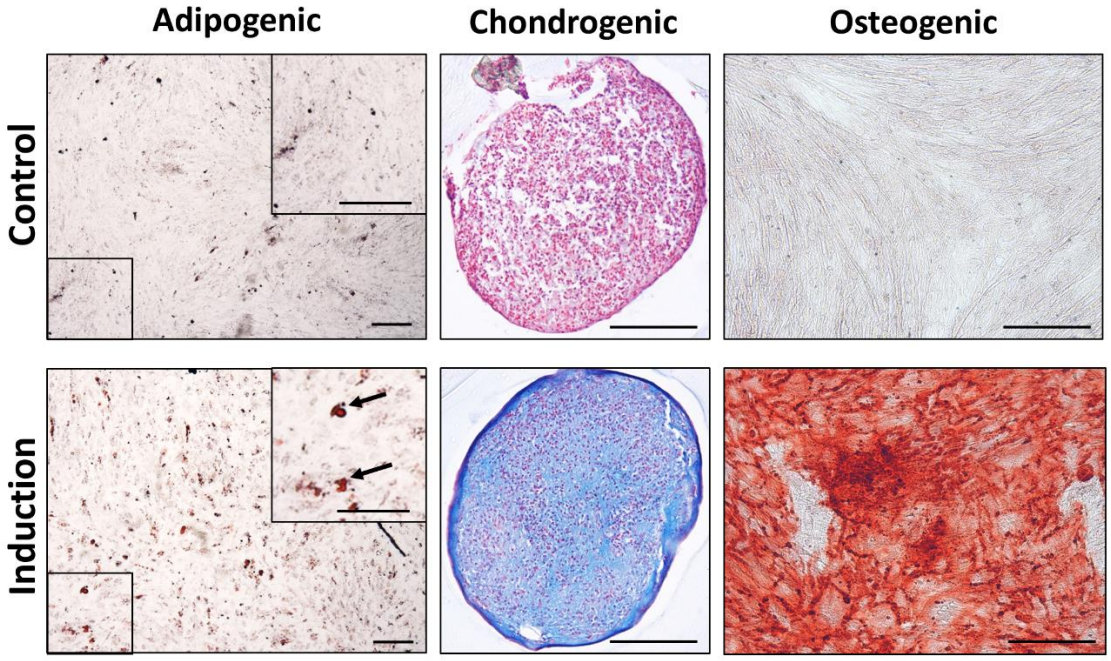
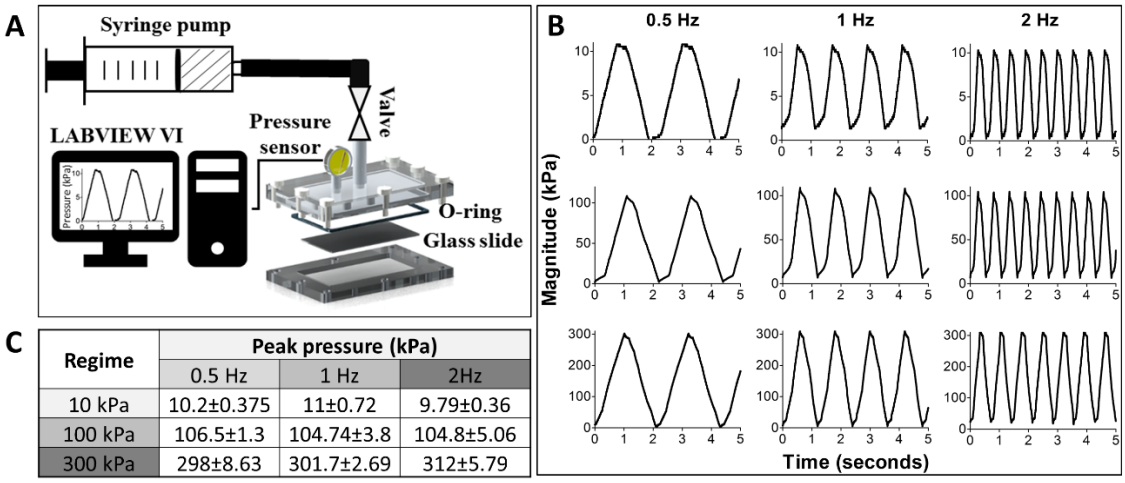


Figure 4-1. Validation of trilineage potential of hMSCs for adipogenesis (Oil red O, A), chondrogenesis (Alician Blue, B) and osteogenesis (Alizarin Red S, C) after 21 days culture. Zoomed in images point to triglyceride accumulation in adipogenic conditions. Scale bar=200 µm

4.2.2 Pressure bioreactor design

The design of the pressure bioreactor is based on the principle of fluid incompressibility whereby delivery of a finite bolus of fluid at various flow rates in a closed system elicits a time-dependent pressure differential. The bioreactor system configuration is composed of a syringe pump (NE1660, New Era Pump Systems) which holds the syringe connected to the custom pressure bioreactor via tubing and a valve, a port on the pressure chamber for real-time pressure oscillations measurement (optional) and a pressure sensor (0-10kPa: HSCMANV015PGAA5, 0-100kPa: SSCDANV150PGAA5, Honeywell) interfaced with LABVIEW Virtual Instrument to monitor and record pressure oscillations over time (Figure 4-2 A). The pressure bioreactor system was empirically validated for pressure magnitudes of 10kPa, 100kPa and 300 kPa at frequencies of 0.5Hz, 1Hz and 2Hz. These pressure magnitudes have been predicted to occur within the bone marrow cavity and lacunar-canalicular system of bone (Ren et al., 2015, Qin et al., 2003) whilst the employed frequencies are representative of human locomotion (Figure 4-2 B, C) (Danion et al., 2003, Nagaraja and Jo, 2014). Given that fluid pressure is delivered to create a transient cyclic pressure differential, the shear stress was assumed to be negligible as volumes less than 2 ml were infused in a system of 50ml of the medium. This was validated using blue dye to visualise the fluid streams during infusion/withdrawal phase where the dye reached only 1/3 of tubing and was not present within the chamber itself (data not presented). Hence, this allows delineating the effect of pressure independent of fluid shear on the osteogenic commitment of hMSCs.



C

Regime	Peak pressure (kPa)		
	0.5 Hz	1 Hz	2Hz
10 kPa	10.2±0.375	11±0.72	9.79±0.36
100 kPa	106.5±1.3	104.74±3.8	104.8±5.06
300 kPa	298±8.63	301.7±2.69	312±5.79

Figure 4-2. Pressure bioreactor design configuration (A) and validation of cyclic hydrostatic pressure regimes of 10 kPa, 100 kPa and 300 kPa at frequencies of 0.5Hz, 1 Hz, 2 Hz. C) Average peak pressures measured at room temperature represented as mean± SD, n=3.

4.2.3 Cyclic hydrostatic pressure (CHP) mechanical stimulation

To systematically delineate the effect of cyclic hydrostatic pressure magnitude, frequency and duration on the early osteogenic response of hMSCs, a series of CHP regimes were employed (Table 8). The range of pressure magnitudes selected represent the physiological pressures elicited within marrow just by muscle contraction independent of whole bone loading (≤10 kPa) and pressure elicited within the lacunar canalicular system due to whole bone loading (300 kPa) (Kumar et al., 1979, Ren et al., 2015). The frequencies of 0.5Hz-2Hz range are representative of human locomotion whereas the duration of mechanical stimulation is based on previous mechanobiology studies (Nagaraja and Jo, 2014, Li et al., 2012, Stavenschi et al., 2017). The CHP parameters were grouped such as to examine the independent effect of peak shear stress, frequency and duration of early osteogenesis. In addition, frequency and CHP duration were coupled such that comparisons of CHP regimes with a constant number of loading cycles can be made i.e. 0.5Hz, 4hrs against 1Hz,2hrs and 2Hz,1hr. This approach is based on a previous systematic investigation of oscillatory fluid flow on mesenchymal stem cells (Stavenschi et al., 2017). The static condition for both short and long-term stimulation consisted of culture slides assembled within chambers and were left open to atmospheric pressure (control). To determine whether cyclic hydrostatic pressure can induce osteogenic lineage commitment, hMSCs were subjected to three separate CHP

regimes over a long-term duration, based on our results from the short-term systematic analysis. The three chosen CHP regimes of 10 kPa,2Hz, 100kP,2Hz and 300kPa 2Hz were applied intermittently on Day 3,5,7,9 for 2 hrs/day and subsequently cultured statically for an additional 7 and 14 days (Figure 4-3).

Table 8. Experimental conditions for short-term fluid pressure stimulation

	Frequency			**Infused volume/cycle**
Shear stress	0.5 Hz	1 Hz	2 Hz	
10 kPa	2,4 hrs	2 hrs	1,2 hrs	≤ 0.03 ml
100 kPa	2,4 hrs	2 hrs	1,2 hrs	≤ 0.375 ml
300 kPa	2,4 hrs	2 hrs	1,2 hrs	≤ 1.5 ml

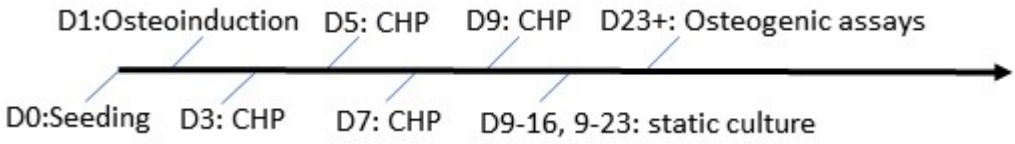


Figure 4-3. Schematic of long-term pressure mechanical stimulation regime.

4.2.4 Quantitative real-time PCR

Cells were lysed using TRI Reagent® (Sigma Aldrich) and mRNA was isolated according to the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Life Technologies). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Select Mastermix (ThermoFisher 4472903). The expression of *18S* ribosomal RNA (*18S*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Cyclooxygenase 2 (*COX2*), Runt Related Transcription Factor 2 (*RUNX2*) and OSTEOPONTIN (*OPN*) were quantified using primers detailed in Table 9 (Sigma). The amplification was performed with an ABI7500 Fast Real-Time PCR machine. Each sample was normalised to reference genes *18S* and *GAPDH*, and to static control using relative quantification method.

Table 9. Human primers and experimental conditions used for qPCR analysis

Gene	Tm (°C)	Primer concentration	Sequence	PCR product size (bp)
18S	60	300 nM	5'- ATCGGGGATTGCAATTATTC -3'	130
			3'- CTCACTAAACCATCCAATCG -5'	
GAPDH	60	300 nM	5'- ACAGTTGCCATGTAGACC -3'	95
			3'- TTTTTGGTTGAGCACAGG -5'	
COX2	60	400 nM	5'- GGAGAAAAGGAAATGTCTGC -3'	186
			3'- GTAGGCAGGAGAACATATAAC -5'	
RUNX2	60	400 nM	5'- GCAGTATTTACAACAGAGGG -3'	112
			3'- TCCCAAAAGAAGTTTTGCTG -5'	
OPN	60	400 nM	5'- GACCAAGGAAAACTCACTAC -3'	84
			3'- CTGTTTAACTGGTATGGCAC -5'	

4.2.5 Adenosine triphosphate assay

After mechanical stimulation for each time point (1,2 and 4 hrs for Day 3,5,7,9), the cells were incubated in 1 ml of medium after which the medium was collected and snap frozen in liquid nitrogen following storage at -80oC. Adenosine triphosphate (ATP) metabolites within media were measured using Molecular Probes® ATP Determination Kit (A22066, Invitrogen) according to manufacturer's protocol. Luminescence was measured using Luminoskan TM Ascent Microplate Luminometer (MTX LAB SYSTEMS).

4.2.6 Osteogenic assays

Cells were fixed in formalin for 10 minutes. For collagen staining, cells were incubated in 0.1% Picro-Sirius Red solution for 1 hour at room temperature. After washing twice in 0.5% acetic acid and water, samples were mounted with DPX mounting medium. Calcium staining was performed using 2% Alizarin Red solution. The bound dye for both calcium and collagen was observed under light microscopy. The bound Alizarin Red was used to quantify the calcium content by extraction using 10%v/v acetic acid and measuring the absorbance at 405nm.

4.2.7 Statistical analysis

All data presented as mean±SEM. For qRT-PCR analysis, each condition was compared to matched static control using a two-tailed student's *t*-test with Welch's correction. CHP to static control (C) is denoted as *p<0.05, **p<0.01, ***p<0.005. One-way ANOVA with Tukey post hoc test was used to compare the effect of magnitude, frequency and duration and significant differences are indicated as # p<0.05, ## p<0.01, ### p<0.005 except for the 2-hour group, where a two-way ANOVA analysis was employed with Bonferroni post hoc test. For calcium quantification, a one-way ANOVA with Dunnett's post-test was performed.

4.3 Results

4.3.1 Cyclic hydrostatic pressure bioreactor design and validation

The design of the pressure bioreactor system was modelled on our previously developed fluid shear bioreactor due to its ease of use and compatibility of glass slides for cell culture and syringe pumps for mechanical stimulation (Figure 4-2 A). The custom pressure bioreactor was designed to allow for pressure stimulation of cell seeded 2D substrates (i.e. glass or PDMS), in addition to cell seeded 3 dimensional scaffolds. By harnessing the power of fluid incompressibility, cyclic pressure transients can be achieved by applying a cyclic oscillatory fluid flow of finite volumes of fluid in a closed system filled with culture medium. Using an external port which allows for real-time measurement of pressures, the pressure bioreactor was validated to generate pressure transients of 10kPa, 100kPa and 300kPa at frequencies of 0.5Hz, 1Hz and 2Hz with a triangular waveform (Figure 4-2 B). The average peak pressures achieved using this system are within 15% of nominal pressures (Figure 4-2 C).

4.3.2 Effect of CHP magnitude on early osteogenic gene expression in hMSCs

Stimulation of hMSCs with cyclic hydrostatic pressure displays a variable osteogenic response based on mRNA expression of osteogenic markers *COX2*, *RUNX2* and *OPN* compared to static conditions. *COX2* mRNA expression is upregulated in response to CHP in a magnitude dependent manner (Figure 4-4 A). This is particularly evident at 0.5Hz,2hr, 0.5Hz,4hr and 2Hz,2hr groups, significant only for the two latter groups ($p<0.001$). In addition, the highest expression within these groups is achieved at 300kPa magnitude and is significantly different to 10kPa and 100kPa ($p<0.05$). Interestingly, for the 2Hz,1hr and 1Hz,2hr group, a consistent ~2-fold change is maintained, displaying little effect of magnitude. This may be attributed to a synergistic time effect as significant magnitude effects are present at a longer duration of stimulation. Overall, CHP did not cause any changes in *RUNX2* expression except an inhibitory magnitude effect was present at 0.5Hz,4hrs time point ($p<0.05$) and a significantly decreased expression at 10kPa and 300kPa at 1hz,2hrs ($p<0.05$) (Figure 4-4 B). *OPN* mRNA expression displayed an inhibitory effect at lower magnitudes of CHP, with a positive magnitude effect for the 2Hz,2hrs and 0.5Hz,4hrs groups ($P<0.05$) (Figure 4-4 C).

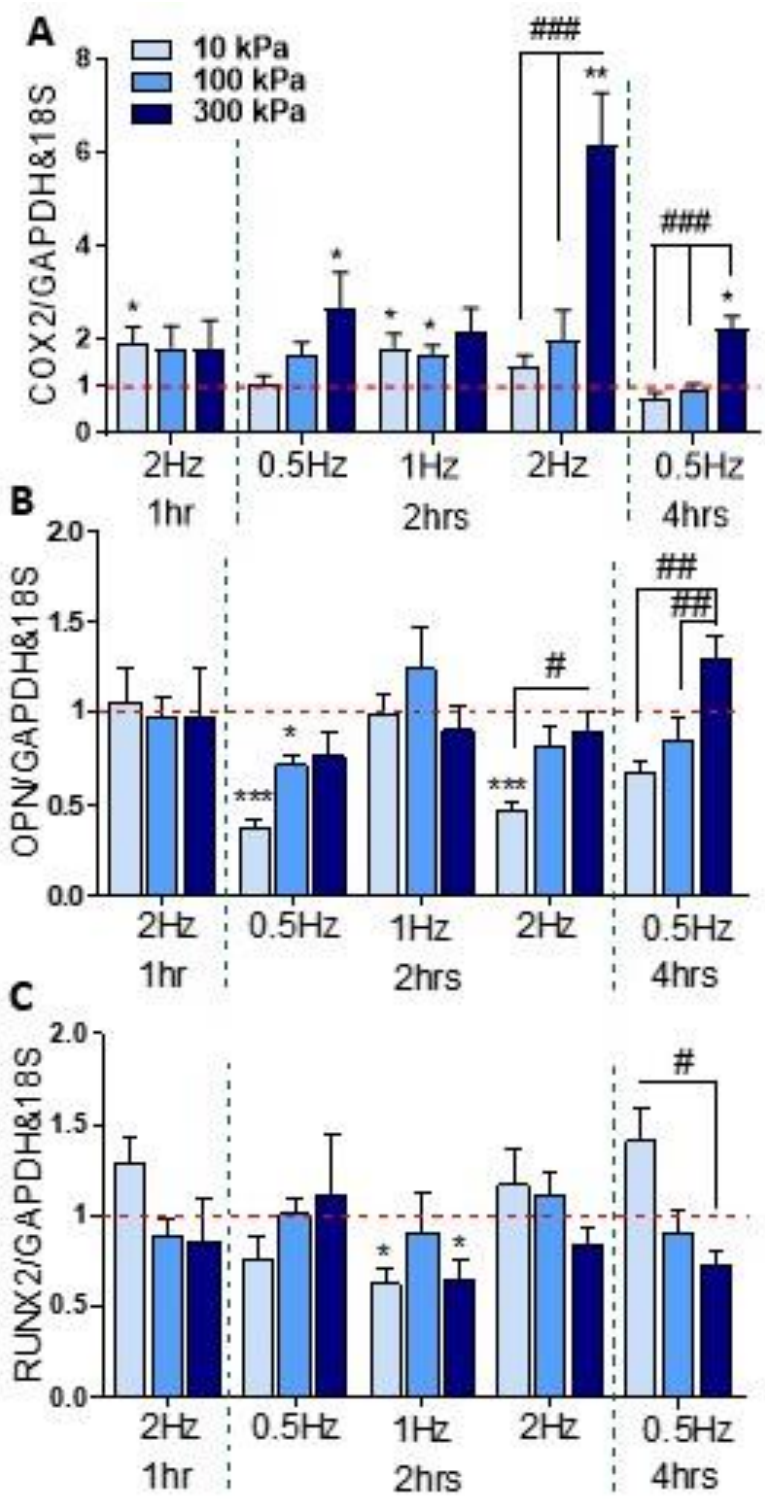


Figure 4-4. Effect of cyclic hydrostatic pressure magnitudes of 10kPa, 100kPa and 300 kPa on early osteogenic gene expression in human skeletal stem cells at frequencies of 0.5Hz, 1Hz and 2Hz. N=2, n=3-4. *p<0.05, ***p<0.001 compared to static control; #p<0.05, ##p<0.01, ### p<0.001 for magnitude effect.

4.3.2 Effect of CHP frequency on early osteogenic gene expression in hMSCs

To identify whether CHP frequency affects early osteogenic mRNA expression, the 2hr pressure group was analysed for 0.5Hz, 1Hz and 2Hz frequencies. Pressure-induced *COX2* mRNA expression displays a similar level of upregulation at all magnitudes irrespective of frequencies except at 300kPa, where a frequency effect is present ($p<0.001$) (Figure 4-5 A). Interestingly, for *RUNX2* and *OPN* mRNA expression, a frequency effect is present only at 10kPa magnitude ($p<0.05$) (Figure 4-5 B, C). Specifically, *OPN* mRNA expression at 10kPa was least inhibitory at 1Hz compared to 0.5Hz and 2Hz ($p<0.001$). This effect is also present at 100kPa, although not significant for frequency effects. Hence, the frequency of pressure stimulation plays a role at higher magnitudes for *COX2* expression and at lower magnitudes for *RUNX2* and *OPN* mRNA expression.

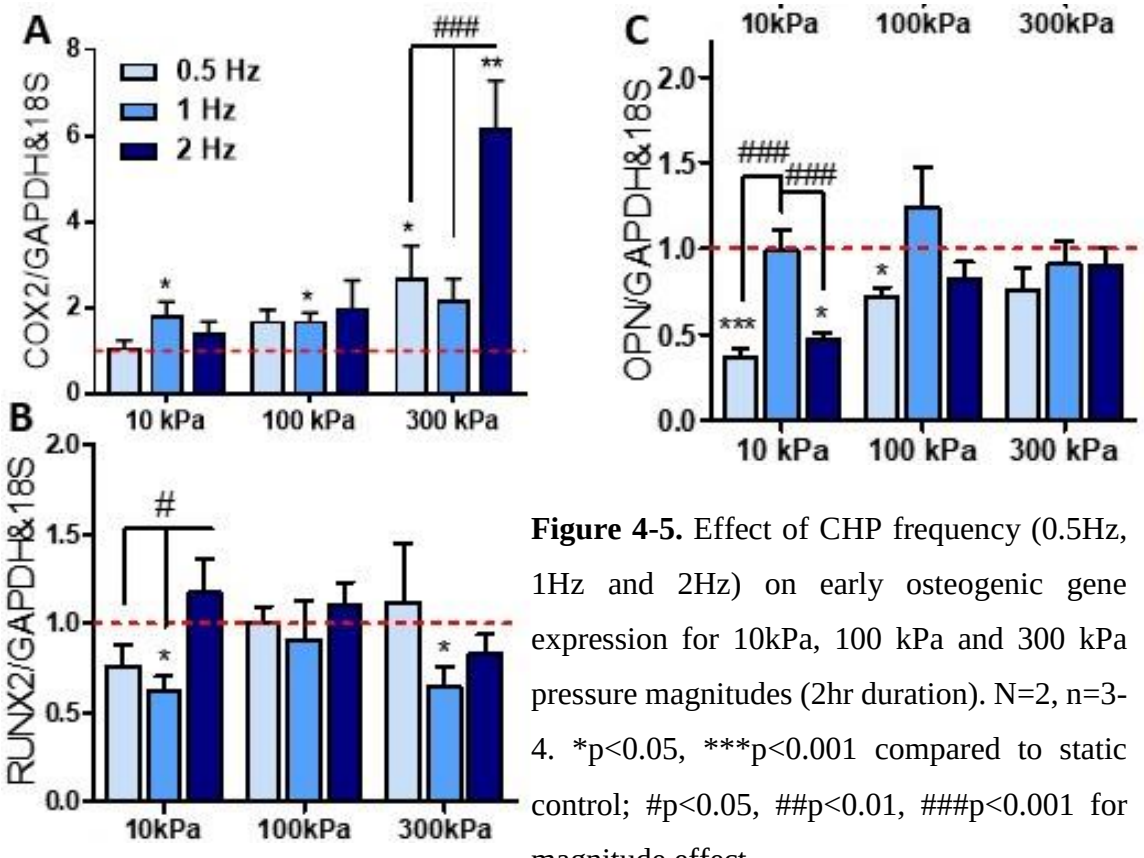


Figure 4-5. Effect of CHP frequency (0.5Hz, 1Hz and 2Hz) on early osteogenic gene expression for 10kPa, 100 kPa and 300 kPa pressure magnitudes (2hr duration). N=2, n=3-4. *p<0.05, ***p<0.001 compared to static control; #p<0.05, ##p<0.01, ###p<0.001 for magnitude effect.

4.3.3 Effect of CHP duration on early osteogenic gene expression in hMSCs

To identify the effect of duration on early osteogenic expression, 1hr and 2hrs, as well as 2hr and 4hrs of stimulation, were compared while other parameters were held constant. Overall, *COX2* mRNA expression displayed similar levels of upregulation over time, except at 300 kPa, 2Hz frequency where a higher response was observed when

stimulated for longer durations (p<0.001) (

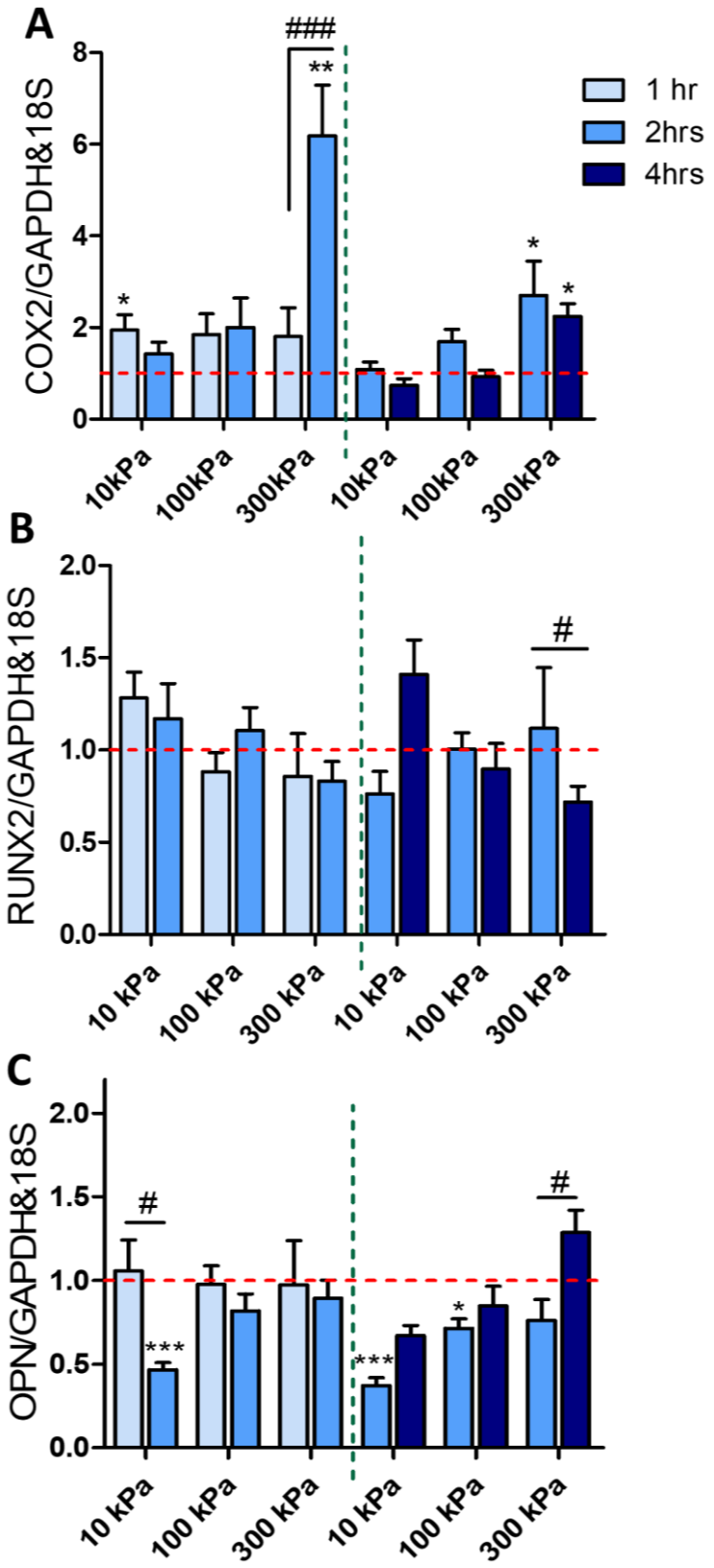
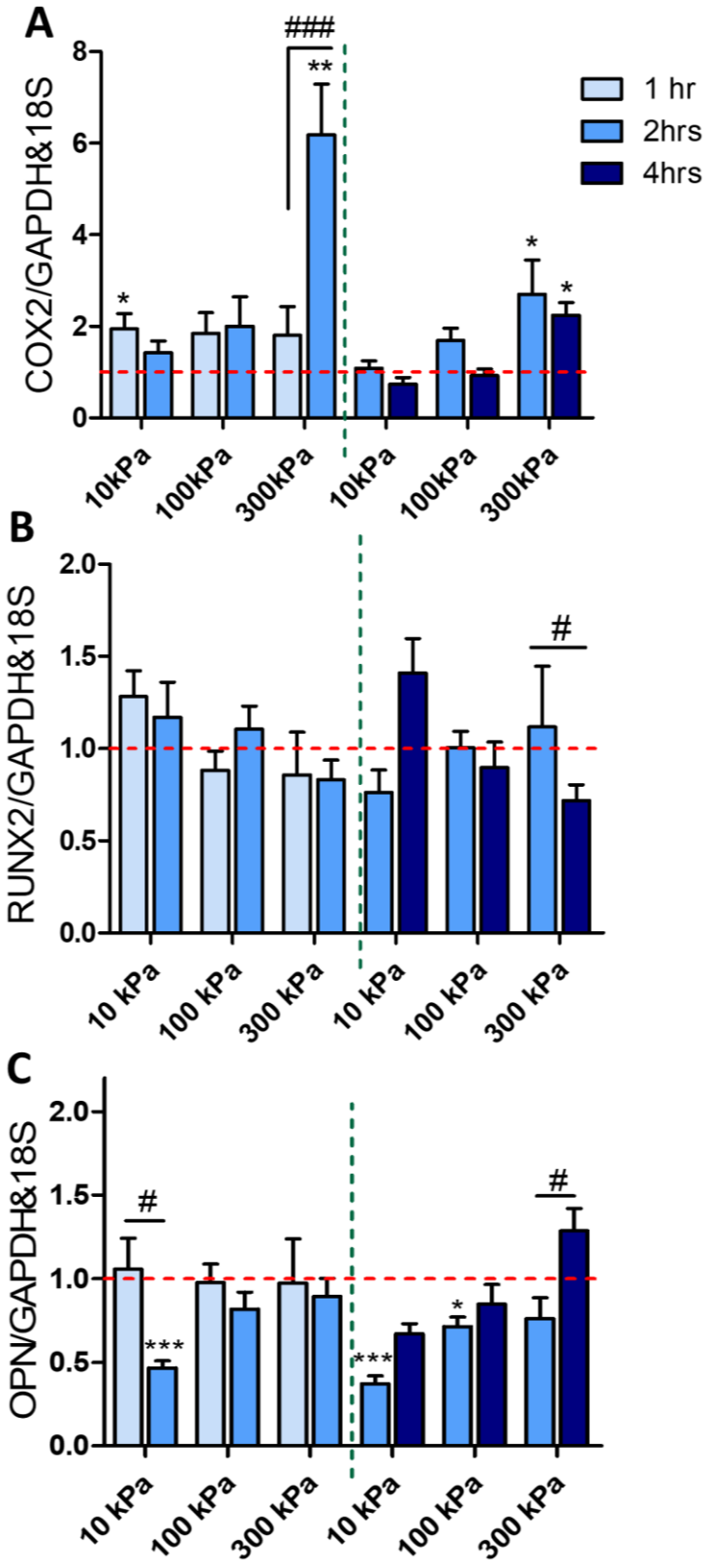


Figure 4-6 A). In contrast, *RUNX2* mRNA expression displayed no changes over time for the compared groups, except at 300 kPa where a significant decrease at 4hrs

versus 2hrs was noted ($p<0.05$). When comparing the 1hr, 2Hz with 2hrs,1Hz and 4hr, 0.5Hz groups, all of which experience the same cyclic hydrostatic pressure magnitude and number of oscillating cycles (7,200), there is a clear abrogated response with the longer duration for 10 kPa and 300kPa suggesting that *RUNX2* expression may have a



time dependency effect (

Figure 4-6 B). No changes over time were observed for *Opn* mRNA expression except an inhibitory effect at 10kPa at 2hrs versus 1 hr ($p<0.05$) and a significant upregulation at 4hrs versus 2 hrs at 300kPa ($p<0.05$) (

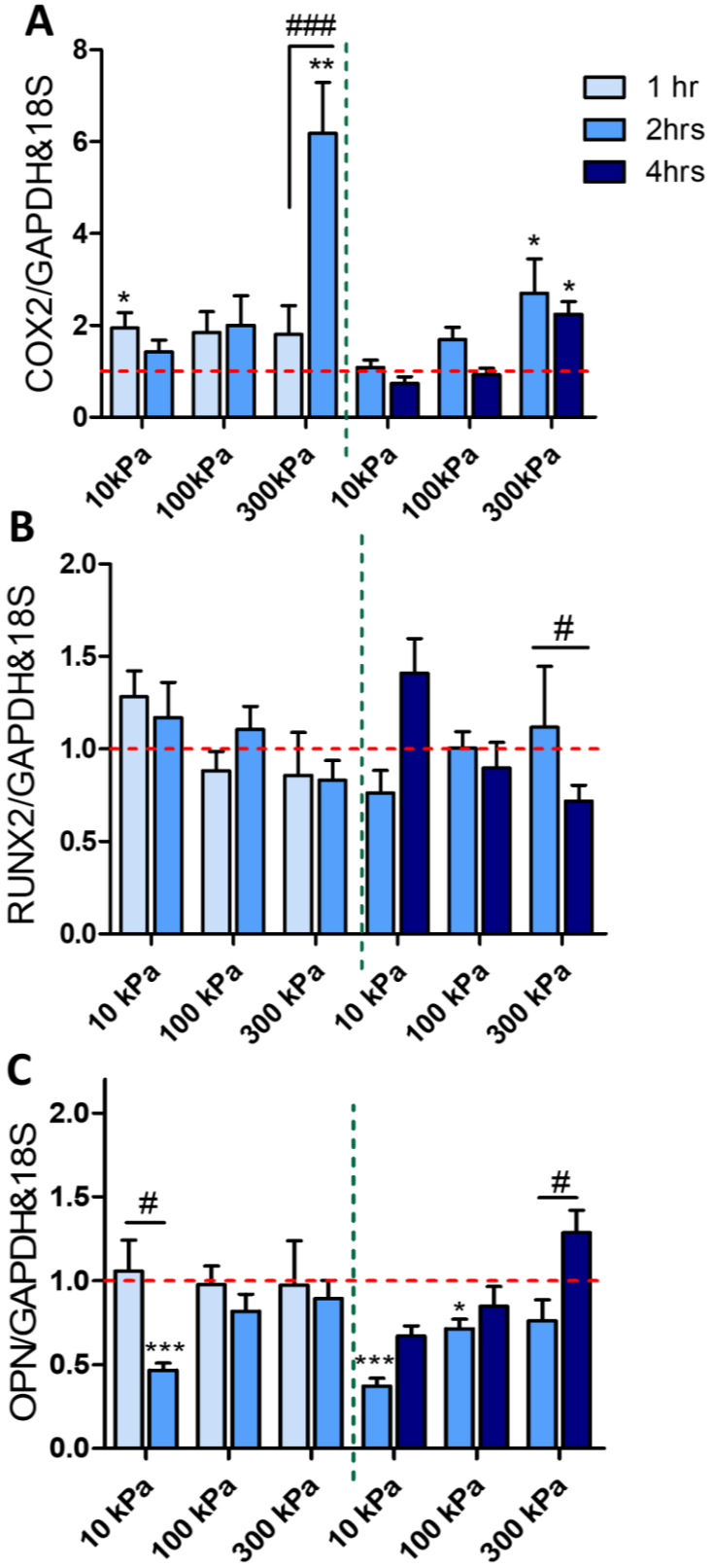


Figure 4-6 C). Therefore, duration of mechanical stimulation plays a role in the level of upregulation of osteogenic markers although this effect is gene and magnitude dependent.

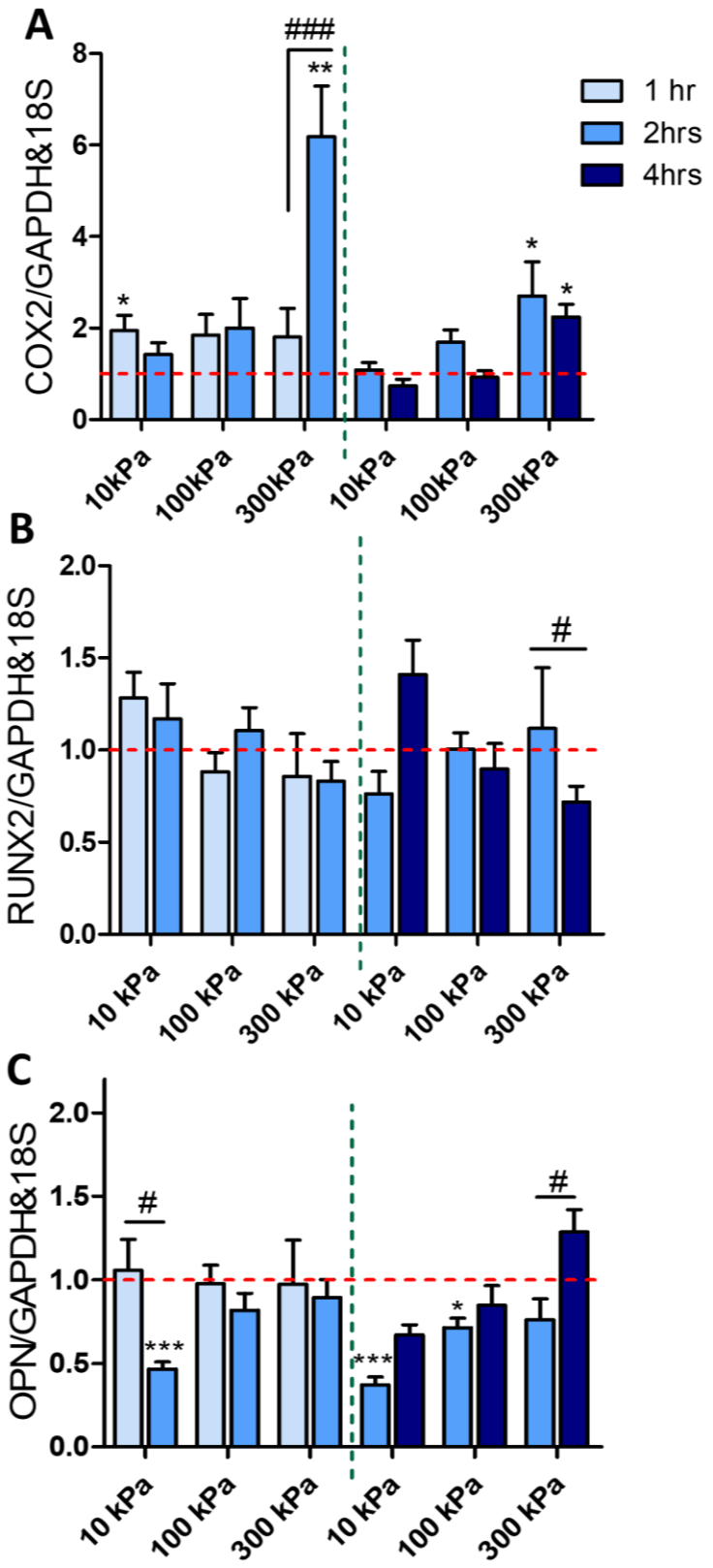


Figure 4-6. Effect of duration of CHP stimulation on early osteogenic gene expression at frequencies of 0.5Hz (2 and 4hrs) and 2 Hz (1 and 2hrs). N=2, n=3-4. *p<0.05, ***p<0.001 compared to static control; #p<0.05, ##p<0.01, ###p<0.001 for magnitude effect.

4.3.4 Effect of long-term intermittent CHP on the osteogenic lineage commitment of hMSCs

Based on the systematic analysis performed above, where the most robust osteogenic gene expression was observed at 2Hz frequency for all pressure magnitudes, these three pressure regimes were brought forward to verify whether CHP can induce the osteogenic lineage commitment of hMSCs. Therefore, the pressure regimes of 10kPa, 100kPa and 300kPa at 2Hz frequency were applied once per day for a total of 4 days followed by 1 and 2 weeks of static culture. Furthermore, to determine whether the hMSCs were responsive throughout the long-term CHP stimulation, ATP release after 1hr of stimulation was determined on each day of pressure stimulation. ATP release within the media of the stimulated hMSCs when compared to static controls, displayed a pressure magnitude effect ($p<0.05$) for all days except Day 7 (Figure 4-7). 10kPa pressure did not induce ATP release at any timepoint. There was a trend towards significance at 100kPa at D3 and D5 ($p<0.07$ at D3, $p<0.14$ at D5), while there was a consistent increase in ATP release following 300kPa pressure ($p<0.05$) at Days 3, 5, and 9. The continuous ATP release over-time confirms that hMSCs maintained their mechanoresponsiveness over extended periods of pressure stimulation and confirms the magnitude effect of pressure stimulation demonstrated above.

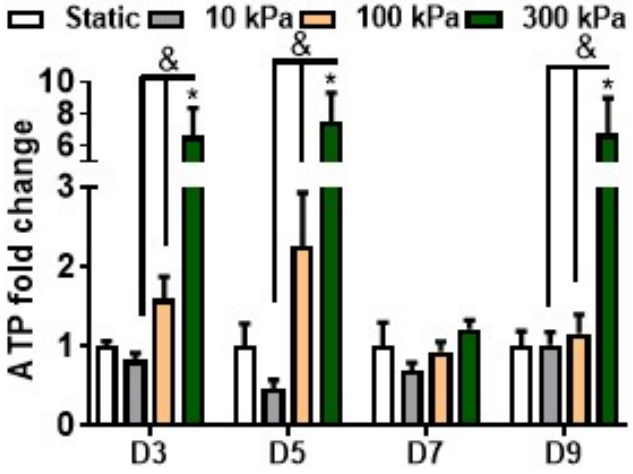


Figure 4-7. ATP release over time during long term intermittent pressure stimulation. The effects of intermittent pressure on ATP release at days 3,5,7 and 9 after 1 hr of mechanical stimulation. n=4-6. *$p<0.05$ compared to either S; &$p<0.05$ for effect of pressure magnitude effect.

After a relatively short-term CHP treatment (4 days), followed by a 2-week static culture, collagen synthesis is observed in all groups. However, the pressure stimulated groups displayed regions of increased collagen deposition when compared to static conditions (Figure 4-8). No distinct qualitative differences in collagen synthesis are noted between the CHP groups.

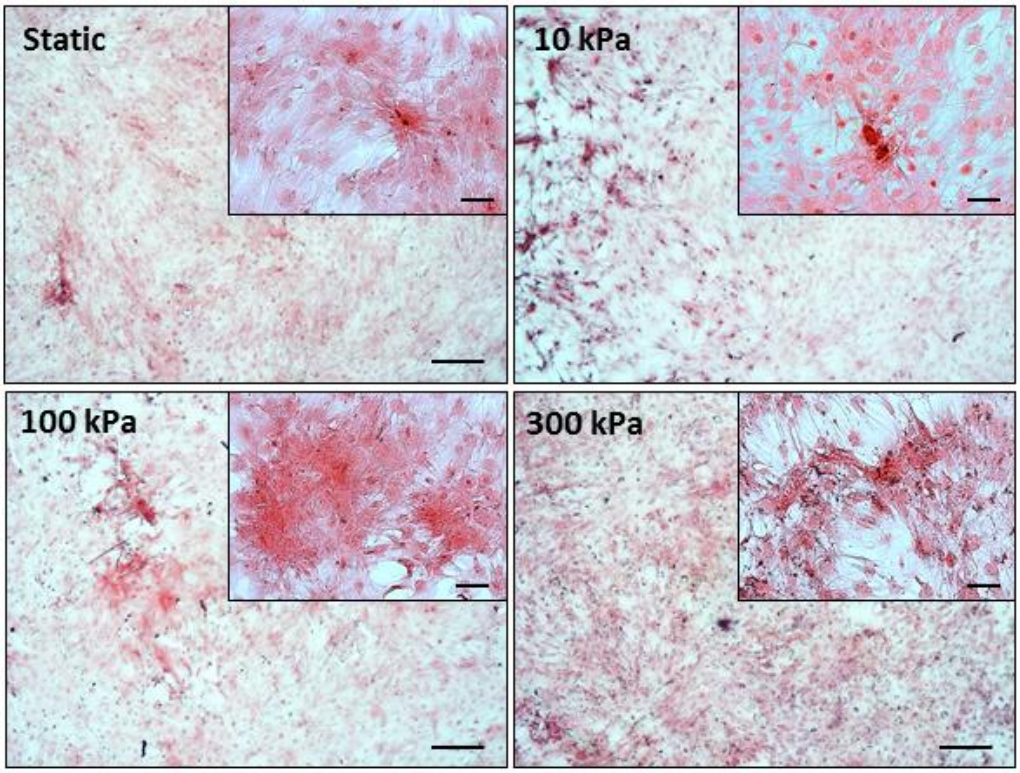


Figure 4-8. PicroSirius Red staining for collagen after long term pressure stimulation (4 days loading and 17 days static). Static- static condition in minimum osteogenic conditions, pressure mechanical stimulation in minimum osteogenic conditions at 10 kPa, 100 kPa and 300 kPa at 2Hz frequency. Scale bar= 250 µm.

Regarding mineralisation, after 1-week post CHP stimulation, there is a slight but significant increase in mineral deposition for the high magnitude 300 kPa,2 Hz group ($p<0.01$) (Figure 4-9 A). However, at 2 weeks post pressure stimulation, quantification of Alizarin Red S staining shows a significant increase in calcium deposition with CHP in all groups ($p<0.05$), with no difference between each magnitude of pressure. The presence of mineralised nodules is detected for all the CHP regimes as illustrated in Figure 4-9 C. Therefore, physiologically relevant cyclic hydrostatic pressures predicted to occur within the bone and marrow cavity is sufficient to directly drive osteogenic

lineage commitment of human stem cells independent of pressure magnitude in the long term, despite demonstrating early magnitude dependent effects.

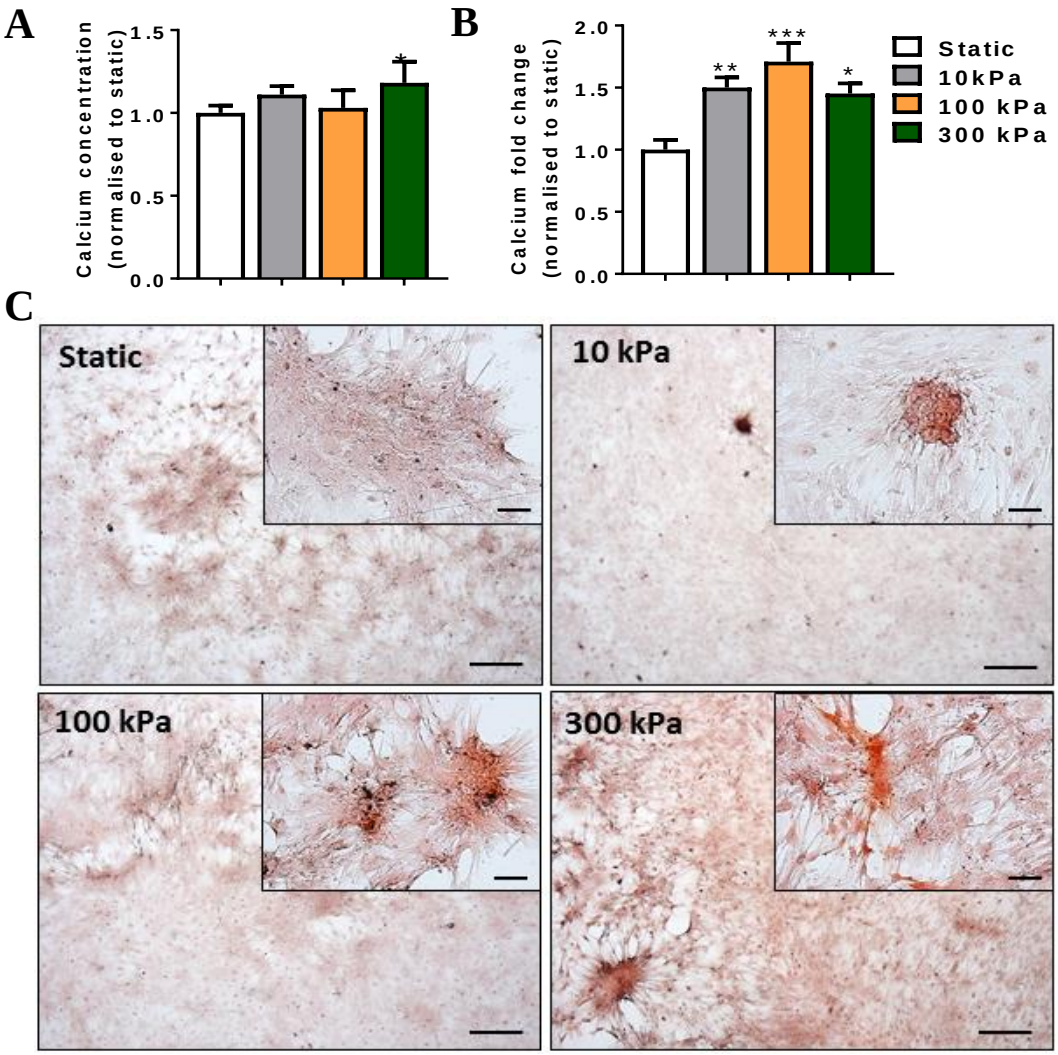


Figure 4-9 Cyclic hydrostatic pressure stimulation induces mineralisation of hMSCs. A) Calcium concentration after 4 days of CHP+10 days static culture (A) and 4 days of CHP+17 days static culture (B). Alizarin S staining for mineralisation after long term pressure stimulation show presence of mineral nodules with CHP (4 days CHP+17 days static culture) (C). Static- static condition in minimum osteogenic conditions, pressure mechanical stimulation in minimum osteogenic conditions at 10 kPa, 100 kPa and 300 kPa at 2Hz frequency. Scale bar= 250 µm. *p<0.05, **p<0.01, ***p<0.001.

4.4 Discussion

In the previous chapter it was demonstrated that physiological levels of fluid flow that are predicted to occur within bone marrow niche, drives stem cell osteogenic lineage commitment in a shear stress and magnitude dependent manner (Stavenschi et al., 2017). However, the effect of pressurisation of the intraosseous fluid, which drives this fluid flow, and its effects independent of fluid shear on stem cell osteogenesis is poorly understood. For the first time, it is reported a systematic in vitro analysis on the effect of physiological CHP magnitude, frequency and duration on human bone marrow stem cell osteogenic responses. Through the utilisation of custom-built pressure bioreactors, it was demonstrated that CHP elicits a variable, yet, positive osteogenic response in hMSCs in a magnitude, frequency, and duration-dependent manner, that is gene specific. Furthermore, long-term mechanical stimulation utilising 10-300kPa pressure magnitudes at 2Hz frequency promoted collagen synthesis and significant mineral deposition compared to static control, proving that physiological levels of pressure elicited within the marrow and bone with loading is sufficient to directly drive osteogenic lineage commitment. Moreover, mineral deposition due to CHP stimulation was found to be independent of pressure magnitude, despite early osteogenic magnitude dependent effects. These findings highlight that bone marrow physiological CHP can directly drive stem cell osteogenesis, independent of fluid flow, which should be considered when studying pressure driven fluid shear effects in hMSC mechanobiology. Moreover, these findings may open new avenues for orthopaedic regenerative medicine strategies, in addition to providing novel platforms to characterise loading induced stem cell osteogenesis.

Human MSCs were found to be mechanosensitive to pressure stimuli and elicited magnitude, frequency and duration dependent osteogenic responses to physiological bone CHP. *COX2* and *OPN* mRNA expression were found to be magnitude dependent at 2Hz,2hrs and 0.5Hz,4hrs whereas *RUNX2* expression did not demonstrate this dose-dependent effect, possibly highlighting activation of alternative pathways. Moreover, when normalising for the number of oscillations comparing 1Hz,2hrs versus 2Hz,1hr (7200 oscillations), *COX2* and *OPN* mRNA expression is similar and independent of pressure magnitude, whereas, when doubling the amount of oscillations at 2Hz,2hrs the magnitude effects become significant. This may indicate that for magnitude dependent effects a cumulative threshold of stimulation must be reached for differential expression

of osteogenic genes to be elicited. *COX2* expression was shown to be sensitive to the magnitude of fluid flow stimulation in both osteocytes and stem cells and its expression has been highlighted as a precursor for osteoblastogenesis (Stavenschi et al., 2017, Li et al., 2012, Zhang et al., 2002). Pressure-induced *COX2* expression was found to be elicited in osteocytes (68kPa, 0.5Hz) and osteoblasts (68kPa, 0.5Hz) but not hMSCs (50kPa,2Hz), which may indicate that the pressure response was either frequency or lineage dependent (Becquart et al., 2016, Liu, 2010, Gardinier et al., 2009). Given that CHP induced a magnitude dependent response in our experimental setup, this could be attributed to the prior biochemical priming of hMSC for 2 days before mechanical stimulation. However, pressure also induces *Cox2* upregulation in a mesenchymal stem cell line (MSC), C3H10T1/2 using the same system, highlighting the possibility in the difference of the bioreactor set up and mode of pressurisation in other studies (Chapter 5).

Interestingly, the overall expression of *OPN* seemed unaltered or inhibited when compared to static culture, with several exceptions of positive magnitude effect at 0.5Hz and 2Hz frequencies. Changes in *OPN* mRNA levels have been reported to depend on the stage of osteogenic differentiation, as osteoprogenitor cells were found to exhibit lower basal levels and no changes in *Opn* mRNA expression in response to 13kPa, 0.3Hz CHP when compared to late-stage osteoblasts (Klein-Nulend et al., 1997b). Similarly, the observation that the master transcription factor *RUNX2* remained unaltered or downregulated in some CHP regimes, may indicate that either CHP inhibits osteogenesis or alternatively results in changes in the translation of the protein, as has been previously shown in osteoblasts using a cyclic stretch model (Zuo et al., 2015). Interestingly, CHP was previously shown to stimulate collagen synthesis and bone mineral deposition in chick femurs, although no changes in *Runx2* expression was observed (Henstock et al., 2013). Similar to our observations, pressure induced osteogenic lineage commitment of hMSCs, in spite of unaltered *RUNX2* expression, alluding to the changes in protein translational activity.

Pressure induced an early osteogenic response in hMSC showing frequency dependent effects which were magnitude-gene coupled. Specifically, *COX2* mRNA expression displayed a frequency dependent effect at 300kPa magnitude, whereas *RUNX2* and *OPN* at 10kPa CHP. Interestingly, fluid flow induced *Cox2* mRNA expression in both MSCs and osteocytes was found to have weak frequency effects,

except at high magnitudes, demonstrating this mechanically driven *COX2* response is consistent across all forms of mechanical stimulation. Increase in bone formation due to CHP stimulation in chick femurs was found to be proportional to frequency but independent of pressure magnitude applied (Henstock et al., 2013). Similarly, 2Hz was found to be the optimum frequency for loading-induced mineralisation in an osteoblast model of dynamic compression (Tanaka and Tachibana, 2015). This effect may be attributed to a universal magnitude-frequency response, possibly related to human kinematics and loading-induced bone formation (Li et al., 2012, Stavenschi et al., 2017, Zhao et al., 2014, Hsieh and Turner, 2001).

During long-term stimulation, using the most pro-osteogenic magnitude dependent 2Hz frequency regime, it was observed that hMSCs were mechanoresponsive to CHP loading overtime by secreting ATP metabolites into the medium. Moreover, this ATP release was magnitude dependent over time at each loading event. To date, only osteoblasts have been shown to elicit an ATP response to CHP, whereas, hMSCs were found to secrete ATP in a fluid flow magnitude dependent manner (Gardinier et al., 2014, Riddle et al., 2008, Riddle et al., 2007). Purinergic signalling plays a crucial role in bone anabolism, therefore ATP synthesis in response to mechanical stimulation may highlight initiation of mechanotransduction events, irrespective of type of stimulus (Ciciarello et al., 2013, Noronha-Matos and Correia-de-Sa, 2016, Li et al., 2015). Although hMSCs display a pressure magnitude dependent sensitivity for ATP release, this did not correlate to the ability to synthesise bone mineral indicating that other mechanisms may be at play.

Cyclic hydrostatic pressure induces osteogenic lineage commitment of hMSCs independent of magnitude of stimulation. This supports various reports, where pressure induced mineralisation in osteoblasts and bone marrow derived stem cells, was shown to be modulated in vitro at low (<40kPa) and high (200kPa) magnitudes, either statically or dynamically (Nagatomi et al., 2003, Roelofsen et al., 1995, Burger et al., 1991, Klein-Nulend et al., 1997b, Sugimoto et al., 2017, Kim et al., 2010). *Ex vivo* intramedullary pressurisation of ulnae, independent of matrix deformation, showed a positive correlation between transcortical pressure gradients and enhanced bone formation. This effect was associated to fluid flow, as transcortical pressure gradients are related to fluid flow velocity (Qin et al., 2003). Similarly, magnitude of shear stress, at the same frequency (2Hz) of CHP stimulation, was shown to elicit a positive effect on mineralisation in stem cells (Stavenschi et al., 2017). Biomechanically, bone

deformation causes pressurisation of intraosseous fluid which forces it to flow within the bone. Given the incompressibility of the water based intraosseous fluid, small pressure gradients can elicit large inertial fluid effects due to the architecture of bone tissue. From this perspective, and the biological effects reported above, it can be concluded that pressure gradients play a role in hMSCs osteogenesis, but this effect may be secondary to fluid flow. However, since fluid flow bioreactors rely on pressure driven flow that at often times reach pressure differentials higher than 10kPa, these pressure effects should be accounted for in mechanobiology studies. A limitation of this study is that only the 2Hz frequency CHP regime was used for long term pressure induced osteogenesis, hence these effects may be specific to this frequency alone.

4.5 Conclusion

In conclusion, it was demonstrated that hMSCs are mechanosensitive to pressure stimuli with a magnitude dependent *COX2* mRNA expression and ATP associated purinergic signalling. Although *RUNX2* and *OPN* mRNA expression remained unaltered with short term CHP stimulation, application of physiologically associated cyclic hydrostatic pressures, over a long term, was found to induce osteogenic lineage commitment of hMSCs, which was independent of pressure magnitude and frequency. This highlights that physiologically low marrow pressures can also affect the hMSCs osteogenic potential, similar to fluid flow, however, its effects may be secondary. The systematic approach taken enabled the identification of early pressure differential effects which can be used to delineate the mechanisms of pressure induced osteogenesis. This can also serve as a platform to discover novel targets for bone therapies (Sugimoto et al., 2017). Moreover, these findings may support the use of mechanotherapies in clinical applications for patients displaying osteopenia/bone fragility, in addition to optimisation of bioreactor design for bone regenerative strategies.

Chapter 5.
Pressure induced osteogenesis in MSCs is dependent on the remodelling of intermediate filaments

5.1 Introduction

Physical forces play a paramount role in the maintenance of skeletal mass and structure. This mechanical-biological coupled relationship is facilitated by the translation of macroscale loading forces to the bone and marrow micromechanical milieu, where resident bone and mesenchymal stem cells (MSCs) sense and translate these finely orchestrated cues into a bone anabolic biochemical response (Robling et al., 2006, Hu et al., 2012, Kwon et al., 2010a). In particular, loading induced stem cell osteogenesis is essential in this process to ensure replenishment of the osteoprogenitor population (Tewari et al., 2015, Chen et al., 2016a). Bone associated mechanical cues predicted to occur in vivo such as matrix deformation (strain), pressure and fluid shear have been shown to modulate stem cell osteogenesis in a controlled in vitro setting (Feria-Diaz et al., 2018, Qin et al., 2003, Liu et al., 2009). Despite great strides being taken in our path to identify the associated loading induced mechanotransduction mechanisms pertaining to osteogenesis in response to strain and fluid flow, little work has explored the role of pressure transients in bone physiology (Feria-Diaz et al., 2018). Given that pressure transients act as an intermediary stimulus to fluid flow, it is necessary to understand how these mechanical cues are translated into an osteogenic response to discern the mechanism of biophysical regulation of stem cell osteogenesis.

Pressure stimulation can modulate osteogenic signalling in MSCs. A number of recent studies have demonstrated that pressures up to 300 kPa can drive MSC osteogenesis as depicted by upregulation of the osteogenic associated genes *Runx2*, *Dlx5*, *Osx*, and ALP (Sugimoto et al., 2017, Liu et al., 2009). Moreover, low bone physiological pressures (10 kPa) were sufficient to promote mineralisation of hMSCs

further supporting the importance of pressure in regulating osteogenic lineage commitment (Liu, 2010, Liu et al., 2009, Burger et al., 1992, Burger et al., 1991, Klein-Nulend et al., 1997b, Roelofsen et al., 1995, Sugimoto et al., 2017). These observations are consistent with the data presented in the previous chapter demonstrating that physiological levels of pressures typical in bone (kPa range) elicit an early osteogenic response which is magnitude dependent for both ATP release and *Cox2* gene expression and that long-term pressure stimulation can drive full osteogenic lineage commitment of hMSCs, but surprisingly ECM deposition is magnitude independent. Despite this clear osteogenic response to pressure stimulation, little is known about how MSCs sense and transduce pressure mechanical cues with only one study to date highlighting the involvement of mechanosensitive ion channel Piezo 1 (Sugimoto et al., 2017). Observations of cytoskeletal remodelling in response to pressure stimuli in bone cells showcase its mechanosensitivity to such mechanical cues (Gardinier et al., 2009, Gardinier et al., 2014, Liu, 2010, Mathieu and Loboa, 2012). However, the importance of cytoskeleton in mediating pressure responses is yet to be explored. The cytoskeleton is a three-dimensional scaffolding which compartmentalizes the organisation of cellular organelles in addition to modulation of intracellular trafficking and presence of surface receptors amongst others (Doherty and McMahon, 2008). The dynamics of the structural cytoskeletal elements described through the tensegrity model, is represented by the tension bearing elements of actin and intermediate filaments and compression resisting microtubules (Ingber, 1997). Modification of this finely tuned balancing act has been attributed to changes in cellular activity such as mitosis, mechanotransduction, differentiation and senescence (Rodriguez et al., 2004, Nishio et al., 2001). Moreover, MSC differentiation and associated mechanotransduction mechanisms are intrinsically coupled as the cytoskeletal architecture constantly evolves during this process (Myers et al., 2007, Malone et al., 2007, Espinha et al., 2014b). Hence, it is pertinent to identify the individual contribution of each cytoskeletal element during MSC osteogenesis, especially in response to mechanical cues.

Mechanical driven cytoskeletal adaptation has been reported to occur in all bone cells, including MSCs (Mathieu and Loboa, 2012, McBeath et al., 2004). However, it is yet to be fully understood how each cytoskeletal component contributes to the osteogenic response, and in particular the influence of mechanical cues such as pressure in this process. Actin remodelling is a distinct cytoskeletal hallmark of osteogenesis and it is

paramount for fluid flow-induced osteogenesis (McBeath et al., 2004, Arnsdorf et al., 2009, Sen et al., 2015). Although the microtubule network can regulate actin dynamics, it was found to not impact MSC osteogenesis, yet is important in osteoblast fluid flow mechanotransduction (Rodriguez et al., 2004, Myers et al., 2007). The cytoskeletal changes such as actin stress fibre formation in both MSCs and osteoblast in addition to spreading of the microtubule network beyond the perinuclear region in osteoblasts was reported in response to hydrostatic pressure (Gardinier et al., 2009, Zhao et al., 2016). However, the biological significance of these architectural changes to pressure is undetermined. It is imperative to note that little is known about the role of intermediate filaments (IF) in modulation of MSC differentiation although it has been shown to interact with the actin network during cell morphogenesis and is associated with chemically induced osteoblastogenesis (Lian et al., 2012, Jiu et al., 2015). In addition, MSCs cultured in hydrogels and exposed to high magnitude hydrostatic pressure known to induce chondrogenesis, display disorganisation in intermediate filaments (Steward et al., 2013). However, the involvement of IF in any osteogenic associated mechanotransduction mechanism is yet to be elucidated.

5.2 Methods

5.2.1 Cell culture

All materials were purchased from Sigma unless otherwise stated. Murine mesenchymal stem cells (C3H10T1/2) were cultured on fibronectin (10 µg/ml) coated glass slides in low glucose DMEM supplemented with 10% foetal bovine serum (FBS: Biosera) and 1% penicillin-streptomycin unless otherwise stated. Prior to mechanical stimulation, cells were cultured for 24 hours under standard conditions followed by 48 hours of serum starvation (0.5% FBS). To disrupt each cytoskeletal element, the cells were treated for a total of 1.5 hrs using pharmacological agents 2 µM Cytochalasin D (CD), 4 µM Nocodazole (NC) and 10 nM Withaferin A (WA) (Cayman Chemical) diluted in 0.1% DMSO (dimethyl sulfoxide), which also acts as a control. Cytochalasin D at low doses inhibits actin polymerization by capping the barbed end of F-actin polymers (Malone et al., 2007). Nocodazole is a synthetic drug which affects the polymerization of microtubule network. Withaferin A has been demonstrated to disassemble intermediate filaments through interaction with vimentin (Grin et al., 2012).

5.2.2 Hydrostatic and cyclic hydrostatic pressure mechanical stimulation

A previously described custom pressure bioreactor was utilised to administer hydrostatic and cyclic hydrostatic pressures (Chapter 4). The bioreactor consists of a rectangular chamber that houses a glass slide onto which cells are cultured, an inlet for infusion of finite volumes of fluid to achieve transient or static pressurisation via a syringe pump. Fluid shear stress is minimal as no valves are open to allow fluid to flow (Gardinier et al., 2009). The pressure bioreactor was validated for a pressure of 100kPa at 1Hz frequency, which is a magnitude predicted to occur within bone and bone marrow whereas the chosen frequency is associated with human locomotion (Ren et al., 2015, Danion et al., 2003). In addition, a constant 100kPa hydrostatic pressure was employed, as it is commonly used mechanical regime for *in vitro* pressure mechanobiology studies (Liu et al., 2009, Zhao et al., 2016, Zhao et al., 2015). Control slides, which were not subjected to pressure mechanical stimulation, were assembled within the chambers, and exposed to atmospheric pressure (37oC, 5% CO2) for the matched duration of mechanical stimulation.

5.2.3 Quantitative real-time PCR

Immediately post mechanical stimulation, cells were lysed using TRI Reagent® and mRNA was isolated per the manufacturer's protocol. The 260/280 and 260/230 absorbance ratios were measured for verification of the purity and concentration of RNA. The RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Life Technologies). Quantitative polymerase chain reaction (qPCR) was executed using SYBR Select Mastermix with ROX passive dye (ThermoFisher 4472903). The expression of osteogenic markers *18S*, *Cox2*, *Runx2* and Osteopontin (*Opn*), SRY (Sex-Determining Region Y)-Box 9 (*Sox9*) and Peroxisome Proliferator Activated Receptor Gamma (*Pparg*) was quantified using primers detailed in Table 7 and Table 10 (Sigma). The amplification was performed with an ABI7500 Fast Real Time PCR machine and melt curve analysis was implemented as a control for primer dimer formation. Each sample was normalised to reference gene *18S* and static control using the relative quantification method.

Table 10 Mouse primers and experimental conditions used for qPCR analysis

Gene	Tm (^{o}C)	Primer concentration	Sequence
Pparg	60	400 nM	5'- AAAGACAACGGACAAATCAC-3'
			5'-GGGATATTTTTGGCATACTCTG -3'
Sox9	60	400 nM	5'-CTCATTACCATTTTGAGGGG -3'
			5'-AAAATACTCTGGTTGCAAGG -3'

5.2.4 Immunocytochemistry and imaging

Briefly, cells were fixed in 4% paraformaldehyde for 10 min, permeabilised in 0.1% Triton-X for 10 min followed by 2-hour incubation in 0.1% BSA. The following antibodies were used: anti-vimentin (Abcam ab92547, 1:500 dilution) for intermediate filaments, anti-α-tubulin (Abcam ab80779, 1:500 dilution) for microtubules, Alexa Fluor 488 Phalloidin (Life Technologies A12379, 1:40 dilution) and Rhodamine Phalloidin (BTIU00027, VWR, 1:40 dilution) both against f-actin. Secondary antibodies Alexa Fluor 488 (Life Technologies, A11008) and Alexa Fluor 594 (Life Technologies, A21203) were used at 1:500 dilutions. Nucleus was stained with DAPI (Sigma 32670-5MG-F, 1:2000 dilution). Coverslips were mounted with Prolong mounting medium (ThermoFisher). For representative images of the cytoskeleton structure, a maximum projection was taken with Leica SP8 scanning confocal fitted a 63x oil (NA 1.4)

objective. The same imaging strategy was employed for the high definition super resolution images of intermediate filaments using Leica SP8 Gated Stimulated Emission and Depletion confocal microscope fitted with a 592nM laser and 100x objective. Deconvolution of images was performed using Huygens Professionals software.

5.2.5 SDS-Page and Western Blot

Immediately post mechanical stimulation cells were scraped in PBS, centrifuged for 5-10min and then lysed in Cell Lysis Buffer (Cell Signalling Technology, #9803) supplemented with phenylmethylsulphonyl fluoride (1:200). Total protein concentration was quantified using Pierce™ BCA Protein Assay Kit following the manufacturer's instructions (ThermoFisher). Samples were diluted in SDS reducing Laemmle sample buffer and boiled for 5 min at 95^{o}C. Protein samples were resolved by SDS-PAGE using the Mini-6 PROTEAN® Tetra Cell Electrophoresis System (Bio-Rad). 15-20 µg of proteins were loaded in 12.5% polyacrylamide gels and the migration was run in Tris-Glycine-SDS running buffer. Proteins were transferred using electrophoresis onto 0.45µm PVDF membranes (Amersham) using the Pierce G2 Semi Dry Transfer System with Pierce™ 1-Step Transfer Buffer (Biosciences). Membranes were blocked with 5% BSA in TBST (Tris Buffer Saline–0.1% Tween20) and stained overnight at 4°C for vimentin (Abcam ab92547, 1:4000) and β-actin (Sigma Aldrich A5441, 1:1000) in TBST buffer containing 5% BSA. Membrane stripping was performed using Restore™ PLUS Western Blot Stripping Buffer. The blots were revealed using an anti-rabbit HRP antibody (Abcam ab97051, 1:20000 incubated 1 hour at room temperature) and ECL Select detection kit (Amersham) according to the manufacturer's instruction. The membranes were imaged using Gel Doc™ XR+ System (Bio-Rad).

5.2.6 Statistical analysis

All data presented as mean ± SEM. A two-tailed student's t test with Welch correction was employed for gene expression to compare the effect of HP or CHP against static control (*$p<0.05$, **$p<0.01$). N - represent experimental repeats, n - represents total sample numbers.

5.3 Results

5.3.1 Pressure elicits an early osteogenic response that is dependent on the dynamic nature of the stimulus

Overall, the application of pressure mechanical stimulation to MSCs resulted in a positive yet variable temporal osteogenic response. The most robust osteogenic response is elicited after one hour of stimulation with both hydrostatic (HP) and cyclic hydrostatic pressure (CHP) (Figure 5-1). *Cox2* mRNA expression was increased 3-fold ($p>0.05$) with HP and 5-fold ($p<0.05$) with CHP at the 1hr timepoint, with no changes present in either mode of pressure at 2 and 4hrs of stimulation (Figure 5-1 A). HP maintains a stable ~1.5 increase ($p>0.05$) for *Runx2* mRNA expression over time, whereas CHP elicits a significant 2.5-fold and 3.7-fold increase after 1hr and 4hrs of stimulation, respectively (Figure 5-1B). *Opn* mRNA expression is elevated 2-fold ($p>0.05$) after 2hrs of stimulation with HP, whereas CHP elicits a 2.5-fold increase at 1hr of stimulation, which decays over time (Figure 5-1C). Interestingly, chondrogenic and adipogenic transcription factors Sox9 and Pparg, respectively, show the most robust effect at 1 hr of CHP stimulation (Appendix 1). Since, one hour of cyclic hydrostatic pressure treatment gave rise to the most robust osteogenic response for all studied genes, this mechanical regime was used as a model to explore the role of the cytoskeleton in MSC pressure mechanotransduction in all further experiments.

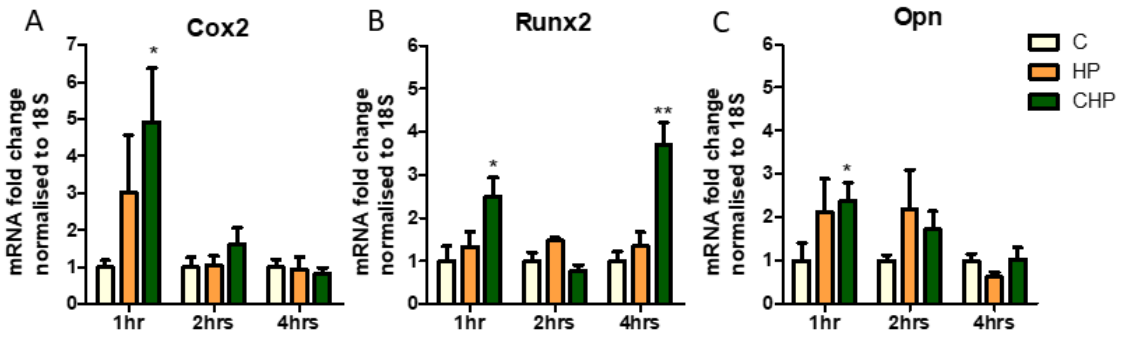


Figure 5-1. Effect of hydrostatic (HP) and cyclic hydrostatic pressure (CHP) on the osteogenic markers (*Cox2*, *Runx2* and *Opn*) in MSCs after 1,2 and 4hrs of stimulation. Mean±SEM. *$p<0.05$, **$p<0.01$, N=2, n=3-4.

5.3.2 Pressure initiates cytoskeleton remodelling in MSCs

To identify whether pressure stimulation drives alterations in the cytoskeleton, 1hr of both HP and CHP was applied to MSCs, as this time course elicited the most robust osteogenic response. After one hour of pressure stimulation, the cytoskeleton was found to display distinct changes specific to the cytoskeletal element and the nature of the stimulation. In static controls, the actin network displayed presence of distinct stress fibres in all directions. The application of hydrostatic and cyclic pressure did not dramatically alter this organisation, although the fibrous nature of the actin elements was marginally more evident following mechanical stimulation. In response to hydrostatic and cyclic hydrostatic pressure, the microtubule network was indistinguishable from that of static controls, indicating the pressure mechanical regime utilised in this study does not affect the microtubules. Intermediate filaments (IF) stained by total vimentin exhibit architectural reorganisation depicted by transition from a filamentous structure in static culture to a more diffusive and disorganised structure with hydrostatic pressure. This effect on intermediate filament structure is further augmented with cyclic hydrostatic pressure (Figure 5-2). Overall, pressure mechanical stimulation alters the architecture of the cytoskeleton with most distinguishable changes specific to intermediate filaments under cyclic loading.

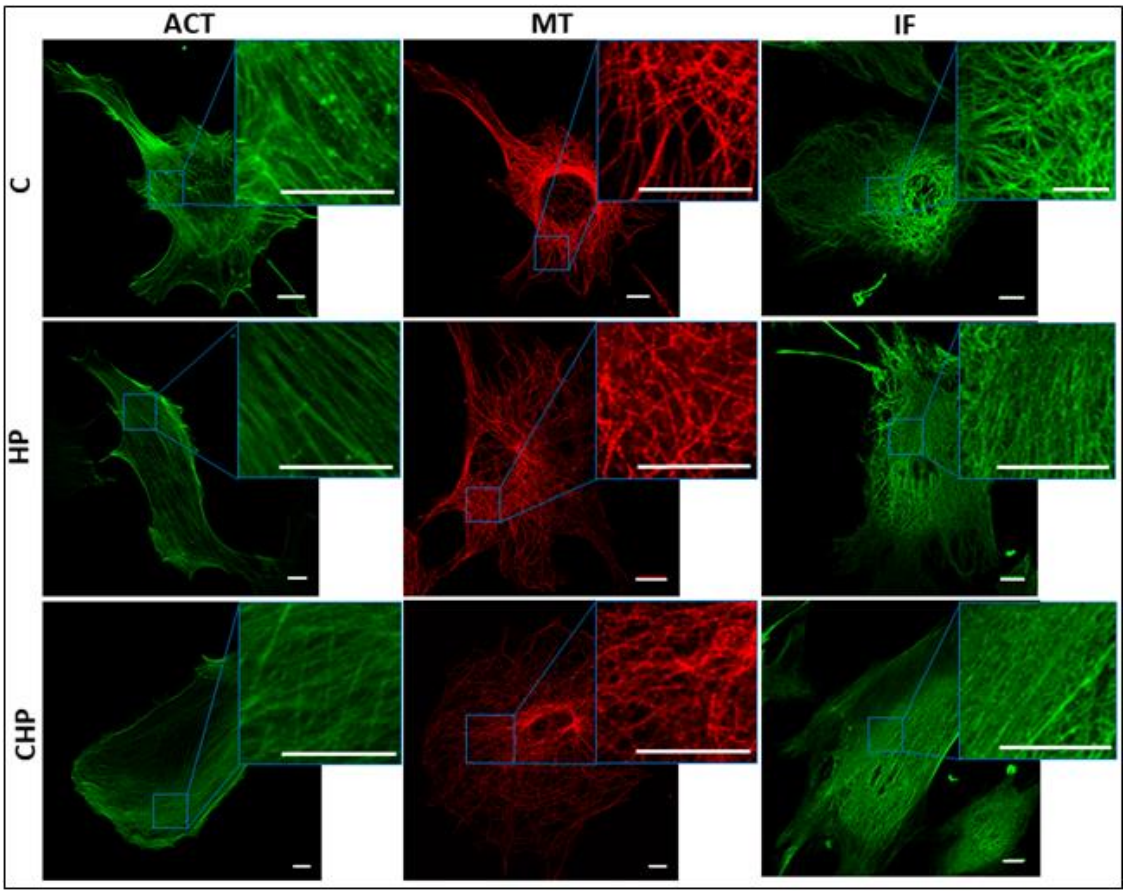


Figure 5-2. The effect of pressure on the cytoskeletal structures depicted by intermediate filaments (IF), microtubule network (MT) and f-actin (FA). C-control, HP-hydrostatic pressure, CHP-cyclic hydrostatic pressure. Scale bar=10μm.

5.3.3 Pharmacological disruption of the cytoskeleton highlights intermediate filaments involvement in stem cell osteogenesis

To explore the involvement of each cytoskeletal structure in MSC osteogenesis, given the display of varying degrees of architectural reorganisation in response to pressure, each cytoskeletal element was pharmacologically disrupted. Validation of disruption of actin with Cytochalasin D (CD), Nocodazole (NC) for microtubules and Withaferin A (WA) for intermediate filaments is presented in Figure 5-3 A. Importantly, the disruption of each individual cytoskeletal network, has not affected significantly the integrity of the remaining two cytoskeletal structures. The only exception was found when disrupting the microtubule network, which resulted in an increased staining of the actin network demonstrating the crosstalk between these two elements (Preciado Lopez et al., 2014). Following the disruption of each element, mRNA expression of osteogenic markers *Cox2*, *Runx2* and *Opn* was investigated. Interestingly, chemical disruption of the actin (CD) and microtubule (NC) networks had no effect of osteogenic gene expression. However, disruption of intermediate filaments (IF) elicited a significant ~3-fold increase ($p<0.05$) in all three osteogenic markers analysed (Figure 5-3 B), demonstrating a similar trend to that seen with cyclic hydrostatic pressure. This therefore highlights that remodelling or breakdown of intermediate filaments is involved in stem cell osteogenesis.

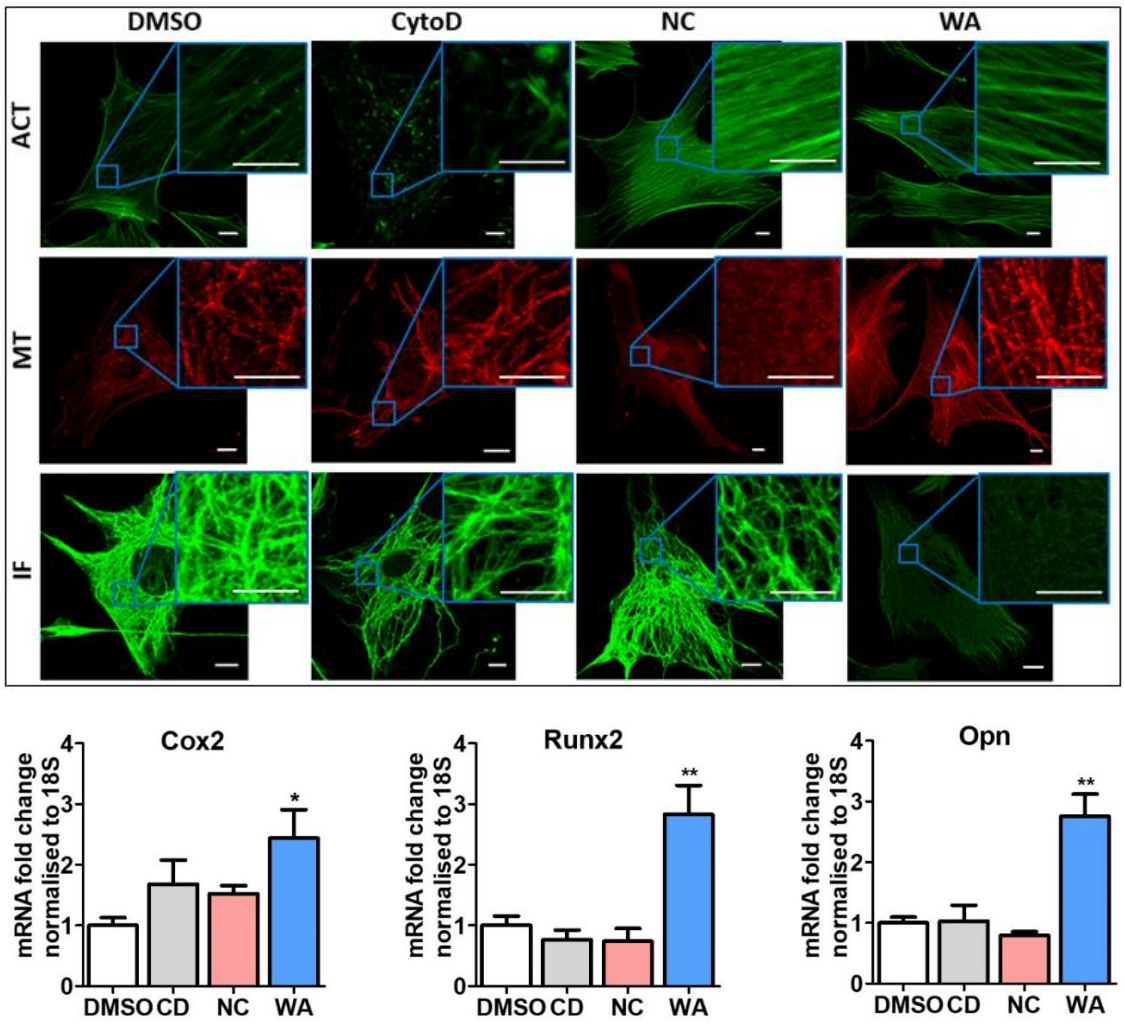


Figure 5-3. Representative images of validation of pharmacological disruption of cytoskeletal components such as Cytochalasin D (CD) for f-actin, Nocodazole (NC) for microtubule network and Withaferin A (WA) for intermediate filament. 0.1% DMSO treatment acted as a control (A). Expression of osteogenic markers *Cox2*, *Runx2* and *Opn* in response to pharmacological disruption of individual cytoskeletal components; (B). Scale bar=10µm. *p<0.05, **p<0.01 N=2, n=3-4

5.3.4 Cyclic hydrostatic pressure drives intermediate filament network remodelling

Given the association between intermediate filament (IF) breakdown/remodelling and MSC osteogenesis described above, the role of intermediate filaments in pressure mechanotransduction was further explored. This was determined using western blot to quantify the IF associated vimentin protein levels as well as high and super resolution (STED) confocal microscopy to visualise the ultrastructure of IF network in response to pressure. Total vimentin protein levels were significantly reduced in MSCs following 1hr of cyclic hydrostatic pressure ($p<0.05$), indicating that that there is a breakdown of the intermediate filament network. Further exploring this phenomenon visually, it was found that the IF network extends throughout the area of the cell in static culture, as demonstrated by co-staining with the actin network. Furthermore, IF co-localised at a number of locations particularly around the periphery of the cell

demonstrating a potential cross-talk between these two cytoskeletal elements (

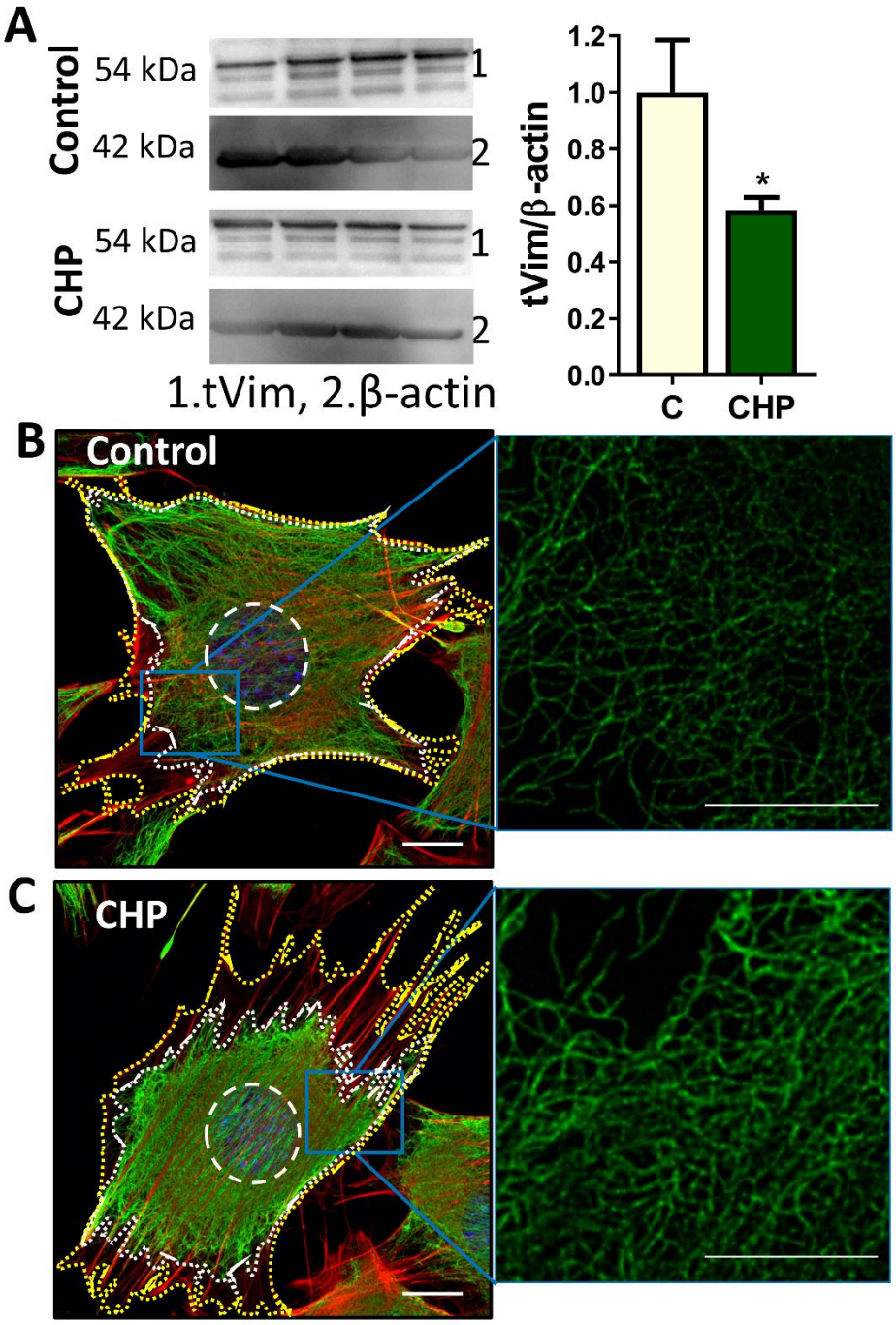


Figure 5-4 B) (Jiu and Lappalainen, 2016). Using STED microscopy, the intermediate filament ultrastructure can be clearly visualised as rods forming a lose mesh

throughout the cell (

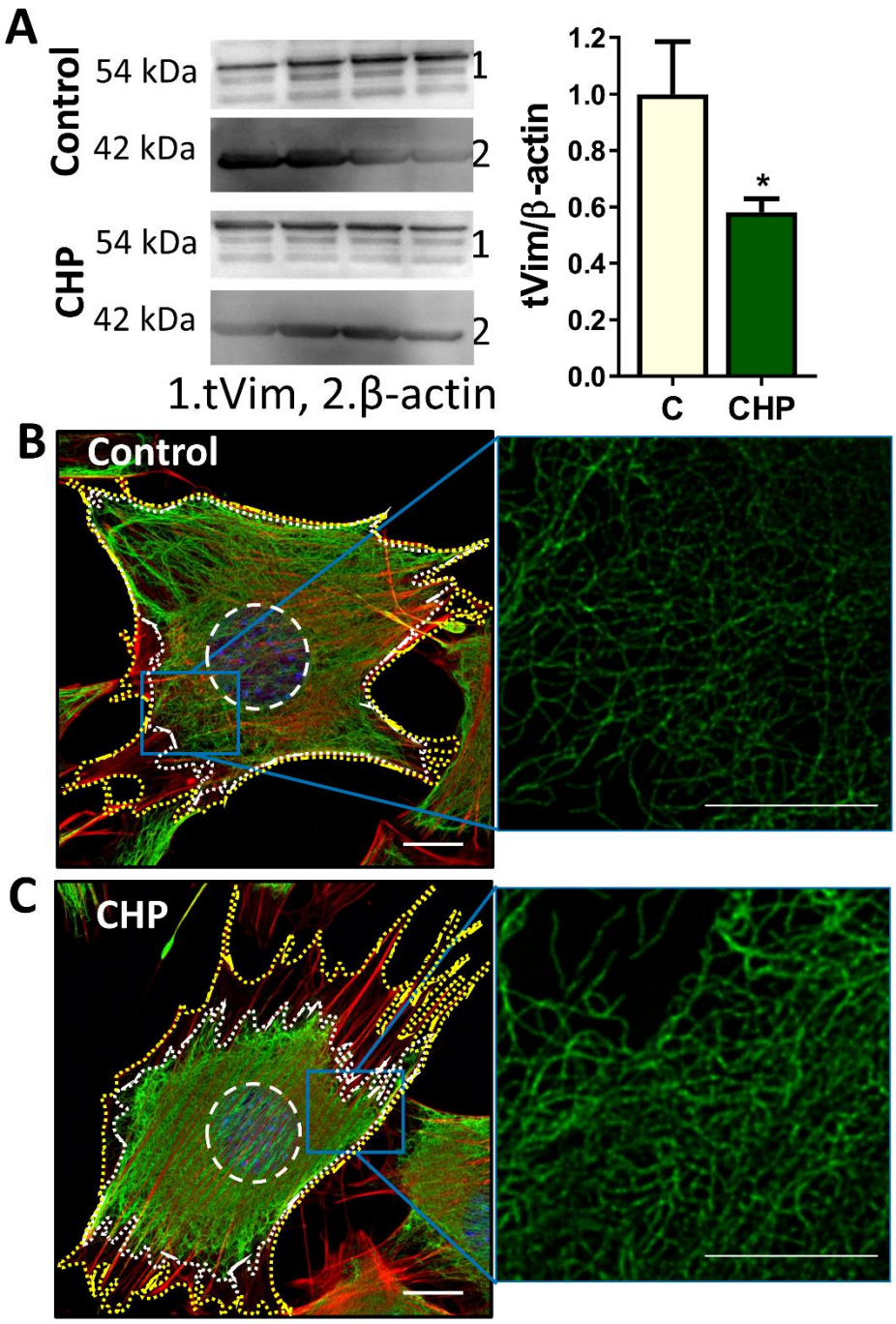


Figure 5-4 B). Interestingly, following 1hr CHP there is a pronounced alteration of the IF network, with a recoiling of the intermediate filaments from the periphery of

the cell to the perinuclear region (

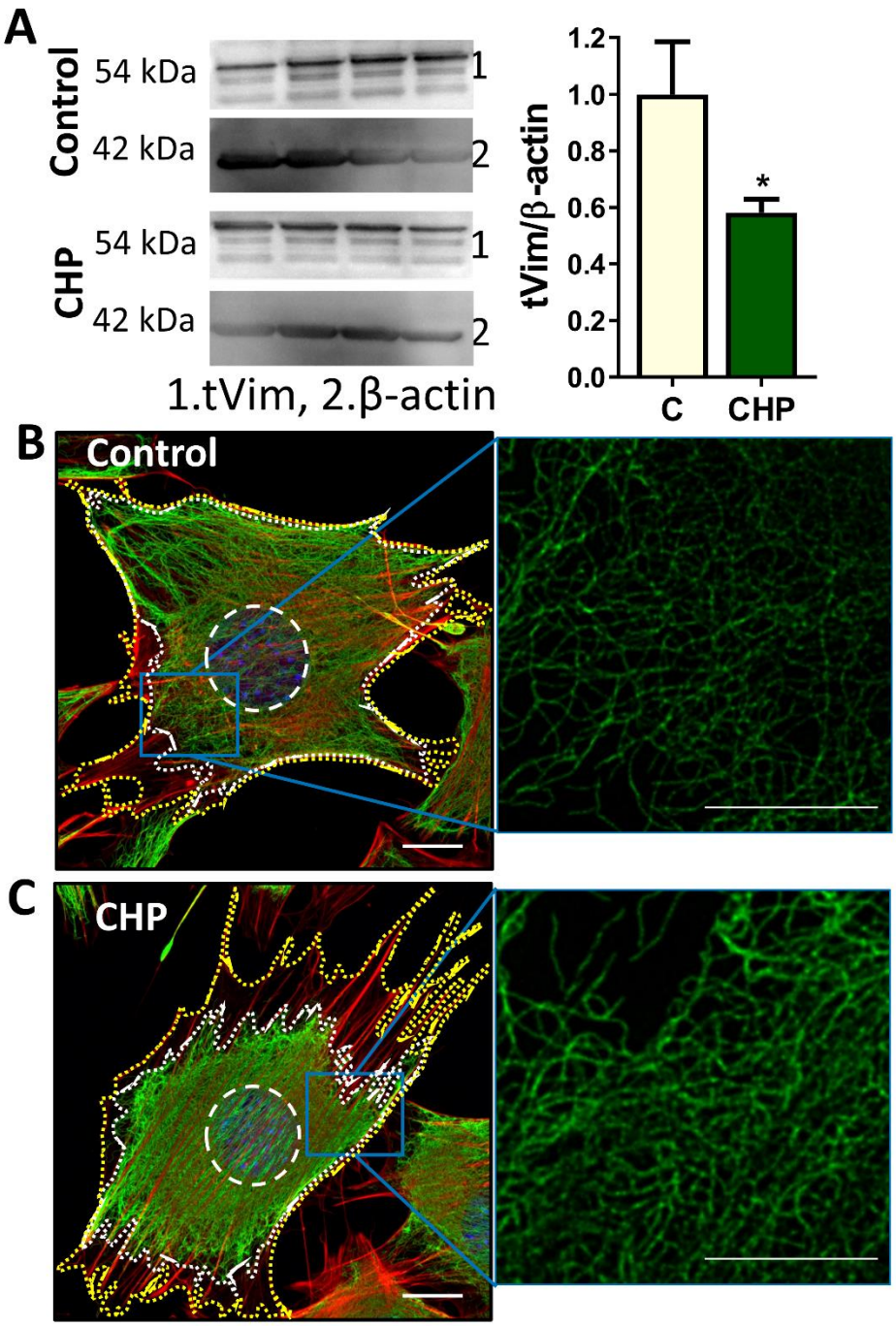


Figure 5-4 C). This recoiling effect is also evident using STED microscopy where a more compact IF network is seen in this region. In summary, following pressure stimulation there is a breakdown of vimentin protein and recoiling of the IF network towards the perinuclear region of the cell.

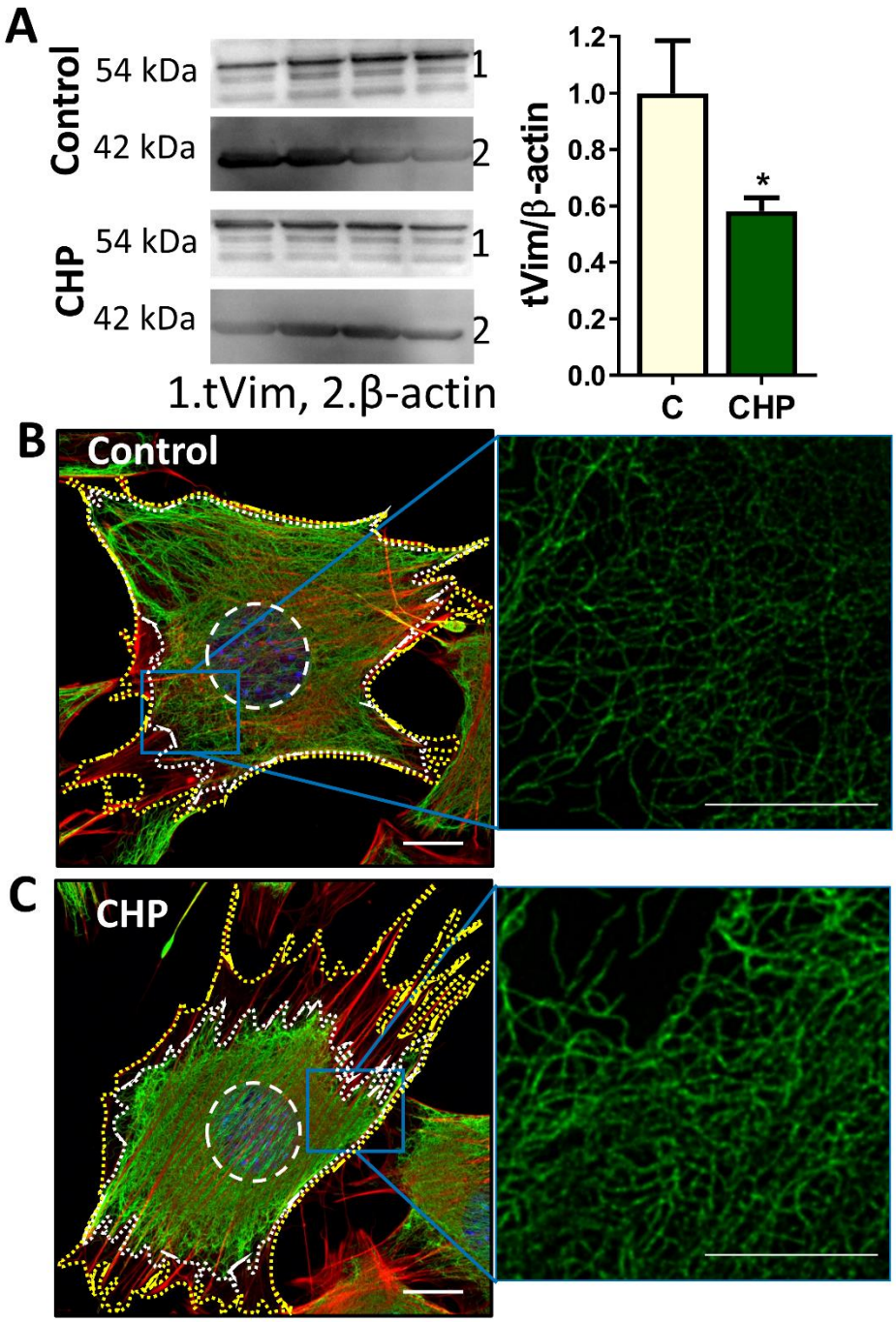


Figure 5-4. Pressure causes alteration in the intermediate filament quantity and structure. (A) Total vimentin (tVim) protein level is downregulated in response to CHP. β-actin acted as the endogenous protein control. N=4, n=3-4, *p<0.05 C (control) vs CHP. B) Cell cytoskeleton stained for intermediate filaments (green), f-actin (red) and nucleus (blue, outlined by dashed white line) shows how in the presence of cyclic hydrostatic pressure (CHP) treatment the intermediate filament network recoils towards the perinuclear region. Scale bar for whole cell and zoomed-in STED images are 15µm and 5µm, respectively.

5.3.5 Pressure induced MSC osteogenesis requires the remodelling of the intermediate filament network

To identify whether this remodelling of the intermediate filament network is required for the earlier demonstrated pressure induced stem cell osteogenesis, a strategy whereby the IF network was stabilised was implemented. Through a serendipitous discovery, it was identified that 0.1% DMSO reinforces the IF network by preventing it from undergoing structural breakdown in response to pressure stimulation. With 0.1% DMSO treatment, the reduction in vimentin protein levels following 1hr of cyclic hydrostatic pressure demonstrated previously is lost (Figure 5-5 A). Furthermore, the recoiling of the IF network from the cell periphery and aggregation in the perinuclear region is also absent (Figure 5-5 B). Although, DMSO treatment reinforces the IF network, it does not alter the basal level expression of osteogenic genes (Figure 5-5 C). The application of 1hr of cyclic hydrostatic pressure elicits a significant upregulation ($p<0.05$) in osteogenic markers *Cox2* (5.2-fold), *Runx2* (1.8-fold) and *Opn* (2.2-fold), as previously demonstrated. However, this pressure-induced increase in osteogenic genes is completely abrogated in presence of 0.1% DMSO (Figure 5-5 D). This demonstrates that breakdown and the remodelling and aggregation of the intermediate filament network is necessary for pressure induced MSC osteogenesis.

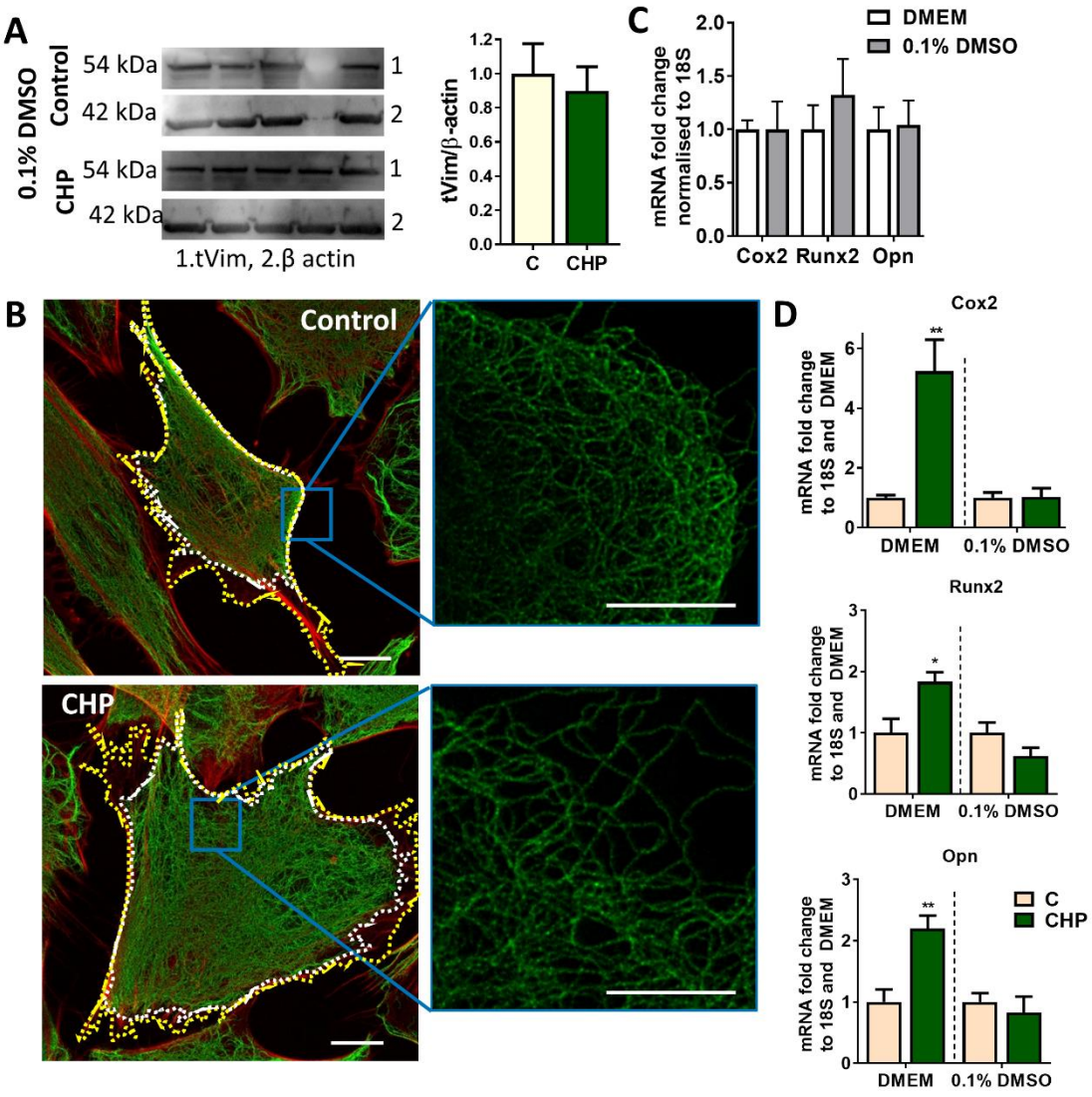


Figure 5-5. 0.1% DMSO treatment prevents pressure induced downregulation of intermediate filament (IF) associated protein vimentin (A) and remodelling of IF structure (B). Cell cytoskeleton stained for intermediate filaments (green) and f-actin (red) in presence of 0.1% DMSO demonstrates the absence of IF recoiling effect in response to CHP stimulation. DMSO reinforcement of intermediate filament network does not affect basal mRNA expression of osteogenic genes *Cox2*, *Runx2* and *Opn* (C) but inhibits pressure induced increase in early osteogenic genes in MSCs (D). Scale bar for whole cell and zoomed-in STED images are 15µm and 5µm, respectively. A) N=1-2, n=3-4 B) N=2, n=3-4 D) N=2, n=4. *p<0.05, **p<0.01.

5.3.6 Withaferin A mimics the effect of CHP, driving MSC osteogenesis via an intermediate filament mechanism

Based on previous observations, whether withaferin A acts via a similar mechanism to that of cyclic hydrostatic pressure to enhance MSC osteogenesis was further explored. MSCs were treated with WA under the same conditions which elicited increases in osteogenic gene expression and total vimentin protein levels were quantified by western blot in addition to the intermediate filament network organisation by high and super resolution confocal microscopy. WA treatment resulted in a significant decrease ($p<0.05$) in total vimentin protein level to a similar degree as observed with CHP (Figure 5-6 A). Furthermore, WA treatment resulted in a remodelling of the IF network with a recoiling of filaments from the periphery to the perinuclear region (Figure 5-6 B), demonstrating a similar mechanism as seen with CHP.

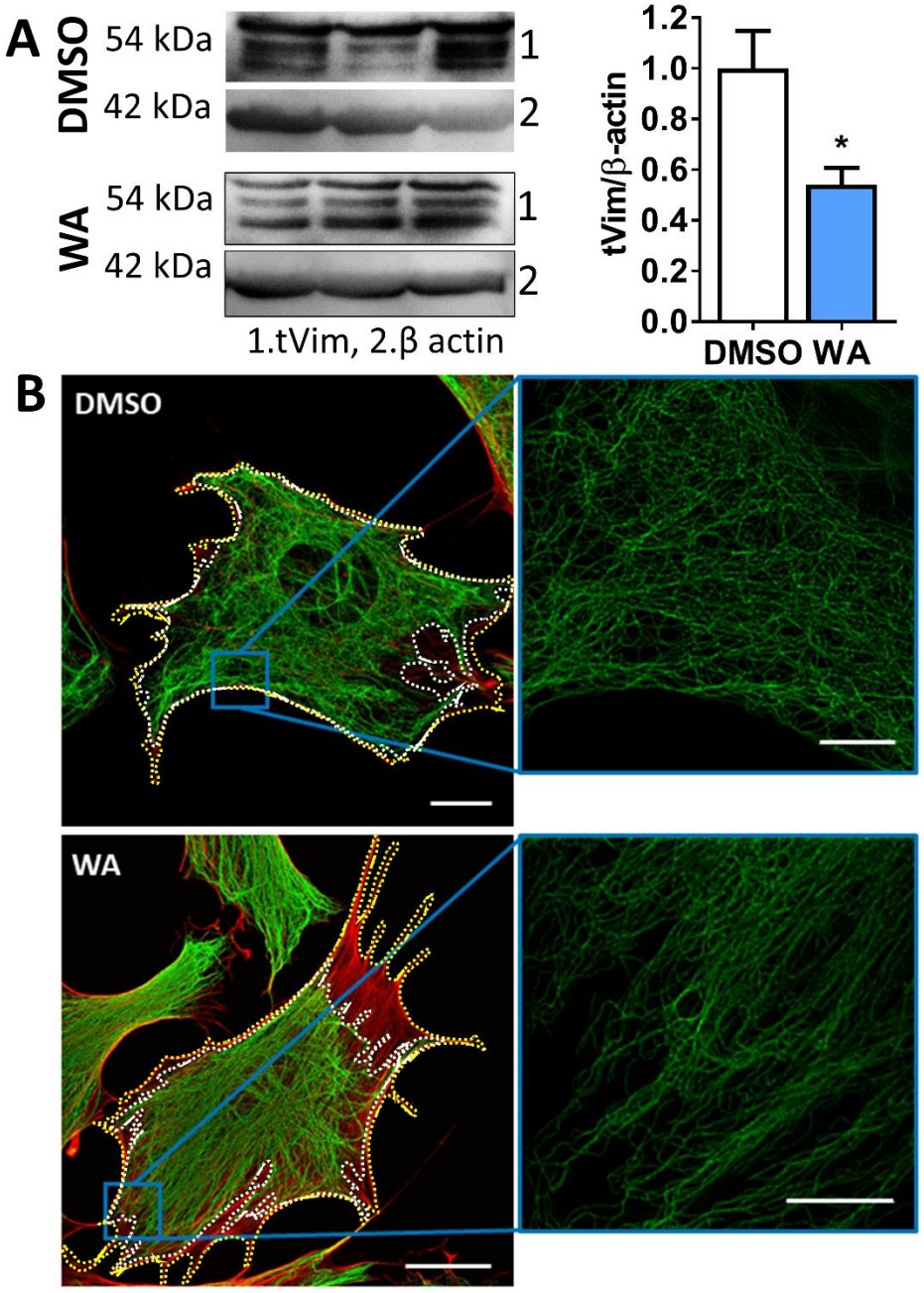


Figure 5-6. WA treatment mimics the effect of pressure induced cytoskeletal remodelling of IF on a structural and morphological scale. A) 1 hr treatment with 10 nM Withaferin A (WA) causes downregulation of total vimentin levels compared to DMSO control. β-actin acted as the endogenous protein control. N=2, n=3, *p<0.05. B) Imaging of the morphological changes of IF in response to WA treatment shows the recoiling mechanism of IF (green) with respect to whole cell area depicted by f-actin (red). This recoiling mechanism towards the perinuclear region was not present in the DMSO control treatment. Scale bar=15µm. Side images are zoomed in sections representing ultrastructure of IF using stimulated emission and depletion microscopy (STED), scale bar=5µm.

5.4 Discussion

Macroscale mechanical loading of bone generates a complex local mechanical microenvironment which drives osteogenesis and bone mechanoadaptation. One such mechanical stimulus generated is hydrostatic pressure (HP), yet the effect of HP on resident cells such as MSCs and the mechanotransduction mechanisms utilised by these cells to sense this stimulus are yet to be fully elucidated. In this study, it was demonstrated that cyclic hydrostatic pressure is a potent mediator of cytoskeletal reorganisation and potentiates osteogenic responses in MSCs. In particular, here it is shown that the intermediate filament (IF) network undergoes breakdown and reorganisation with a recoiling of the network from the periphery of the cell to the perinuclear region. Furthermore, for the first time it is demonstrated that this remodelling of the intermediate filaments is required for loading induced MSC osteogenesis, demonstrating a novel mechanism of MSC mechanotransduction. Moreover, chemical disruption of intermediate filaments with Withaferin A treatment induces a similar mechanism of IF breakdown and remodelling and subsequent increase in osteogenic gene expression in MSCs, demonstrating a potential mechanotherapeutic mechanism to enhance MSC osteogenesis. Hence, this study highlights a novel mechanotransduction mechanism of pressure-induced MSC osteogenesis involving the understudied cytoskeletal structure, the intermediate filament, highlighting a potential new therapy to enhance bone formation in bone loss diseases such as osteoporosis.

A low magnitude, dynamic hydrostatic pressure stimulus, consistent with that found within bone, is sufficient to drive MSC osteogenesis *in vitro*. MSCs were stimulated with both static and dynamic (cyclic,1Hz) pressures of 100kPa magnitude which has been predicted to occur *in vivo* (Ren et al., 2015). It was found that pressure, independent of fluid shear, elicits a pro-osteogenic response in MSCs depicted by upregulation of bone markers *Cox2, Runx2* and *Opn*. Furthermore, this pressure induced osteogenic response is more robust under cyclic loading. Pressures of less than 100 kPa were shown to upregulate bone markers such as *Runx2, Osx, Dlx5, Msx2, Bmp2* under both static and dynamic regimes (Sugimoto et al., 2017, Liu et al., 2009). In contrast, hMSCs stimulated with 10-100 kPa under 2Hz regime were found to express no changes in mRNA expression of bone associated markers (Becquart et al., 2016). One significant difference between these studies is the use of osteogenic biochemical induction medium, which has been shown previously to modulate the mechanical induced osteogenic response

(Yourek et al., 2010). In the cell models described in this thesis, MSCs with and without biochemical induction medium elicit an early osteogenic response when stimulated with a dynamic pressure rather a static regime. This response can be contributed to a possible physiological mechanism, as pressure transients fluctuate within the bone in response to mechanical loading (Qin et al., 2003, Gurkan and Akkus, 2008b). However, it is recognised that differences in the bioreactor set up and stimulation protocols may also contribute to divergent observations. Nonetheless, current observations that physiological low magnitude cyclic pressure transients, that represent the precursor to fluid shear *in vivo*, are sufficient to directly drive MSC osteogenesis independent of fluid flow.

Cyclic hydrostatic pressure initiates a breakdown and remodelling of cytoskeletal filaments. Given the limited knowledge of the role of pressure on cell behaviour, it is not surprising that the mechanisms of pressure mechanotransduction remain elusive. Many mechanotransduction mechanisms have been discovered within bone in response to fluid shear and several of these represent an extension of the cytoskeleton, such as microtubule based primary cilia, or focal adhesion-integrin binding of the actin network (Corrigan et al., 2018, Hoey et al., 2012). Moreover, modulation of cytoskeletal architecture with bone lineage commitment has been indicated as a marker of mechanoadaptation such as delayed actin remodelling and formation of dendritic processes in response to oscillatory fluid flow in osteocytes versus osteoblasts (Ponik et al., 2007, Klein-Nulend et al., 2012). To date, MSC osteogenesis, either mechanically or biochemically induced, has been attributed mostly due to changes in actin architecture, whereas change in microtubule dynamics have been related particularly to bone committed cells such as osteoblasts and osteocytes (Liu, 2010, Rubin and Sen, 2017, Espinha et al., 2014b, Gardinier et al., 2009, Arnsdorf et al., 2009). Yet, in this study, low magnitude cyclic hydrostatic pressure, which exhibited a robust osteogenic response, resulted in a distinct disassembly and remodelling of the intermediate filament network with only a slight and no alteration in actin filaments and the microtubule network respectively. This minimal effect of pressure on actin and microtubule networks is consistent with previous observations following pressure stimulation in mature bone cells (Gardinier et al., 2009, Zhao et al., 2016). Interestingly, the role of intermediate filaments in MSC differentiation and mechanotransduction has been largely unexplored although downregulation of intermediate filament associated protein vimentin is thought to be a required mechanism

to promote terminal osteoblastogenesis (Lian et al., 2009, Zhang et al., 2014). Moreover, in high magnitude pressure induced chondrogenesis model of MSCs, the IF were found to undergo structural reorganisation suggesting that this understudied cytoskeletal component can be mechanically modulated (Steward et al., 2013). However, in this study it has been shown that low magnitude stimulation is sufficient to drive IF breakdown and have demonstrated that IF remodels by recoiling towards the perinuclear region of the cell. This perinuclear aggregation is similar to that recently demonstrated in human osteosarcoma cells during migration where the formation of actomyosin arcs across actin stress fibres drives the retrograde flow of vimentin and perinuclear aggregation of intermediate filaments via the cytolinker protein, plectin (Jiu et al., 2015). Whether a similar mechanism for pressured induced IF organisation is at play here is yet to be determined, however given the enhanced expression of actin stress fibres following pressure stimulation, this may suggest a similar cross-talk mechanism in pressure mechanotransduction.

Disruption, remodelling and perinuclear recoiling of the intermediate filament network is a key component of MSC pressure mechanotransduction and osteogenesis. From serendipitous empirical observations, dimethyl sulfoxide (DMSO, concentration dependent) was found to affect IF dynamics by preventing pressure induced downregulation of the IF protein, vimentin. Although, the mechanism by which DMSO 'reinforces' the IF network is unknown, other studies also reported that DMSO modulates IF associated protein expression (Melguizo et al., 1994). As a side note, this should be considered when utilising DMSO as a solvent in stem cell mechanotransduction studies. Interestingly, by stabilising the IF network with DMSO, the pressure induced osteogenesis was abrogated, demonstrating for the first time that mechanical breakdown and remodelling of IFs is necessary for MSC osteogenesis. This finding is supported by Lian et al. who demonstrated that vimentin acts as a break for osteoblastogenesis and inhibition of this protein promotes osteogenic differentiation (Lian et al., 2009). Interestingly, pharmacological disruption of IF using Withaferin A, at sub-cytotoxic levels, elicited an upregulation of osteogenic markers *Cox2*, *Runx2* and *Opn* similar to pressure mechanical stimulation. Yet microtubule and f-actin breakdown with Nocodazole and Cytochalasin D, respectively, did not alter the expression of these osteogenic genes, highlighting a unique role for IFs in MSC osteogenesis. Furthermore, Withaferin A treatment drove an IF network remodelling event that mirrored that seen

with loading suggesting that this drug may act via a similar IF based mechanism of pressure mechanotransduction to enhance osteogenesis and thus represents a potential novel mechanotherapeutic avenue for bone regeneration. This can have clinical translational potential as Withaferin A administration to osteopenic ovariectomized mice was shown to promote osteoblastogenesis and supress osteoclastogenesis, in addition to enhancing bone microarchitecture and mechanical properties in vivo (Khedgikar et al., 2013).

5.5 Conclusion

In this study, it was demonstrated that cyclic hydrostatic pressure elicits an early osteogenic response in MSCs and introduces a novel pressure mechanotransduction mechanism whereby the intermediate filament network plays a key role. Specifically, vimentin based intermediate filaments remodel and recoil towards the perinuclear region following pressure simulation and this phenomenon is required for downstream osteogenesis. Furthermore, importantly it was demonstrated that this mechanism can be mimicked pharmacologically using Withaferin A to enhance MSC osteogenesis and thus represents a new anabolic mechanotherapeutic to enhance bone regeneration in diseases such as osteoporosis.

Chapter 6.
Pressure mechanotransduction in mesenchymal stem cells is mediated by the primary cilium

6.1 Introduction

Bone integrity is dependent on the coupling of mechanical inputs during locomotion as well as transduction of these cues into a biological response by the cellular effectors (Frost, 2003). Previously, it was demonstrated that pressure plays a key role in loading-induced MSC osteogenesis, with cyclic hydrostatic pressure enhancing osteogenic gene expression and the secretion of adenosine triphosphate (ATP) metabolite in a magnitude dependent manner, while the deposition of bone extracellular matrix is magnitude independent. Furthermore, pressure induced osteogenesis in mesenchymal stem cells (MSCs) was shown to require the remodelling of the cytoskeleton and the breakdown of intermediate filaments. However, cytoskeletal remodelling, although critical for mechanotransduction, is often a downstream effect following activation of an upstream mechanosensor. Therefore, further research is required to identify this upstream mechanosensor in stem cell pressure mechanotransduction.

Primary cilia are non-motile solitary organelles, emanating from an invagination of the plasma membrane as an extension of the microtubule-based cytoskeleton into the pericellular environment. It is a rod-like structure, several microns in length and enriched with receptors allowing it to serve as a signalling hub, mediating chemosensation, mechanosensation and downstream signalling (Nachury, 2014, Hoey et al., 2012, Brown et al., 2014). Primary cilia were shown to play a pivotal role in bone formation as conditional impairment of ciliogenesis in periosteal progenitors, osteocytes and osteoblasts prevented loading-induced bone anabolism (Temiyasathit et al., 2012, Moore et al., 2018b). Moreover, presence of a functional primary cilium in MSC was demonstrated to be paramount for loading induced-bone formation *in vivo* (Chen et al.,

2016a). *In vitro* studies demonstrated that biophysical regulation of MSC osteogenesis in response to fluid shear as well as paracrine signalling from flow stimulated osteocytes is dependent on this ubiquitous organelle (Hoey et al., 2012, Hoey et al., 2011). However, much is unknown about the primary cilium's capability to sense and translate pressure mechanical cues. In trabecular meshwork cells, primary cilia become shortened in response to atypical intraocular pressures (<7 kPa) in addition to presence of a functional cilium was essential for pressure sensation and downstream transcriptional programs (Luo et al., 2014). However, the role of bone cell cilia in mechanotransduction of skeletal associated pressure cues is currently unknown.

Accumulating evidence highlights that the release of purine extracellular nucleotides are one of the key primary messengers involved in early loading induced osteogenesis and are important mediators of bone cell metabolism (Ciciarello et al., 2013, You et al., 2002, Genetos et al., 2007, Kringelbach et al., 2015, Noronha-Matos and Correia-de-Sa, 2016). Adenosine triphosphate (ATP) represents one form of purine nucleotides that are triggered by bone associated mechanical stimuli such as fluid flow and pressure (Kringelbach et al., 2015, Genetos et al., 2005). In bone committed cells, fluid flow induced extracellular ATP release was found to mobilise intracellular calcium (You et al., 2002), regulate transcription activity of osteogenic genes (Costessi et al., 2005) and prostaglandin E2 signalling (Genetos et al., 2005, Reich and Frangos, 1991). Moreover, extracellular ATP has been associated with increases in osteoblast proliferation (Shimegi, 1996), regulation of MSC osteogenic/adipogenic lineage commitment (Ciciarello et al., 2013), activation key signalling pathways necessary for bone formation such as cAMP/PKA (Wang et al., 2016, Ogasawara et al., 2001) as well as modulation of mineralization (Burnstock et al., 2013). In stem cells, ATP release in response to fluid shear was found to be dependent on the flow rate and a precursor for intracellular calcium signalling modulating calcineurin activity, nuclear translocation of NFATc1 and proliferation (Riddle et al., 2007, Riddle et al., 2008). The effect of bone associated pressures on ATP signalling in bone cells is understudied. Osteoblasts were shown to secrete ATP in response to pressure cues, however much is unknown about the effect of pressure on MSC behaviour (Gardinier et al., 2009). In chapter 4, it was demonstrated that MSCs stimulated with bone associated pressure transients prompted a magnitude dependent ATP secretion. However, the mechanism that enables this rapid ATP secretion in response to pressure stimulation in MSCs is yet to be identified.

Fluid flow release of ATP in osteocytes was found to occur via hemichannels although this mechanism was not present in osteoblasts (Genetos et al., 2007). Interestingly, in a study by Steward et al. (2016), high pressure magnitudes were found to induce MSC chondrogenesis as demonstrated by sulphated glycosaminoglycan synthesis which was dependent on extracellular ATP. Moreover, they demonstrated that intermediate filament remodelling in response to pressure was also ATP dependent (Steward et al., 2016, Garcia and Knight, 2010). In chondrocytes, compression was shown to induce calcium transients and aggrecan mRNA expression that was also mediated by the ATP release, but the autocrine sensing of extracellular ATP was dependent on the presence of the primary cilium, demonstrating a novel mechanotransduction mechanism of cilia-mediated control of ATP reception (Wann et al., 2012). Primary cilia are paramount for fluid shear mechanotransduction in bone cells including MSCs (Hoey et al., 2012, Espinha et al., 2014b, Delaine-Smith et al., 2014), however the cilium's capability to sense pressure stimuli and mediate purinergic signalling in MSCs is unknown. Therefore, the objective of this study is to determine the role of the primary cilium in MSC pressure mechanotransduction, specifically investigating its involvement in pressure mediated ATP associated purinergic signalling, proliferation and cytoskeleton reorganisation. Identification of the upstream mechanosensor in MSC mechanotransduction would reveal a new target for therapeutic development to enhance MSC osteogenesis and bone formation.

6.2 Methods

6.2.1 Cell culture

All materials were purchased from Sigma unless otherwise stated. Mouse mesenchymal stem cells (C3H10T1/2) were cultured on fibronectin (10 µg/ml) coated glass slides in low glucose DMEM supplemented with 10% foetal bovine serum (FBS: Biosera) and 1% penicillin-streptomycin unless otherwise stated. Prior to mechanical stimulation, cells were cultured for 24 hours under standard conditions followed by 48 hours of serum starvation (0.5% FBS). ATP treatment was performed using adenosine 5′-triphosphate disodium salt hydrate (A6419, Sigma) dissolved in water.

6.2.2 Ift88 knock down (KD) for primary cilia abrogation

The formation of functional primary cilia was abrogated by small-interfering RNA (siRNA)-mediated depletion of Intraflagellar Transport 88 protein (Ift88). IFT88 protein is required for functional ciliogenesis (Taulman et al., 2001). C3H10T1/2 cells were transfected with 32 nM siRNA targeting Ift88 (1320001, ThermoFisher Scientific) or with a scrambled siRNA (12935300, ThermoFisher Scientific) for 24 hours using Lipofectamine RNAi Max (BioSciences, 13778-150) in Opti MEM (BioSciences, 31985062). The cells were maintained in growth media for 24 hours, before seeded on glass slides and cultured for an additional 48hrs in serum starvation conditions before mechanical simulation.

6.2.3 Hydrostatic and cyclic hydrostatic pressure mechanical stimulation

Cyclic hydrostatic pressure was applied to MSCs cultured on glass slides using custom pressure bioreactor described in Section 4.2.2. Briefly, MSCs were mechanically stimulated with 100kPa, 1Hz for an hour, except for short term stimulation to determine pressure induced ATP release were CHP was applied for 5, 10 and 15 minutes.

6.2.4 ATP assay

After mechanical stimulation for each time point, the medium was collected from the bioreactor and snap frozen in liquid nitrogen followed by storage at -80ºC. Adenosine triphosphate metabolites within media were measured using Molecular Probes® ATP Determination Kit (A22066, Invitrogen TM) according to manufacturer's protocol. Luminescence was measured using Luminoskan TM Ascent Microplate Luminometer

(MTX LAB SYSTEMS). The ATP volume released in the medium was normalised to total protein levels measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific).

6.2.5 Quantitative real-time PCR

Immediately post mechanical stimulation, cells were lysed using TRI Reagent® and mRNA was isolated per the manufacturer's protocol. The 260/280 and 260/230 absorbance ratios were measured for verification of the purity and concentration of RNA. The RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Life Technologies). Quantitative polymerase chain reaction (qPCR) was executed using SYBR Select Mastermix with ROX passive dye (ThermoFisher 4472903). The expression of osteogenic markers *18S*, was quantified using primers detailed in Table 7. Mouse primers and experimental conditions used for qPCR analysis. Ift88 mRNA expression (5'-CTTAACCTACTCCGTTCTTTTC-3'; 3'-CATTCGGTAGAACTTAATGGC-5', 400 nM, 60oC) was used to validate primary cilia abrogation via siRNA against Ift88. The amplification was performed with an ABI7500 Fast Real Time PCR machine and melt curve analysis was implemented as a control for primer dimer formation. Each sample was normalised to reference gene *18S* and static control using relative quantification method.

6.2.6 Immunocytochemistry and imaging

Briefly, cells were fixed in 4% paraformaldehyde for 10 min, permeabilised in 0.1% Triton-X for 10 minutes followed by 2-hour incubation in 0.1% BSA. The following antibodies were used: vimentin (Abcam ab92547, 1:500 dilution) against intermediate filaments, α-tubulin (Abcam ab80779, 1:500 dilution) against microtubules, Rhodamine Phalloidin (BTIU00027, VWR, 1:40 dilution) against f-actin, pericentrin (Abcam, ab4448, 1:1500 dilution) against centrosome and acetylated α tubulin against primary cilia (Abcam, ab24610, 1:1500 dilution). Secondary antibodies Alexa Fluor 488 (Life Technologies, A11008) and Alexa Fluor 594 (Life Technologies, A21203) were used at 1:500 dilutions. Nucleus was stained with DAPI (Sigma 32670-5MG-F, 1:2000 dilution). Coverslips were mounted with Prolong mounting medium (ThermoFisher). For representative images of the cytoskeleton structure, a maximum projection of 3D scans was taken with Leica SP8 scanning confocal fitted a 63x oil (NA 1.4) objective. Similar imaging strategy was employed for primary cilia and centrosome

imaging using an Olympus IX83 fitted with a 100x objective. Cilia length was measured using Pythagorean Theorem on 3D projection of cilia as previously described in (Dummer et al., 2016). Intermediate filament distribution was quantified as % area of IF compared to total cell areas stained by f-actin.

6.2.7 Proliferation

The rate of proliferation was determined using commercial kit Click-iT EdU (Invitrogen, C10637). Detection of new DNA synthesis is based on incorporation of the nucleoside analog EdU (5-ethynyl-2'-deoxyuridine) into DNA. After mechanical stimulation, cells were incubated in DMEM supplemented with 0.5% FBS and 1% P/S for 24 and 48 hours followed by 1-hour incubation in the same media supplemented with a 10 µM EdU solution. Cells were then processed for immunofluorescence as above. EdU incorporation was detected using an Alexa Flour 488 azide, and total cell number was determined using Hoechst 33342. Twenty representative images were captured per slide using an Olympus IX83 inverted microscope fitted with a 10x objective (70+ cells per image). Ratio of total number of EdU positive cells against total cells is reported.

6.2.8 Statistical analysis

All data presented as mean ± SEM. A two-tailed student's t test with Welch correction was employed for gene expression to compare the effect of HP or CHP against static control (*$p<0.05$, **$p<0.01$). Non-parametric statistical analysis using Chi-square was used for primary cilia length.

6.3 Results

6.3.1 The effect of cyclic hydrostatic pressure on primary cilia expression and length

To determine whether pressure mechanical cues affect primary cilia morphology in MSCs, primary cilia incidence and length were quantified after 1 hour of cyclic hydrostatic pressure (CHP) stimulation. Primary cilia stained with acetylated α tubulin, display extended rod-like structures within the perinuclear region (Figure 6-1 A). The primary cilium incidence displays no changes in response to pressure with an incidence of 68% in static conditions and 72% with CHP (Figure 6-1 B). However, the primary cilium length in response to pressure acquires a greater range of variability with a median of 1.42 μm at static conditions and 1.39 μm with CHP (Figure 6-1 C). Non-parametric analysis using Chi-square for defined length ranges indicates that primary cilia tend to acquire longer lengths, although not statistically significant ($p=0.08$). Overall, CHP stimulation does not affect cilia incidence, but influences its length, with increasing numbers of cells with elongated cilia length.

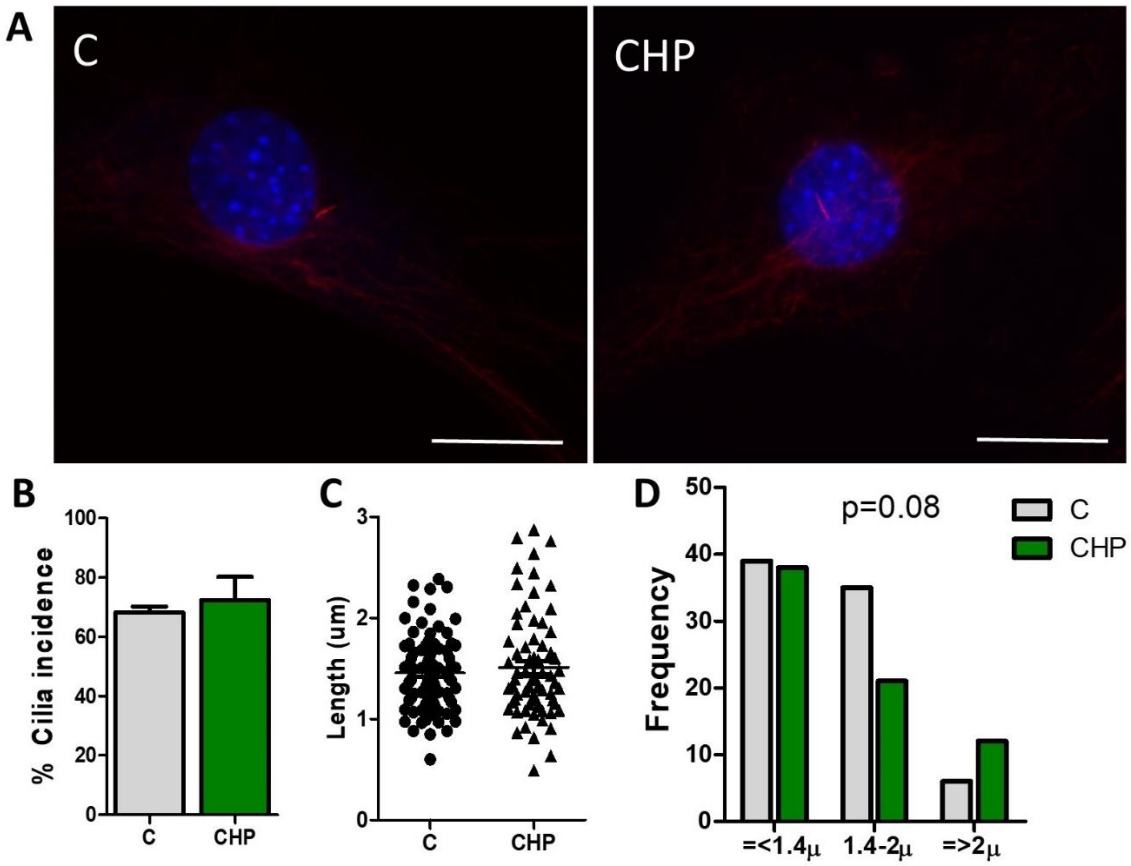


Figure 6-1. Primary cilia expression in response to pressure mechanical stimulation. Representative images (A) % cilia incidence (B) and length of primary cilium (C) in response to static control and 100 kPa,1Hz cyclic hydrostatic pressure (CHP), Chi-square analysis of the frequency distribution of cilium length (D). Blue-DAPI and red-acetylated alpha tubulin. Statistical test employed Chi-square with difference between frequency distributions of cilia length ranges. Scale bar 5μm. n=71-80 cells/condition.

6.3.2 Validation of stem cell primary cilia knock down

The formation of primary cilia was inhibited by using siRNA technology targeting the ciliary protein Intraflagellar transport protein 88 (Ift88), which is a principal motor protein required for ciliogenesis (Figure 6-2 A). Transfection with Ift88 siRNA resulted in a significant 72.3% decrease in the mRNA expression of Ift88 (Figure 6-2 B) and cilia incidence (84.53% Scr versus 23.96% Ift88 KD, Figure 6-2 C) as demonstrated by the immunocytochemistry (Figure 6-2 A). Of the few cells that maintain a cilium following siRNA treatment, the ciliary axoneme length is similar to that of scrambled control (Figure 6-2 D). However, these cells may have altered IFT88 and thus dysfunctional primary cilia.

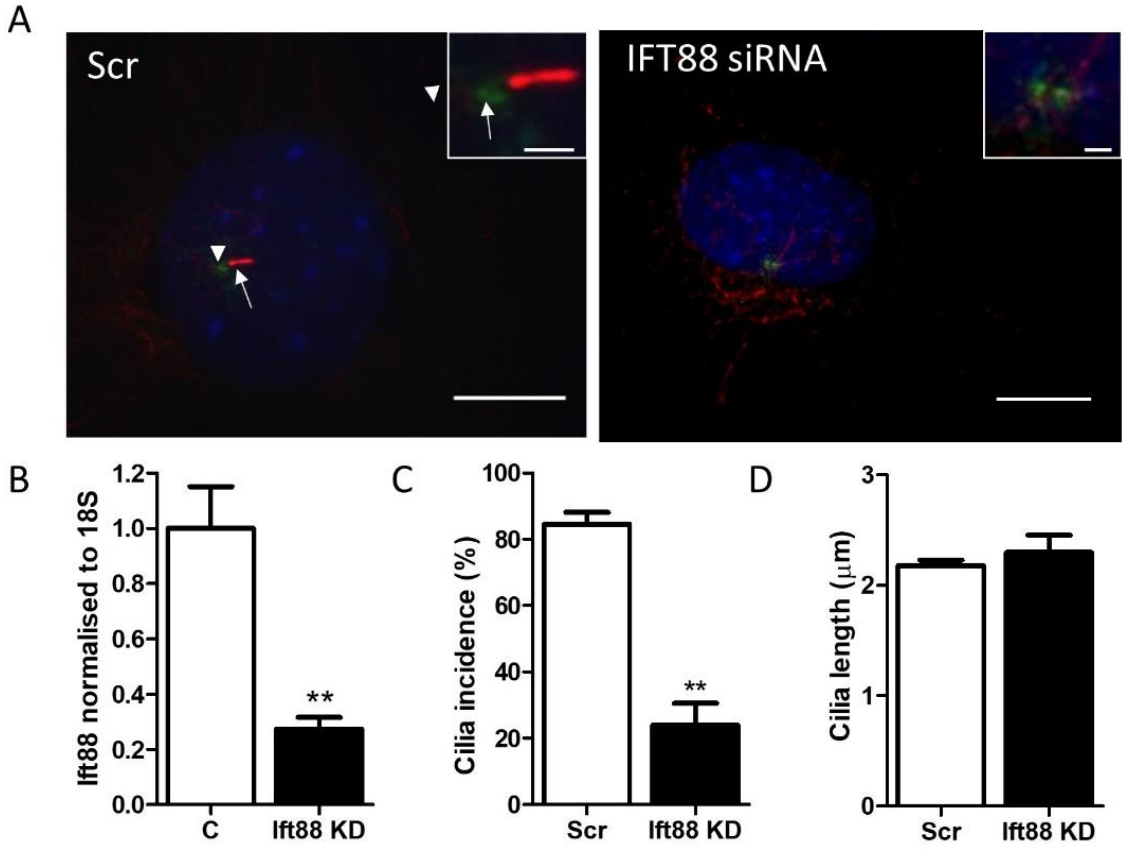


Figure 6-2. Intraflagellar transport protein 88 (IFT88) expression was successfully knocked down in C3H10T1/2 mesenchymal stem cell line using IFT88 siRNA as seen by immunocytochemistry (A), qRT-PCR (n=7-8) (B), cilia incidence (n=154-159) (C) and length (n=44 for IFT88 siRNA and n=130 for Scr) (D). Statistical test employed-an unpaired two tailed student t-test with Wilcoxon correction **p<0.1. Scale bar 5μm and 1μm. Scrambled siRNA (Scr) was used as a negative control. Triangle points to the centrosome which forms a part of cilia basal body.

6.3.3 Cyclic hydrostatic pressure induced ATP release is dependent on the primary cilium

To determine the role of the primary cilium in pressure MSC mechanotransduction, presence of purinergic extracellular signalling following mechanical stimulation was assessed. The application of 100 kPa cyclic hydrostatic pressure was found to elicit a robust and rapid ATP release response from MSCs, with ATP secretion determined to increase by 3-fold ($p<0.05$), 4.2-fold ($p<0.001$) and 2.89-fold ($p<0.01$) compared to static controls after 5, 10 and 15 minutes of stimulation (reported as concentrations, Figure 6-3 A) respectively.

To investigate whether the primary cilium is involved in this early mechano-signalling, CHP was applied for 10mins (shown to elicit the most robust ATP release), following the abrogation of the primary cilium. Scrambled control elicited a significant 3.4-fold increase ($p<0.001$) in ATP concentration (16.88 nM/µL/µg control vs 57.8 nM/µL/µg CHP) following CHP, demonstrating that the transfection procedure did not inhibit this response. However, following cilia abrogation, the ATP release following CHP was significantly attenuated (Figure 6-3 B) to a 1.83-fold change (18.83 nM/µL/µg control vs 34.47 nM/µL/µg CHP, not significant). This data therefore demonstrates that the primary cilium is involved in MSC pressure mechanotransduction.

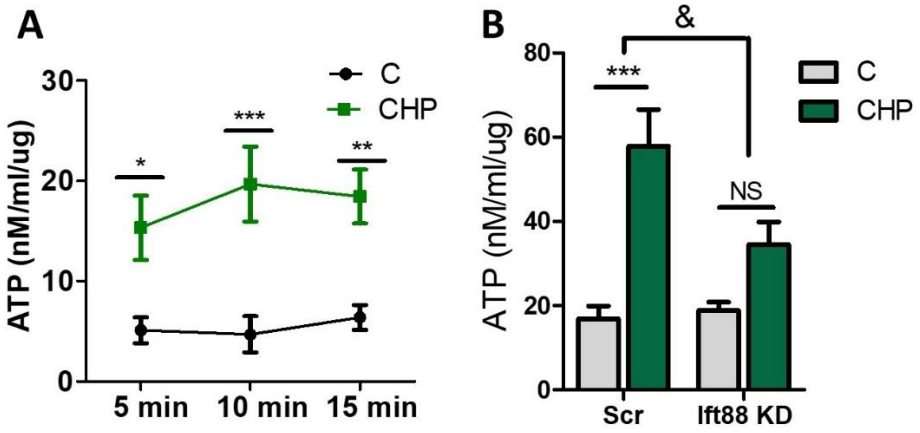


Figure 6-3. Time course of ATP release in response to cyclic pressure mechanical stimulation (A, N=2, n=3-4); ATP release after 10 min of CHP is attenuated when primary cilium expression is abrogated (A, N=2, n=3-4). For A) a student t-test with Welsh correction was employed: *$p<0.05$, **$p<0.01$, ***$p<0.005$; B) two-way ANOVA with Bonferroni post-hoc test was employed: ***$p<0.001$ between C and CHP; &$p<0.01$ for KD interaction

6.3.4 Cyclic hydrostatic pressure attenuates proliferation independent of the primary cilium

Previous studies have demonstrated that fluid shear enhances the proliferation of MSCs and that this response was surprisingly enhanced following abrogation of the primary cilium, demonstrating a role for the cilium in controlling stem cell proliferation. To determine whether a similar mechanism exists under pressure stimulation, MSCs were exposed to 1hr of CHP and proliferation was assessed using EdU incorporation at 24 and 48 hrs post mechanical stimulation. At 24 hrs post mechanical stimulation, the % of EdU cells between static control and pressure groups was similar (17.89% Control vs 18.88% CHP, Figure 6-4 A). Even though the cells were maintained in serum starved conditions throughout the treatment time, the cells ability to proliferate was not hindered as a significant time dependent effect was observed in the static control group (Control: 17.78% at 24 hrs and 24.43% at 48 hrs, &<0.05). However, examining the effect of CHP 48 hrs after stimulation revealed a decrease in MSC proliferation rate with a significant inhibition of positive EdU cells compared to static control, demonstrating that pressure arrests MSC proliferation (24.43% control versus 17.38% CHP).

Using the 48 hr timepoint, it was further investigated whether the primary cilium may be involved in this pressure mediated decrease in proliferation (Figure 6-4 B). Similar to previous observations, CHP downregulated % EdU positive cells compared to static control following scrambled negative control siRNA transfection (15.39% Scr versus 11.69% Ift88 KD, p=0.0546). Interestingly, abrogation of primary cilia alone increased significantly the proliferation rate of MSCs (15.39% Scr versus 20.72% IFt88 KD, p<0.01), which is consistent with a role for the cilium in controlling stem cell proliferation. Moreover, following CHP treatment, Ift88 depleted cells did not respond with a significant decrease in the rate of proliferation (p>0.05). Taken together, this data demonstrates that pressure induced inhibition of MSC proliferation may be in part mediated by the primary cilium.

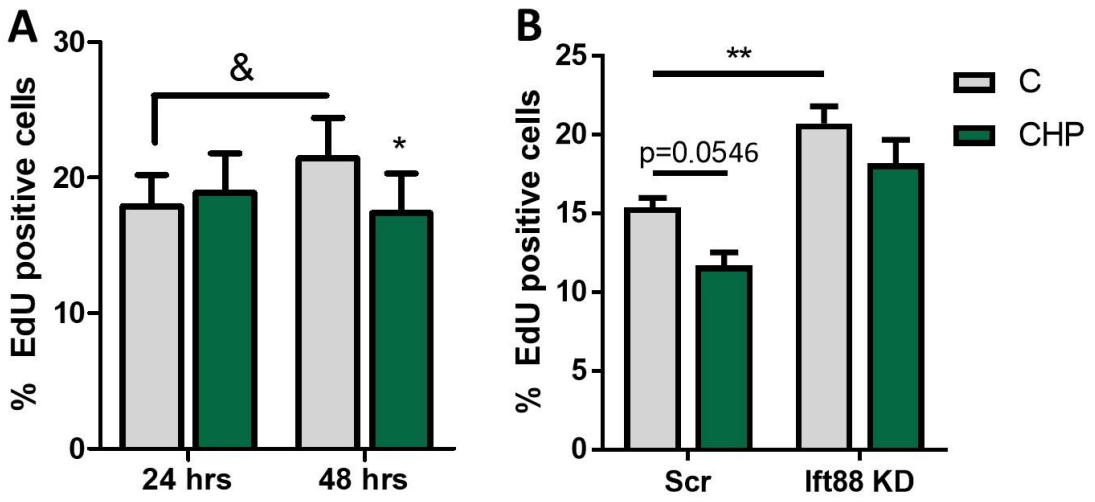


Figure 6-4. Proliferation is attenuated in response to CHP as observed 48hrs after CHP mechanical stimulation (A, N=2, n=3-4, 6700-7800 cells/condition). Abrogation of primary cilia expression via Ift88 siRNA (Ift88 KD) increases proliferation over time with no change in proliferation after 48 hrs post mechanical stimulation (B, N=2, n=3-4). For statistical analysis a two-way ANOVA with Bonferroni post-hoc test was employed. *$p<0.05$, **$p<0.01$; &$p<0.01$ for time interaction.

6.3.5 The primary cilium is required for pressure induced remodelling of the intermediate filament network

Given the demonstrated role of the intermediate filament (IF) network in MSC pressure mechanotransduction in Chapter 5, next step was to examine whether primary cilium mediated pressure mechanotransduction is associated with the IF mechanism described previously. Initially, physical association of the primary cilium with the intermediate filament network was explored using qualitative observations from high resolution confocal imaging (Figure 6-5). No co-localisation of IF was observed with the IF network spread uniformly throughout the cell. To further investigate a potential link, primary cilia formation was abrogated with Ift88 siRNA resulting a blunted primary cilium axoneme, as expected. However, despite defective primary cilium, the intermediate filament network shows similar morphological architecture compared to scrambled (Scr) control.

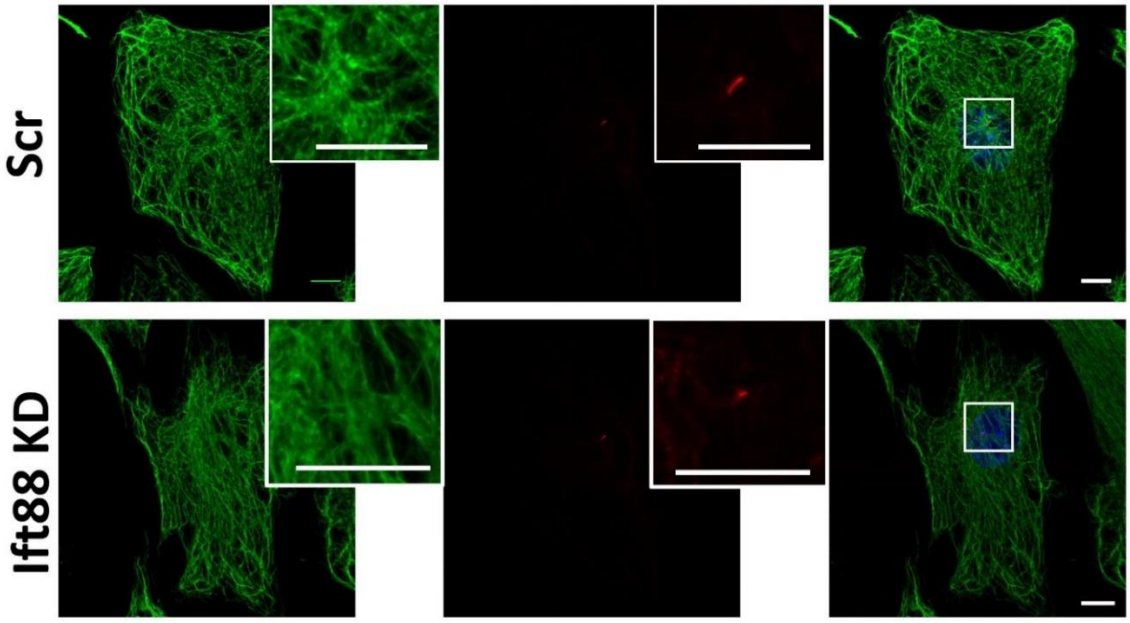
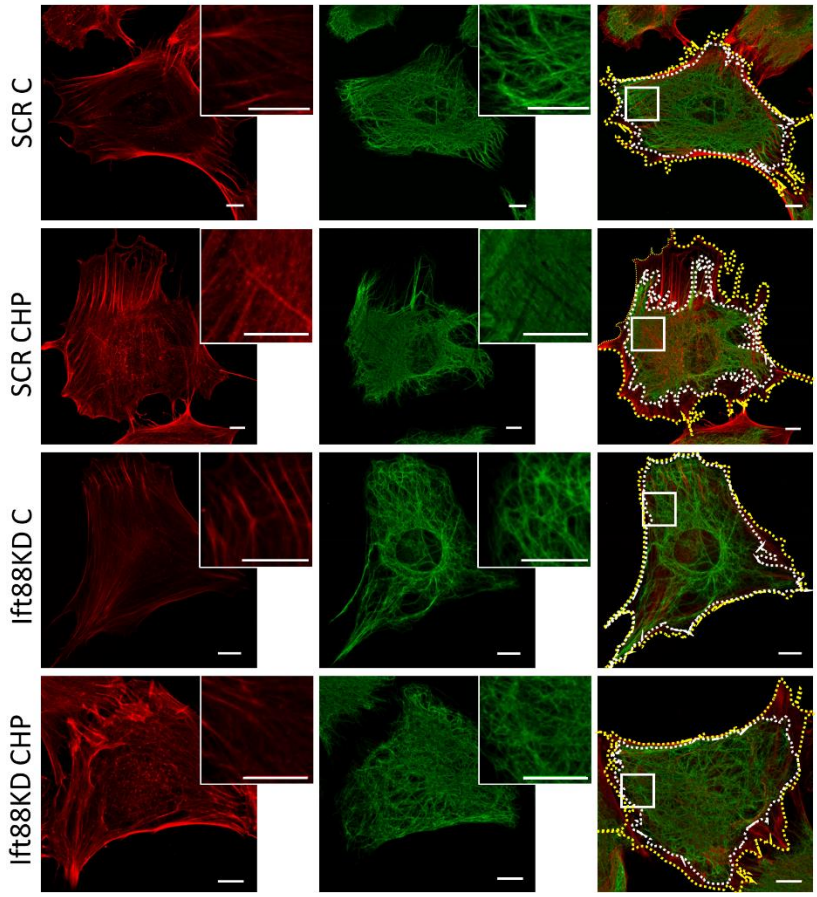


Figure 6-5. Representative images of intermediate filaments network (green) and primary cilia morphology (red) with Ift88 KD. Scr- scramble siRNA acted as control. No qualitative morphological distinctions were observed for intermediate filaments with IFT88KD whereas primary cilia length is blunted as expected with siRNA Ift88 treatment.

In the previous chapter, it was shown that 1hr of pressure stimulation induces disassembly of IF network with a recoiling effect towards the perinuclear region. To determine whether this mechanism could be downstream of cilium-mediated pressure mechanotransduction, CHP was applied to MSCs following primary cilia inhibition. In the scrambled negative control group, co-staining of IF with actin displayed the recoiling and breakdown of filamentous IF structure with CHP stimulation, highlighting that transfection treatment did not affect the MSC capability to respond to this type of stimulation, as previously noted (Figure 6-6 A). Preliminary quantification of this recoiling mechanism indicates that IF area distribution is reduced by 13.6% ($p<0.05$; 85.4% static control to 71.8% CHP, Figure 6-6 B). When primary cilium expression was abrogated via siRNA against Ift88, the recoiling mechanism in response to CHP was not evident, although only a small number of independent samples were measured. Quantification of IF area distribution displays a higher variability with 10.6% reduction in the dispersion area with CHP treatment (89.2% static control to 78.6% CHP, Figure 6-6 B). These observations suggest that the primary cilium may possibly play an upstream role in IF recoiling mechanism in response to CHP.



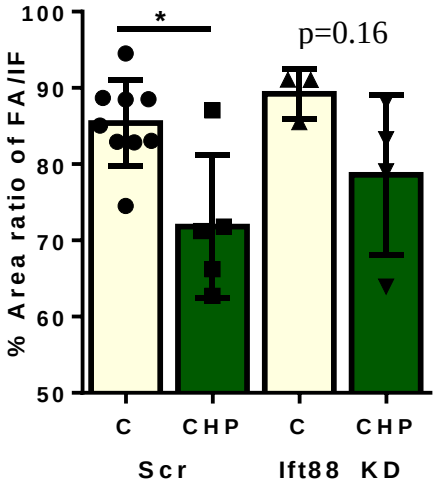


Figure 6-6. Changes in vimentin architecture in response to CHP and cilia abrogation via Ift88 KD. Cilia abrogation prevents CHP induced recoiling of IF, highlighting its possible involvement in IF mechanotransduction. F-actin depicted by red and intermediate filaments by green. (A) Quantification of % area dispersion of intermediate filaments to f-actin. n=3-9 cells, average± SEM. $p<0.05$ (B). Scale bar=10µm. Scr-scramble siRNA.

6.4 Discussion

Loading-induced bone formation is intrinsically coupled to the ability of MSCs to undergo osteogenesis in response to bone associated mechanical cues. However, much is unknown about how MSCs sense pressure transients within bone and what are the early signalling events that would trigger the downstream osteogenic processes. In this study, it was demonstrated that cyclic hydrostatic pressure (CHP) stimulation of MSCs elicits a rapid release of ATP into the extracellular space, a reorganization of the intermediate filament network with concentration in the perinuclear region and an inhibition of proliferation after 48hrs. Furthermore, it was demonstrated that CHP results in a modulation of the length of the cytoskeletal extension and known mechanotransducer, the primary cilium. Importantly, the integrity of the primary cilium was found to be paramount for pressure induced ATP release and inhibition of proliferation, demonstrating a novel role for the cilium in MSC pressure mechanotransduction. Furthermore, preliminary observations highlighted that primary cilium expression may play a pivotal role in pressure induced remodelling of intermediate filament (IF) network, with functions upstream of this IF dependent pressure mechanotransduction mechanism demonstrated previously. Overall, this study highlights a novel pressure induced mechanotransduction mechanism pertaining to the expression of the primary cilium, which mediates purinergic signalling, anti-proliferative and cytoskeletal remodelling effects in response to a pro-osteogenic pressure stimuli.

One of earliest signalling events in response to mechanical loading within the bone microenvironment attributed to MSC activity is the purinome cascade, such as ATP signalling and its associated purinoceptors (Noronha-Matos and Correia-de-Sa, 2016). In this study, it is shown that MSCs exposed to in vivo locomotion associated pressure gradients cause the release of ATP in the extracellular environment. The release of ATP metabolites in response to mechanical stimulation was previously found to occur in osteoblast with cyclic hydrostatic pressure stimulation (Gardinier et al., 2009). Interestingly, MSCs mechanically stimulated with chondrogenic associated pressures (MPa) also exhibit a robust ATP response as well as fluid flow stimulation of MSCs, osteoblasts and osteocytes (Riddle et al., 2007, Gardinier et al., 2009, Genetos et al., 2007, Steward et al., 2016). Hence, loading induced ATP purinergic signalling may be viewed as an initial response to mechanical stimulation rather than a lineage specific

response. Furthermore, Riddle et al. (2008) elegantly demonstrates that fluid flow induced ATP release in MSCs does not depend on the magnitude of the shear stress but rather flow rate associated chemotransport effects (concentration of serum, amino acids, glucose and rate of replenishment) which in turn affects both fluid flow induced calcium signaling and proliferation. Hence, the dynamic property of mechanical stimulation (oscillatory/cyclic versus steady) can significantly impact the purinergic signalling and metabolic activity of the MSC, however the significance on the downstream lineage effects (osteogenesis vs chondrogenesis) may differ due to other synergistic signalling effects (Steward et al., 2016, Wann et al., 2012). Overall, bone associated pressure mechanical cues has similar capabilities as fluid flow to initiate purinergic signalling in MSCs which may contribute to downstream osteogenesis.

The mechanism of loading induced ATP release and subsequent reception in MSCs is yet to be fully identified. Previously, loading induced ATP release, such as fluid flow, has been attributed to hemichannel activity in osteocytes, with sensing occurring via purinergic receptors such as P2X and P2Y in both osteocytes and osteoblasts (Genetos et al., 2007, You et al., 2002). In MSCs, purinergic receptor P2XR7 has been demonstrated to be necessary for the exogenous ATP induced osteoblastogenesis although its involvement in loading induced ATP mechanotransduction is unknown (Li et al., 2015, You et al., 2002). Similarly, fluid flow stimulation was shown to instigate a vesicular release of ATP in both osteoblasts and MSCs although the mechanism mediating this process is unknown (Riddle et al., 2007, Genetos et al., 2005). Here, a novel mechanism of pressure induced ATP release mediated by the primary cilium is demonstrated. This organelle, which is an extension of the microtubule-based cytoskeleton, was found to be necessary for the release of ATP with CHP stimulation. In contrast, pressure induced ATP release in chondrocytes was found to be independent of the primary cilium expression, but paramount for its reception to activate downstream chondrogenesis (Wann et al., 2012). This highlights that ATP associated mechanosignalling is possibly cell specific and functionally tailored although the biological significance of it is yet to be determined.

Interestingly, the primary cilium sensitivity to pressure was reflected by a trend towards an increase in the cilium axoneme length. In trabecular meshwork cells, which are responsible for the drainage of aqueous fluid in the eye and thereby regulating intraocular pressure, the cilia of these cells were found to shorten in response to pressure

stimulation (Luo et al., 2014). Similarly, fluid flow stimulation in kidney epithelial cells as well as chondrocytes exposed to strain and compression mechanical stimulation were found to shorten the ciliary axoneme as a potential mechanism to modulate cell sensitivity to the signalling cues in the extracellular environment (Thompson et al., 2014, McGlashan et al., 2010, Besschetnova et al., 2010). Lengthening of the cilia in response to pressure seems to be counterintuitive based on previous observations and these effects may be attributed either to the methodology of cilium length measurement (length of a 2D projection versus Pythagorean approach developed by Dummer et al. 2016 employed in this study), short term simulation (1 hr) and cell lineage (mesenchymal stem cells versus committed tissue specific cells). Cilia lengthening to CHP stimulation represents an intriguing finding and potentially indicates an adaptive mechanotransduction mechanism to mechanical loading.

Pressure stimulation was found to arrest proliferation which was also dependent on the primary cilium. Previously, 10 kPa continuous static or intermittent pressure stimulation was found to promote proliferation in human bone marrow cells whereas intermittent negative 50 kPa pressure inhibited proliferation of hMSCs (Yang et al., 2009, Rottmar et al., 2011, Sugimoto et al., 2017). Moreover, other types of mechanical stimulation such as fluid flow and strain have been shown to promote proliferation, therefore current disparity may be attributed to the nature of the cells, culturing conditions or method of quantification (metabolic assay versus EdU incorporation) (Choi et al., 2007, Hoey et al., 2012). Interestingly, primary cilium abrogation enhanced proliferation over time, and this effect has been previously reported also in hMSCs (Hoey et al., 2012). This is not surprising as primary cilium assembly/disassembly dynamics is tightly regulated in the cell cycle, and interference of this process may modulate cell cycle progression (Goto et al., 2013). Intriguingly, fluid flow stimulation of hMSCs with silencing of IFT88 further enhanced proliferation, whereas with pressure stimulation the inhibitory effect on proliferation was abolished (Hoey et al., 2012). The loss of mechanical modulation of pressure antiproliferative effects could be attributed to the increased cell cycle progression due to cilia abrogation.

The remodelling of intermediate filaments in response to pressure stimulation was also found to be affected by the primary cilium expression. Abrogation of the primary cilium alone did not affect the cytoskeletal network as shown by the fibrillar nature of intermediate filament network and presence of actin stress fibres. As described in the

previous chapter, application of cyclic hydrostatic pressure causes a breakdown and recoiling of intermediate filament network. Surprisingly, preliminary investigation found that the degree of recoiling/pericellular aggregation of IF was diminished in MSCs where primary cilium expression was inhibited. This represents an intriguing finding, as primary cilium inhibition does not cause any disturbance to IF network in static normal conditions, however it exhibits diminished network remodelling in response to mechanical stimulation. Changes in the cytoskeleton mainly as remodelling of actin and microtubule networks have been related to modulation of primary cilia length (Goto et al., 2013). However, this is the first report to show the potential that intermediate filaments pressure induced mechanotransduction mechanism may be downstream of primary cilia mediated mechanotransduction.

Although the observations of pressure induced ATP and cytoskeleton remodelling mediated by primary cilia are independent observations, current literature provides basis for a potential unifying mechanotransduction mechanism. ATP is an essential part of actin remodelling kinetics as well as the hydrolysis of this molecule has been shown to modulate myosin II activity thereby generating tension within the actin network (McBeath et al., 2004, Arnsdorf et al., 2009, Kudryashov and Reisler, 2013). Furthermore, actomyosin association with IF via plectins can modulated IF dynamics, with enhanced actin cytoskeletal tension resulting in the retrograde flow of intermediate filaments to the perinuclear region (Jiu et al., 2015). Therefore, it can be speculated that pressure induced ATP and/or its degradation products act back on MSCs, enhancing actin stress fibre formation which in turn initiates breakdown and remodelling of the intermediate filament network and osteogenesis. Given that we have shown that the primary cilium is required for both the ATP release and IF remodelling, suggests that the cilium is a primary pressure mechanosensor mediating this downstream mechanosignalling. Other mechanisms may also be at play such as activation of purinergic receptors as ATP was not completely abolished with primary cilia abrogation as well as focal adhesions and integrins involvement in pressure transduction (Thamilselvan and Basson, 2005). Further studies are required to identify the relation of pressure induced ATP release mediated by the primary cilium in regard to loading-induced MSC osteogenesis given the role of primary cilium in loading-induced bone formation.

6.5 Conclusion

In conclusion, this study demonstrates that cyclic hydrostatic pressure (CHP) stimulation of MSCs elicits a rapid release of ATP, a reorganization of the intermediate filament network and an inhibition of cell proliferation, all of which was dependent on the primary cilium, demonstrating a novel role for this organelle in stem cell pressure mechanotransduction.

Chapter 7.
Thesis discussion

7.1 Summary

The objective of this thesis was to investigate how changes in the bone marrow mechanical environment under physiologically relevant loading regimes can affect the osteogenic potential of mesenchymal stem cells; with a further objective to determine what biological mechanisms allow for transduction of these biophysical cues into an osteogenic response. To answer these questions, an in-depth literature review understanding the marrow mechanical environment under physiological loadings and how it relates to current *in vitro* mechanobiological practices were assessed. Initially, the effect of bone associated physiologically relevant fluid flow on early osteogenic responses and osteogenic lineage commitment of MSCs was evaluated. To do that, a custom fluid shear bioreactor was built and validated to be able to perform fluid shear regimes that have been predicted to occur within the bone and marrow niche during mechanical loading. Using a systematic approach, it was identified that physiologically mimetic oscillatory fluid flow induced shear stress elicits a positive osteogenic response in MSCs in a shear stress magnitude, frequency, and duration dependent manner that is gene specific. Based on the mRNA expression of osteogenic markers *Cox2*, *Runx2* and *Opn* after short-term fluid flow stimulation, it was identified that a regime of 2Pa shear stress magnitude and 2Hz frequency induces the most robust and reliable upregulation in osteogenic gene expression. Furthermore, long-term mechanical stimulation utilising this regime, elicits a significant increase in collagen and mineral deposition when compared to static control demonstrating that mechanical stimuli predicted within the marrow is sufficient to directly drive stem cell osteogenesis.

Fluid flow streams are generated by pressure gradients that are built up during locomotion within the bone architecture. Given that fluid flow was demonstrated to influence osteogenic lineage commitment of MSCs, the role of physiologically related pressure gradients in loading induced MSC osteogenic lineage commitment was yet to

be determined. To answer this question, a custom pressure bioreactor that can mimic cyclic pressure transients which have been predicted to occur within the bone and marrow niche was built and validated. Employing a similar systematic approach to fluid flow study, it was identified that CHP elicits a positive but variable early osteogenic response in human MSCs in a magnitude and frequency dependent manner that is gene specific. Moreover, the most robust pro-osteogenic response was found at the highest magnitude (300kPa) and frequency regimes (2Hz). In addition, for the first time it was determined that long-term mechanical stimulation was found to induce sustained magnitude dependent purinergic ATP signalling. However, all magnitudes of CHP promoted similar levels of collagen synthesis and significant mineral deposition, demonstrating that lineage commitment is magnitude independent. This therefore demonstrates that physiological levels of pressures, as low as 10kPa, within the bone can drive stem cell osteogenic lineage commitment independent of fluid shear stimuli.

To further explore what biological mechanisms enable stem cells to sense and translate pressure mechanical cues into an osteogenic response, mechanotransduction mechanisms related to the cytoskeleton and cytoskeleton related organelle, the primary cilium was explored. Initially, it was observed that cyclic hydrostatic pressure is a potent mediator of cytoskeletal reorganisation complemented by an early osteogenic response in MSCs. Using validated pharmacological agents to disrupt specific cytoskeletal elements and high-end confocal and super resolution microscopy techniques, it was discovered that CHP stimulation causes breakdown and remodelling of intermediate filament (IF) network. Furthermore, this restructuring of IF in response to pressure was necessary to elicit an osteogenic response in MSCs due to mechanical loading. Moreover, I was able to identify a mechanism to mimic pressure induced osteogenesis by chemically disrupting intermediate filaments using pharmacological agent Withaferin A, highlighting a potential mechanotherapeutic effect to enhance MSC osteogenesis.

Lastly, the role of cilia in pressure mechanotransduction was explored given its role in loading induced bone anabolism and fluid shear mechanotransduction. Firstly, it was demonstrated that primary cilia in MSCs, as an extension of cytoskeleton, is sensitive to pressure cues by exhibiting longer axonemal length. In addition, CHP stimulation was found to initiate a purinergic ATP response, anti-proliferative effects and remodelling of intermediate filaments which were dependent on the primary cilium presence. This highlights primary cilium as potential mechanotransducer involved in the upstream

signalling in response to pro- osteogenic pressure cues as well as demonstrating a novel mechanism of pressure induced mechanotransduction in MSCs.

7.1.1 Fluid flow induced MSC osteogenesis

The thesis began by exploring how changes in the bone and marrow mechanical environment during bone physiological loading can directly regulate the ability of MSC to commit towards the osteogenic lineage. Initially, the effect of fluid flow stimulation on MSCs osteogenic lineage commitment was assessed given that fluid flow is thought to be the mechanical stimulus with the most stimulatory effects (direct and paracrine) as well as several reports showing that short term stimulation can instigate an osteogenic response in MSCs (summarised in **Table 4**). From a review of the literature and recent computational models of bone marrow mechanics, these studies were found to employ fluid flow regimes that underrepresent the physiological flow induced shear stresses (<2 Pa) that may occur within the marrow niche during locomotion (<8 Pa) (Metzger et al., 2015a). Moreover, the use of different dynamic properties of fluid flow (steady state, pulsatile and oscillatory) added to the disparity to the osteogenic responses demonstrated due to different chemotransport and diffusional effects, with only oscillatory fluid flow recognised as being physiologically relevant (Jacobs et al., 1998, Riddle et al., 2008). Therefore, the objective of this study is to conduct a systematic analysis of physiologically relevant oscillatory fluid shear stress magnitude, frequency and duration on early osteogenic responses and to determine whether these mechanical stimuli are sufficient to drive osteogenic lineage commitment of mesenchymal stem cells during long term stimulation.

Initially, a custom parallel plate flow chamber (PPFC) bioreactor was built based on best microfluidic practices reported and validated using computational fluid dynamics (Chung et al., 2003, Nauman et al., 1999, Doorly and Sherwin, 2009). Many PPFC bioreactor configurations have been reported to date and the lack of design standards or requirement of minimal engineering performance reporting makes it difficult for technology assessment for differences in biological outputs. This is also applicable for bioreactors employing scaffold culture where fluid inertial effects will depend on pumping mechanism/pressure differential in addition to biomechanics and architecture of the scaffold which will dictate shear stress distribution on the cells and any other fluid flow associated effects. For parallel plate flow chambers a recommendation minimum reporting of chamber height, flow rate and pumping system should be included. Using

the custom made PPFC, the effect of physiologically relevant shear stress displayed mostly a magnitude dependent effect with strong correlation for *Cox2* mRNA expression, but a weaker effect on *Runx2* and *Opn* was observed. It can be postulated that different mechanotransduction mechanisms may be involved in the expression of osteogenic genes to various mechanical regimes however, the biological significance of the level of the expression is yet to be determined. The most intriguing observation was that 2Hz frequency regime at 2Pa and 5Pa exhibited the most robust early osteogenic response. This gives a new perspective on what regimes may be best for osteogenic studies exploring mechanobiology and mechanotransduction associated osteogenic effects in MSCs or in bone, given that most commonly used oscillatory fluid flow regime is 1 Pa, 1 Hz (Arnsdorf et al., 2010, Arnsdorf et al., 2009, Espinha et al., 2014b, Hoey et al., 2012). Furthermore, comparing long term fluid flow stimulation of 2 Pa, 2 Hz to 1 Pa, 1 Hz regime, both displayed presence of collagen synthesis whilst, the 2 Pa, 2Hz regiment had the highest mineral content present. This provides evidence that the ability of MSCs to undergo osteogenic lineage commitment by functional assessment to synthesise organic and inorganic bone matrix can be tailored using various magnitudes of fluid flow induced shear stress. Moreover, it highlights osteogenic lineage commitment can be directly regulated by biophysical cues independent of osteocyte and osteoblast activity in response to mechanical cues which have also been demonstrated recently in a model of low magnitude mechanical stimulation in vertebral bone explants (Curtis et al., 2018).

7.1.2 Cyclic hydrostatic pressure induced MSC osteogenesis

In the previous study it was demonstrated that physiologically mimetic bone marrow associated fluid flow can induced osteogenic lineage commitment of MSCs. However, the role of physiologically relevant pressures in this event is unknown although flow streams are dependent on pressure gradients developed during bone loading. Surprisingly, the role of pressure in bone anabolism is understudied: only 5 studies related to stem cells, 2 to osteocytes and majority osteoblasts (<20) have been reported to date (Table 3). Furthermore, similar to fluid flow, the nature of mechanical regimes employed to determine the involvement of pressure in osteogenic events vary in terms of combination of pressure magnitude, dynamic properties (static, intermittent), duration as well other culturing conditions resulting in conflicting observation (Becquart et al., 2016, Sugimoto et al., 2017, Liu et al., 2009). Therefore, the aim was to conduct a

systematic analysis of cyclic hydrostatic pressure magnitude, frequency and duration on early osteogenic responses and to determine whether pressure independent of fluid flow can drive osteogenic lineage commitment of mesenchymal stem cells.

To achieve this, initially a custom pressure bioreactor was built and empirically validated to produce pressure gradients that have been measured and predicted to occur during locomotion. The pressure bioreactor system represents a modified version of fluid flow bioreactor, as an elegant and simplified engineering solution that mimics pressurisation of marrow and interstitial fluid during bone loading. The modification to the system represents a closed chamber-bioreactor system to allow for pressurisation of fluid by the syringe pump in a triangular wave mode whereby infusion/extraction of a finite bolus of fluid is related to a defined pressure gradient. The simplicity of the system comes with limitations such as depending on the pump performance, over pressurisation may occur therefore validation of the time before such an event happens is necessary. Hence, this system can be used for only short-term studies (<2 hrs) with manual resets or inclusion of independent mechanical relief valves. Using this bioreactor system, it was discovered that human MSCs are mechanosensitive to pressure mechanical stimulation with a positive magnitude effect which was gene specific. In particular, positive magnitude dependent effects were found only at 2Hz, 2hrs and 0.5Hz, 4hrs CHP regimes for *Cox2* and *Opn* mRNA expression which was not present for *Runx2* expression. Several factors can be alluded to play a role in these observations: 1) CHP inhibits osteogenesis 2) expression of the studied genes are dependent on activation of different pathways to pressure cues and 3) biochemical priming modulates the threshold of protein translational activity (Yourek et al., 2010, Henstock et al., 2013). These statements are based on studies showing that 10 kPa static pressure increases *BMP2, RUNX2, OSX*, ALP, Col1α1 in hMSCs and 68 kPa, 0.5 Hz elicit *Runx2, Dlx5, Msx2, Osx* in rat MSCs using different doses of osteogenic compounds, in particular Dexamethasone, which is known to influence *Runx2* transcription (Liu et al., 2009, Sugimoto et al., 2017, Langenbach and Handschel, 2013). This is further demonstrated by the absence of pressure induced osteogenesis in hMSCs using 10-100 kPa,2Hz CHP without osteogenic biochemical priming (Becquart et al., 2016). Therefore, further analysis on the interplay between biochemical induction and pressure stimulation on downstream osteogenic program in MSC is required.

One interesting finding was that osteogenic commitment of hMSCs was found to be independent of pressure magnitudes although pressure induced ATP purinergic signalling during long term pressure stimulation displayed consistent magnitude effects. This alludes to the fact that the cells are mechanosensitive to pressure magnitudes, but it does not influence their ability to synthesise more inorganic matrix, unlike fluid shear stimulation. These effects are reflected by a previous study showing 10 kPa static pressure driving mineral deposition in hMSCs (Sugimoto et al., 2017). Therefore, it can be concluded that pressure induced osteogenic lineage commitment of MSCs is magnitude independent of physiologically relevant cyclic pressure gradients.

From a biomechanics perspective, pressurisation of the intraosseous fluid and marrow due to strain induced deformation of bone can elicit large fluid inertial effects dictated by the architectural anatomy of the bone. Therefore, the effect of pressure gradients in MSCs osteogenesis may be secondary to fluid flow. An interesting and possibly relatable example is the development of high intracranial pressure leading to thickening of the calvaria in microgravity conditions highlighting the interplay between pressure and fluid shear mechanical cues in bone anabolism (Zhang et al., 2013, Kramer et al., 2012).

7.1.3 Intermediate filament mechanotransduction mechanism of pressure induced osteogenesis

Despite previous demonstration of osteogenic responses to pressure stimulation, little is known about how MSCs sense and transduce pressure mechanical cues with only one study to date highlighting the involvement of the mechanosensitive ion channel Piezo 1 (Sugimoto et al., 2017). Previous observations of cytoskeletal remodelling in osteoblasts and osteocytes to pressure stimulation as well as mechanically driven cytoskeletal adaptation of bone cells to fluid flow and strain has spurred the exploration of the MSC cytoskeleton involvement in mediating the response to pressure cues (McBeath et al., 2004, Mathieu and Loboa, 2012, Liu, 2010, Gardinier et al., 2009). Here it was observed that short term pressure (100 kPa, 1Hz, 1hr) stimulation that enhances early osteogenic response in MSCs depicted by upregulation of *Cox2*, *Runx2* and *Opn*, also causes remodelling of the cytoskeleton, with presence of actin stress fibres and intermediate filaments undertaking a fibrillar architecture indicative of disassembly. Employing pharmacological agents against each type of cytoskeletal element

(Cytochalasin D- f-actin; Nocodazole- microtubules, Withaferin A- intermediate filaments) it was identified that only disassembly of intermediate filaments (IF) yielded an osteogenic response almost identical to pressure stimulation in addition to similar fibrillar architectural arrangement of IF network. Further investigating the correlation of IF filament breakdown and pressure induced osteogenesis, it was found that pressure causes decrease in total vimentin associated IF protein as well as perinuclear aggregation of IF via a recoiling mechanism in response to pressure cues. Moreover, through serendipitous discovery, DMSO was found to reinforce the IF network and thereby prevented pressure induced breakdown of IF which also abolished pressure induced osteogenesis. This is the first report implicating mechanical modulation of IF in loading induced stem cell osteogenesis in MSCs as previously cytoskeleton related osteogenic effects have been attributed mainly to actin remodelling (Arnsdorf et al., 2009). Intermediate filament is rather an understudied cytoskeletal component within the musculoskeletal field, with only two reports to date highlighting that downregulation of IF protein total vimentin is necessary during chemically induced osteoblastogenesis (Lian et al., 2012, Lian et al., 2009). IF research has been highlighted mostly in cancers due to its involvement in epithelial to mesenchymal transition and migration (Liu et al., 2015a, Ivaska et al., 2007). One such report demonstrated that crosstalk between IF and actin stress fibres occur during migration whereby the formation of actomyosin arcs across actin stress fibres drives the retrograde flow of vimentin and perinuclear aggregation of intermediate filaments via the cytolinker protein, plectin (Jiu et al., 2015). Furthermore, vimentin depletion was found to promote RhoA activity and actin stress fibre assembly by causing phosphorylation of the microtubule-associated GEF-H1 (Jiu et al., 2017). Previously, Arnsdorf et al. (2009) demonstrated that fluid flow induced early osteogenic response depends on GTPase RhoA and its effector ROCKII which generates actin cytoskeleton tension. Although a complex crosstalk between actin and IF seems to exist, in this chapter mechanical modulation of intermediate filaments with pressure stimulation proved to be critical for onset of early osteogenic response in MSCs, providing a new insight into the cytoskeletal contribution MSC osteogenesis.

Lastly, the use of the pharmacological agent Withaferin A to disrupt the intermediate filament elicited an increase in *Cox2*, *Runx2* and *Opn* mRNA expression, effectively mimicking the pressure induced osteogenic response, thus highlighting this drug as a potential anabolic agent for bone associated diseases such as osteoporosis. This is

demonstrated in preclinical models of osteopenic ovariectomized mice whereby administration of Withaferin A was shown to promote osteoblastogenesis and supress osteoclastogenesis, in addition to enhancing bone microarchitecture and mechanical properties (Khedgikar et al., 2013).

7.1.4 Primary cilia mediated pressure mechanotransduction in MSCs

Primary cilium as an extension of the cytoskeleton in the pericellular milieu, has been shown to be an important organelle in loading-induced bone anabolism and fluid shear induced MSC mechanotransduction (Hoey et al., 2012, Chen et al., 2016a, Moore et al., 2018b). Exploring the role of cilium in MSC pressure induced mechanotransduction, it was found that its presence was necessary for pressure induced ATP release. ATP represents one of the key primary messengers of purinergic signalling which is secreted in response to loading and has been associated with downstream osteogenic signalling (Noronha-Matos and Correia-de-Sa, 2016, Kringelbach et al., 2015, Costessi et al., 2005). In the previous study it was shown that at each bout of pressure loading during long term stimulation, the MSCs will retain their mechanosensitivity and the amount of ATP secreted depends on magnitude of pressure applied. Taking these observations together, it can be speculated that mechanically mediated purinergic signalling that modulates osteogenic downstream signalling may be taking place via a cilia related mechanism. This hypothesis differs to the observations of cilia mediate purinergic signalling in chondrocytes, as primary cilia was not necessary for pressure induced ATP release, but important for ATP reception and downstream chondrogenesis (Wann et al., 2012). Future studies will have to determine whether primary cilium mediated pressure induced ATP release is also related to the downstream pressure induced osteogenesis.

Primary cilium was also discovered to modulate pressure associated proliferation effects and remodelling of intermediate filaments. The effect of pressure on proliferation in this mouse stem cell model (C3H10 T1/2) was found to be, surprisingly, anti-proliferative unlike previous observations related to fluid flow stimulation and pressure in human MSCs (Riddle et al., 2006, Sugimoto et al., 2017, Rottmar et al., 2011). To discern the disparity in the results, a side by side comparison of pressure induced mitogenic effect of these two MSC species and cell types should be considered. The involvement of cilium in cell cycle regulation was confirmed based on increase in proliferation after Ift88 abrogation (Goto et al., 2013) and intriguingly the pressure

induced arrest in proliferation was abolished, highlighting a possible role for cilium in modulating pressure associated anti-proliferative effects.

Lastly, pressure induced remodelling of intermediate filaments (IF) which was shown to be necessary for downstream osteogenic responses, was found to be dependent on the primary cilium presence. This is an intriguing finding as primary cilium abrogation does not cause any structural changes in the cytoskeleton, but the remodelling of IF with pressure stimulation was abolished. Several interlinking mechanisms are postulated to be at play: 1) the primary cilium is necessary for upstream pressure induced mechanotransduction given its modulatory effect on ATP release and 2) remodelling of IF via modulation of actin dynamics. ATP plays an important role in actin kinetics since ATP hydrolysis is indirectly involved in generation of actin tension (Kudryashov and Reisler, 2013). Furthermore, IF was found to associate with actin through the cytolinker protein plectin thereby actin remodelling causing restructuring of IF network (Jiu et al., 2015).

Overall, this thesis represents an exploration of how physiological changes in the bone marrow micromechanical milieu can drive MSC osteogenic lineage commitment. Moreover, novel mechanisms pertaining to pressure mechanotransduction was discovered involving the cytoskeletal associated intermediate filament network and primary cilium, which has revealed new targets and pharmacological agents to mimic the effect of loading to enhance MSC osteogenesis and potentially bone formation.

7.2 Limitations and future directions

For the fourth chapter exploring the role of physiologically relevant oscillatory fluid flow in influencing MSC osteogenic lineage commitment, an MSC model represented by C3H10T1/2 mouse embryonic mesenchymal stem cell line derived from C3H mice were used. Although, this cell line has been shown to be responsive to fluid flow stimuli (Arnsdorf et al., 2009) and pressure stimuli (Chapter 6 and 7), and has been consistently used as a model of mesenchymal stem cells, it has also been shown to have limited mineralisation ability (Zhao et al., 2009, Hu et al., 2013). To verify translation of these effects in human species, human mesenchymal stem cells should be used in the future. Furthermore, all mechanical studies employed only 2D systems therefore a more physiological approach of 3D spatial distribution on various extracellular matrices are worth exploring. Using the developed bioreactor systems, the mechanosensitivity of stem cells derived from various tissues (i.e. synovium, muscle, adipose) can be studied which will enable the discovery of the molecular mechanisms enabling the transduction of fluid flow and pressure cues contributing to the functional maintenance of a tissue.

In chapter 5, exploring the effect of physiologically relevant cyclic hydrostatic pressure on MSCs ability to undergo osteogenesis, the custom pressure bioreactor was not validated for presence of flow streams using computational modelling. An empirical approach of observing the fluid stream and diffusional pattern with a food colour dye displayed that the fluid flow inertial effects lasted for only a third of the tube length, therefore an assumption of a hydrostatic pressure effect with minimal shearing effects was employed. From a design perspective, the use of a clamping system over a screw-in system would be more user friendly to clamp the bioreactor and minimise occurrence of leakages/depressurisation if a screw was not secured properly.

For both, fluid flow and pressure systematic studies, a protocol of short-term intermittent stimulation followed by 2-3 weeks static culture stimulation was employed. Due to facility limitation, the bioreactors were designed primarily for short term studies and modified for long term culture. Future studies exploring a protocol whereby intermittent mechanical stimulation over 2-3 weeks on MSCs osteoblastogenesis should be considered. Furthermore, the cells were cultured on fibronectin on glass slides where an inherent bias towards stiffness of the glass slide (50-90 GPa) and cell-matrix interaction such as primarily αvβ3 integrin for fibronectin may be present. Future

consideration towards more biomimetic substrates in the range of osteoid and bone stiffness in addition to use of organic matrix interaction such as collagen should be included.

A limitation of the pressure systematic study is that only 2Hz frequency CHP group regime was used for long term pressure induced osteogenesis hence these effects may be specific to this frequency alone. Comparison between 1Hz and 2Hz long term stimulation should be considered for future studies.

In chapter 6, pressure induced osteogenesis was found to occur via a novel mechanotransduction mechanism utilising the breakdown and remodelling intermediate filament network. Vimentin kinetics are related to phosphorylation of serine residues therefore identifying phosphorylation of serines in response to pressure stimulation may be an avenue to explore molecular kinetics associated with this mechanism (Cytoskeleton). Phosphorylation of Serine56 in this process was explored in this study without success because of the lack of reactivity of the antibody and general lack of antibodies availability for IF associated phosphorylated serines.

Lastly, in Chapter 7 further exploration of the relationship between pressure induced ATP release and downstream osteogenesis is required such as whether pressure induced osteogenesis is affected by primary cilium abrogation. Moreover, identification of the intermediary mechanisms that may revolve around ATP signalling and its by products such as cAMP to instigate loading induced osteogenesis may reveal novel therapeutic targets.

Primary cilium is a microtubule-based organelle and since primary cilium abrogation prevented pressure induced remodelling of IF, the involvement of microtubule network in regulating this process requires further study. From observational imaging studies, no changes were found in the microtubule network stained by α tubulin, however lengthening of primary cilium with pressure alludes to possible restructuring of microtubule network. Employing quantitative methods and other microtubule-based or primary cilia antibodies will provide a better understanding related to this process. The cytoskeleton represents a complex interaction between three cytoskeletal elements: actin, intermediate filaments and microtubules, in addition to matrix-based communication with focal adhesion complexes, primary cilium in the apical orientation of the cell and cell adhesion junctions. Hence, changes to such an intricate system in

response to mechanical cues would require a pragmatic approach to discern the mechanism of pressure based or other mechanically coupled osteogenic responses in MSCs.

7.3 Thesis Conclusions

- MSCs were found to be mechanosensitive to bone and marrow biophysical cues developed during physiological mechanical loading such as fluid shear and pressure stimuli.
- These physiological mechanical stimuli displayed early osteogenic responses which were mainly magnitude dependent, at 1-5Pa fluid flow shear stresses and 10-300 kPa cyclic hydrostatic pressures with frequency interplay at magnitudes above 1 Hz.
- MSC osteogenic lineage commitment was found to be magnitude dependent only for fluid shear stimulation whereas pressure induced osteogenic lineage commitment was magnitude independent and can be sustained at physiological pressures as low as 10kPa.
- Mechanism of pressure induced osteogenesis was demonstrated to be driven via cytoskeletal remodelling such as breakdown and recoiling of intermediate filament network and this effect can be mimicked pharmacologically using Withaferin A agent.
- Pressure was found to elicit a purinergic response such as extracellular release of ATP by the MSCs during long term pressure induced osteogenic lineage commitment as well as short term stimulation.
- Short term ATP purinergic signalling was identified to be dependent on the expression of cytoskeletal extension and mechanotransducer, the primary cilium.
- Presence of the primary cilium was paramount for pressure associated anti-proliferative effects and remodelling of intermediate filaments.
- Overall, these studies demonstrate the importance of physiologically relevant cues in modulating MSC ability to undergo osteogenic lineage commitment in addition to discovery of novel mechanotransduction mechanism of pressure induced osteogenesis in MSCs pertaining to the cell cytoskeleton.
- These studies provide new indicators of biophysical guidelines for bioreactor based orthopaedic strategies and how it can be circumvented using pharmacological agents to target the mechanotransduction pathways involved in osteogenesis, such as intermediate filaments.
- This thesis also highlighted the need to establish a more harmonised approach to bone related mechanobiology studies to understand bone physiology and how

alterations in reception of these cues can lead to debilitating diseases such as osteoporosis.

Appendix 1

Pressure stimulation of mouse MSCs as described in Chapter5, was also found to elicit positive changes for chondrogenic and adipogenic lineages. mRNA expression of chondrogenic marker, Sox9, in response to HP exhibits a 1.5-1.8-fold change over time. However, with CHP treatment, it displays a biphasic elevated expression with a 2.8-fold change at 1hr ($p<0.05$) and 1.6-fold change at 4hrs, although not significant. Similarly, Pparg mRNA expression displays a 4-fold increase at 1 hr and a 1.8-fold increase at 4 hrs ($p<0.05$) only with CHP.

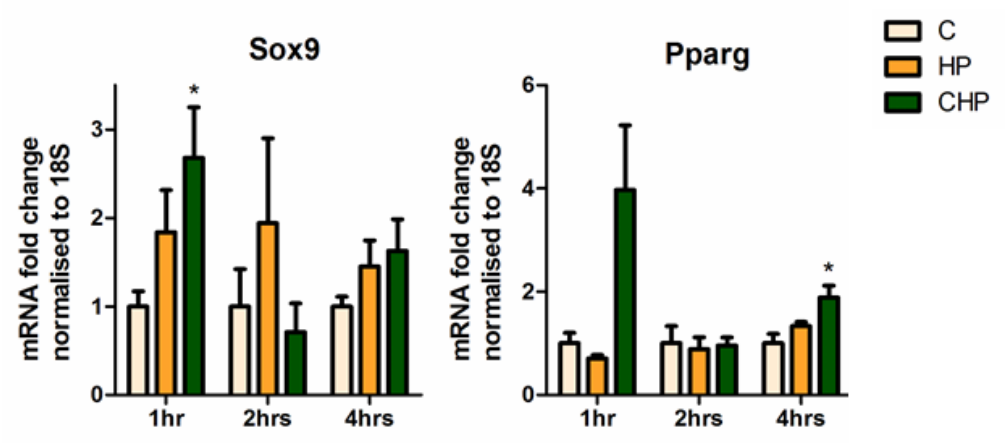


Figure A1 2 Expression of transcription markers for chondrogenesis (Sox9) and adipogenesis (Pparg) in response to cyclic hydrostatic (100 kPa, 1Hz) pressure and hydrostatic pressure (100 kPa). Both pressure groups were compared to static group (atmospheric pressure). Two tailed student t-test with Welch's correction was employed to compare each pressure group against static control. *$p<0.05$, **$p<0.01$. n=3-7

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