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**Comparison of primary and stem cell-derived  
osteoblasts and the influence of nanoscale  
mechanotransduction on cell behaviour**

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## Abstract

The use of stem cells in regenerative bone strategies holds great promise, however it is becoming increasingly clear that stem cell-derived osteoblasts differ in behaviour and function to native osteoblasts. Pluripotent embryonic stem cells (ESCs) present a useful model to study developmental biology and in particular, osteogenesis *in vitro*; information which can be used to further inform and develop regenerative strategies for bone repair. Here, the differentiation of ESCs is compared to primary calvarial osteoblasts. Whilst both cell types exhibit mineralisation, expression of bone specific proteins and increased ALP activity, marked differences are observed in these characteristics over 35 days, particularly in mineral formation.

The range of factors influencing osteogenesis is well known, but recently the effects of nanoscale mechanotransduction have been observed; allowing osteogenesis of mesenchymal stem cells in the absence of conventional osteogenic medium supplements. Here we study for the first time the effect of nanoscale mechanotransduction on ESCs, comparing established ESC differentiation techniques to 1000Hz, 30nm vibrational stimulation, alongside similar studies of calvarial osteoblasts. Although nanovibration allows formation of mineralised osteoblasts cultures indicative of bone phenotype as assessed by immunocytochemistry and qPCR, this differentiation is delayed compared to conventional medium supplementation. However 1000Hz stimulation promoted higher ALP activity and produced mineral composition more similar to mature bone tissue, revealing its importance in osteogenesis.

Mechanisms by which nanovibration influences cell behaviour and differentiation were also explored, revealing influence over cell morphology, spreading and actin cytoskeleton organisation in both cell types. 1000Hz stimulation also promoted focal adhesion complex maturation, with focal adhesion kinase (FAK) activity implicated in this process, particularly

in mESCs. This mechanosensing effect was confirmed by metabolomics analysis, suggesting FAK activity plays a significant role in ESC fate whilst not as important in calvarial osteoblasts. The influence of nanovibration over migratory behaviour of osteoblasts was also observed; an important part of bone remodelling that is less well characterised than other aspects of osteoblast behaviour. Migratory ability developed following conventional osteogenic culture, however 1000hz stimulation had an overriding influence over this; delaying migratory behaviour in a similar manner to osteogenesis.

This work reinforces differences in stem cell-derived osteoblasts and primary calvarial osteoblasts; which has significant implications on the use of stem cells for regenerative bone therapies. However we also highlight the importance of nanoscale mechanotransduction in osteogenesis; 1000Hz stimulation helping to improve osteogenic outcomes particularly when combined with conventional methods. It is hoped this combination approach will help bridge the current gap between stem cell-derived osteoblasts and their native counterparts.

## Publications and Presentations

### Conference oral presentations

**Hollingworth, M.,** Alexander, M. and Buttery, L. 'Precision, 3D bioengineering of the bone microenvironment and control of bone biology in health and disease'. (CDT Joint conference, Leeds, 9<sup>th</sup> October 2017.)

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### Conference publications

**Hollingworth, M.,** Tsimbouri, M., Paul Campsie P., Alexander, M., Reid, S., Dalby, MJ., Buttery L. Osteogenesis of embryonic stem cells by nanoscale mechanotransduction. (TERMIS World Congress, Kyoto, September 2018)

**Hollingworth, M.,** Tsimbouri, M., Paul Campsie P., Alexander, M., Reid, S., Dalby, MJ., Buttery L. Osteogenesis of embryonic stem cells by nanoscale mechanotransduction. European Cells and Materials; eCM meetings abstracts 2018, collection 4. (TCES, Keele University, 2-4<sup>th</sup> July 2018)

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## List of Abbreviations

|               |                                                     |
|---------------|-----------------------------------------------------|
| 1000Hz        | 1000Hz nanovibration                                |
| ALP           | Alkaline Phosphatase                                |
| BGLAP         | Bone gamma-carboxyglutamic acid-containing protein  |
| BMP           | Bone morphogenic protein                            |
| BMU           | Bone multicellular unit                             |
| BSA           | Bovine serum albumin                                |
| Cbfa1         | Core-binding factor subunit alpha-1                 |
| cDNA          | Complementary Deoxyribonucleic Acid                 |
| DMEM          | Dulbecco's modified eagle's medium                  |
| DMSO          | Dimethyl sulfoxide                                  |
| DNA           | Deoxyribonucleic Acid                               |
| EB            | Embryoid body                                       |
| ERK1/2        | Extracellular signal-related kinase                 |
| ESC           | Embryonic stem cell                                 |
| FA            | Focal adhesion                                      |
| FAK           | Focal adhesion kinase                               |
| FBS           | Fetal Bovine Serum                                  |
| g             | Centrifugal G force                                 |
| LIF           | Leukemia inhibitory factor                          |
| mESC          | Mouse embryonic stem cell                           |
| mPC           | Mouse primary calvarial                             |
| MSC           | Mesenchymal stem cell                               |
| OCM           | Osteogenic culture medium                           |
| OCN           | Osteocalcin                                         |
| OPN           | Osteopontin                                         |
| OSX           | Osterix                                             |
| PBS           | Phosphate buffered saline                           |
| PDGF-BB       | Platelet derived growth factor BB                   |
| PFA           | Paraformaldehyde                                    |
| PPAR $\gamma$ | Peroxisome proliferator-activated receptor $\gamma$ |
| qPCR          | Quantitative polymerase chain reaction              |
| RNA           | Ribonucleic acid                                    |
| RT            | Reverse Transcriptase                               |
| RUNX2         | Runt-related transcription factor 2                 |
| TAZ           | Transcriptional coactivator with PDZ-binding motif  |
| U             | units                                               |
| w/v           | Weight per volume                                   |
| $\alpha$ -MEM | Minimum essential medium alpha                      |
| $\beta$ GP    | Beta-glycerophosphate                               |
| 2D            | Two-dimensional                                     |
| 3D            | Three-dimensional                                   |
| $\mu$ g       | micrograms                                          |
| $\mu$ l       | Microlitres                                         |
| $\mu$ m       | Micrometres                                         |
| nM            | Nanomolar                                           |
| ECM           | Extracellular matrix                                |
| ml            | Millilitres                                         |

# Chapter 1 - Introduction

## 1.1 Tissue engineering and regenerative medicine

### 1.1.1 General Background

The damage or loss of human tissues and organs is one of the major challenges in modern healthcare due to the lack of donors. The field of tissue engineering presents an interdisciplinary approach to overcome this issue; combining the principles of engineering and life sciences in an attempt to repair, maintain or replace tissue and restore function. Tissue engineering approaches are typically based upon the use of appropriate isolated cells, scaffolds and other tissue supporting materials, or a combination of both [1]. Tissue engineering strategies can either be applied *in vivo* in order to directly induce regeneration within the body from, or *in vitro* to create a suitable replacement material or tissue that can later implanted into the desired repair site [2] [3]. *In vitro* tissue engineering approaches also aim to develop cellular models of human biology in order to better understand tissues in both healthy and diseased states, thus providing us with valuable insights to aid the development of treatments in the future.

In recent years the term regenerative medicine has been more commonly used, often interchangeably with tissue engineering. However this term is often associated with the specific use of stem cells as the source of tissue regeneration [2]. The term also encompasses biotech, pharmaceutical and device approaches/products that also aim to restore function of human tissue and/or organs in a similar way to tissue engineering [4].

### 1.1.2 Cell therapies

The use of cell therapies dates back to the first bone marrow transplant in 1956 [5], however interest in the field has grown rapidly with the advances of stem cell research in the 1990s. The possibility of growing replacement tissues from isolated adult cells or from stem cells

holds great promise for the treatment of a wide range of clinical needs. Although considered part of the regenerative medicine field, the cell therapy industry is often evaluated as its own entity due to the success of therapies such as Provenge (Dendreon), a cellular immunotherapy for metastatic prostate cancer treatment [4]. Whilst several products are now on the market and many show great promise, a deeper understanding of cell behaviour is required in order to allow the success of cell therapy products and the regenerative medicine field as a whole, and to allow these products to become available to the mass market. In particular, the use of stem cells for regenerative medicine therapies requires efficient differentiation procedures in order to generate committed cells for use in a therapy. Differences between stem-cell-derived cells and native human cells still remain, and characterising these differences is essential to developing successful cell-based regenerative strategies.

### **1.1.3 Cells in regenerative medicine**

A number of cell therapies approved for market are based on autologous cell isolation, *ex vivo* expansion and implantation back into the injury site to promote repair [6]. For example, Carticel™ (Genzyme Biosurgery) relies on chondrocytes harvested from articular cartilage that are expanded *ex vivo* and implanted into damaged cartilage to produce recovery comparable to the “gold standard” repair strategies of mosaicplasty and microfracture [7]. Whilst this approach has shown some success, it is not without drawbacks. Firstly cell isolation causes donor site morbidity and there is a limit to the amount of cells available. Secondly, the need to expand cells means a delay in the treatment for enough cells to be generated. The efficacy of autologous therapies can also range significantly depending upon the health of the patient’s isolated cells and their ability to proliferate.

Cell therapies that rely on isolated stem cells are subject to further variability since the repair often relies upon the successful differentiation to a desired phenotype, either induced *ex*

*vivo* or occurring after reintroduction to a repair site [8]. Effective differentiation is not always possible, particularly in older patients where aging of stem cells can impair proliferative activity and efficacy of repair [9]. This obstacle can result in unpredictability in efficacy of the treatment, often a major hurdle in the approval of cell therapy product by the regulatory authorities.

In order to generate a consistent, repeatable regenerative medicine product much focus has been placed, in recent years, on cell manufacturing; upscaling the production of stem cells to produce sufficient quantities of cells in an reproducible and cost-effective way. Historically most cell therapies have been patient-specific and manufacturing has therefore focused on expansion of batches of cells for each individual case. However, several therapies currently in development are based on cell types that give a low immune response even when used as an allogeneic product. For example, Apligraf (Organogenesis) is based upon mass expansion of allogeneic fibroblasts and has shown low immunogenicity and success in treating ulcers [6]. These therapies lend themselves to large scale manufacturing processes and could be seen as pharmaceutical-like “off the shelf” products [10]. In order to develop these “off the shelf” products derived from stem cell sources, studies must compare the phenotype and behaviour of the resulting cells compared to native human cell/tissue samples.

#### **1.1.4 Progress in regenerative medicine**

Whilst progress with tissue engineering and regenerative medicine show promise for the production and/or repair of human tissues and organs, only a small number of discoveries have been successfully translated to the market. The inability to reliably generate and characterise specific cell populations is potentially the greatest hurdle to the success of cellular therapy products [11]. For this reason, more rigorous and clinically-relevant derivation protocols and characterisation techniques are required. It has also become apparent that defining and controlling the microenvironmental niche is pivotal to ensuring

successful tissue regeneration [12], thus future research should take into account all the relevant factors and signals experienced by cells in native tissue. Replicating these environmental niches *in vitro* is a major target for regenerative medicine, and can help identify appropriate signals that affect cells once transplanted *in vivo*. Accurate *in vitro* systems can be applied to study cell behaviour; representative cellular models can allow observation of key events in development biology, whilst also permitting the study of cell-cell interactions in tailored, reproducible environments. More complex models can better inform about cell behaviour and differentiation *in vitro*, allowing users to better predict the efficacy of cells used for regenerative therapies; thus increasing the likelihood of their overall success.

Advances in the field are also being applied to other disciplines, in particular the production of *in vitro* models for the evaluation of potential therapeutic molecules for pharmaceutical applications. Cellular models to study drug behaviour have the potential to bridge the gap between benchtop testing and animal models, allowing repetitive, high-throughput screening of drug molecules [13]. This in turn can reduce or replace the need for animal models thus reducing costs to pharmaceutical companies and alleviating ethical concern surrounding animal testing [14].

## **1.2 Bone biology and repair**

Bone is a dynamic tissue that continuously remodels throughout life and has remarkable ability to repair following injury [15]. However, like any other human tissue, bone disorders and trauma can disrupt bone biology and architecture, and some form of intervention is required. Knowledge of bone biology has directed repair strategies as discussed below.

### **1.2.1 Bone structure**

Bone is a porous, mineralized tissue with a hierarchical structure, providing the body with structural support, a frame to allow movement as well as protecting vital organs [16]. Long bone structure also protects bone marrow which contains stem cell populations, cytokines and is the site for haematopoiesis [17]. Much of the structural integrity of bone is owed to the bone matrix

deposited by osteoblasts; mainly consisting of collagen type 1 fibres with associated hydroxyapatite crystals [15]. The adult human skeleton consists of 80% cortical bone (also known as compact bone), and 20% trabecular bone (cancellous or spongy bone). These two bone types have significantly different structure and matrix content, and their ratio and arrangement varies depending upon the site within the skeleton.

Cortical bone is dense, with high mineral hydroxyapatite content. Its structure consists of cylindrical units called osteons (Fig. 1.1B), formed from collagen fibres in concentric lamellae that provide the cylindrical structure and resulting mechanical strength. Trabecular bone is less dense with a mesh structure (Fig. 1.1 C) of packets containing blood vessels and marrow.

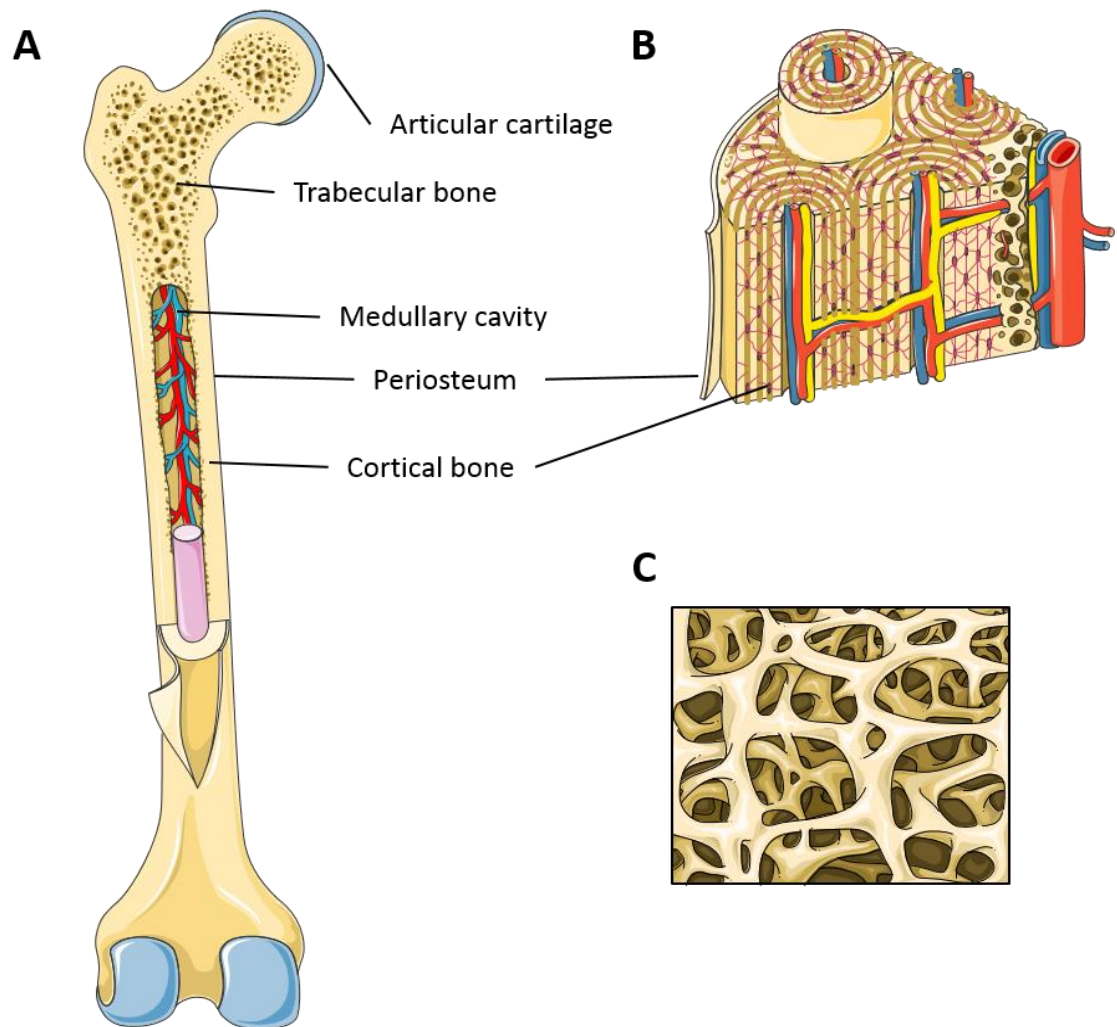


**Figure 1.1 Structure of bone**

A: long bone structure. B: Osteon structure. C: Trabecular bone mesh structure. Figure made with the use of images from Servier medical art.

### 1.2.2 Bone cells

There are several cell types involved in bone development and its hierarchical structure. As discussed in more detail below osteoblasts and osteocytes develop from osteoprogenitor cells (derived from mesenchymal stem cells), whilst osteoclasts form from the haematopoietic stem cell lineage.

#### 1.2.2.i Stem cells

Embryonic stem cells (ESCs) are pluripotent cells able to differentiate into all of the somatic cell types from the three primary germ layers; mesoderm, ectoderm and endoderm [18]. After formation of the blastocyst during development, the inner cell mass consists of ESCs which are commonly used to study embryological development *in vitro*, for which ESC cell lines have been established [19] [20]. In natural embryonic development the pluripotency of ESCs is maintained by a complex of factors. To replicate this effect *in vitro*, leukaemia inhibitory factor (LIF) has been employed; maintaining pluripotency of ES cells by the JAK/STAT pathway [21] [22].

Mesenchymal stem cells (MSCs) are multipotent stem cells that are well known to give rise to osteogenic, chondrogenic and adipogenic cell lineages, as seen in figure 1.2. However differentiation into other cell types has been observed including cardiomyocytes [23] [24]. Clinical trials incorporating MSCs for ischaemic heart failure have implicated immunoregulatory/paracrine functions rather than differentiation mechanisms [25][26]. A population of MSCs found in the bone marrow stroma are recruited during the bone remodelling process after which they differentiate to committed osteoprogenitor cells. Several transcription factors are responsible for MSC fate; including RUNX2 and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) which drive osteogenesis and adiposeness respectively [27][28]. Transcriptional coactivator with PDZ-binding motif (TAZ), activates

RUNX2 whilst suppressing PPAR $\gamma$ , thus directing MSCs towards osteoprogenitor commitment [29].

The use of mesenchymal and embryonic stem cells within regenerative medicine research is extensive, with cell lines enabling the research of human development where regulatory restrictions limit research by other means [30]. More recently, induced pluripotent stem cells (iPSCs) technology has allowed the development of pluripotent cells from reprogramming of somatic cells [31] [32]. This allows production of a pluripotent stem cell similar to ESCs in differentiation capability which overcomes some of the ethical concerns regarding use of embryonic tissues, however their similarity to ESCs is not fully characterised.

#### 1.2.2.ii Osteoprogenitor cells

Osteoprogenitors are an early stage bone cell derived from MSCs, which retain ability to proliferate during remodelling, often also referred to as pre-osteoblasts. Whilst regulated by RUNX2 – also known as core-binding factor alpha 1 – MSC commitment to osteoprogenitors is also promoted by Msx2, which acts upon several downstream factors in osteoblast development [33].

#### 1.2.2.iii Osteoblasts

Osteoblasts are the bone forming cell type, responsible for depositing bone ECM and later allowing mineralisation. Following commitment of osteoprogenitors and expression of RUNX2, Osterix (OSX) marks development of osteoblast phenotype and is involved in matrix formation [34]. Osteoblasts display strong alkaline phosphatase activity [35], and secrete collagen I (Col-I) as well as collagen IV (Col-IV). Bone specific proteins are also secreted by osteoblasts; with bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN) considered significant markers of mature osteoblast phenotype [36] [37].

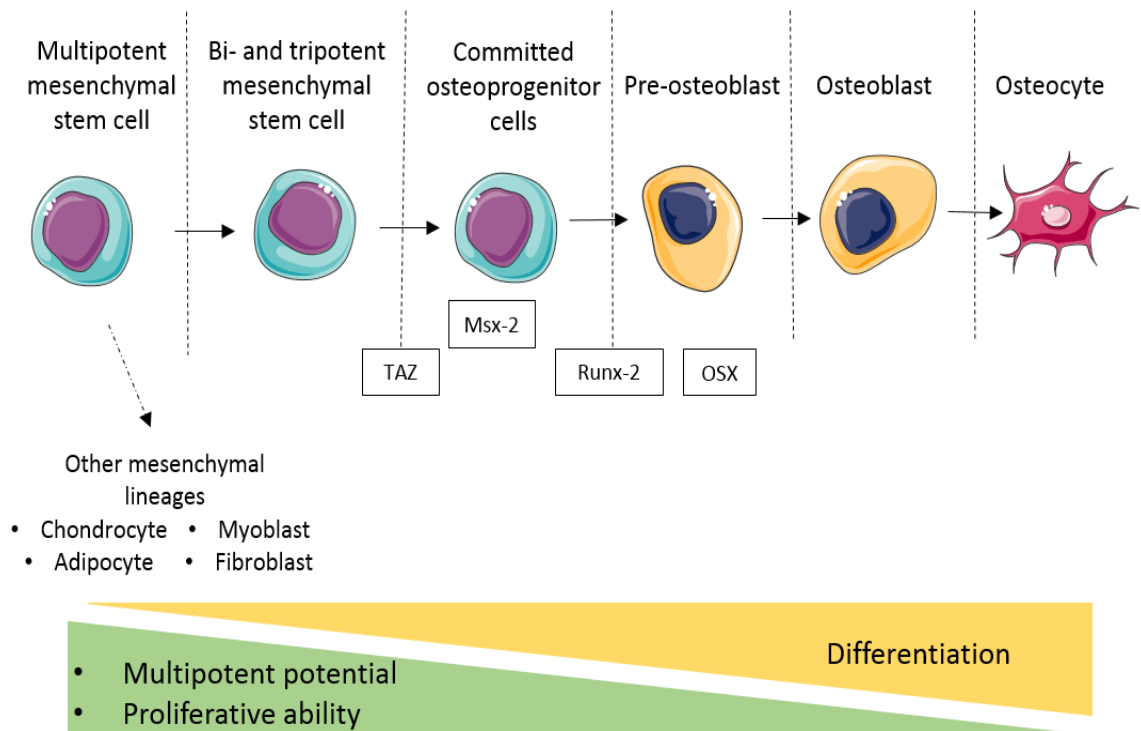


**Figure 1.2 Osteoblast development**

Stages during osteogenesis from MSCs, showing some of the key transcription factors involved in development of the osteoblast lineage. Figure made with the use of images from Servier medical art.

#### 1.2.2.iv Osteocytes

Osteocytes – the most abundant cell type in bone - are terminally differentiated osteoblasts found embedded in mature bone tissue [38]. Osteocytes reside in lacunae of mineralised bone, connected by dendrites through canaliculi, allowing communication to other osteocytes and to osteoblasts [39]. Loading and deformation to bone causes a mechanotransduction effect which is thought to be initiated by osteocytes sensing interstitial fluid flow [40], making them a key regulator in the remodelling process. As terminally differentiated cells, osteocytes lose their ability to proliferate unlike less committed cell types before them, as seen in figure 1.2.

#### 1.2.2.v Osteoclasts

Osteoclasts are large multinucleated cells formed by fusion of multiple precursor cells, and are responsible for bone resorption remodelling process [35]. Resorption occurs by acidification and proteolysis of matrix at a resorption pit (Howship's lacunae) by enzymes including tartrate-resistant acid phosphatase (TRAP), pro-CATK (cathepsin K) [41]. Osteoclast activity is regulated by local cytokines as well systemic hormones such as calcitonin and parathyroid hormone [15].

### **1.2.3 Bone formation**

Bone formation occurs by two different processes; endochondral and intramembranous ossification [42]. Most bones (including long bones) are formed by endochondral ossification in which growing cartilage is remodelled into bone, whilst flat bones and the bones of the skull are formed by intramembranous ossification in which stem cells differentiate directly into osteoblasts without an intermediate cartilaginous step.

#### 1.2.3.i Endochondral ossification

During endochondral bone formation, chondrocytes within the proliferative zone towards the end of a cartilaginous structure divide and deposit extracellular matrix rich in collagens

and proteoglycans, allowing the template for bone future bone structure to grow [43]. Chondrocytes in the mid portion of the structure become hypertrophic, organising into columns which are then invaded by blood vessel formation. Invading blood vessels bring bone precursor cells, resulting in bone matrix formation at the site of the organised chondrocytes. This mixture of calcified cartilage and immature bone matrix is then gradually remodelled to form bone structure of the long bones [44]. Alongside this, cells differentiate into osteoblasts in the region surrounding the organised chondrocytes, forming the dense outer envelope of the long bone. The remaining chondrocytes at either end of the bone form the basis for further remodelling, further organising and allowing ossification of existing cartilage matrix to gradually increase the length of the bone [45]. Eventually cartilage and immature bone matrix is completely remodelled to form mature long bone structure with bone marrow cavity (see figure 1.1)

#### 1.2.3.ii Intramembranous ossification

Intramembranous bone formation is specific to flat bones and the calvariae in the formation of the skull, and does not involve remodeling of previously organized cartilage. Instead, condensations of mesenchymal stem cells differentiate directly into osteoblasts which secrete extracellular matrix to form bone structure [46]. Actively proliferating cells are present at the bone front, allowing the flat bone to increase in size. In the human calvaria, overlapping bone plates grow independently (allowing their movement in response the growth of the brain), and form the complete skull after birth.

Whilst the mechanism of intramembranous and endochondral bone formation are different, both result in newly formed bone which will be continually remodelled throughout life, as discussed in section 1.2.4.

### 1.2.4 Bone remodelling

During the bone remodeling process existing bone matrix is replaced by new bone matrix; an ongoing process which is essential to the maintenance of adult bone tissue. A number of different cell types (described in section 1.2.2) are involved in bone remodeling, often collectively described as the bone multicellular unit (BMU). Effective remodeling relies upon the coordination between the cells of the BMU and importantly, requires the balance between bone resorption by osteoclasts and formation of new bone by osteoblasts [45]. This balance is commonly termed “coupling” and allows for the bone mass to be maintained during healthy bone turnover, whilst an imbalance in resorption/formation can contribute to a loss in bone mass and cause pathologies such as osteoporosis [38] [47]. Whilst bone remodeling has been previously divided into two phases – resorption and formation - these phases are separated by an intermediate period in which no resorption or new bone matrix formation occurs. This intermediate phase is referred to as the “reversal phase” during which the resorbing osteoclasts have migrated away leaving remodeling site which must be prepared for new matrix formation by osteoblasts [48]. It follows that coupling is not achieved by direct signaling mechanisms between osteoclasts and osteoblasts; in fact these cells experience little direct cell-cell contact during the bone remodeling process. Thus the activity of osteoblasts and osteoclasts is influenced by a multitude of interactions with other cell types, growth factors and their physical environment in order to coordinate bone matrix resorption and subsequent formation at the correct site and activity [49] [50].

The process by which bone remodeling is initiated is not fully understood, however many signals have been implicated in osteoclast recruitment and activation; basic fibroblastic growth factor involved in both the recruitment and activity of osteoclasts [51] whilst monocytes have been shown to migrate towards osteocalcin and collagen I fragments. Receptor activator nuclear factor- $\kappa$ B ligand (RANKL) also plays an important role in

osteoclastogenesis, interacting with RANK receptor on osteoclast precursors resulting in activation and fusion of hematopoietic cells to form osteoclasts capable of resorption [15] [41] whilst osteoprotegerin signaling also increases osteoclast activation and activity [52]. It has also been hypothesized that osteocytes or bone-lining cells that are damaged and become apoptotic signal to adjacent cells allowing osteoclast recruitment to begin [50]. It has been shown that fatigue loading can cause loss of osteocyte integrity and microdamage to bone matrix, which is followed by elevation in matrix resorption in these regions [53]. Osteoclasts differentiate from the hematopoietic stem cell lineage [41] however it remains unclear if resorbing osteoclasts originate from circulating monocytes [54] or if specific osteoclast precursors exist in the BMU and at what stage they become differentiated into bone-resorbing cells [55] [56].

### **1.2.5 Osteoblast recruitment**

Whilst the recruitment and localization of osteoclasts allows for bone resorption to take place, it is also necessary to recruit osteoblasts in order to allow new bone formation. The coupling of signals between osteoclasts and osteoblasts is complex, osteoclast-derived signals that may promote subsequent osteoblast activity can be divided into four categories; (1) matrix-derived signals released during resorption, (2) factors secreted by osteoclasts, (3) factors expressed on the surface of osteoclast cells, (4) topographical cues from the bone surface caused by osteoclast resorption [57]. These signals have multiple and cooperative effects on the BMU including the recruitment of osteoblasts (and/or their precursors) to allow new matrix formation. This localization of bone forming cells at the repair site is key to remodeling and repair and it has been suggested that the recruitment of osteoblast progenitors is largely responsible for new bone formation [58]. The temporal expression and/or release of recruitment factors allow for recruitment of specific cell types throughout the different stages of the bone remodeling process [59]. It is well understood that bone

marrow is rich in osteoprogenitors and is thus a likely candidate for the source of osteoblasts recruited during remodeling. However quiescent bone lining cells on reversal surfaces also have osteogenic potential and osteoblast progenitors can be found in the bone remodeling canopies that cover remodeling sites [60]. It is clear that remodeling requires migration of osteoblasts and/or their precursors, and a plethora of signals are responsible for this action.

### 1.2.6 Bone injury and repair

Instances of significant trauma can result in bone fracture, increasingly common in old age due the deterioration of bone density resulting in weaker, more brittle bone [61]. The normal fracture healing process can be split into four phases as outlined below [62].

- 1) **Haematoma formation** – Blood vessels broken during result in haematoma formation at the injury site. Inflammation initiated by cell death.
- 2) **Fibrocartilaginous callus formation** – Fibroblast and osteoblasts/pre-osteoblasts are recruited to the area and begin reconstruction process. Chondrocytes secrete cartilage matrix and the primary fibrocartilaginous callus is formed.
- 3) **Bone callus formation** – Fibrocartilaginous callus is converted to bony callus or trabecular bone, firm union developed (over weeks to months) allowing force to be applied.
- 4) **Bone remodelling** – Bony callus is remodelled over a period of months to complete healing process, trabecular bone is converted to cortical bone in shaft wall to generate tissue that resembles unbroken bone.

It is clear that efficient recruitment of osteoblasts is important to fracture repair as well as bone remodeling. This healing process can be interrupted by large trauma resulting in a non-union where surgical intervention is necessary [63], or by bone diseases such as osteoarthritis or rheumatoid arthritis in which normal cellular activity in the healing processes is affected.

### **1.2.7 Current strategies to bone repair**

As previously discussed an interruption or imbalance of the bone remodelling process can result in loss of tissue. Furthermore, significant trauma can result in delayed union, non-union and malunion fractures that require surgical intervention to aid repair, whilst large defects can be left after tumour removal or as a result of periprosthetic osteolysis. The current 'gold standard' for repair lost tissue or unhealed bone defects tissue is an autologous graft used to aid repair of a defect, however this presents issues of donor site morbidity [64], and relies on the availability of healthy tissue which is not always the case in patients who are aged or have lost tissue as a result of disease. Allogenic grafts can provide structural support for repair whilst avoiding issues surrounding donor morbidity and are more readily available. However allogenic tissues are less osteoinductive than an autologous graft and typically result in less complete healing [65]. Bone graft substitutes based on calcium phosphate, hydroxyapatite and collagen amongst others have been applied in clinic, providing structural support for repair that can be partially remodelled however these materials typically remain in a patient for several years. Injectable calcium phosphate and bioactive glass beads have also been described for bone repair with some clinical success however these interventions provide less structural support for healing [66].

## **1.3 Bone Tissue Engineering**

### **1.3.2 The tissue engineering approach to bone**

Tissue engineering has the potential to overcome shortfalls of current repair strategies and allow reliable regeneration of tissues. Specifically to bone, tissue engineering approaches may utilize stem cell transplantation into a repair site in order to encourage regeneration of bone tissue or may allow production of accurate, mimetic bone constructs that can replace lost tissue [67]. Regenerative approaches to bone are typically based on MSCs due to their

differentiation potential and their ability to regulate immune response [68] [69]. MSCs and have shown some success in pre-clinical and clinical models for repair of cartilage [70], showing safety and reduced pain in osteoarthritis patients. Similarly the application of MSCs has demonstrated some success in bone repair [71], typically producing bone formation and integration into host tissue similar to graft approaches. However with over 100 clinical trials based on MSCs for bone repair clinical results have varied significantly and concerns remain over ectopic bone formation [72]. Furthermore clinical studies mainly focus on transplantation of autologous MSCs, however if an “off the shelf” regenerative therapy is to be widely applicable the development of an allogeneic therapy is required.

To this end tissue engineering also focuses on *in vitro* differentiation from various stem cell sources in order to develop consistent and scalable cells for use on a regenerative bone therapy. *In vitro* differentiation towards osteoblasts may provide suitable cell for a repair strategy and would evade some concerns around MSCs since osteoblasts are a less proliferative cell type and would not be at risk of committing to other undesired lineages. Whilst progress has been made, in order to produce tissue engineered osteoblasts and/or bone that mimics all the characteristics of their native counterparts a more comprehensive understanding of osteoblast behavior during differentiation and remodeling is required. To achieve this, *in vitro* osteogenesis must be studied in relevant models, incorporating all factors influencing osteogenesis, and using relevant characterization techniques to compare functionality of stem-cell-derived bone to native tissue.

#### **1.3.4 Osteogenic differentiation techniques**

Tissue engineering approaches for bone typically involve the culture of MSCs or preosteoblasts under osteogenic conditions, with *in vitro* bone-like nodule formation and mineralisation reported after 21-35 days. *In vitro* osteogenesis of ESCs is also described however these often involve additional differentiation steps. It has been shown that

aggregation to form embryoid bodies (EBs) for 3 days and dissociation prior to osteogenic culture allows the formation of mineralised bone in vitro [73] [74].

#### 1.3.4.i Osteogenic supplements

The majority of *in vitro* osteogenic protocols are based on the supplementation of culture media with dexamethasone, ascorbic acid and  $\beta$ -glycerophosphate. These supplements have allowed for osteogenesis of MSCs [75] [68] and ESCs [76] [77], shown by expression of bone specific proteins and mineralisation to form bone-like nodules. Bone morphogenic protein 2 (BMP-2) is also well known for supporting osteogenesis in vitro [78], and was widely used in reconstructive surgeries following FDA approval in 2004 [79] however, long-term studies have shown mixed outcomes and potential safety concerns around ectopic bone formation remain [80]. Statins (regularly prescribed to reduce serum cholesterol) such as lovastatin and simvastatin have also been shown to increase bone formation by increasing BMP-2 expression [81].

#### 1.3.4.ii Scaffold materials

Scaffold materials have been employed to provide a carrier for cells but also to provide architectural cues for osteogenesis [82]. Micro-fibre polymer scaffolds generated by electrospinning and jet-spraying have allowed the culture of mesenchymal stem cells and their differentiation in osteoblast lineages, showing promise as tissue engineered constructs for bone repair [83]. 3D printing has also been used to produce scaffold materials that allow cell culture in a 3D environment [84], precise 3D scaffold architecture has shown promise for tissue engineered constructs for bone repair [85]. 3D printed composite calcium phosphate and collagen scaffolds allowed for levels of new bone formation similar to allografts [86]. Porous scaffolds based on hydroxyapatite present a biomimetic approach, strong enough for load bearing application in bone repair [84][87]. Bioglass 45S5 is a glass with similar composition to hydroxyapatite resulting in osteoinductive properties and has been in clinical

use since 1985 [88]. Materials advances have allowed formation of porous scaffolds from Bioglass however this often results in loss of some osteoinductive properties and overall load bearing strength [89]. Hydrogel materials have received much attention for tissue engineering applications; allowing hydrated 3D environments for cell culture [90][91]. Hydrogels formed from natural polymers can be employed to mimic the native cell matrix, whilst more inert synthetic hydrogels can produce more permissive cell-culture environments [92]. Hydrogels can be engineered to incorporate cell-instructive motifs; factors promoting bone regeneration can be tethered to hydrogel to present and released over time or liberated by cell-mediated degradation of the material [93]. A distinct advantage of hydrogels is their ability to completely degrade over time, however this often means compromising on load-bearing ability.

#### 1.3.4.iii Bioreactors

A range of bioreactors have been designed for regenerative medicine applications. Stirred suspension bioreactors such as the spinner flask reactor and rotating wall reactors are the simplest in design; allowing nutrient exchange in a homogenous cell microenvironment, and are therefore the easiest to scale up for cell manufacture [94]. However for specific tissue engineering applications such as osteogenesis, a more instructive microenvironment is required for cell growth and differentiation [95]. Several bone specific bioreactors have been designed to promote osteogenesis in vitro including perfusion/scaffold systems that provide nutrient transfer to static scaffolds via hollow tubes that mimic vascular structures and the bone marrow of bone tissue [96]. Fluid shear forces exerted by perfused media had been shown to promote osteogenesis and matrix deposition of MSCs [97][98]. The role of mechanical stimulation in osteogenesis and maintaining bone homeostasis is well reported [99], and many bioreactors providing stimulation by hydrostatic compression have been described. Intermittent compression has been shown to increase osteoblast calcification as well as alkaline phosphatase activity and Col-1 expression and ERK1/2 (extracellular signal-

related kinase) activation [100][101]. Mechanical stimulation of MSC loaded 3D scaffolds by a four-point bending bioreactor also showed increased ALP and mineralisation [102]. Slow turning lateral vessel (STLV) bioreactors initially developed by NASA (National Aeronautic and Space Administration, USA) have allowed culture of cells on scaffold materials under constant freefall conditions, improving cell seeding distribution within scaffolds [103]. These 'microgravity' culture systems have shown also shown positive effects on osteogenesis of MSCs and ALP activity of osteoblasts, presenting a more tissue-specific development on scalable bioreactor design [104]. Specifically, culture of mESC under microgravity has produced constructs with morphology and phenotype indicative of osteogenesis as well as mineralised calcium/phosphate deposition [105].

#### 1.3.4.iv Nanoscale stimulation

Recently, the effect of vibrational stimulation on fibroblasts and MSCs has been described [106]. The influence of nanoscale vibrations on cell behaviour has also been noted; during adhesion nanoscale vibrations of the plasma membrane can be observed, which are likely to influence the way cells interact with a surface [107] [108]. Mimicking this via external stimulation by nanoscale sinusoidal vibrations – a technique termed 'nanokicking' – has been shown to influence cell behaviour *in vitro*, causing alterations in cell proliferation, morphology and expression of endothelin-1, a potent vasoconstrictor [109] [110].

Nanovibrational stimulation has also been shown to influence phenotype of stem cells *in vitro*. Stimulation of MSCs by nanoscale vibrations has been shown to direct osteogenesis, demonstrated by upregulation in RUNX2 and osteocalcin [111], without the need for traditional osteogenic supplements. Nanoscale stimuli (in particular nanoscale topographies) have been shown to exert changes in cell morphology and alter in cell signalling and gene transcription, which may account for phenotypic effects of nanokicking by a 'nanomechanotransduction' effect [112].

## **1.4 Mechanotransduction**

### **1.4.1 Overview**

Cells attach to the extracellular matrix (and to culture substrates *in vitro*) via focal adhesions (FAs), structures which vary in size and composition depending upon the cell type [113] [114]. External stimuli – including topography, substrate stiffness and mechanical loading lead to changes in cell behavior by alteration of internal cell signaling pathways and gene transcription [115], a phenomena collectively described as mechanotransduction [116]. Mechanotransduction is effective either by direct processes wherein the nucleus is physically distorted by cytoskeletal rearrangement as a result of mechanical force, or by indirect processes wherein biochemical pathways are influenced as a result of mechanosensing by force-responsive proteins - often initiated at focal adhesion complexes.

Focal adhesion protein composition alters depending upon their size, with small FAs consisting of talin, vinculin, paxillin and integrins, and more mature FAs also incorporating zyxin and tensin amongst others. Integrins form the base of FAs, forces derived from connection to the ECM trigger integrin activation and formation of FA structures [117]. Integrins on the cell exterior connect to the intercellular actin cytoskeleton via focal adhesions which act as a hub for mechanosensing of external stimuli [118] [119]. In order to relay these external mechanical messages a cell cytoskeleton must be able to convey force. It remains unclear how this happens within a cell, however, Ingber (1993) suggested tensional integrity or 'Tensegrity' model in which interplay of tension and compressive forces provided by tubulin and actin creates overall stability of the cell structure. This allows strength and elasticity of cells but also allows relay of mechanical effects throughout via these structures [120]. Focal adhesion kinase (FAK) plays an important role in biochemical mechanotransduction signalling by phosphorylation of substrates for a variety of

downstream signalling whilst also influencing FA dynamics and structure [121][122]. The role of this will be further explored in chapter 4.

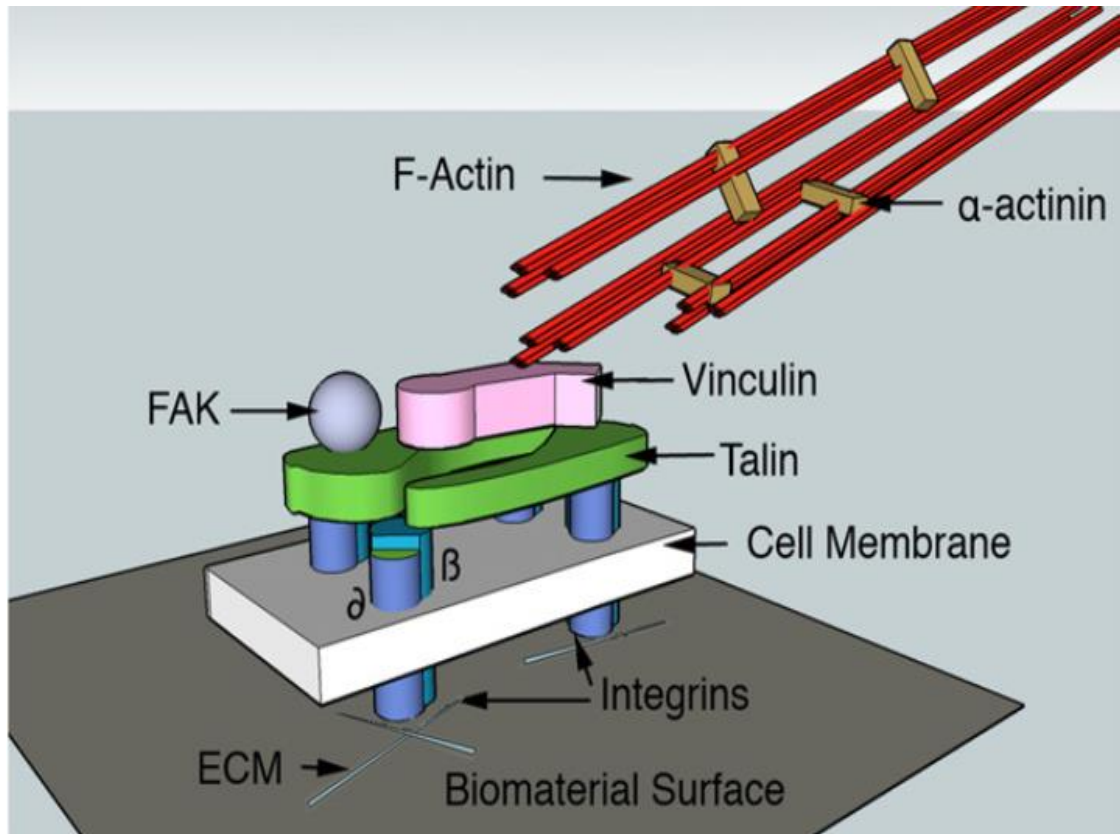


**Figure 1.3 Focal adhesion structure**

A simplified overview of the proteins involved in focal adhesion structure and signalling. Extracellular matrix (ECM) connected to cell cytoskeleton (F-actin) via focal adhesion complexes. Common focal adhesion proteins displayed as well as focal adhesion kinase (FAK). Not to scale, taken from Biggs, *et al.* (2010) [123].

### 1.4.2 Mechanotransduction in bone

The importance of mechanotransduction in bone has been observed with mechanical strain shown to influence resorption and formation of bone tissue *in vivo* [124]. Osteocytes sense mechanotransduction by fluid flow as a result of bone loading, and in turn produce signaling molecules that regulate osteoclast and osteoblast activity [40]. It is clear that mechanotransduction plays a key role in maintenance of adult bone tissue however, the effect on stem cells during osteogenesis is less well defined. *In vitro* studies of MSC confinement have shown that when limited to printed fibronectin squares of  $1000\mu\text{m}^2$ , MSC spreading was inhibited and differentiation tended towards adipose tissue. However on larger  $10000\mu\text{m}^2$  squares MSCs were allowed to spread and form large adhesion complexes with extensive cytoskeleton organization and thus differentiated to osteoblasts [125]. This supports the idea osteoblast differentiation occurs with highly spread cells which undergo extensive cytoskeletal reorganization and therefore exhibit high intracellular tension. Furthermore, larger and more mature focal adhesion structures are typically observed in osteoblasts, thought to support the higher state of tension exerted by this phenotype [126] [127]. Studies have shown that mechanotransduction affects proliferation and differentiation of osteoblasts via integrins, cadherins and stretch activated  $\text{Ca}^{2+}$  channels, ultimately leading to changes in gene expression and phenotype [128].

## **1.5 Chemotaxis**

Regenerative medicine therapies generally rely upon transplanting cells into the patient, often accompanied by a scaffold material to provide mechanical support and to localize the implanted cells to the desired repair site. The tracking of cells within the body has received much attention, often labelling cells with magnetic nanoparticles in order to detect migration of injected cells and to track their final destination within the body [129] [130]. However these studies are not able to distinguish signal produced by transplanted cells or signal transferred to bystander macrophages [131].

For bone repair therapies this issue is particularly important since osteoblast precursors are highly migratory by nature and their recruitment to the repair site is pivotal to bone repair. Migratory behavior of osteoblasts (and osteoblast precursors) should be further characterized *in vitro* in order to better predict the activity of cells once implanted into the body.

Soluble factor signalling and gradients are essential to many biological processes including cellular development [132] [133] [134], cell polarisation[135] [136] and cell migration [137] [138]. As discussed above, soluble factor signalling is also an essential part of bone remodelling, promoting chemotaxis of bone cells and their recruitment to repair sites following injury or during normal bone turnover. If osteoblasts are to be used in a cell therapy product, chemotactic behaviour should be characterised since successful regeneration will rely upon the migration to the specific repair site.

Whilst several growth factors have been identified as chemoattractants for osteoblasts, their interactions with each other and with multiple cell types of the BMU means osteoblast recruitment is a complex process. For example, it has been shown that osteoclasts secrete platelet derived growth factor BB (PDGF-BB) which acts as a chemoattractant for MCT3T3 osteoblasts [139] suggesting that PDGF-BB is part of the mechanism coupling osteoclasts and

osteoblasts during remodeling. Leukemia inhibitory factor (LIF) promotes resorption by osteoclast cells [140] but has also been shown to enhance osteoblast chemotaxis towards PDGF-BB [141]. PDGF-BB is also a chemoattractant for mesenchymal progenitors cells [142], and is also thought to influence their differentiation to osteoblast lineages [143]. In this way, one growth factor has several roles in the bone remodeling process, and their effect on cell activity and differentiation must be considered when identifying them as chemotactic factors.

Many other growth factors are implied in the chemotaxis of bone cells. TGF- $\beta$ 1 has been identified as chemotactic towards osteoblasts [144] and bone mesenchymal stem cells [145], modulating their migration via influencing CXCL16 and LIF expression in osteoclasts [146]. BMP-2 has also been shown to recruit osteoblast progenitors [142], whilst IL-4 & IL-13 are chemotactic towards osteoblasts [147].

## **1.6 Project Aims and Objectives**

Since it has been previously shown that there is significant difference between the behaviour of primary osteoblasts and osteoblasts derived from stem cells *in vitro*, the objective of this project was to compare the behaviour of mouse primary calvarial cells (mPCs) and mouse embryonic stem cells (mESCs) to osteogenic stimuli.

This thesis presents work towards three main aims. The first aim was to observe osteogenesis in response to nanovibrational mechanical stimulation as compared to standard medium supplementation, the second aim was to dissect the cellular mechanisms by which nanovibration influences osteoblast phenotype, and the final aim was to study chemotaxis of cells throughout differentiation considering the effects of nanovibration. These aims are detailed below.

- 1) Compare osteogenesis in response to nanovibration and conventional medium supplementation
  - Isolation of mouse primary calvarial cells (mPCs)
  - Calibration of nanovibrational bioreactor (Nanokick technologies)
  - Osteogenesis of mPCs by medium supplementation and/or nanovibration
  - Osteogenesis of embryonic stem cells (mESCs) by medium supplementation and/or nanovibration
  - Osteogenic evaluation
    - Mineralisation assay
    - Immunocytochemistry for protein level analysis
    - qPCR for gene level analysis
    - Alkaline phosphatase assay
    - Raman spectroscopy

2) Investigate mechanisms of nanovibrational osteogenesis as compared to medium supplementation

- Observe changes in cell morphology and cytoskeletal structure
- Measure focal adhesion signalling dynamics
- Investigate changes in cellular metabolomics profile
- Predictive signalling analysis based on metabolite changes
- Study effects of nanovibration on 3D cell aggregates

3) Observing osteoblast chemotaxis *in vitro* and influence of nanovibration

- Optimisation of Boyden chamber assay
- Optimisation of chemotaxis slide assay
- Dose range chemotaxis studies using PDGF-BB
- Evaluation of chemotaxis throughout differentiation of mPCs
- Comparison of chemotaxis following medium supplementation and/or nanovibration stimulation

## Chapter 2 - Materials and Methods

This chapter details general materials and methods fundamental to all or multiple chapters of this thesis. Specific techniques discussed in methods sections at the beginning of the relevant results chapter.

### **2.1 Materials**

#### **2.1.1 Cells**

Mouse primary calvarial cells (mPCs) were extracted from CD-1 neonatal mouse pups, supplied by Biomedical Science Unit, University of Nottingham. Mouse embryonic stem cells (mESCs) were the cell line denoted CGR8.

#### **2.1.2 Chemicals and cytokines**

##### **2.1.2.i General chemicals**

Several chemicals used in this work were part of the Tissue Engineering group general stock. Unless stated otherwise, these chemical were purchased from Sigma. These included 1x PBS solution, Trypsin/EDTA solution (0.25% trypsin, 0.02% EDTA), 3.7% paraformaldehyde, 10% formalin solution.

##### **2.1.2.ii Foetal bovine serum**

Foetal bovine serum (FBS) batch testing was performed to assess growth and osteogenic differentiation of mPCs and mESCs, details of this process around found in appendix 7.1. The serum batch with optimum growth and osteogenesis (F9665, Sigma) was selected and used for all studies.

##### **2.1.2.iii Cytokines**

Recombinant cytokines for use during cell culture were purchased from R&D system, UK. Recombinant human PDGF-BB (cross-species reactive) was used during chemotaxis studies.

2.1.2.iv Antibodies and stains

The following antibodies and other stains were used for Immunocytochemistry and cytoskeletal staining.

**Table 2.1 Primary antibodies**

| Antibody              | Host   | Supplier / product code | Working dilution |
|-----------------------|--------|-------------------------|------------------|
| RUNX2                 | Rat    | R&D systems (Mab2006)   | 1:25             |
| Osteocalcin           | Rabbit | Millipore (AB10911)     | 1:200            |
| Vinculin              | Mouse  | Sigma (V9264)           | 1:150            |
| FAK                   | Mouse  | Invitrogen (39-6500)    | 1:250            |
| Phospho-FAK (Tyr397)  | Rabbit | Invitrogen (700255)     | 1:500            |
| p44/42 MAPK (Erk 1/2) | Rabbit | Cell Signalling (4377)  | 1:200            |
| Phospho ERK 1/2       | Mouse  | Cell Signalling (4696)  | 1:200            |

**Table 2.2 Secondary antibodies**

| Antibody                   | Host   | Supplier / product code | Working |
|----------------------------|--------|-------------------------|---------|
| Alexaflour 488 anti-rabbit | Donkey | Invitrogen (A21206)     | 1:200   |
| Alexaflour 546 anti-rat    | Goat   | Invitrogen (A11081)     | 1:200   |
| IRDye 800CW Anti-rabbit    | Goat   | LI-COR (926-32211)      | 1:5000  |
| IRDye 680CW Anti-mouse     | Goat   | LI-COR (926-68070)      | 1:5000  |
| Biotinylated Anti-Mouse    | Horse  | Vector (BA-2000)        | 1:50    |

**Table 2.3 Other staining reagents**

| Name                       | Dilution | Supplier/ product code |
|----------------------------|----------|------------------------|
| Rhodamine Phalloidin       | 1:300    | Invitrogen (R415)      |
| Alexa flour 488 Phalloidin | 165nM    | ThermoFisher (A12379)  |
| Hoescht                    | 5µg/mL   | Sigma (94403)          |
| FITC-Streptavidin          | 1:50     | Vector (SA-5001)       |

2.1.2.v Consumables

General consumables used for this project included tissue culture treated T25 and T75 flasks (Nunc, Thermo Fisher), tissue culture treated well-plates (Falcon, BD Biosciences), non-tissue

culture treated Petri dishes (Thermo Scientific) and 'CellView' glass bottom culture dishes (Greiner Bio-One).

## **2.2 Methods**

### **2.2.1 Cell Culture**

All cell culture was performed in a class 2 microbiological safety cabinet fitted with a (high efficiency particulate air) HEPA filter. Cell cultures were maintained within incubators set to 37°C and 5% CO<sub>2</sub>. The various culture media used are detailed in table 2.2, all supplements passed through a 0.22µm sterile filter when added to base medium, and stored at 4°C.

**Table 2.4 Cell culture media recipes**

| Medium                      | Base medium                      | Supplements                                                                                                                                                                                                                                                                                                 |
|-----------------------------|----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| mPC growth medium           | alpha modified Eagle's medium    | <ul style="list-style-type: none"> <li>• 10% v/v FBS (Sigma Aldrich)</li> <li>• 2mM L-glutamine (Gibco)</li> <li>• Penicillin (100 units/ml) /streptomycin (50µg/mL) (Gibco).</li> </ul>                                                                                                                    |
| mPC osteogenic medium (OCM) | alpha modified Eagle's medium    | <ul style="list-style-type: none"> <li>• 10% v/v FBS (Sigma Aldrich)</li> <li>• 2mM L-glutamine (Gibco)</li> <li>• Penicillin (100 units/ml) /streptomycin (50µg/mL) (Gibco).</li> <li>• 50 µg/mL L-ascorbic acid</li> <li>• 50 mM β-glycerophosphate</li> <li>• 10 µM dexamethasone.</li> </ul>            |
| mESC growth medium          | Dulbecco's modified Eagle medium | <ul style="list-style-type: none"> <li>• 10% v/v FBS (Sigma Aldrich)</li> <li>• 0.1mM 2-mercaptoethanol (Gibco)</li> <li>• 2mM L-glutamine (Gibco)</li> <li>• Penicillin (100 units/ml) /streptomycin (50µg/mL) (Gibco).</li> <li>• 1000 units/ml Leukaemia inhibitory factor (LIF) (Calbiochem)</li> </ul> |

|                         |                                  |                                                                                                                                                                                                                                                                                                                                              |
|-------------------------|----------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| mESC aggregation medium | Dulbecco's modified Eagle medium | <ul style="list-style-type: none"> <li>• 10% v/v FBS (Sigma Aldrich)</li> <li>• 0.1mM 2-mercaptoethanol (Gibco),</li> <li>• 2mM L-glutamine (Gibco)</li> <li>• Penicillin (100 units/ml) /streptomycin (50µg/mL) (Gibco).</li> </ul>                                                                                                         |
| mESC osteogenic medium  | Dulbecco's modified Eagle medium | <ul style="list-style-type: none"> <li>• 10% v/v FBS (Sigma Aldrich)</li> <li>• 0.1mM 2-mercaptoethanol (Gibco),</li> <li>• 2mM L-glutamine (Gibco)</li> <li>• Penicillin (100 units/ml) /streptomycin (50µg/mL) (Gibco).</li> <li>• 50 µg/mL L-ascorbic acid</li> <li>• 50 mM β-glycerophosphate</li> <li>• 10 µM dexamethasone.</li> </ul> |
| Cryopreservation medium | FBS                              | <ul style="list-style-type: none"> <li>• 10% DMSO (Sigma)</li> </ul>                                                                                                                                                                                                                                                                         |

### 2.2.1.i Mouse primary calvarial cells

Mouse Primary Calvarial cells (mPCs) were extracted from CD-1 neonatal mouse pups (1-3 days old) by dissection to remove the calvariae, followed by collagenase digestion. Euthanized pups (obtained from Biomedical Services Unit, University of Nottingham) were dissected by incision from front to back of the head to separating the two calvariae, followed by incision to separate skull from the facial bones. After removing brain tissue, calvariae could be gripped with forceps and removed.

Extracted calvariae were washed in PBS containing 100 U/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL amphotericin B. Calvariae were then cut into small pieces with sterilised scissors before several treatment steps, each consisting of 12 minute incubation on a roller in 1.4 mg/mL collagenase 1A and 0.5 mg/mL trypsin II S (Sigma Aldrich) solution. After the first two digestions under these conditions the released cells were discarded. For the

following three digestion steps the solution containing released cells were isolated in an equal volume of FCS. These three populations were pooled together, filtered through a 70µm strainer and centrifuged at 300g for 5 minutes. Cells were then allowed to attach for 24 hours in mPC growth medium before being washed with PBS to remove non-adherent haematopoietic cells. Fresh growth medium was added and cells cryopreserved once 90% confluency was reached (detailed in section 2.2.1.iii).

Once in culture, mPCs were grown in T75 flasks and split every 3-4 days once they reached 80-90% confluency. Cell passaging was achieved by first washing with PBS, then treating with trypsin/EDTA for 5 minutes at 37C. Once trypsin was neutralised with medium growth medium mPCs were then centrifuged at 300g for 5 minutes before being resuspended in growth medium seeded at ratios between 1:3 and 1:4.

#### 2.2.1.ii Mouse embryonic stem cells

Mouse embryonic stem cells (mESCs) were the cell line denoted CGR8; a feeder-free cell line originally established by Nichols et al which exhibit normal differentiation *in vitro* differentiation and retain capacity for germ-line transmission [20].

mESCs culture flasks were coated with sterile filtered 0.1% gelatin (Sigma, G1393) in PBS for 24 hours at room temperature prior to use. mESCs were cultured in T25 flasks, and passaged once cell colonies were perceived to have reached 80-90% confluency. To passage, mESCs were washed in PBS then treated with trypsin/EDTA solution at room temperature for 5 minutes. Trypsin was then neutralised by adding culture medium and mESCs were centrifuged at 180g for 5 minutes. The cell pellet was then resuspended in fresh mESC growth medium and cells seeded.

### 2.2.1.iii Cryopreservation and reanimation

Cells were cryopreserved by trypsinising a confluent flask and resuspending a cell pellet in cryopreservation medium to give a cell density of  $1 \times 10^6$  cells/ml. 1ml of this cell suspension was then transferred into a cryo-vial which was placed in CellCool cell and stored at  $-80^\circ\text{C}$  for 48 hours. Cell vials were then transferred to liquid nitrogen tanks for long term storage. Cells were reanimated by thawing a vial half-submerged in  $37^\circ\text{C}$  water bath, constantly moving by hand. 1ml of cryopreserved cell suspension was then added to 9ml of pre-warmed growth medium, and centrifuged at relevant settings in order to generate a cell pellet and allow DMSO-containing cryopreservation medium to be aspirated. Cell pellets were then resuspended in relevant growth medium and seeded into culture flasks.

## **2.2.2 Osteogenic stimulation**

### 2.2.2.i Osteogenesis by supplemented medium

During osteogenic differentiation of mPCs, cells at passage 2 or 3 were seeded into well plates at a density of 8,000 cells/cm<sup>2</sup> in mPC growth medium and allowed to attach. After 24 hours, growth medium was replaced with mPC osteogenic culture medium (defined in table 2.4), this being defined at 'day 0' starting point for osteogenic cultures. Osteogenic medium was changed every 2-3 days.

For osteogenic differentiation of mESCs, CGR8 cells were first allowed to form embryoid bodies (EBs) by mass suspension aggregation for 72 hours in aggregation medium. mESCs were aggregated in non-tissue culture treated dishes to prevent adherence during aggregation. Embryoid bodies were then dissociated and cells seeded at 10,600 cells/cm<sup>2</sup> and cultured in aggregation medium for 24 hours. After this time growth medium was replaced with mESC osteogenic culture medium (table 2.4).

### 2.2.2.ii Nanoscale vibration

Nanoscale mechanotransduction was delivered to cells using the 'Nanokick' bioreactor. The bioreactor relies upon the reverse piezo electric effect in which application of a voltage causes predictable deformation of the piezo actuators [148]. The bioreactor consists of an array of piezo actuators with a vibration plate attached on top which will undergo predictable nanoscale displacement when sinusoidal voltage is applied, thus delivering nanovibrations to the cells. Cells were seeded into well plates and allowed to attach for 24 hours, as per osteogenic differentiation using osteogenic culture medium. Well plates were then attached to the Nanokick bioreactor using one-sided adhesive magnets (F4MA43MHP-1-AMZ, Magnet Expert Ltd) that allowed adhesion to the well plate, and magnetic attachment to the bioreactor vibration plate (see figure 3.1). Cells were then stimulated continuously at settings optimised to deliver vibrations of 30nm amplitude at 1000Hz.

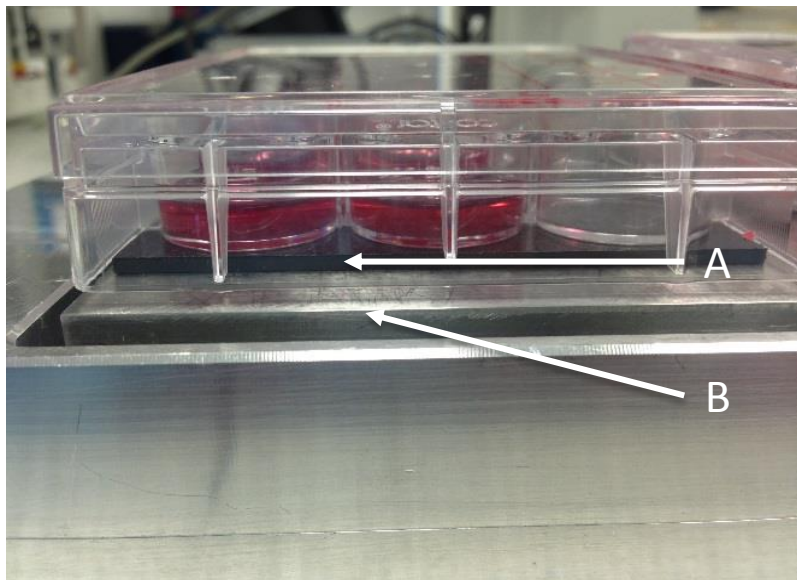


**Figure 2.1 Nanovibrational bioreactor**

12 well plate attached to Nanokick bioreactor via sheet magnet (A). Adhesive on one side of magnet allows attachment to bottom of wells, other (magnetic) side attached to vibration plate of bioreactor (B).

### 2.2.3 Cell fixation

Cell fixation was used prior to immunocytochemistry and other histological techniques. Depending upon the technique, cell monolayers were fixed in either 3.7% (v/v) paraformaldehyde or in 10% Formalin solution (Sigma). Both allow preservation of sample architecture, however PFA fixation creates a strong, highly cross-linked structure which is suitable for immunocytochemistry. Before fixation monolayers were washed twice in PBS before being fixed for the desired time. Samples were then rinsed in PBS twice to remove all fixative, then stored in PBS until used for analysis.

### 2.2.4 Immunocytochemistry

First monolayers were fixed in 3.7% PFA, then samples were permeabilised using 0.1% (v/v) triton X-100 (Sigma) for 20 minutes. Monolayers were then washed three times in PBS before blocking with 3% (v/v) serum taken from the secondary antibody host species. After blocking solution was removed, monolayers were cultured overnight at 4°C with appropriate primary antibody solution, diluted in 1% (w/v) BSA in PBS. Table 2.1 details primary antibodies used and their dilutions for immunocytochemistry. After overnight incubation monolayers were washed 3 times with 1% BSA in PBS, and monolayers were incubated with secondary antibody solution (also diluted in 1% BSA in PBS) for 2 hours at room temperature, protected from light (secondary antibodies listed in table 2.2) Secondary antibody solutions were removed, samples washed 3 times with 1% BSA in PBS and imaged using fluorescent microscopy (detailed in section 2.2.9). Hoechst counterstaining for cell nuclei were used in some cases, for which monolayers were stained with 5µg/mL hoechst solution for 30 minutes at room temperature, protected from light (following secondary staining and washes). If unable to image immediately, samples were mounted under Prolong Gold Antifade mountant (Thermo Fisher Scientific, UK) by adding reagent drop wise to cover well. Samples were then stored protected from light until imaging.

### 2.2.5 qPCR

Quantitative (real-time) polymerase chain reaction (qPCR) was used to evaluate relative expression of key osteogenic genes. Following culture under osteogenic conditions, cell monolayers were trypsinized and centrifuged as per usual cell culture conditions. Culture medium was then removed and cell pellets stored in RLT buffer (RNeasy kit, Qiagen) containing 10µl/ml β-mercaptoethanol at -80°C until beginning qPCR.

#### 2.2.5.i RNA isolation and cDNA synthesis

RNA isolation from cell pellets was performed using QIAshredder (Qiagen) and RNeasy Mini Kit (Qiagen). Lysed cells were pipetted directly onto QIAshredder spin column atop a collection tube and centrifuged at 8000g for 2 minutes. 70% ethanol was added to tube (equal to volume of lysate), mixed and left for 15 minutes. Entire volume was then added to RNeasy spin Column atop a collection tube and centrifuges for 15 secs (8000g), flow through discarded. 350µl RW1 buffer was added to columns then centrifuged 15 secs (8000g). DNase I was added to columns (diluted 1:7 in RDD buffer) and left for 15 minutes. 350µl RW1 buffer was added to columns then centrifuged 15 secs (8000g), flow through discarded. 500µl RPE buffer was added to each RNeasy column, centrifuged 15 secs (8000g) and flow through discarded. 500µl RPE buffer was added to each RNeasy column, centrifuged 2 mins (8000g), flow through discarded and column placed in a new collection tube. 30µl of RNase-free water was then added to column and centrifuged 1min at 8000g to elute RNA. Samples were then immediately used for cDNA synthesis or stored at -80 °C until use.

Prior to cDNA synthesis RNA yield was assessed by Nanodrop. Samples were diluted in nuclease free water accordingly to ensure each sample matched concentration of the lowest yield sample. 2µl of sample was then added the RNase free Eppendorf, and 1µl of 10mM dNTP mix and 1µl of Oligo(dT)<sub>20</sub> added (both from SuperScript® III First-Strand Synthesis kit

(Invitrogen)). Samples were then incubated at 65 °C for 5 minutes before being placed on ice. Superscript III first-strand synthesis master mix was then generated containing contents seen in table 2.5 and 12µl was added to each sample reaction tube before placing them all in thermal cycler programmed as following; 10 mins at 25 °C, 50 mins at 50 °C and 5 mins at 85 °C. For RT- control samples SuperScript® III RT was replaced with nuclease free water. cDNA was then immediately used for qPCR reaction or stored at -20 °C until use.

**Table 2.5 First strand synthesis master mix**

| Component                      | Volume per reaction |
|--------------------------------|---------------------|
| 10X RT buffer                  | 2µl                 |
| 25mM MgCl <sub>2</sub>         | 4µl                 |
| 0.1M DTT                       | 2µl                 |
| RNaseOUT™ (40U/µl)             | 1µl                 |
| SuperScript® III RT (200 U/µl) | 1µl                 |

#### 2.2.5.ii qPCR

For each sample 1.25µl of cDNA was added to PCR mixture containing TaqMan™ Gene Expression Master Mix (12.5µl), Taqman assay of interest (1.25 µl) and dH<sub>2</sub>O (10µl). Taqman assays used were RUNX2 (Mm00501584\_m1) and GAPDH (Mm99999915\_g1), supplied by ThermoFisher. 20µl of this mix was pipetted in PCR plate (MicroAmp™ Fast Optical 96-well, ThermoFisher) in triplicate and plate sealed using MicroAmp™ Optical Adhesive Film (ThermoFisher). qPCR run was performed using 7500 FAST real-time PCR system (Applied Biosystems), TaqMan Standard 2 hour ramp speed. For each osteogenic condition qPCR analysis was run in triplicate. Gene of interest C<sub>T</sub> values of were compared to GAPDH values to generate  $\Delta C_T$  values, from which  $\Delta\Delta C_T$  values were generated by comparing treatment groups to untreated control conditions. Data is presented as RQ values, where RQ is equal to  $2^{-(\Delta\Delta C_T)}$ , with error bars to represent standard deviation.

### **2.2.6 Osteoblast characterisation**

Osteoblast phenotype was determined by immunocytochemistry and qPCR discussed above, and also by mineralization assay and alkaline phosphatase activity, discussed in chapter 3. Scanning electron microscopy was used to assess morphology whilst Raman spectroscopy was used to analyze mineral content in osteogenic cultures, these methods are also discussed in detail in chapter 3.

### **2.2.7 Evaluation of osteogenic mechanisms**

Osteogenic mechanisms of OCM and/or nanovibration were studied by vinculin and actin staining to observe focal adhesion and cytoskeleton morphology respectively. An in-cell western blot approach was also used to observe FAK and ERK activity, whilst untargeted metabolomics analysis was used to observe metabolic changes induced by OCM and/or nanovibration. These methods are discussed at length in chapter 4.

### **2.2.8 Osteoblast migration studies**

Migration of osteoblasts was observed using an adapted Boyden chamber assay, as well as by a 2D adherent chemotaxis model (Ibidi chemotaxis slides). These are discussed in detail in chapter 5 along with associate cytoskeletal staining of osteoblasts.

### **2.2.9 Microscopy**

During cell culture an Olympus CKX21 was used to visualise cells and observe and non-fluorescent samples. Brightfield images were captured using a Nikon Eclipse TS100 inverted microscope (Nikon Instruments) with an attached Nikon digital sight DS-L3 screen for imaging. For fluorescent imaging a Leica DM-IRB microscope was used with X-cite series 120 light source.

### **2.2.10 Statistical Analysis**

Statistical significance between groups was analysed using GraphPad Prism 7 software by unpaired student T-Test. Multiple groups were compared using one-way ANOVA with Tukey multiple comparison test with a single pooled variance.

## **Chapter 3 - Osteogenesis by nanovibration and/or osteogenic culture medium**

### **3.1 Introduction**

#### **3.1.1 Overview**

*In vitro* osteogenesis of stem cells typically employs mesenchymal stem cells (MSCs) and the use of osteogenic medium supplements as discussed above. However in order to properly understand the mechanisms of osteogenesis, it is important to consider the plethora of factors that influence cell behavior and differentiation *in vitro*. In particular, mechanical stimulation plays a key role in bone homeostasis as discussed in chapter 1. This chapter describes the application of nanoscale mechanotransduction using a nanovibration bioreactor, assessing its effects on osteogenesis of mouse embryonic stem cells (mESCs) and mouse primary calvarial cells (mPCs), whilst comparing to the more conventional method of medium supplementation with osteogenic factors. The mPCs used herein are extracted from 1-3 day old CD-1 mouse pups by digesting of the newly formed calvarial plate as described in section 2.2.1.i. Therefore the population are osteoblasts and/or pre-osteoblasts recently differentiated from mesenchymal stem cells, providing an 'early stage' osteoblast cell source that have not yet formed extensive extracellular matrix and traditional bone mineral.

#### **3.1.2 Nanovibrational bioreactor**

The nanovibrational or 'Nanokick' bioreactor (Nanokick Technologies) provides a simple benchtop system to apply nanoscale mechanotransduction to cells seeded within standard well plates, discussed in detail in section 2.2.2ii. This bioreactor has been shown to promote osteogenesis in human MSCs [111], however its effects on ESCs and mPCs have not been studied. This chapter describes in part describes the study of osteogenesis effects nanoscale mechanotransduction using this bioreactor system.

### 3.1.3 Osteoblast characterisation

To confirm *in vitro* osteogenesis, cells are typically assessed by a number of parameters. Perhaps most importantly, ability to form mineralised bone nodules by the accumulation of calcified matrix is detected by staining with alizarin red for calcium deposition [149]. Alkaline phosphatase (ALP) activity is also often measured as an indicator of osteoblast activity, playing a critical role in formation of mineralised deposits [150] [151].

Studies of differentiating rat calvarial osteoblasts *in vitro* have revealed sequential expression of bone specific proteins [152]. These markers associated with bone development are often assessed during stem cell osteogenesis at a protein or gene level by immunocytochemistry or polymerase chain reaction (PCR) respectively. Typical characterisation includes RUNX2 and Osterix in early stages of osteogenic culture followed by osteocalcin and osteopontin which give an indication of mature osteoblast phenotype [75] [153] [154].

More in depth analysis of the mineral formed during *in vitro* differentiation can be performed by Raman spectroscopy. Raman spectroscopy provides a non-destructive technique for analysing composition of mineral; measuring the inelastic scattering of monochromatic laser light resulting from the interaction of the light with molecular vibrations within the material structure [155] [156]. The technique has been used to successfully characterise bone mineral by producing a structural fingerprint based on Raman scattering associated with identifiable molecules within the material [157] [158]. Phosphate and carbonate species are incorporated into mineral samples in a variety of ways, leading to diversity in naturally occurring mineral and thus a diversity of Raman signatures [159] [160]. Characterisation of bone tissue by Raman spectroscopy largely focuses on the identification of prominent peak phosphate  $\nu_1(\text{PO}_4^{3-})$  symmetric stretch around  $960\text{cm}^{-1}$  [161]. However the position of this peak differs slightly depending upon the composition of the apatite (as discussed in section 3.4.3) and can reveal differences in the material. Analysis of the developing mouse calvaria

has shown the ratio of these different apatite species alters during bone formation and changes continuously during remodelling of existing bone tissue [161]. A summary of the peaks typically used to identify bone mineral is found in table 3.1, forming the basis for spectral analysis in section 3.4.3. Other relevant peaks are described in the 1300-1500  $\text{cm}^{-1}$  region however these were not used in our analysis due to interference with substrate material.

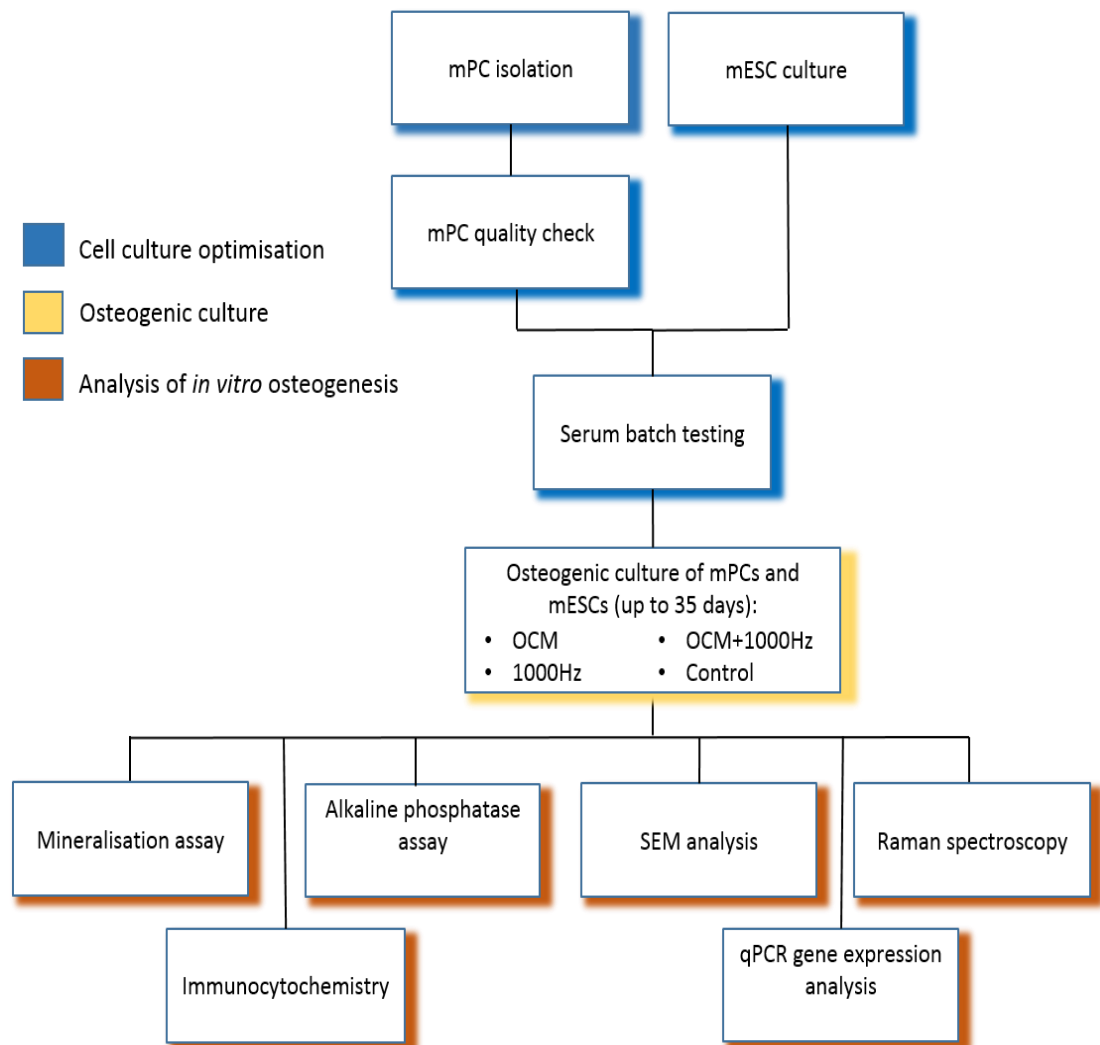
**Table 3.1 Raman shifts of bone mineral**

Typical Raman shifts in spectra of bone [157] [161] [162] [163].

| Expected peak position, Raman shift ( $\text{cm}^{-1}$ ) | Peak assignment                                    |
|----------------------------------------------------------|----------------------------------------------------|
| 853                                                      | hydroxyproline $\nu(\text{C-C})$                   |
| 950-964                                                  | $\nu_1(\text{PO}_4^{3-})$ (P-O symmetric stretch)  |
| 1003                                                     | Phenylalanine $\nu(\text{C-C})$                    |
| 1030                                                     | $\nu_3(\text{PO}_4^{3-})$ (P-O asymmetric stretch) |
| 1065-1076                                                | $\text{CO}_3^{2-}$ (C-O symmetric stretch)         |

### **3.2 Objectives and experimental design**

The objectives of this chapter were to compare the *in vitro* osteogenesis of mouse embryonic stem cells (mESCs) to mouse primary calvarial cells (mPCs), and investigate the effects of nanovibration stimulation (1000Hz) on osteogenesis as compared to osteogenic culture medium (OCM). To complete these studies, this chapter followed the experimental plan shown below.



### **3.3 Materials and methods**

#### **3.3.1 Osteogenic stimulation**

To promote osteogenic differentiation cells were seeded and stimulated by osteogenic culture medium (OCM), continuous nanovibration (1000Hz) or a combination of the two (OCM+1000Hz), as per section 2.2.2. The beginning of differentiation (day 0) defined as when osteogenic medium was added, or when nanovibration was started.

To ensure that the Nanokick bioreactor was delivering the desired level of vibration, it was calibrated using an interferometer (SP series, SIOS). The interferometer laser beam was reflected off of the reflective disk attached to the Nanokick bioreactor during vibration, the reflected light producing a real-time reading of altered interferometer voltage. Voltage is then converted to relative displacement of the vibration plate, and a Fast Fourier Transform (FFT) used to isolate of displacement only associated the chosen output frequency (1kHz). Example transformation data found in appendix 7.6.

#### **3.3.2 Mineralisation assay**

Mineralisation was assessed by alizarin red S staining. Samples fixed in 10% formalin for 30 minutes were washed twice in deionised water before staining with alizarin-red S solution (Millipore) for 15 minutes. Samples were rinsed twice in deionised water to wash away excess staining before imaging.

#### **3.3.3 Scanning electron microscopy**

Scanning electron microscopy (SEM) was used to visualise cell morphology and bone nodule structure following osteogenic culture. To allow SEM analysis monolayers were cultured on glass, mPCs seeded directly onto glass and mESCs seeded onto glass coated with gelatin (as per usual differentiation protocol). For OCM stimulation sterilised glass coverslips were placed in well plates and cells seeded on top. For nanovibrational stimulation, cells were

seeded within CellView glass-bottom cell culture wells, and magnets attached to the underside to allow transition of nanokicking signal to monolayers. To check suitability of this set up, mPCs were cultured on glass for 21 days and assessed using mineralisation assay to check normal osteogenic behaviour on CellView wells. These results are found in appendix 7.3. Monolayers were cultured for up to 35 days under osteogenic conditions before medium was removed and monolayers were washed twice in PBS before being fixed for 1 hour in 3.7% PFA. Fixed monolayers were then dehydrated in increasing ethanol concentrations; placed in 10, 25, 50, 75, 90, and 100% (v/v in water) sequentially for 20 minutes each, using 100% ethanol twice to finish dehydration. Dehydrated monolayers were then gold coated for SEM analysis (25mA, 180 seconds) before being mounted on stubs and imaged using a JEOL 6060LV scanning electron microscope.

### **3.3.4 Adipogenic and chondrogenic staining**

Osteogenically stimulated cells were also stained for adipogenesis and chondrogenesis markers to assess levels of differentiation to other mesenchymal lineages. For adipogenic positive control, MSCs were seeded at 15,000 cells/cm<sup>2</sup>, and cultured using StemPRO Adipogenesis Kit (Gibco), changing medium every 2 days. For chondrogenic positive control MSCs were seeded at 10,000 cells/cm<sup>2</sup>, and cultured growth medium supplemented with 10ng/ml TGF- $\beta$ 1 and 100nM dexamethasone, changing media 3 times per week. Adipogenesis was assessed by staining with AdipoRed (Lonza, PT-7009) for 30 mins, protected from light. Fat globules/vesicles visible under light microscopy were imaged, capturing fluorescent AdipoRed staining of the same areas. Chondrogenesis was assessed by staining with 1% Alcian blue in 3% acetic acid (Sigma, B8438) overnight.

### **3.3.5 Alkaline phosphatase activity**

Alkaline phosphatase activity was measured using p-nitrophenol phosphate (pNPP). The assay is based on the catalytic activity of phosphatases during the hydrolysis of pNPP to form

p-nitrophenol. P-Nitrophenol is a chromogenic product which absorbs at 405nm. Colour change during this assay was used to assess levels of alkaline phosphatase in the sample. Cell culture media was removed from monolayers and pNPP was added at 1.0mg/ml in 0.2M Tris buffer (Sigma) in deionised water. Samples were incubated for 30 minutes before reading absorbance at 405nm using plate reader (TECAN Infinite 200). ALP concentration was calculated from relative absorbance values using the standard curve generated by serial dilution of alkaline phosphatase standard from calf intestine (see appendix 2).

### **3.3.6 Immunocytochemistry**

Stimulated cells were stained for bone specific markers as per immunocytochemistry method described in section 2.2.4. Antibodies used were RUNX2 and osteocalcin which are typically expressed during early and late stages of *in vitro* osteogenesis respectively.

### **3.3.7 Raman spectroscopy**

For Raman analysis of mineral structure cells were first cultured under osteogenic conditions for up to 35 days. Cell monolayers were then washed once in PBS and then detached by gentle scraping into PBS. Cell/matrix suspensions were then collected and centrifuged at 300g for 5 minutes and cell pellets resuspended in 20µl of 4% formaldehyde solution. 3-5µl of these suspensions was then spotted onto CaF<sub>2</sub> substrates and allowed to air-dry before Raman analysis. Prior to use CaF<sub>2</sub> slides were cleaned by sonication in ethanol for 20 minutes and allowing them to dry under sterile conditions to avoid contamination. Spectra were obtained using a Renishaw inVIA spectrometer with attached Leica microscope. The 785nm laser was focused through a 100x microscope lens for analysis and spectra obtained using 100% laser power, 5 second exposure time and 20 repeated measurements. As positive control, cortical bone from mouse femur was used; bone tissue dissected from animal and all non-bone tissue scraped/washed away. Bone tissue was cut into small pieces and washed in sterile PBS until clean then allowed to dry under sterile conditions. Wire 4.0 software was

used to subtract baseline interference from spectra and for peak picking and labelling. Mineral to matrix ratio was calculated as a phosphate peak ( $960\text{cm}^{-1}$ ) intensity divided by hydroxyproline ( $853\text{cm}^{-1}$ ) peak intensity.

### **3.4 Results and discussion**

#### **3.4.1 Nanovibrational bioreactor calibration**

To observe the level of stimulation delivered by the nanovibrational bioreactor displacement amplitude was measured using an interferometer as described in section 3.3.1. Amplitude was measured at the center of individual wells in both 6-well and 12-well cell culture plates, with two well-plates placed side by side on the bioreactor. For this work nanovibration parameters were set to 1000Hz frequency and 30nm amplitude displacement based on previous results achieving osteogenesis in mesenchymal stem cells, so calibration was performed at these settings.

Measuring the amplitude of displacement delivered by the bioreactor at room temperature revealed some variation across the well plate (Table 3.2) and a mean amplitude of 20.5nm, substantially lower than the programmed 30nm output. However at incubator temperature ( $37^{\circ}\text{C}$ ) the bioreactor delivered a mean amplitude of 32.5nm, significantly higher than room temperature (Figure 3.2). Within 6 well plates the mean amplitude was slightly lower at 27.5nm however this was deemed acceptable level of displacement in regards to programmed output. Differences between different 6 and 12 well plates is likely due to resonant effects of the plasticware. As the 'Nanokick' system is still continually being developed, the consistency of nanovibration amplitude should be monitored throughout the use of the bioreactor. The bioreactor used in this project was tested at manufacture, and was tested after 6 months of use during this project (results discussed above). Whilst this calibration time should be reduced, the bioreactor variation fell within acceptable limits.

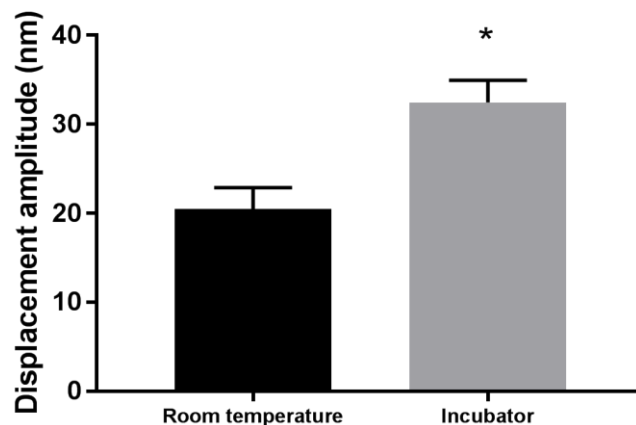
**Table 3.2 Measured displacement during bioreactor calibration**

Displacement delivered by nanovibration bioreactor (nm), measured by laser interferometry.

Measurements taken in two side by side 12 well plates at either room temperature or by

| Room temperature |      |      |              |      |      | Incubator (37°C) |      |      |              |      |      |
|------------------|------|------|--------------|------|------|------------------|------|------|--------------|------|------|
| Well plate 1     |      |      | Well plate 2 |      |      | Well plate 1     |      |      | Well plate 2 |      |      |
| 8.7              | 15.7 | 28.9 | 29.1         | 28.6 | 33.1 | 18.2             | 16.5 | 35.2 | 47.4         | 45.3 | 43.5 |
| 11.2             | 19.6 | 22.8 | 20.6         | 29.1 | 46.3 | 9.8              | 19.2 | 31.1 | 39.9         | 45.5 | 52.4 |
| 15.6             | 12.5 | 5.6  | 4.1          | 15.9 | 47.1 | 15.6             | 21.1 | 27.4 | 30.9         | 41.8 | 53.1 |
| 13.9             | 3.3  | 17.8 | 25.4         | 11.1 | 25.6 | 27.7             | 28.5 | 31.5 | 26.4         | 28.4 | 42.4 |

warming bioreactor to 37°C overnight. Programmed displacement amplitude was 30nm.

**Figure 3.1 Measured nanovibration displacement amplitude**

Displacement amplitude within 12 well plates at room temperature and at 37°C as per a cell culture incubator. Error bars show standard error of the mean, n=24 \* indicates significance  $p < 0.0011$ .

### 3.4.2 Mineralization of osteoblast cultures

After 21 days of culture in osteogenic conditions mESCs have been shown to differentiate into osteoblast-like cells with mineralised bone nodules [73]. Here we observe the ability of

mESCs to form mineralised osteoblasts in response to the nanovibrational bioreactor, using osteogenic culture medium as a comparison. We also observe the response of primary calvarial osteoblasts, known to form mineral deposits in response to osteogenic culture medium.

Both mESCs and mPCs were cultured for up to 35 days to assess for *in vitro* mineralization studies in response to OCM and/or 1000Hz stimulation. Alizarin red S staining revealed extensive calcium deposits in mESCs at both 21 and 35 days of OCM culture, indicative of mineralized bone nodules (Fig. 3.2). As expected no staining was visible in control samples (mESC growth medium alone). Nanovibration showed no obvious alizarin red staining at 21 days however under microscopy clustering of cells similar to other conditions could be observed. This morphology could also be related to accumulation of collagen as discussed in section 1.2.3. Nanovibration did however reveal calcified matrix after 35 days, suggesting 1000Hz stimulation promotes osteogenesis of mESCs however its effects are delayed compared to OCM. The combination of OCM and nanokicking produced less calcification at 21 days compared to OCM, suggesting that nanovibration inhibits or delays the OCM-induced mineralization. However after 35 days OCM+1000Hz stimulation revealed extensive alizarin red staining with smaller, more numerous bone nodules compared to OCM alone. The alizarin red staining following OCM+1000Hz also appeared to be darker, showing more intense staining for calcium deposits. In this way, the combination of the two techniques may have a beneficial effect on mineralization of mESCs *in vitro*.

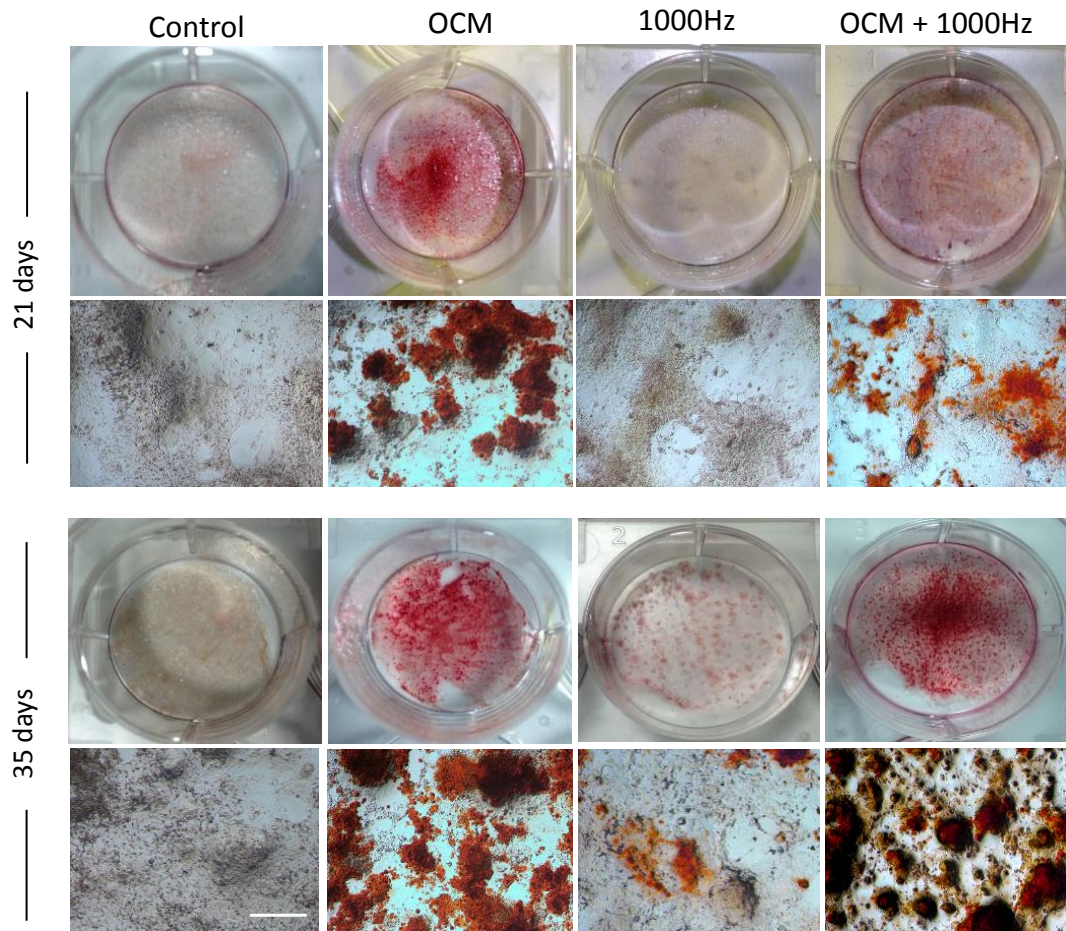


**Figure 3.2 mESC mineralisation**

Representative images (photographs and brightfield) of alizarin red S staining of mESCs following osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only. Scale bar = 200 $\mu$ m.

It is clear nanovibration allows mineralization of mESCs, and influences the morphology of nodules formed by usual osteogenic methods. It also appears that the mechanisms of OCM-induced and 1000Hz-induced mineralization of mESCs may be different and that these mechanisms compete in some way (further explored in chapter 4).

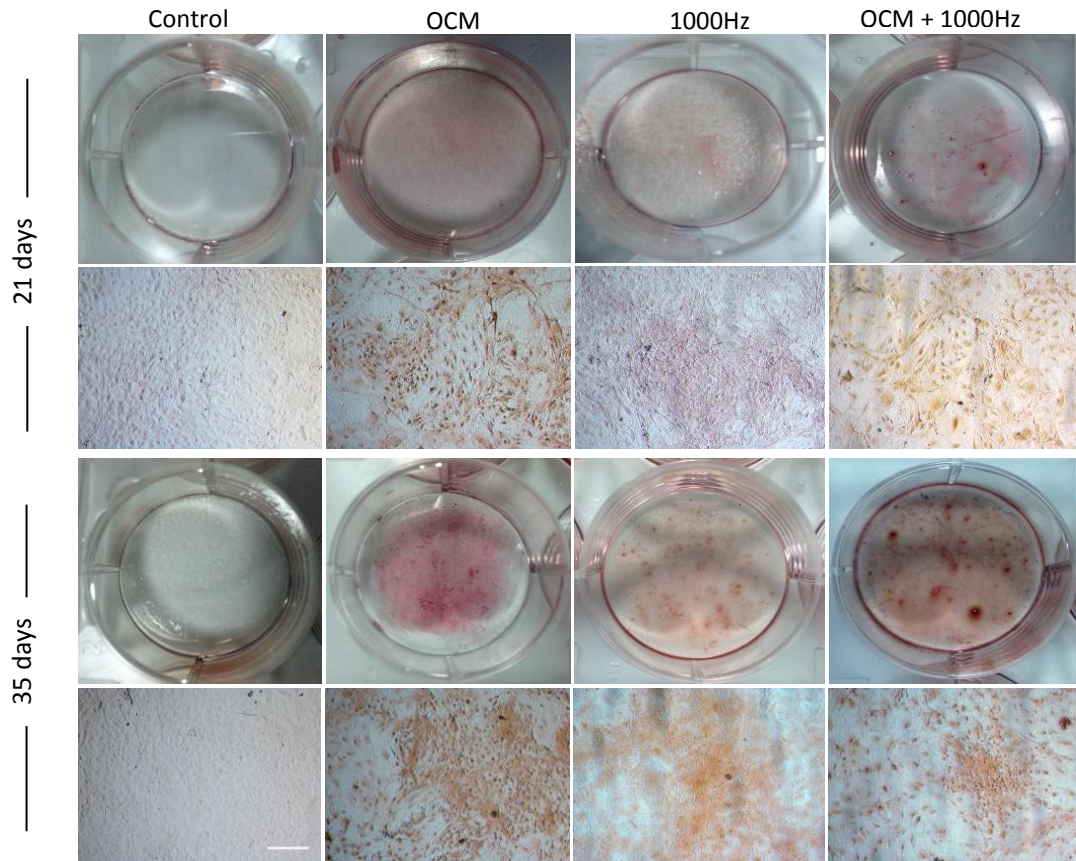


**Figure 3.3 mPC mineralisation**

Representative images (photographs and brightfield) of alizarin red S staining of mPCs following osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only. Scale bar = 200 $\mu$ m.

Similar to mESCs and as expected Alizarin red staining of mPCs revealed calcified matrix at 21 days of OCM culture, with larger more intensely stained areas appearing after 35 days (figure 3.3). 1000Hz stimulated mPCs also showed alizarin red s staining at both timepoints however to a lesser extent. Similarly to mESCs, the combination of OCM and 1000Hz altered morphology of nodules produced, with smaller nodule-like areas visible under brightfield microscopy.

To further analyze the morphology of bone nodules formed as a result of osteogenic culture, scanning electron microscopy was used. During in vitro osteogenesis cells form clusters of densely packed cells trapped within extracellular matrix [73]. Imaging revealed mESCs

cultured under control conditions showed cells spread on a consistent monolayer, with no obvious areas of accumulated collagens or mineral disposition (figure 3.4). Following 21 day OCM culture, mESCs showed changed morphology with dense patches of cells and/or matrix. Brighter areas observed within these morphologies could indicate mineral deposition however this would need investigation to confirm. Nanovibration appeared to show a combination of control and OCM morphologies; no dense patching of cells but with similar accumulation of matrix and/or possible mineral on top. This was also true in the combination of OCM+1000Hz, suggesting that nanovibration interferes with morphological changes that OCM promotes.

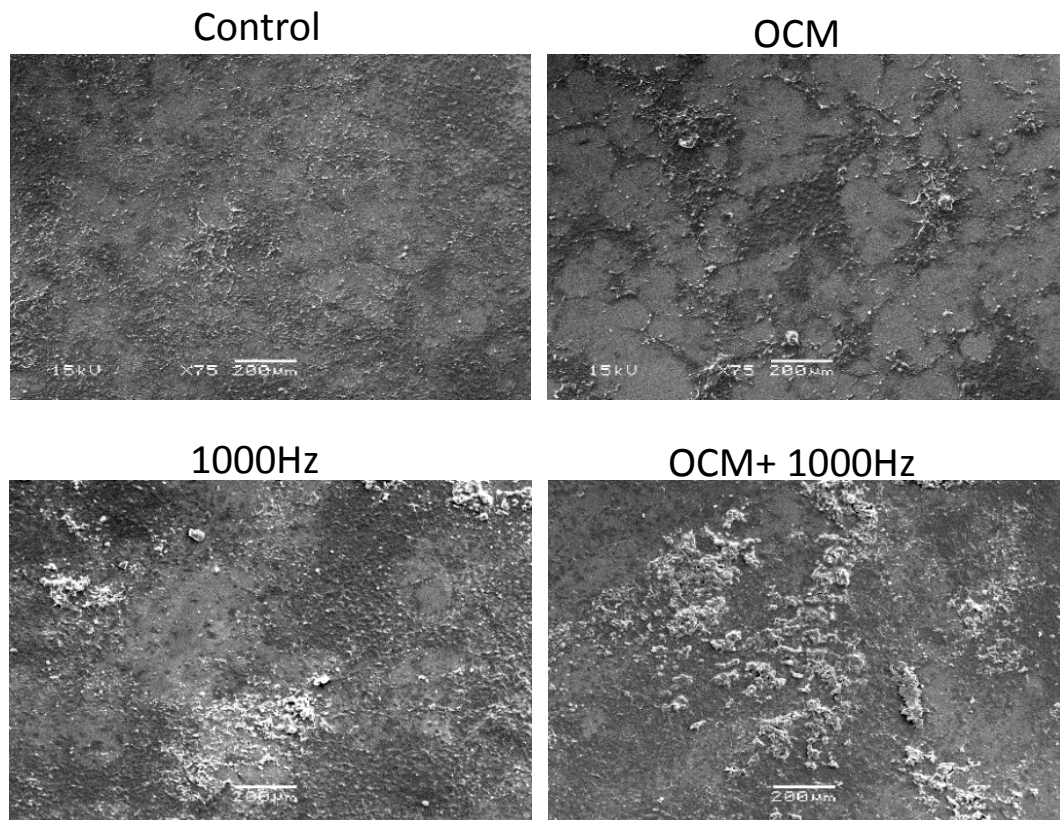


**Figure 3.4 Scanning electron microscopy of mineralised cultures at 21 days**

Representative scanning electron microscopy images of mESCs following osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only.

Further SEM analysis may be beneficial to better understand changes in cell morphology in response to nanovibration. Apparent mineral deposition could also be confirmed utilizing energy dispersive X-ray (EDX) spectroscopy (often linked with SEM instrumentation) which has been previously used to observe mineral within *in vitro* differentiation towards of stem cells [105] [164].

### 3.4.3 Raman spectroscopy of mineralized nodules

Whilst nodule-like structures formed and calcified matrix was present, it was important to characterise the composition of mineral and therefore assess material differences caused by nanovibrational stimulation. Mineralised cultures were analysed after 28 days of osteogenic stimulation using Raman spectroscopy whilst considering typical peaks for mineral and cellular characterisation (summarized in table 3.1).

In positive control of mouse femur cortical bone peak pick analysis revealed characteristic peak for phosphate  $\nu_1(\text{PO}_4^{3-})$  symmetric stretch at  $960.5\text{cm}^{-1}$  (Fig 3.5). Bone also displayed small peak at  $1072\text{cm}^{-1}$  suggesting carbonate  $\nu_1(\text{CO}_3^{2-})$  substitution which can replace  $\text{OH}^-$  and or  $\text{PO}_4^{3-}$  in the mineral component of bone [163].

Undifferentiated mESC and mPC samples acting as negative control were dominated by spectral features related to matrix proteins, notably  $853\text{cm}^{-1}$  related to hydroxyproline collagen proline, phenylalanine peak at  $1003\text{cm}^{-1}$ ,  $\delta(\text{CH}_2)$  deformation at  $1450\text{cm}^{-1}$ , amide band I at  $1660\text{cm}^{-1}$  and amide band III around  $1250\text{cm}^{-1}$  [161] [165]. As expected no detectable phosphate or carbonate species peaks were found in undifferentiated mESC or mPC samples suggesting no mineral was present (Fig 3.5, Fig. 3.6).

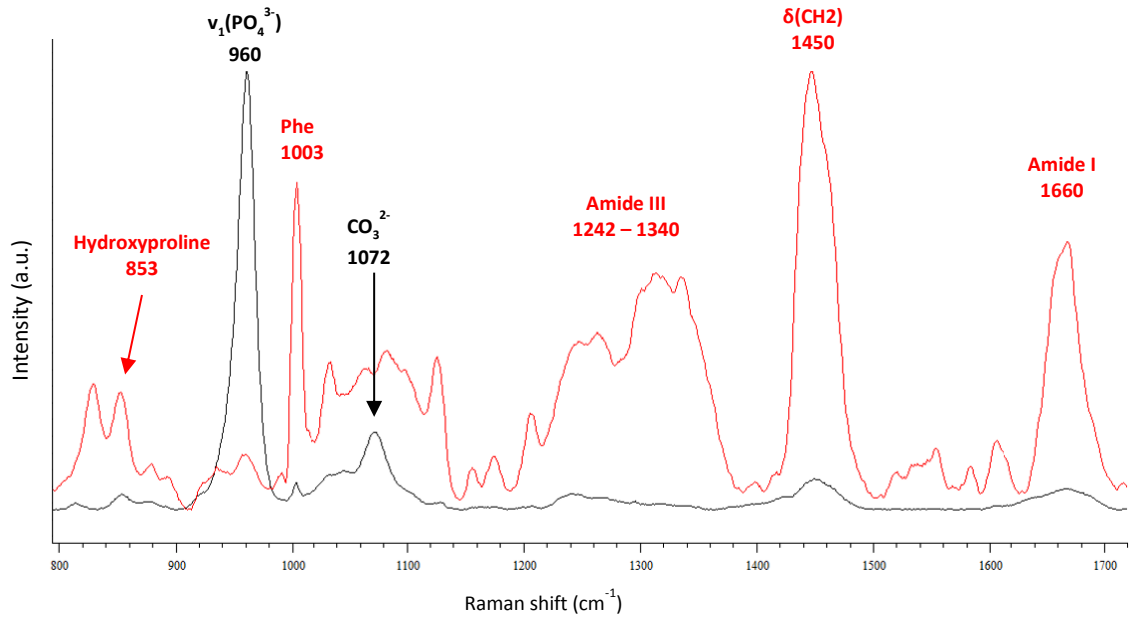


**Figure 3.5 Raman analysis of cortical bone and mESCs**

Raman spectra of mouse cortical bone (positive control, black) and undifferentiated mESCs (negative control, red). Labels above peaks represent significant features for characterisation bone and cellular samples detected by peak pick analysis.

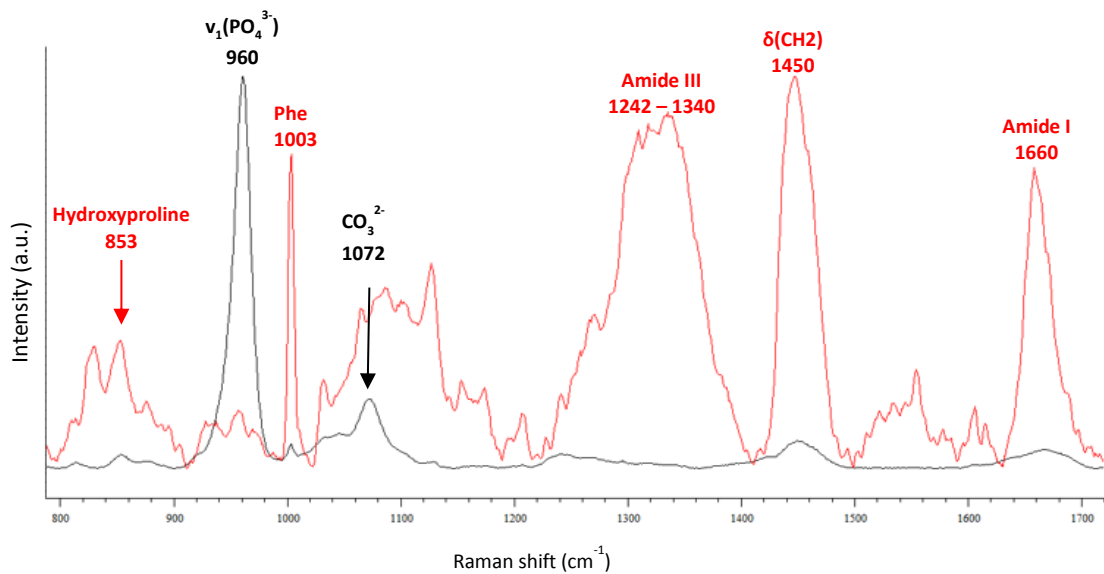


**Figure 3.6 Raman analysis of cortical bone and mPCs**

Raman spectra of mouse cortical bone (positive control, black) and undifferentiated mPCs (negative control, red). Labels above peaks represent significant features for characterisation bone and cellular samples detected by peak pick analysis.

Peak pick analysis of Raman spectra obtained from mESC cultures revealed  $\nu_1(\text{PO}_4^{3-})$  peaks in OCM, 1000Hz and OCM + 1000Hz cultures (Fig. 3.7), with no detectable peak in control samples (growth medium only). Position of this peak can reveal information about crystallinity and species substitution within the mineral. Peaks between 962 and 964  $\text{cm}^{-1}$  represent crystalline non-substituted hydroxyapatite whilst peaks between 955 and 959  $\text{cm}^{-1}$  are indicative of B-type carbonate substitution [161].  $\nu_1(\text{PO}_4^{3-})$  position varied between mESC culture conditions with OCM or 1000Hz alone at 958.7  $\text{cm}^{-1}$  and 959.7  $\text{cm}^{-1}$  respectively, suggesting some carbonate substitution. Interestingly the combination of OCM and 1000Hz showed  $\nu_1(\text{PO}_4^{3-})$  at 961.8  $\text{cm}^{-1}$  implying non-substituted phosphate hydroxyapatite most similar to the positive bone tissue.

Larger, more pronounced  $\nu_1(\text{PO}_4^{3-})$  peaks appeared to be found as a result of 1000Hz stimulation suggesting increased presence of this spectral factor however no significant differences were found in mean  $\nu_1(\text{PO}_4^{3-})$  peak intensity. Similarly OCM + 1000Hz appeared to produce a broader peak implying reduced crystallinity however no significant differences were found between FWHM values of all mESC conditions (table 3.3).

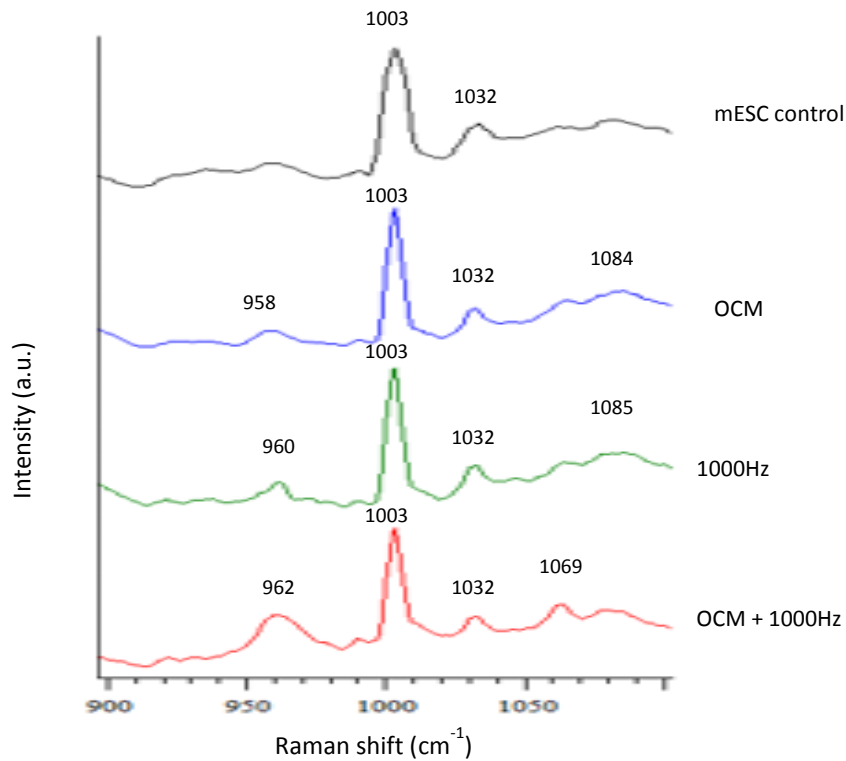


**Figure 3.7 Raman spectra of mESC cultures**

Raman spectra of mESCs stimulated by OCM, 1000Hz or OCM+1000Hz for 28 days, selected region for significant mineral species (full spectra in appendix 7.4). All detected peaks labelled with position. Control cultures in growth medium alone.

**Table 3.3 Peak position and FWHM values of mESC mineral species**

Peak position (+/- standard deviation) and full-width at half maximum (FWHM) values for detected mineral species in mESC cultures at 28 days, n=5.

|              | Phosphate $\nu_1(\text{PO}_4^{3-})$ |                           | Phosphate $\nu_3(\text{PO}_4^{3-})$ |                           | Carbonate $\nu_1(\text{CO}_3^{2-})$ |                           |
|--------------|-------------------------------------|---------------------------|-------------------------------------|---------------------------|-------------------------------------|---------------------------|
|              | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) |
| Bone         | $960.5 \pm 0.1$                     | $17.8 \pm 0.4$            | -                                   | -                         | $1072.3 \pm 0.6$                    | $28.2 \pm 10.5$           |
| mESC control | -                                   | -                         | $1032 \pm 0.1$                      | $10.2 \pm 0.8$            | -                                   | -                         |
| OCM          | $958.7 \pm 0.5$                     | $14.3 \pm 0.7$            | $1032 \pm 0.1$                      | $7.7 \pm 0.1$             | $1083.5 \pm 3.7$                    | $49.2 \pm 19.6$           |
| 1000Hz       | $959.7 \pm 1.3$                     | $13.2 \pm 1.8$            | $1032.1 \pm 0.1$                    | $8.5 \pm 1.4$             | $1084.7 \pm 0.7$                    | $37.2 \pm 5.4$            |
| OCM + 1000Hz | $961.8 \pm 1.6$                     | $17.0 \pm 1.2$            | $1032.3 \pm 0.3$                    | $7.8 \pm 0.5$             | $1069.5 \pm 10.7$                   | $35.4 \pm 18.6$           |

Also detected in mESC cultures were peaks close to  $1035\text{ cm}^{-1}$  which represents  $\nu_3(\text{PO}_4^{3-})$  asymmetric stretch however this region overlaps with proline  $\nu$  C-C component [162] and therefore may be remaining matrix protein signature. Peaks detected in the  $1070\text{-}1085\text{ cm}^{-1}$  region possibly related carbonate  $\text{CO}_3^{2-}$  stretch and B-type carbonate substitution, or immature  $\nu_3(\text{PO}_4^{3-})$  were present in OCM, 1000Hz, and OCM+1000Hz at 1083, 1085 and  $1070\text{ cm}^{-1}$  respectively but not detected in mESC control. Interestingly the combination of nanovibration and OCM produced a peak at a lower region and most similar to mature bone sample tested ( $1072\text{ cm}^{-1}$ ) suggesting these conditions contribute to a more bone-like mineral composition, as implied by  $\nu_3(\text{PO}_4^{3-})$  peak position.

Mineral to matrix ratio was calculated as a ratio of phosphate ( $960\text{ cm}^{-1}$ ) and hydroxyproline ( $853\text{ cm}^{-1}$ ) peak intensities, providing an insight into how much bone mineral is present relative to extracellular matrix. This has been shown to directly correlate to bone mechanical properties [166]. Significantly higher ratio was observed in 1000Hz and 1000Hz+OCM samples compared to OCM (Fig. 3.8), revealing that nanovibration resulted in on the production of more phosphate species in these cultures. This is surprising given the low amount of alizarin red staining observed in 1000Hz cultures in section 3.4.2, but may suggest that whilst less calcium deposition is observed compared to OCM, phosphate formation has still occurred.

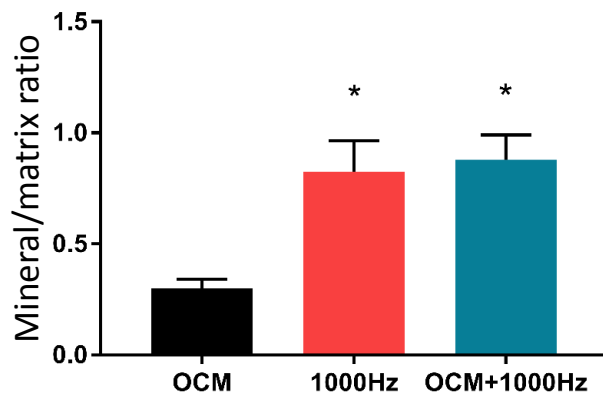


**Figure 3.8 Mineral to matrix ratio of mESCs**

Mineral to matrix ratio of mESCs at 28 days culture; peak intensity ratio of phosphate ( $960\text{cm}^{-1}$ ) to hydroxyproline ( $853\text{cm}^{-1}$ ) species. Error bars show standard error of the mean,  $n=5$ , \* =  $p < 0.03$ .

Raman analysis of mPCs revealed different trends to mESC cultures, most noticeably differences in  $\nu_1(\text{PO}_4^{3-})$  peak position in response to nanovibration. OCM produced a small  $\nu_1(\text{PO}_4^{3-})$  peak at  $958\text{cm}^{-1}$ , most similar in position to bone positive control sample. Whilst the addition of 1000Hz stimulation produced much larger  $\nu_1(\text{PO}_4^{3-})$  peaks (both with and without OCM), these were altered in both peak position and shape; at  $966\text{cm}^{-1}$  and  $974\text{cm}^{-1}$  for 1000Hz and OCM+1000Hz respectively. These results show nanovibration has a larger effect on the  $\nu_1(\text{PO}_4^{3-})$  peak in mPCs than seen in mESC cultures; causing much larger  $\nu_1(\text{PO}_4^{3-})$  peaks but with significantly altered position indicative of altered mineral composition.

Also dissimilar to mESCs was detection of  $\nu_3(\text{PO}_4^{3-})$  asymmetric stretch which was only detected in OCM cultures in mPCs (present in all mESC conditions). Carbonate related peaks were also detected in the  $1070\text{-}1085\text{cm}^{-1}$  region in mPCs however peaks were smaller than seen in mESCs suggesting less carbonate substitution. Mineral to matrix ratio of mPCs appeared to show that production of phosphate species was also increased following nanovibration however significant difference was only observed in OCM+1000Hz samples (Fig. 3.10). It is however important to note that mineral to matrix ratio of OCM+1000Hz

stimulated mPCs was much higher than that exhibited by mESC under the same conditions, highlighting again the differences in mineral formed depending on cell source.

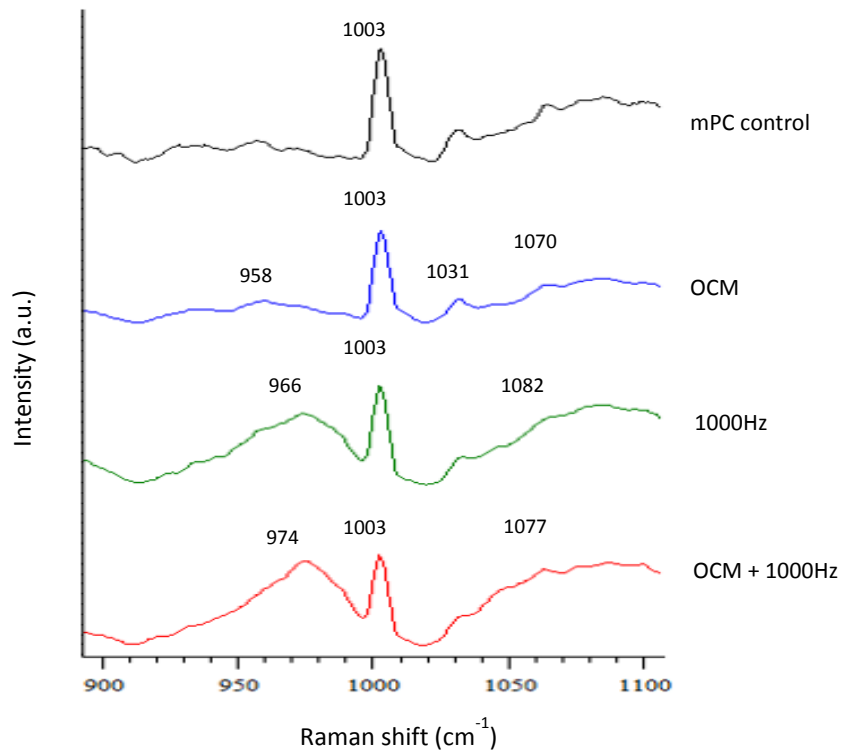


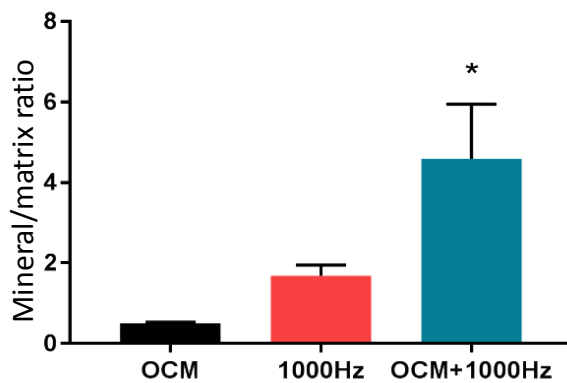
**Figure 3.9 Raman spectra of mPCs**

Raman spectra of mESCs stimulated by OCM, 1000Hz or OCM+1000Hz for 28 days, selected region for significant mineral species (full spectra in appendix 7.4). All detected peaks labelled with position. Control cultures in growth medium alone.

**Table 3.4 Peak position and FWHM value of mPC mineral species**

Peak position (+/- standard deviation) and full-width at half maximum (FWHM) values for detected mineral species in mPC cultures at 28 days, n=5.

|              | Phosphate $\nu_1(\text{PO}_4^{3-})$ |                           | Phosphate $\nu_3(\text{PO}_4^{3-})$ |                           | Carbonate $\nu_1(\text{CO}_3^{2-})$ |                           |
|--------------|-------------------------------------|---------------------------|-------------------------------------|---------------------------|-------------------------------------|---------------------------|
|              | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) | Peak position                       | FWHM ( $\text{cm}^{-1}$ ) |
| Bone         | 960.5 $\pm$ 0.1                     | 17.8 $\pm$ 0.4            | -                                   | -                         | 1072.3 $\pm$ 0.6                    | 28.2 $\pm$ 10.5           |
| mPC control  | -                                   | -                         | -                                   | -                         | -                                   | -                         |
| OCM          | 957.8 $\pm$ 1.0                     | 21.6 $\pm$ 2.5            | 1031*                               | -                         | 1070 $\pm$ 5.1                      | 37.4 $\pm$ 2.25           |
| 1000Hz       | 965.9 $\pm$ 0.7                     | 30.4 $\pm$ 0.6            | -                                   | -                         | 1081.6 $\pm$ 2.4                    | 66.9 $\pm$ 8.2            |
| OCM + 1000Hz | 973.6 $\pm$ 0.5                     | 32.8 $\pm$ 3.9            | -                                   | -                         | 1077 $\pm$ 5.5                      | 71.0 $\pm$ 6.1            |

**Figure 3.10 Mineral to matrix ratio of mPCs**

Mineral to matrix ratio of mPCs at 28 days culture; peak intensity ratio of phosphate ( $960\text{cm}^{-1}$ ) to hydroxyproline ( $853\text{cm}^{-1}$ ) species. Error bars show standard error of the mean, n=5, \* =  $p < 0.03$ .

Stevens et al. described significant differences in Raman signature within mineral deposits depending upon the cell source, with MSCs and neonatal calvarial osteoblasts forming complex mineral structures similar to native bone tissue, whilst ESC nodules displayed less extensive mineral content and reduced stiffness [163]. We show here further differences between cell source when cultured under the same osteogenic conditions, most notably the presence of much larger phosphate  $\nu_1(\text{PO}_4^{3-})$  peaks in our primary calvarial osteoblast

cultures compared to mESCs. This is likely to influence cell differentiation behaviour since matrices bearing phosphate moieties have been shown to be osteoinductive for stem cells [167].

The results also reveal extensive differences in mineral composition linked to osteogenic stimulation parameters. Both mESCs and mPCs showed altered mineral composition in response to nanovibration stimulation, most significantly increasing prominence of phosphate  $\nu_1(\text{PO}_4^{3-})$  peaks. This did appear to also produce broader peaks but analysis revealed no significant differences between nanovibration stimulation and OCM. In mESCs 1000Hz produced peak position more similar to femoral bone tissue than OCM stimulation, and also resulted in significantly higher mineral to matrix ratio. Mineral to matrix ratio of mPC cultures was also higher following the combination of 1000Hz and OCM stimulation however not by nanovibration alone. It is important to note however that the amount of phosphate species formed and the mineral to matrix ratio values of these osteogenic cultures is significantly lower than the femoral bone tissue analysed. Whilst a direct comparison to bone tissue is perhaps an unreasonable benchmark for *in vitro* differentiation cultures, the differences observed between nanovibration and conventional osteogenic supplements is helpful in understanding these *in vitro* osteogenesis of stem cells.

#### **3.4.4 Expression of osteogenic proteins: nanovibration vs medium supplementation**

*In vitro* osteogenic cultures have shown mESCs express bone-specific protein RUNX2 from around 7-14 days and osteocalcin at later stages of culture around 21 days [168]. In this work culture of mESCs in a standard osteoinductive medium confirms this, with RUNX2 staining noticeably increased compared to control after 7 days - peaking at 14 days OCM culture - and osteocalcin staining increasing over time in OCM culture (Fig. 3.9). A similar pattern was observed in mPCs; osteocalcin accumulation increased over culture period reaching its

highest at 21 days, whilst RUNX2 staining was increased compared to controls at earlier timepoints (Fig. 3.10). A noticeable difference between mESCs and mPCs is the timing of highest RUNX2 staining; observed at 7 days in mPCs however 14 days in mESCs. This suggests mPCs are quicker to respond to osteoinductive medium however this is to be expected due to their pre-osteoblast state; it is to be expected that the more naïve embryonic stem cells require more time to reach a similar differentiation state.

Nanovibration resulted in increased RUNX2 staining compared to controls in both cell types, however to a lesser extent than OCM cultures. Amount of visible RUNX2 staining was similar at all timepoints during 1000Hz stimulation. Nanovibration also caused increasing osteocalcin staining over the 21 days of stimulation, however overall amount of staining appeared to be slightly less than OCM cultures in mPCs. Interestingly stimulation by nanovibration appeared to promote changes in mPC morphology, promoting cells to form tight clusters (as seen at 14 and 21 days of mPC osteocalcin staining).

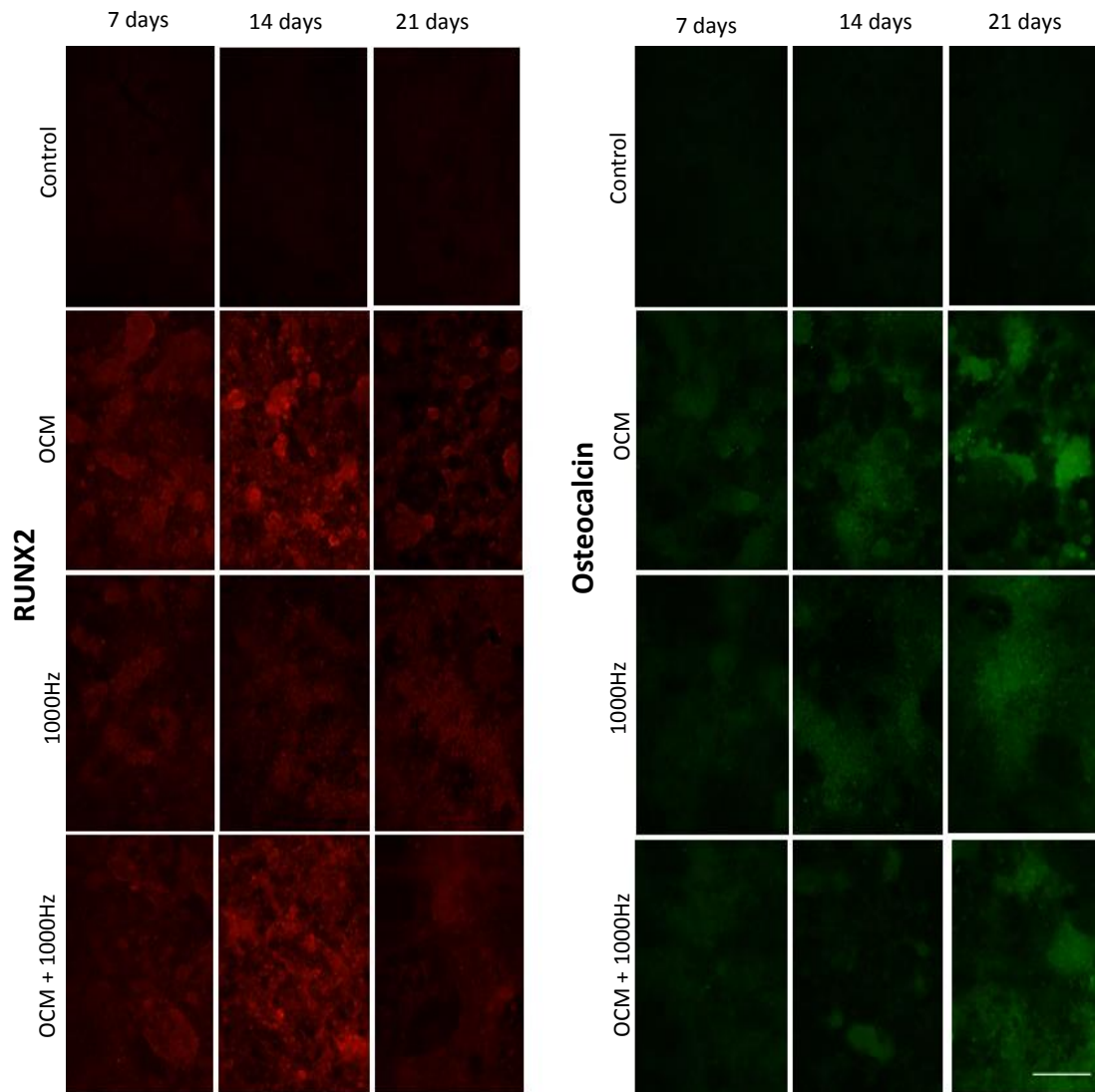


**Figure 3.11 Runx2 and Osteocalcin staining of mESCs**

Immunocytochemical staining for RUNX2 and osteocalcin in mouse embryonic stem cells (mESCs) during osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. All images at same magnification, scale bars = 300 $\mu$ m.

The combination of 1000Hz stimulation with OCM resulted in largely similar staining to OCM cultures in the case of mPC staining, suggesting osteogenic culture medium has the overbearing effect on RUNX2 and osteocalcin expression. In mESC samples OCM+1000Hz was also similar to OCM alone however at 21 days the OCM appeared to produce more concentrated areas of staining whilst OCM+1000Hz staining was more consistent over the whole plate. This suggest nanovibration also has an effect on mESC morphology; producing

less obvious cell clustering which corroborates observations of alizarin red and SEM imaging in section 3.4.2. The effects of nanovibration on cell morphology will be further explored in chapter 4.

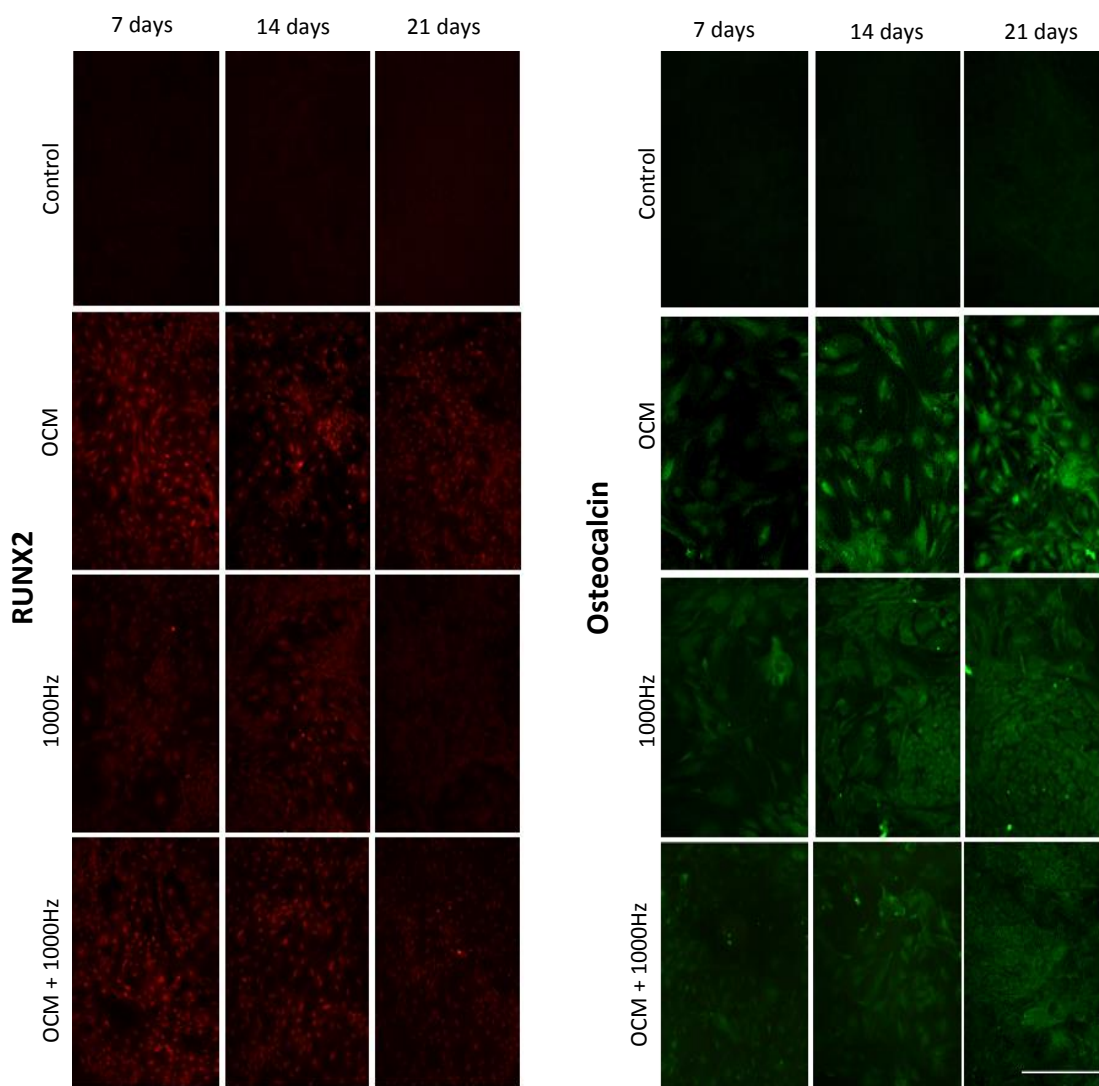


**Figure 3.12 Runx2 and Osteocalcin staining of mPCs**

Immunocytochemical staining for RUNX2 and osteocalcin in mouse primary calvarial cells (mPCs) during osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. All images at same magnification, scale bars = 300 $\mu$ m.

### 3.4.5 Effect of nanovibration on alkaline phosphatase activity

Alkaline phosphatase activity is closely associated with calcification and mineralization [169], and is widely used as an indicator of osteoblast activity during formation of mineralized cultures *in vitro*. mPCs cultured in OCM revealed an increase in ALP, peaking at 14-21 days as expected during *in vitro* osteogenesis. 1000Hz stimulated samples did not reveal any significant increase in ALP activity compared to controls, however the combination OCM and 1000Hz produced a synergistic effect with ALP activity higher than either method alone at 7 and 14 days. Whilst OCM is osteoinductive for mPCs, nanovibration appears to further promote ALP activity.

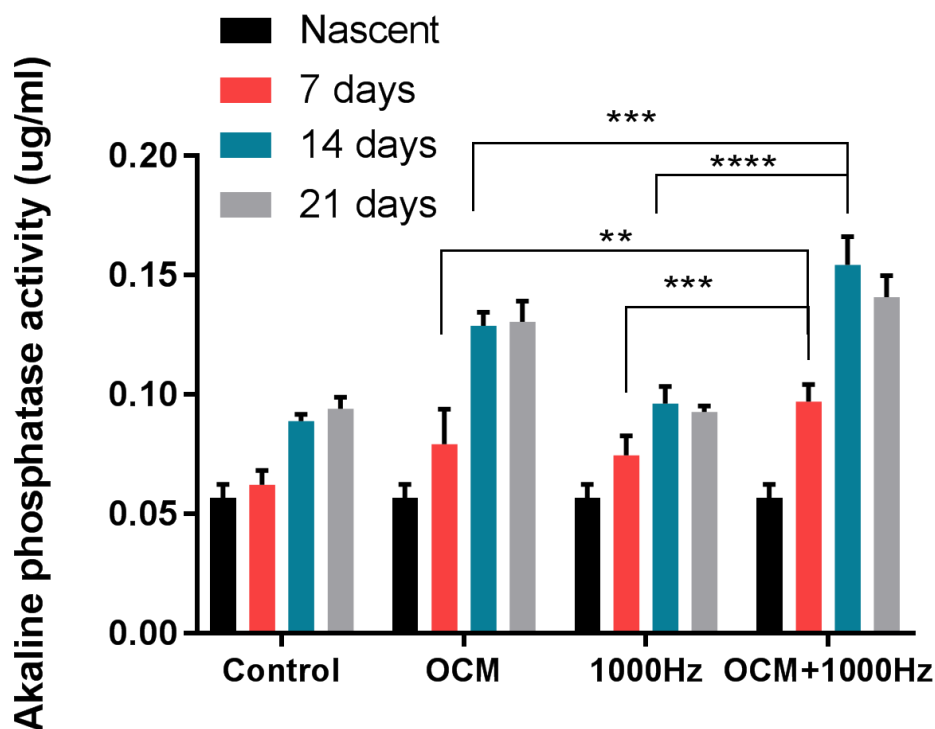


Figure 3.13 Alkaline phosphatase activity of mPCs

Alkaline phosphatase activity of mPCs during osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only. Error bars show standard error of the mean, n=6. \*\*= p<0.0021, \*\*\*=p<0.0002, \*\*\*\*=p<0.0001.

In mESC cultures ALP activity increased over 21 days of OCM culture. Nanovibration caused an initial spike in ALP activity after 7 days much higher than OCM, however this reduced at later timepoints to levels similar to OCM. Similar to mPC results, the combination of OCM and nanovibration caused a synergistic effect on ALP at 14 days, however values were lower than OCM at 21 days. It should also be noted that levels of ALP observed in mESCs were an order of magnitude higher than mPCs. ALP is typically highly expressed in ESCs, however timing of increase observed here suggested bone-specific ALP is activated.

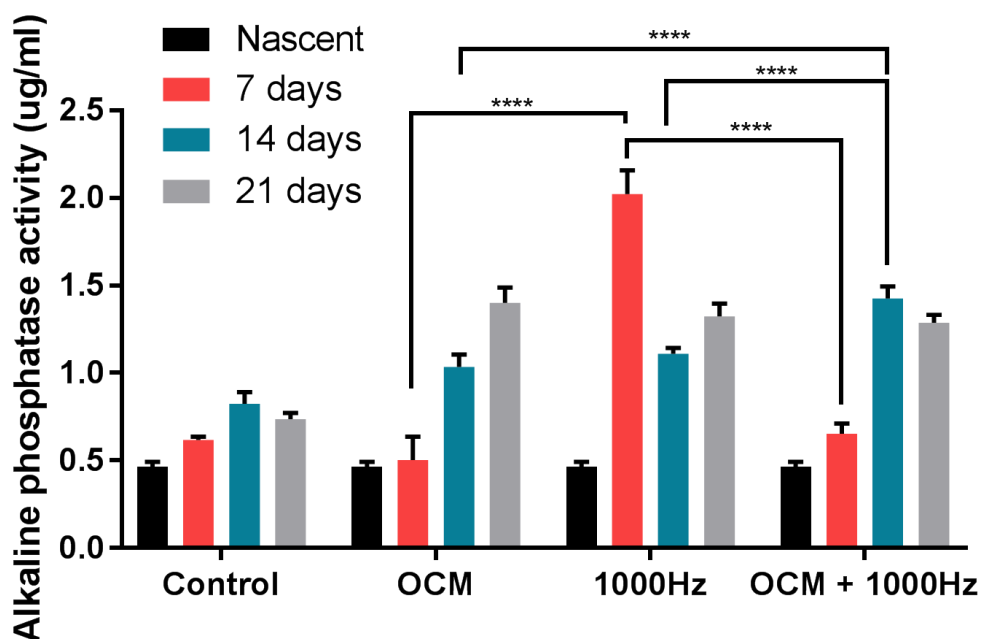


**Figure 3.14 Alkaline phosphatase activity of mESCs**

Alkaline phosphatase activity of mESCs during osteogenic stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only. Error bars show standard error of the mean, n=6. \*\*\*\*=p<0.0001.

Increase alkaline phosphatase activity following nanovibration would increase the amount of available phosphate groups and may account for the increased measurements of phosphate-incorporating mineral species observed during Raman spectroscopy (section 3.4.3). It remains unclear however, how nanovibration promotes ALP activity. This change may be

related to increased expression of ALP on the cell membrane, or by increasing availability of metallic ions required for ALP activity [170] and warrants further study.

### 3.4.6 Gene expression during osteogenesis

To confirm RUNX2 expression observed during immunocytochemical staining of mESCs, quantitative polymerase chain reaction (qPCR – section 2.2.5) was used and relative expression calculated compared to untreated controls. As seen in figure 3.15, relative expression of RUNX2 was increased at 7 days in response to OCM stimulation, in agreement with immunocytochemistry staining and previous osteogenesis studies [105] [168]. After 7 days of 1000Hz stimulation RUNX2 expression was similar to controls, but was increased after 14 days. This may account for delayed RUNX2 staining compared to OCM observed during immunocytochemistry.

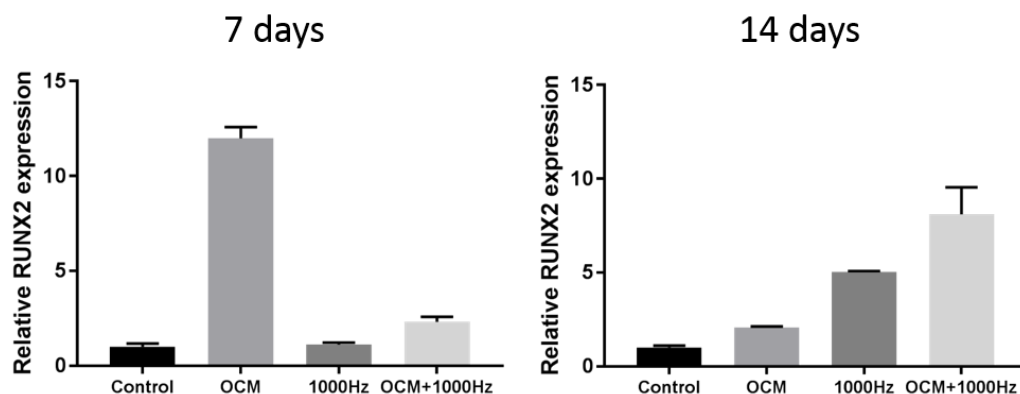


**Figure 3.15 RUNX2 gene expression during osteogenic stimulation of mESCs**

Relative RUNX2 gene expression assessed by quantitative polymerase chain reaction following OCM, 1000Hz and OCM+1000Hz stimulation. Results relative to control (untreated mESC) expression, error bars show standard deviation, n=3.

### 3.4.7 Differentiation to other mesenchymal lineages

Staining for adipogenesis using AdipoRed revealed no characteristic fat staining as seen in positive controls, suggesting no differentiation to adipocyte lineage under any of the

osteogenic conditions used herein (Figure 3.16). In mESCs, culture in OCM produced a small amount of AdipoRed staining which was not present with the addition of 1000Hz stimulation, suggesting nanovibration acts to improve efficiency of osteogenic differentiation.

Alcian Blue staining revealed neither mESCs nor mPCs formed cartilaginous pellets spontaneously as observed with MSCs in positive control studies. This may indicate presence of sulphated glycosaminoglycans present in other tissues at low levels, therefore not indicative of chondrogenesis in these samples [171]. However mESC revealed more visible Alcian blue staining compared to mPCs, which could be indicative of collagen deposition associated with chondrogenesis. If chondrocytes are present in mESC cultures, this suggests bone formation observed in this work could be occurring via remodelling of chondrocyte matrix and therefore endochondral ossification. Conversely the lack of Alcian blue staining in mPCs may confirm bone matrix formation is happening via intramembranous ossification, as expected for calvarial precursor cells.

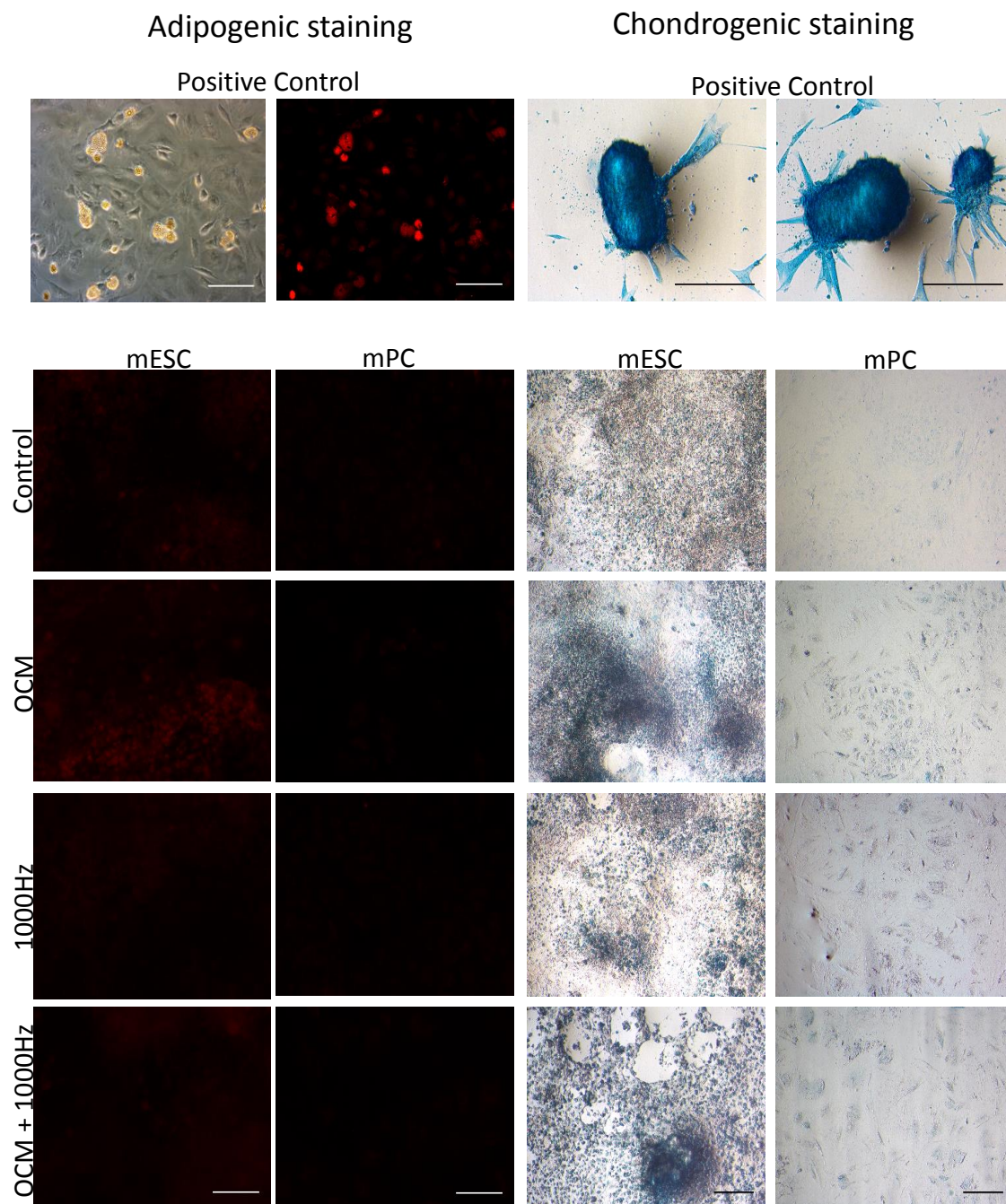


**Figure 3.16 Chondrogenic and adipogenic staining of mineralised cultures**

AdipoRed (adipogenic) and Alcian Blue (chondrogenic) staining of mESCs and mPCs following 30 days of osteogenic culture. Scale bar 200µm.

### **3.5 Conclusion**

This chapter has shown the ability of the nanovibrational simulation at 1000Hz frequency to promote the formation of mineralised osteoblast cultures *in vitro* from both an embryonic stem cell line and a primary pre-osteoblast cell source. However longer stimulation was required to achieve mineralization compared to traditional osteoinductive medium. Further differences were also observed in bone specific protein presentation; with nanovibration seemingly having a delayed effect on RUNX2 expression than OCM. However osteocalcin staining was still observed, indicating a mature osteoblast phenotype [37]. Differences in morphology of mineralized nodules and distribution of staining during immunocytochemistry suggest the osteogenic effect of OCM and 1000Hz act via different mechanisms which may compete. These mechanisms will be explored in detail in the following chapter.

Raman analysis performed here shows nanovibration promotes formation of phosphate  $\nu_1(\text{PO}_4^{3-})$  species, and also increases amount of mineral formed in osteogenic cultures. Combination osteogenic protocols like the combination of osteoinductive medium and nanovibration used here may help to overcome shortcoming in ESC osteogenesis observed by Stevens et al. and others. Furthermore this Raman analysis was performed after 28 days of mESC culture, longer term cultures may reveal more mineralisation in 1000Hz stimulation (as seen with alizarin red staining in section 3.1).

Staining with AdipoRed suggested no adipogenic differentiation in either cell type, however more Alcian blue staining in mESCs suggested more matrix collagen indicative of chondrogenesis. This may indicate bone matrix formation happening by different mechanisms in embryonic stem cells and calvarial cells (endochondral and intramembranous ossification respectively). The combination of the nanovibration with osteoinductive medium also increased alkaline phosphatase activity implying a nanovibration promotes functional osteoblast activity.

It appears 1000Hz and 30nm amplitude stimulation can promote differentiation of mESCs and mPCs towards the osteoblast lineage without need for osteogenic supplements, however the combination of the two methods may provide best overall outcomes. These results highlight the importance of nanoscale mechanotransduction, and will help to further develop *in vitro* osteogenic protocols.

## **Chapter 4 - Mechanisms of nanovibrational osteogenesis**

### **4.1 Introduction**

In the previous chapter the effects of nanoscale mechanotransduction on osteogenesis have been observed in both primary calvarial osteoblasts and embryonic stem cells. This chapter aims to analyse the mechanisms by which the nanovibrational bioreactor directs osteogenesis.

#### **4.1.1 Forces influencing cell morphology**

Mechanical and topographical cues presented by the extracellular matrix play an important role in defining stem cell fate; highlighted by Engler et al. who described cells detecting elasticity of their environment using cortical actin structures via focal adhesions [172]. This study amongst others show that matrix forces, stiffness and topographies influence cell morphology and ultimately directs phenotype [173] [174] [175].

#### **4.1.2 Mechanotransduction in osteogenesis**

The influence of nanoscale cues (in particular nanotopography) on cell behaviour is well known. Nanoscale topographies have been shown to cause longer cellular protrusions and highly organised actin filaments in mesenchymal stem cells, as well influencing focal adhesion complex size and shape [123] which can have downstream effects on stem cell phenotype. The effect of these nanoscale stimuli on stem cell pluripotency has been observed in MSCs; responding by both direct (mechanical) and indirect (biochemical) signalling [127] [176] [177]. Nanoscale stimulation also promoted larger and more numerous focal adhesions typical of osteoblast lineage [126] [127]. By this mechanism, nanoscale effect may regulate phenotype towards osteogenesis [111]. Engineered nanoscale topography replicating nacre promoted mineralisation of osteoblasts with higher crystallinity than samples induced by

osteogenic culture medium, highlighting the importance of nanoscale cues on osteoblast function [165]. Furthermore both focal adhesion kinase (FAK) and extracellular signal regulated kinase (ERK)1/2 have been shown to respond to nanoscale cues and influence osteoblasts adhesion behaviour as well as levels of RUNX2 activation [178] which is critical to osteogenesis. These results implicate focal adhesions act as a mechanoreceptor for nanoscale cues regulating osteogenesis downstream.

Focal adhesion kinase activity is critical to integrin signalling; FAK is phosphorylated following integrin engagement and thus allowing the formation of docking sites for phosphotyrosine motifs on a range of signalling molecules [179]. One of the downstream pathways thought to be regulated by FAK activity is extracellular signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway. Increased FAK Tyr-925 phosphorylation and FAK-stimulated phosphorylation of Ser298 in MAPK/ERK kinase-1 (MEK1) by p-21 kinase (PAK) lead to ERK/MAPK cascade activation [115] [180].

This ERK activation (which in turn phosphorylates other downstream molecules) varies depending upon the amount of cell adhesion signalling; lower adhesion can actually downregulate ERK allowing more PPAR $\gamma$  (active in the unphosphorylated form) and promote adipogenesis, whilst more extensive cell adhesion increases ERK activity producing phosphorylated RUNX2 which becomes active (whilst now phosphorylated PPAR $\gamma$  becomes inactive) and thus osteogenesis is promoted [181] [182]. The role of MAP/ERK pathway in osteoblast differentiation and skeletal development was confirmed by selective expression of constitutively active MAPK/ERK1 in mice; causing accelerated *in vitro* differentiation of extracted calvarial cells by increasing RUNX2 phosphorylation and transcriptional activity. Conversely osteogenesis was inhibited in the cells from dominant-negative MAPK/ERK1 mice [183].

### 4.1.3 Metabolomics and cell pluripotency

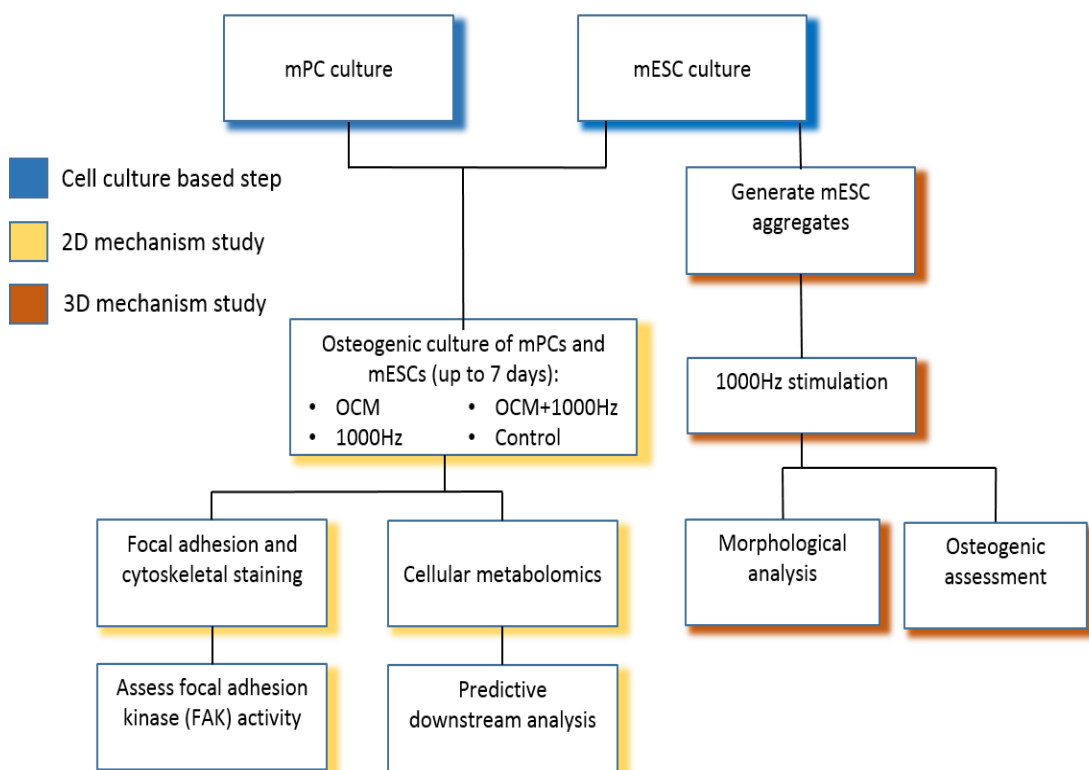
It is becoming increasingly apparent that the analysis of the metabolite population or 'metabolome' of an organism or cell can provide vast information about the biological system, and this metabolic profile can be used to characterise disease states, cell behaviour and even cell phenotype [184] [185].

There is growing evidence that metabolomics analysis can be used to characterise cell pluripotency and/or phenotype; specific metabolites produced by the activity of stem cell self-renewal or differentiation can provide a signature for characterisation of these events [186][187]. Metabolic changes during MSC differentiation towards osteoblast phenotype have been noted and may allow non-invasive phenotypic characterisation [188]. Similarly, high levels of unsaturated metabolic compounds have been observed in ESCs [189] and MSCs [127], the number of which decreasing following differentiation. Detection of this metabolite category could provide a non-invasive technique to characterise ESC differentiation, however using broad categories such as this make it difficult to observe changes in specific metabolic products relevant to the end-cell phenotype.

Other metabolomics studies have revealed ERK1/2 – along with c-Jun N-terminal kinases (Jnk) and low-density lipoprotein (LDL) – as a key signalling hub in modulating MSC multipotency using tailored topographies, and have observed changes in overall metabolite activity associated with differentiating stem cells [190]. Analysis of metabolite population following osteogenesis induced by nanotopographical cues resulted in a higher proportion of significantly altered metabolites compared to chemical osteogenic induction, revealing the impact of mechanotransduction on overall metabolic activity of stem cells [165]. These results further indicate the role of focal adhesion dynamics and ERK/MAPK signalling in mechanotransduction and osteogenesis. In this chapter the effect of 1000Hz nanovibration on these pathways will be considered during metabolomics analysis.

## 4.2 Objectives and experiment design

In this chapter the mechanisms of nanoscale mechanotransduction in mESCs and mPCs are explored by monitoring focal adhesion and cell cytoskeleton characteristics as well as measuring changes in signalling molecules associated with mechanosensing and osteogenesis in response to 1000Hz stimulation. Cellular metabolite population was also analysed to connect cellular event observations to signalling pathways based on metabolic differences. Finally, the response of 3D cell structures to 1000Hz stimulation was also observed. The chapter therefore followed the experimental plan shown below.



## **4.3 Materials and methods**

### **4.3.1 Cell culture**

mPCs and mESCs were cultured as per section 2.2.1, and stimulated as by OCM and/or nanovibration as per section 2.2.2 for up to 7 days for studies performed during this chapter. Subsequent analysis methods used are discussed in detail below.

### **4.3.2 Cytoskeletal and focal adhesion staining**

Monolayers were co-stained for vinculin and F-actin, antibody and reagent details found in section 2.1.2. Samples fixed in 10% (v/v) formalin were permeabilised in PBS containing 0.5% v/v Triton X-100 (100ml of buffer also contained 10.3g sucrose, 0.292g NaCl, 0.006g MgCl<sub>2</sub>, 0.476g Hepes) for 5 minutes at 37°C. Samples were blocked with 1% BSA in PBS solution for 1 hour at room temperature before removing and adding Vinculin primary antibody together with rhodamine Phalloidin and incubated for 1 hour at 37°C. Samples were washed 5 times (each 5 minutes on shaker) in PBS with 0.5% Tween before secondary antibody was added and samples incubated for 1 hour at 37°C protected from light. Samples were washed 5 times (each 5 minutes on shaker) and incubated with Streptavidin-FITC, protected from light at 4°C for 30 minutes. Samples were washed again 5 times (each 5 minutes on shaker) before imaging or protected with a drop of Vectashield antifade (Vector laboratories) for later imaging.

### **4.3.3 In-cell western**

The amount of total and phosphorylated FAK and ERK1/2 were analysed via quantitative “in-cell western blot” method using detection of infrared secondary antibodies using the LI-COR Odyssey imager (BioAgilytix), antibody and reagent details found in section 2.1.2. Samples fixed in 10% formalin (v/v) were permeabilised in using PBS containing 0.5% v/v Triton X-100

(100ml of buffer also contained 10.3g sucrose, 0.292g NaCl, 0.006g MgCl<sub>2</sub>, 0.476g Hepes) for 5 minutes at 37°C. Samples were then blocked with 1% BSA in PBS for 90mins on a shaker at room temperature. Samples were washed 5 times in PBS before primary antibodies were added and left at 4°C overnight. Following primary incubation, samples were again washed 5 times (each 5 minutes on shaker) in PBS before adding secondary antibodies and incubating on a shaker at room temperature for 1 hour, protected from light. Wells were simultaneously stained for total FAK and phospho-FAK using primary antibodies raised in different species to allow calculation of the total/phosphorylated FAK ratio (same method used for total ERK and phospho-ERK on additional wells). Samples were imaged using the LI-Cor Odyssey system and relative infrared emission compared. Samples were then mounted using Vectashield with DAPI (Vector laboratories) for cell counting and amount of FAK and ERK1/2 per cell calculated.

#### **4.3.4 Cellular metabolomics**

Cells were cultured for up to 14 days prior to metabolite extraction using cold chloroform / methanol / water (1:3:1 v/v) by agitation on a shaker at 4°C for one hour. Samples were then centrifuged at 13,000 rpm and 4°C for 3 minutes to separate debris, supernatant was removed and stored at -80°C for later analysis. Untargeted metabolomics analysis was carried out at Glasgow Polynomics via hydrophilic lipid chromatography mass spectrometry (UltiMate 3000 RSLC (Thermo Fisher) with a 150 x 4.6mm ZIC-HILIC column running at 300µl/min and Orbitrap Exactive (Thermo Fisher) respectively). LC-MS results were entered into IDEOM software [191]; a macro enabled, Excel based resource which performs filtering and identification of metabolite peaks. Software utilises XCMS [192] and mzMatch [193] to extract peaks, filter noise and annotate peaks, matching mass and retention time to database of known metabolites.

Metaboanalyst 3.0 [194] was used to perform principal component analysis and generate heat maps representing relative metabolite levels within samples. Ingenuity pathway

analysis (IPA) software [195] was used to generate metabolite networks and predictive analysis of signalling hubs.

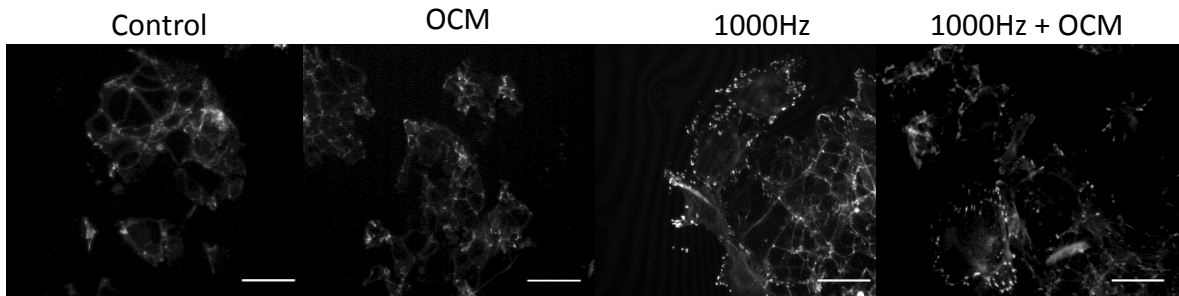
#### **4.3.5 Nanovibrational stimulation of 3D aggregates**

Mouse ESC aggregates were generated as per protocol used during differentiation studies (section 3.1.1.i). After 72 hours of aggregation, single aggregates were pipetted into wells then stimulated by 1000Hz nanovibration (with or without allowing time for aggregates to settle/attach, n=12). Fixed samples (as per section 2.2.3) were permeabilised with triton X-100 for 15 minutes before washing 3x with PBS. 165nM Alexaflour 488 phalloidin for 30 minutes and washed 3x with PBS before imaging. RUNX2 staining was performed as per immunocytochemistry method in section 2.2.4 and representative images presented in this report.

## **4.4 Results and discussion**

### **4.4.1 Effect of nanovibration on cell morphology**

#### **mESC**



#### **mPC**

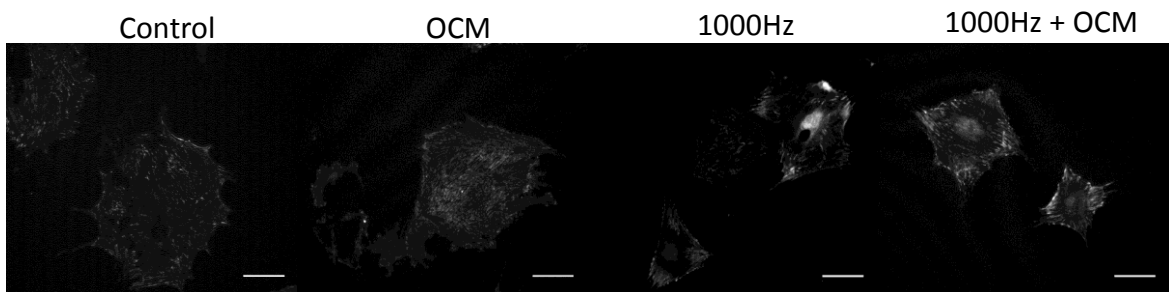


**Figure 4.1 Vinculin staining**

Representative images of vinculin staining of mESCs and mPCs following 4 day stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only, scale bar = 80 $\mu$ m.

In both mESCs and mPCs nanovibration appeared to cause more cell spreading with clustered vinculin staining at cell edges indicative of focal adhesion complexes (Fig. 4.1). This morphology indicates greater interaction with cell culture surface in response to mechanical stimulus by nanoscale vibration, further evidence that nanovibration can influence cell behaviour [109]. In mESCs OCM culture caused similar cell morphology to controls, whilst in mPCs OCM actually revealed less cellular protrusions implying less spreading. 1000Hz and 1000Hz+OCM stimulation resulted in more intense vinculin staining clustered to cell edges in both cell types, suggestive of mature focal adhesion complexes which are supportive of osteogenesis as discussed in section 1.4.2.

As shown by cytoskeletal staining in figure 4.2, OCM stimulation of mESCs appeared to cause more intense actin staining, whilst in mPCs actin staining was comparable to controls. In both cell types 1000Hz stimulation resulted in more intense actin staining and also influence cell morphology; promoting cellular protrusions and a more polygonal shape. This effect was less apparent in the combination of OCM+1000Hz suggesting OCM acts to cancel out some of the effects of nanovibration stimulation.

Taken together, changes in vinculin presentation and actin morphology highlight the influence of nanoscale mechanotransduction on morphology both mESCs and mPCs. This would imply potential mechanotransductive effects on these two cells types which may account for osteogenesis observed in chapter 3. This is in agreement with previous studies that identify force and nanoscale stimuli as regulators of cell shape and downstream behaviour [112] [127].

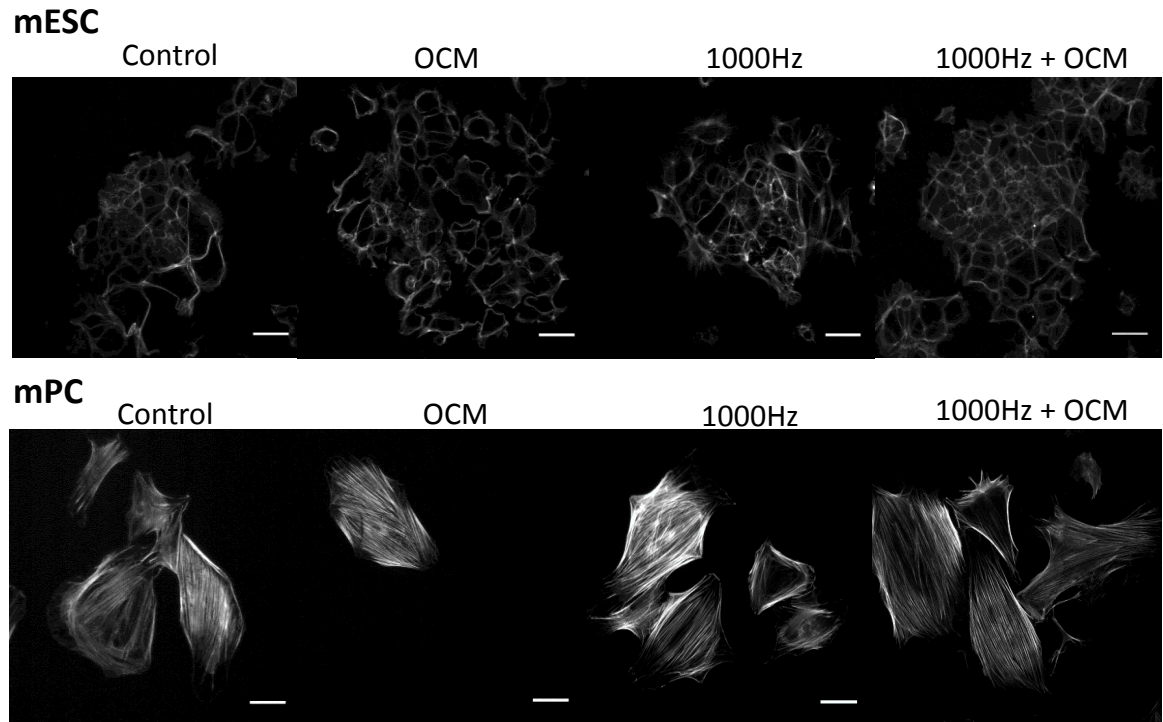


**Figure 4.2 Actin cytoskeleton staining**

Representative images of actin cytoskeleton staining of mESCs and mPCs following 4 day stimulation by OCM, 1000Hz and OCM+1000Hz. Control samples cultured in growth medium only, scale bar = 80 $\mu$ m.

#### 4.4.2 Focal adhesion kinase activity

Levels of both inactive and active (phosphorylated) focal adhesion kinase were measured and a ratio of these generated to give an indication of actual FAK activity following stimulation by nanovibration. In mESCs the proportion of phosphorylated FAK was highest in 1000Hz, significantly higher than OCM or control wells (Fig. 4.3A). This suggests nanoscale vibrational acts to promote FAK activity, and may be responsible for larger focal adhesion complexes observed in section 4.4.1. The combination of OCM and 1000Hz also appeared to produce higher phosphorylated FAK ratio however statistically significant difference was not observed. This result coincides with intermediate levels of vinculin staining observed under OCM+1000Hz conditions and further links FAK activity to development of focal adhesion structures [181].

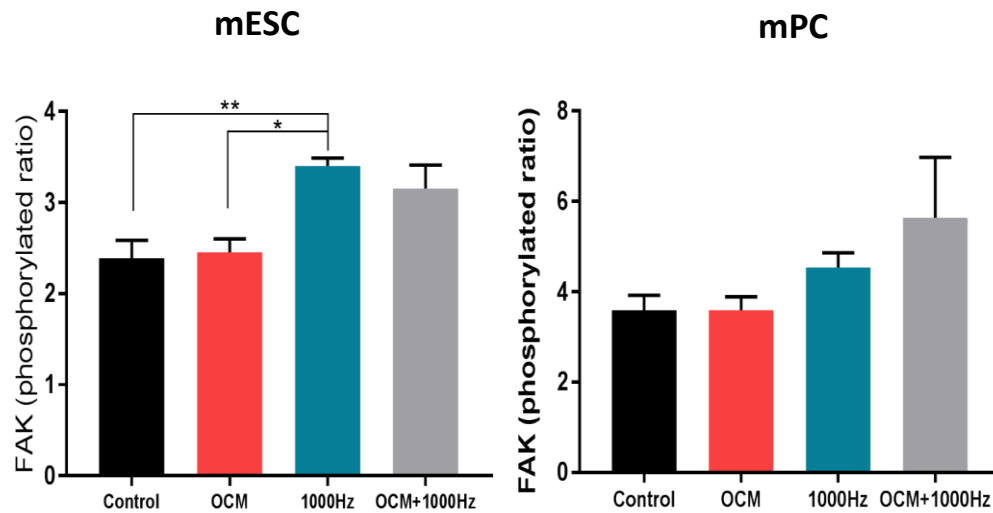


**Figure 4.3 Ratio of phosphorylated FAK**

Focal adhesion kinase activity (ratio of phosphorylated FAK to total FAK) of mESCs and mPCs following 4 day stimulation by OCM, 1000Hz and OCM+1000Hz. Error bars show standard error of the mean, n=4. \* =  $p < 0.015$  \*\* =  $p < 0.0095$ .

In mPCs levels of phosphorylated FAK appeared to be increased compared to controls in response to 1000Hz stimulation however no significant difference was observed (Fig. 4.3B). However similar to the trend seen in mESCs OCM values were nearly identical to control values. It should also be noted that whilst overall levels of phosphorylated FAK were slightly higher compared to the same conditions in mESCs, no significant differences were found.

Conversely to FAK activity, phosphorylated ERK ratio appeared to be lower when either mESCs or mPCs were stimulated by 1000Hz nanovibration (Fig. 4.4), implying lower ERK activity in these conditions. However looking at separate graphs for total ERK and phosphorylated ERK (pERK) revealed increased total ERK following 1000Hz stimulation (Fig. 4.5). This suggests total ERK is in fact promoted by nanovibrational stimulation which may contribute to actual ERK activity at a later timepoint.

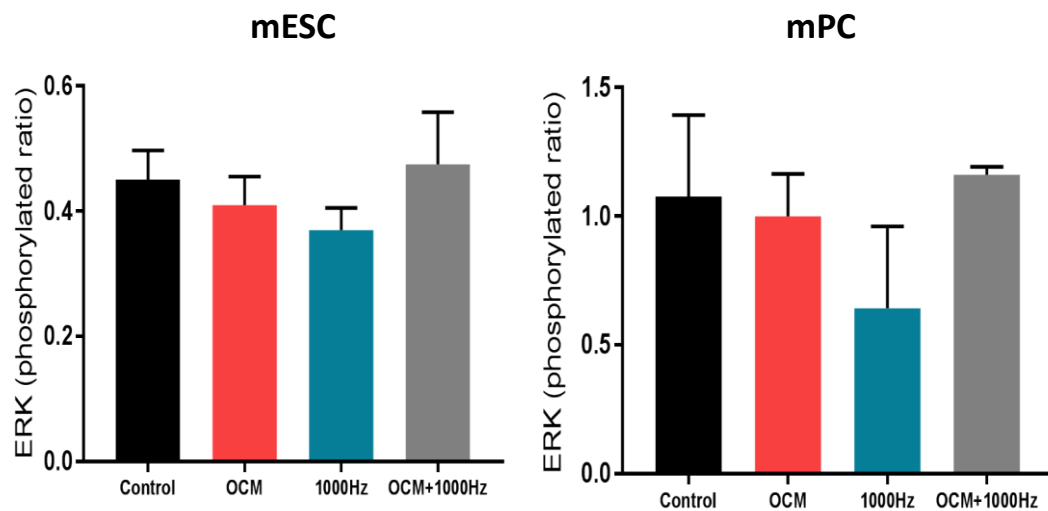


**Figure 4.4 Ratio of phosphorylated ERK1/2**

ERK activity (ratio of phosphorylated ERK to total ERK) of mESCs and mPCs following 4 day stimulation by OCM, 1000Hz and OCM+1000Hz. Error bars show standard error of the mean, n=4.

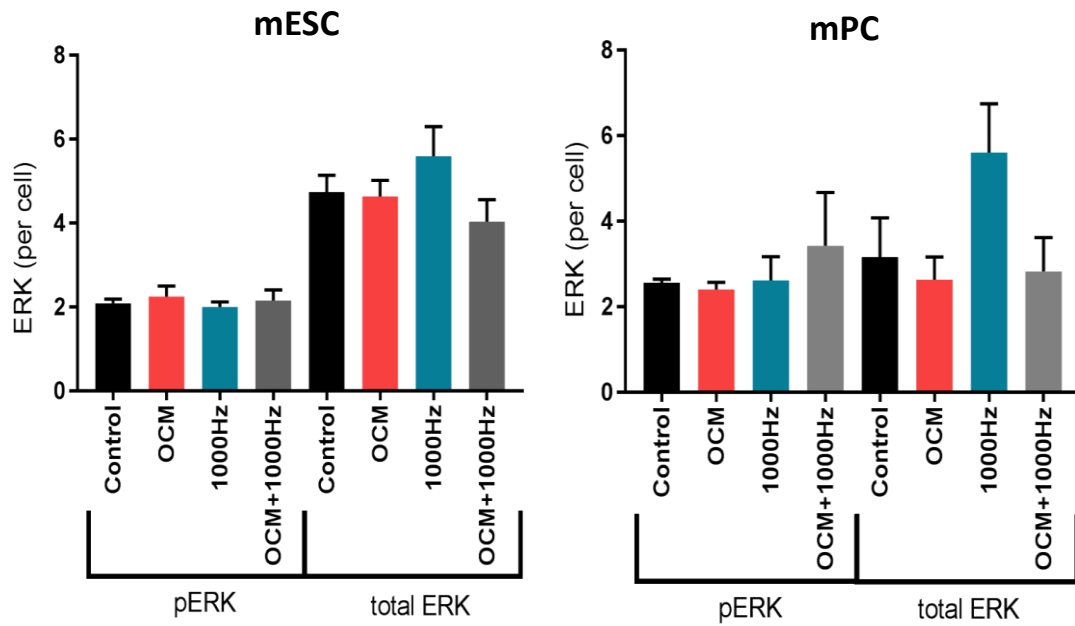


**Figure 4.5 Total and pERK values**

Total and phosphorylated ERK activity calculated per cell of mESCs and mPCs 4 day stimulation by OCM, 1000Hz and OCM+1000Hz. Error bars show standard error of the mean,  $n=4$ .

It seems that nanovibration has a direct effect on focal adhesion kinase activity and may influence ERK1/2 activation downstream as previously observed in osteogenic studies [196] [197]. However it must be noted that only significant upregulation was present in mESC samples, which may indicate FAK activation by nanoscale stimulation is specific to stem cell differentiation or early stages of osteogenesis. However the differences in the two cell types studied here may be due to source of mPCs; a likely pre-osteoblast cell source which are more advanced in osteogenesis development (as described in Figure 1.2) and may already have FAK and ERK1/2 activation triggered during *in vivo* development. This idea is supported by the higher values for ratios of active FAK and ERK observed in mPCs compared to mESCs (Figures 4.3 and 4.4).

### 4.4.3 Metabolic population changes in response to nanovibration

To further understand cellular pathways associated with nanovibrational osteogenesis, total metabolite populations were analysed following OCM, 1000Hz and OCM+1000Hz stimulation whilst comparing to control conditions of unstimulated mESCs. Principal component analysis (PCA) of 7 day mESC and mPC metabolomics results revealed clustering of samples within groups for each culture condition (Fig. 4.6) indicating distinct metabolite populations for each. Of all conditions, control samples showed the greatest inconsistency reflected by further spacing between samples on PCA plot (particularly in mPCs). This suggests a wider range of metabolite activity as expected in cells cultured in growth medium alone without any specific differentiation cues. In both cell types OCM samples were tightly packed suggesting a distinctive metabolite population resulting from cues provided by OCM medium. 1000Hz stimulation caused PCA plot grouping closest to control samples suggesting lower overall change in metabolite activity, whilst 1000Hz+OCM was found in the area between OCM and 1000Hz clusters implying OCM+1000Hz represented a metabolite population consisting of a combination of the two separate conditions.

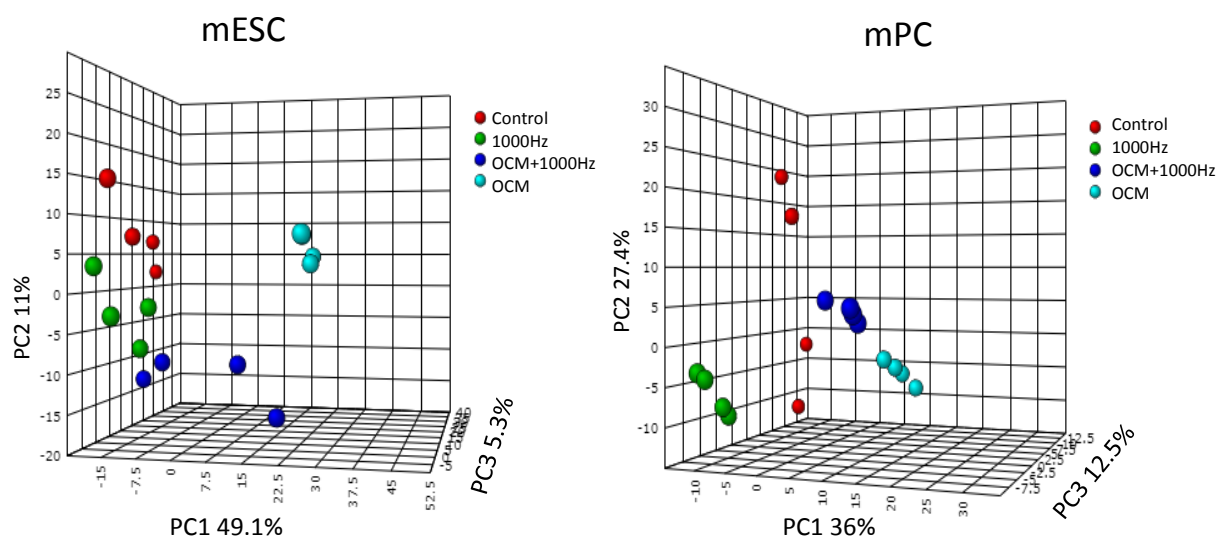


**Figure 4.6 Principal component analysis of metabolomics results**

PCA plots of mESC data (A) and mPC data (B), after at 7 days of stimulation by OCM, 1000Hz nanovibration or OCM+1000Hz. Plotted using Metaboanalyst online resource, n=4.

PCA grouping was slightly broader in mESCs compared to mPCs in all cases except for control, implying that the culture conditions studied here resulted in a wider variation of metabolite activity in ESCs compared to primary calvarial cells. This is to be expected due to the nature of the cell types; ESC differentiation by the techniques used here is likely to cause wider phenotypic variation compared to the culture of a pre-osteoblast cell type and therefore more variation in metabolic activity can be expected.

Heat map plot of mESC metabolite population revealed a range of increased and decreased metabolites varying between conditions however with generally consistent results between repeat samples of the same conditions (Fig. 4.7A). Comparing average change of each group revealed several metabolites highly increased by OCM and decreased by nanovibration and vice versa (Fig 4.7B). Based on this heat map 1000Hz and OCM+1000Hz appeared showed more similarities to each other than between other groups, whilst OCM produced a largely different metabolite population. This suggests 1000Hz stimulation is highly influential over metabolite population even when combined with OCM, directing the metabolite activity towards a similar population to 1000Hz stimulation alone.

mPC heat map revealed higher variation in control sample readings (Fig. 4.8A), consistent with broader spacing observed in PCA plot. Similar to mESCs, OCM appeared to generate a greater change in overall metabolite population in mPCs, with large groups of metabolites drastically increased or decreased. 1000Hz stimulation appeared to generate metabolite populations more highly altered from the control compared to OCM+1000Hz, which had several areas in common with 1000Hz but also OCM suggesting an intermediate response.

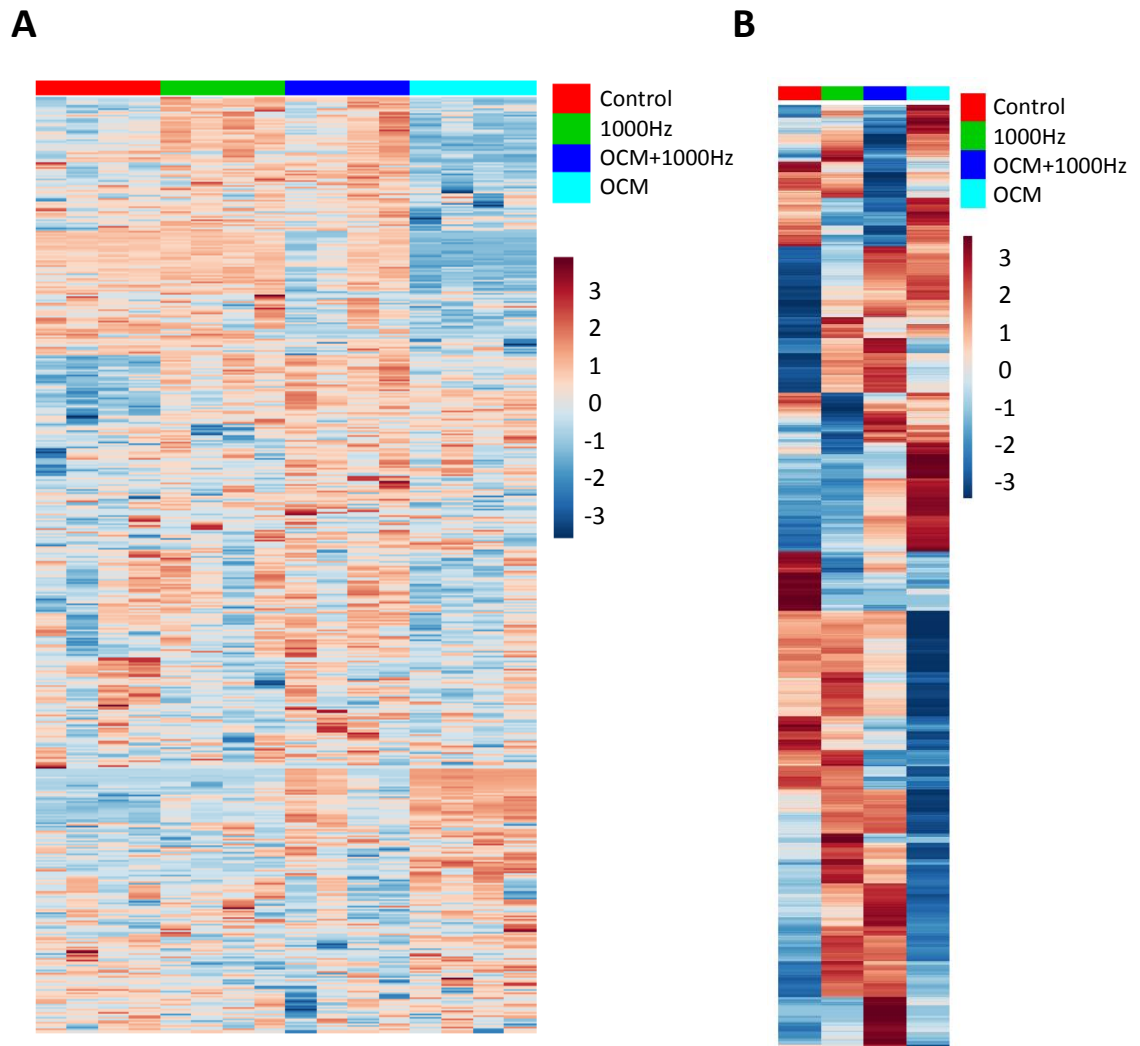


**Figure 4.7 Heat map plots of mESC metabolite analysis**

Heat maps showing relative amount of metabolites detected in mESCs after 7 days of stimulation by OCM, 1000Hz nanovibration or OCM+1000Hz. Red indicating increase in selected metabolite and blue indicating decrease (compared to undifferentiated mESCs at day 0). A: Individual metabolite readings (n=4), B: Average of readings for each metabolite.

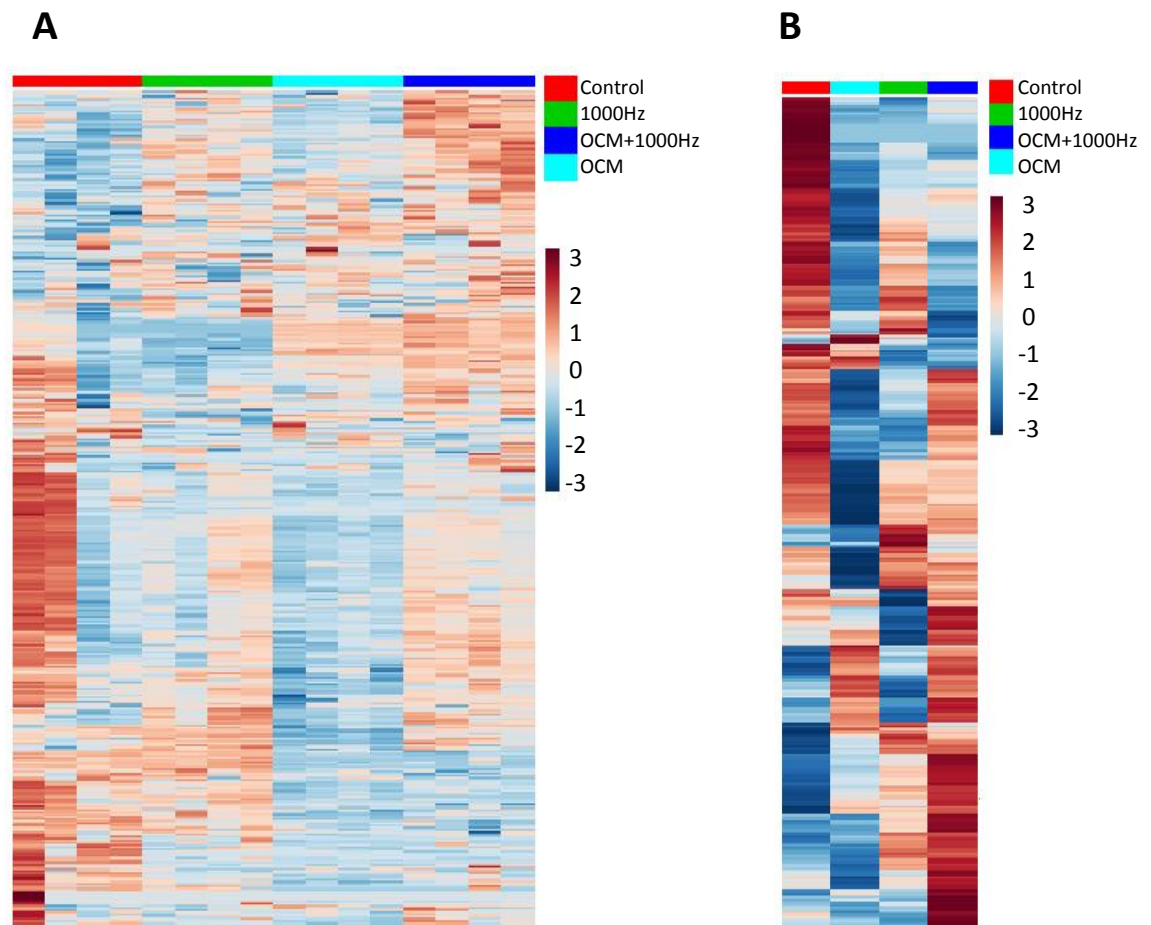


**Figure 4.8 Heat map plots of mPC metabolite analysis**

Heat maps showing relative amount of metabolites detected in mPCs after 7 days of stimulation by OCM, 1000Hz nanovibration or OCM+1000Hz. Red indicating increase in selected metabolite and blue indicating decrease (compared to undifferentiated mPCs at day 0). A: Individual metabolite readings (n=4), B: Average of readings for each metabolite.

Within readings for each metabolite, mPC results appeared to show higher consistency overall compared to mESC variation, corroborating PCA plots. However higher variation in mPC control samples was also observed.

For more informative analysis of cellular metabolomics data, metabolites were grouped based on their main activity upon cell behaviour (as predicted by metabolite identification in IDEOM software). As seen in figure 4.9 a higher proportion of the total mESC metabolite population were altered in response to 7 days OCM stimulation than in response to 1000Hz or OCM+1000Hz (which yielded similar results). This further suggests that OCM has a larger influence on metabolite activity as a whole but the addition of 1000Hz stimulation acts to negate this, bringing levels of metabolic activity back down to levels seen in 1000Hz stimulation alone. Metabolism of carbohydrates was more significantly altered following OCM stimulation implying higher regulation of energetic cell activity. No differences were observed in overall amino acid metabolism. Co-factor and vitamin metabolism was upregulated in 1000Hz stimulation implying higher enzymatic activity of vitamin dependent processes – many of which are linked to bone formation.

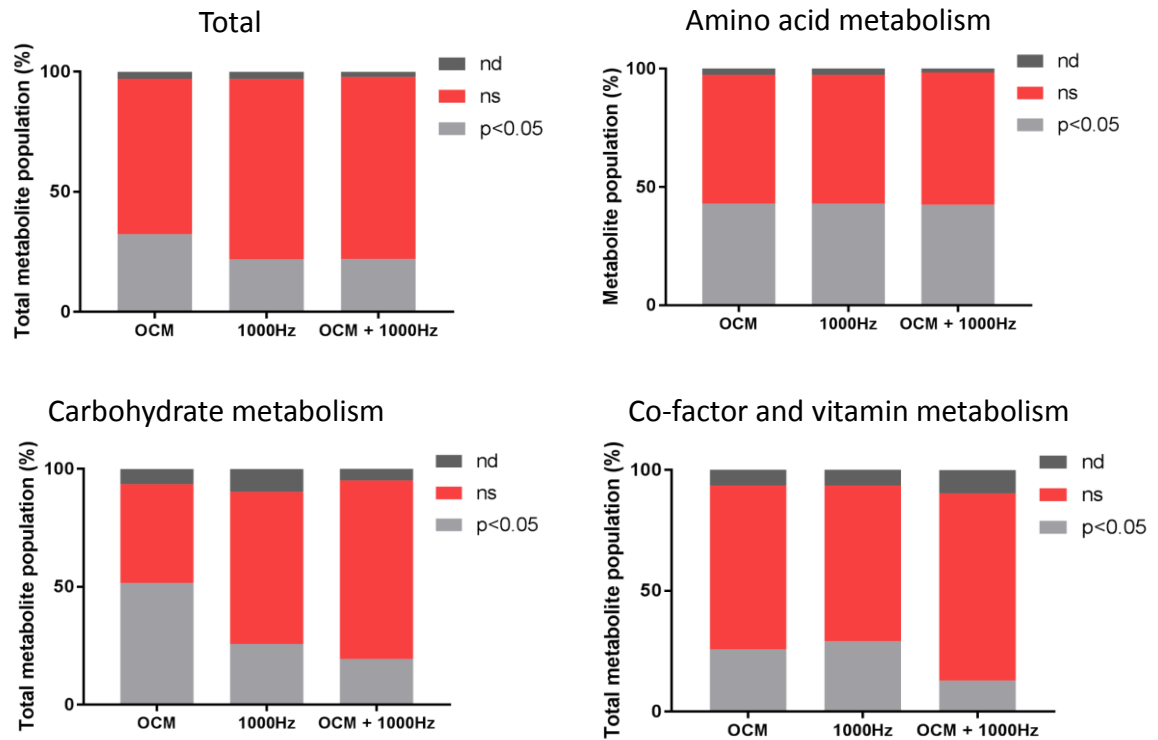


**Figure 4.9 Percentage change of metabolite categories in mESCs**

Percentage of changed metabolites within distinct categories based on metabolite activity in mESCs following culture in by OCM, 1000Hz or OCM+1000Hz for 7 days. Percentage change of metabolite groups compared to control sample at same time point, ns = no significant difference, nd = metabolite not detected.

Proportion of significantly altered metabolites was lower in all mPC categories compared to mESCs (Fig. 4.10), suggesting osteogenic stimulation has less effect on metabolic activity of mPCs. This could be due to mPCs more advanced differentiation state and thus a likely more stable phenotype and thus less changes in metabolite overall activity in response to external stimuli. OCM did still however have a slightly higher overall impact of total metabolite activity compared to nanovibration, as seen in mESCs. OCM did not however result in a large change in carbohydrate metabolism as observed in mESCs, suggesting less overall impact on high energy activities such as differentiation. mPC results also revealed no undetected metabolites in most cases, whilst this accounted for around 5% in each group in mESCs. This group represents metabolites detected in control that were no longer detected in

osteogenically stimulated samples, and may be related to metabolic activity related to multipotency of ESCs. This further corroborates the theory that stem cells produce a detectable population of metabolites that could be used to characterise multipotency [190]. It should be noted however that changes in overall amount of significantly activated metabolites only considers the population or metabolite groups as a whole and does not single out any specific metabolites that are of high importance to osteogenic mechanisms. To assess these specific metabolites Ingenuity Pathway Analysis (IPA) was used.

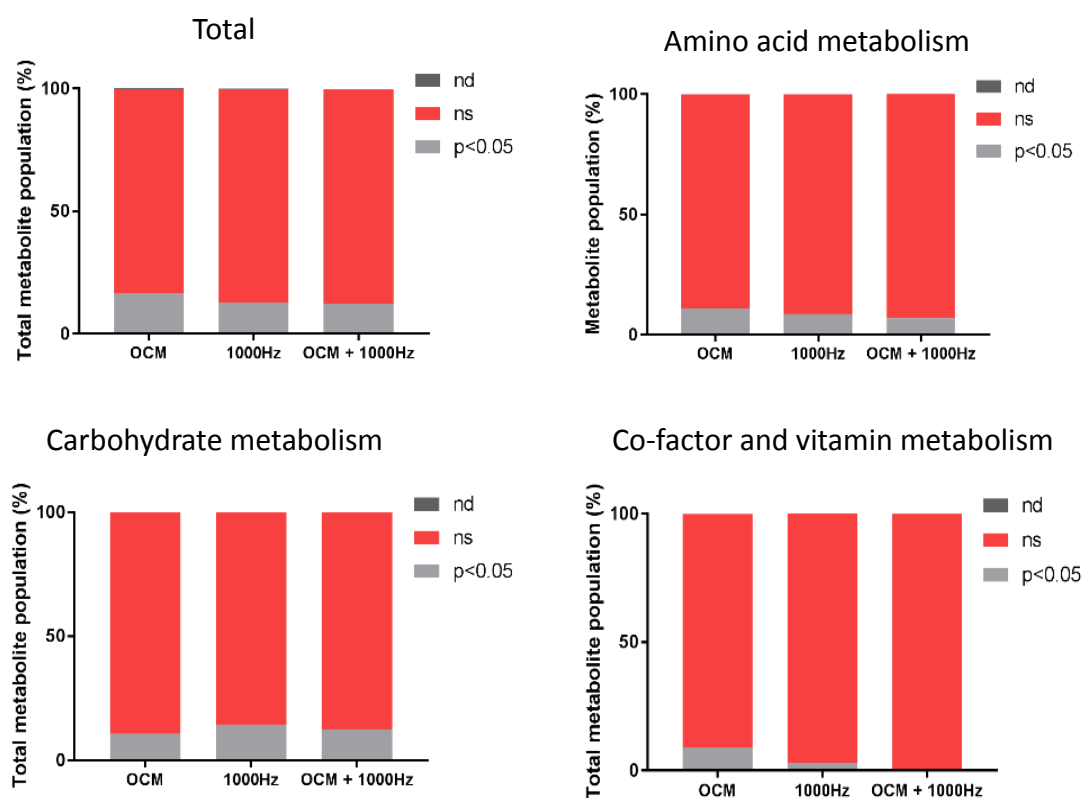


**Figure 4.10 Percentage change of metabolite categories in mPCs**

Percentage of changed metabolites within distinct categories based on metabolite activity in mPCs following culture in by OCM, 1000Hz or OCM+1000Hz for 7 days. Percentage change of metabolite groups compared to control sample at same time point, ns = no significant difference, nd = metabolite not detected.

IPA analysis highlighted upregulation of tRNA charging in both mESCs and mPCs with nanovibration stimulation, implying 1000Hz has a greater effect on RNA translation compared to OCM culture (Figure 4.11). Both cell types also exhibited higher upregulation of metabolic pathways related to glycolysis and Krebs cycle indicating a high energy cell activity in response to 1000Hz stimulation.

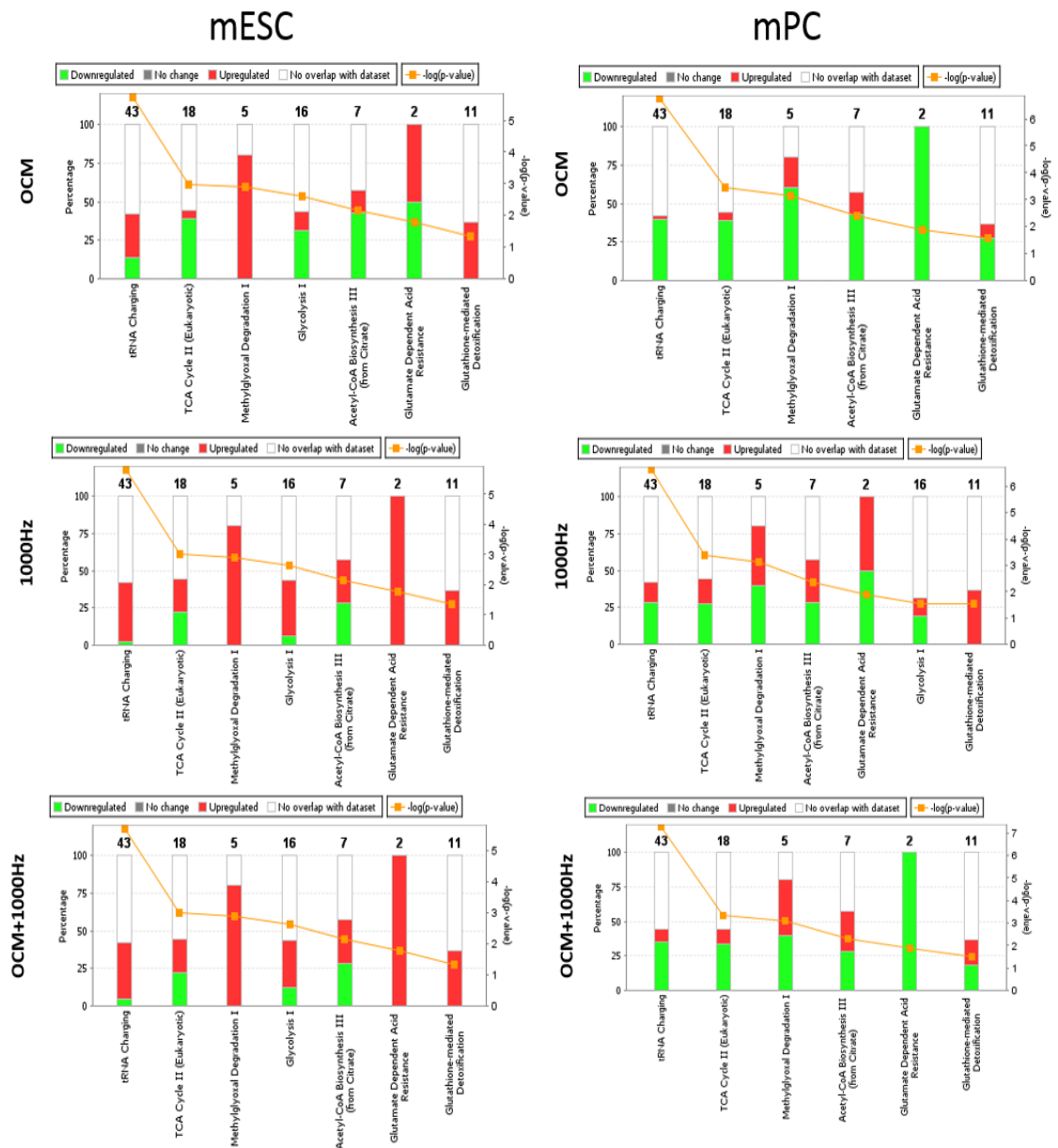


**Figure 4.11 Effect of nanovibration on energy related pathways**

Proportion of up/down regulated metabolites related to specific pathways related to tRNA charging, glycolysis and Krebs cycle, highlighted by IPA.

#### **4.4.4 Predictive metabolic pathway analysis**

IPA organised related metabolites in order to select the most highly influenced cell signalling networks, with ERK/MAPK signalling highlighted in mESC stimulated under all conditions. However as seen figure 4.12 metabolic activity within this network was vastly different between culture conditions. In the case of OCM highly increased measurement of ascorbic acid was observed, as expected due to presence of ascorbic acid with OCM medium. Ascorbic acid increase was also measured in 1000Hz stimulated mESCs however to a lesser degree, and was accompanied by increase in several other metabolites connected to FAK and ERK signalling hubs including L-lysine and riboflavin which were actually decreased following OCM culture. These networks suggest OCM activity on FAK and ERK signalling pathways is largely attributable to ascorbic acid metabolism alone, whilst nanovibration activates these pathways by increasing a wider range of involved metabolites.

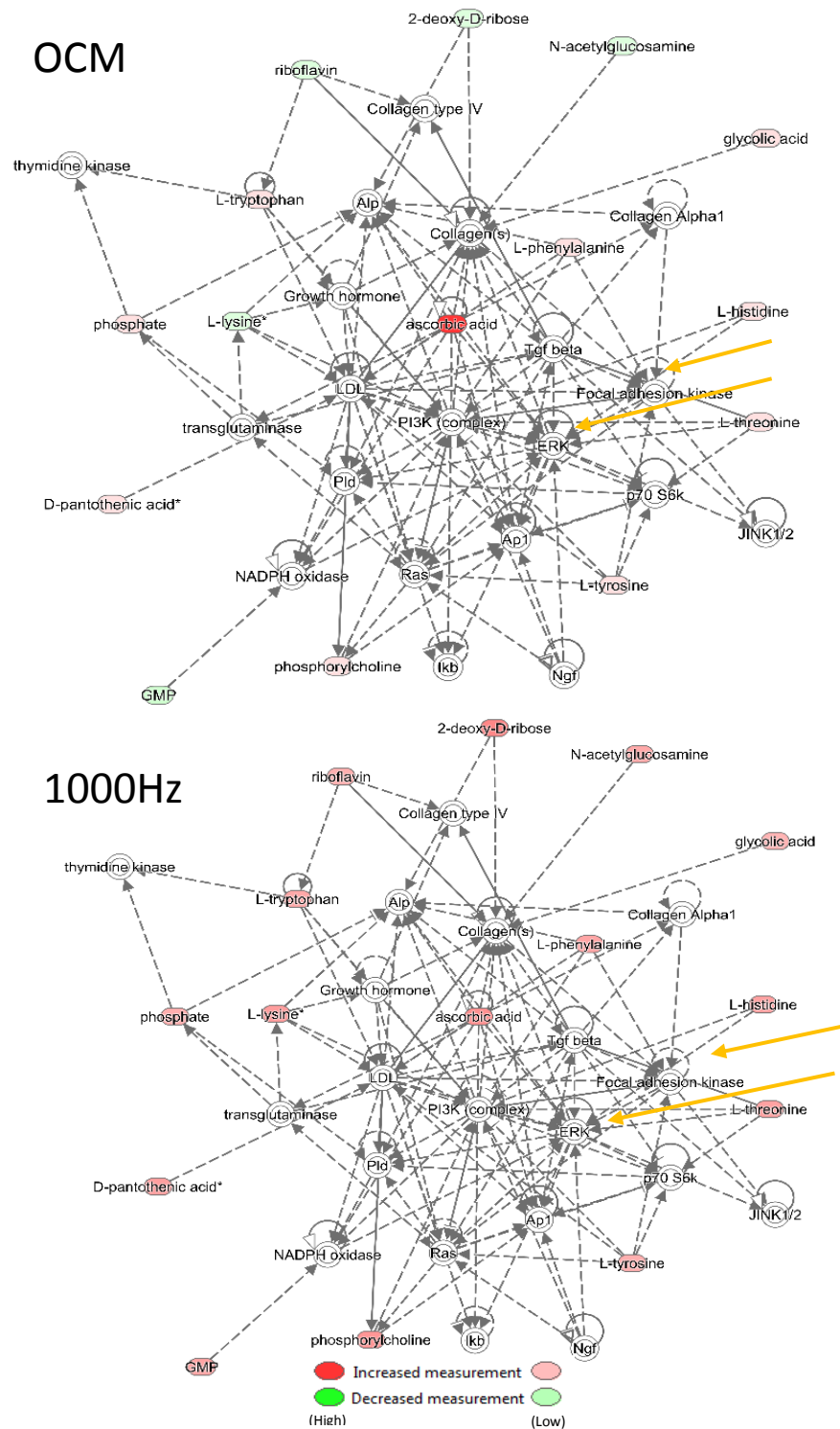


**Figure 4.12 IPA networks involving FAK and ERK signalling hubs**

Key metabolic network highlighted by IPA predictive analysis, involving FAK and ERK signalling hubs (highlighted by yellow arrows). mESCs stimulated for 7 days by OCM or 1000Hz. Red metabolites indicating increased measurement, green decreased measurement compared to control, n=4.

Predictive activity of these signalling hubs (figures 4.13 and 4.14) also highlighted increased ascorbic acid levels in OCM and predicted that this promotes mESC ALP activity and collagens as previously shown [198] [199], contributing to activated FAK signalling hub. FAK hub is also activated by 1000Hz however signalling seems to be associated with a number of metabolites and growth hormone signalling which activates MAPK/ERK pathway and contributes to bone growth via IGF-1 [200] [201], both of which are relatively unchanged by OCM stimulation. These results show activation of FAK activation by different mechanisms depending upon stimuli. OCM+1000Hz showed an intermediate response, with high measurement for ascorbic acid which appeared to dominate the network but also increase of other associated metabolites (Figure 7.5). Interestingly OCM+1000Hz analysis did not highlight FAK signalling hub, but associated metabolites and signalling hubs were implicated.



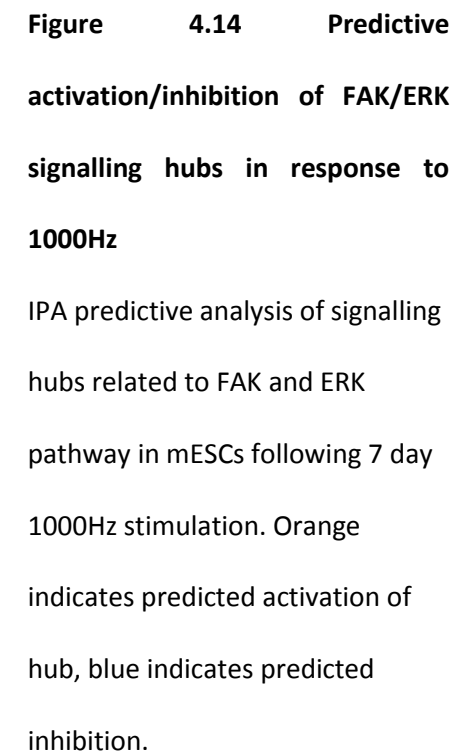
**Figure 4.13 Predictive activation/inhibition of FAK/ERK signalling hubs in response to OCM**  
IPA predictive analysis of

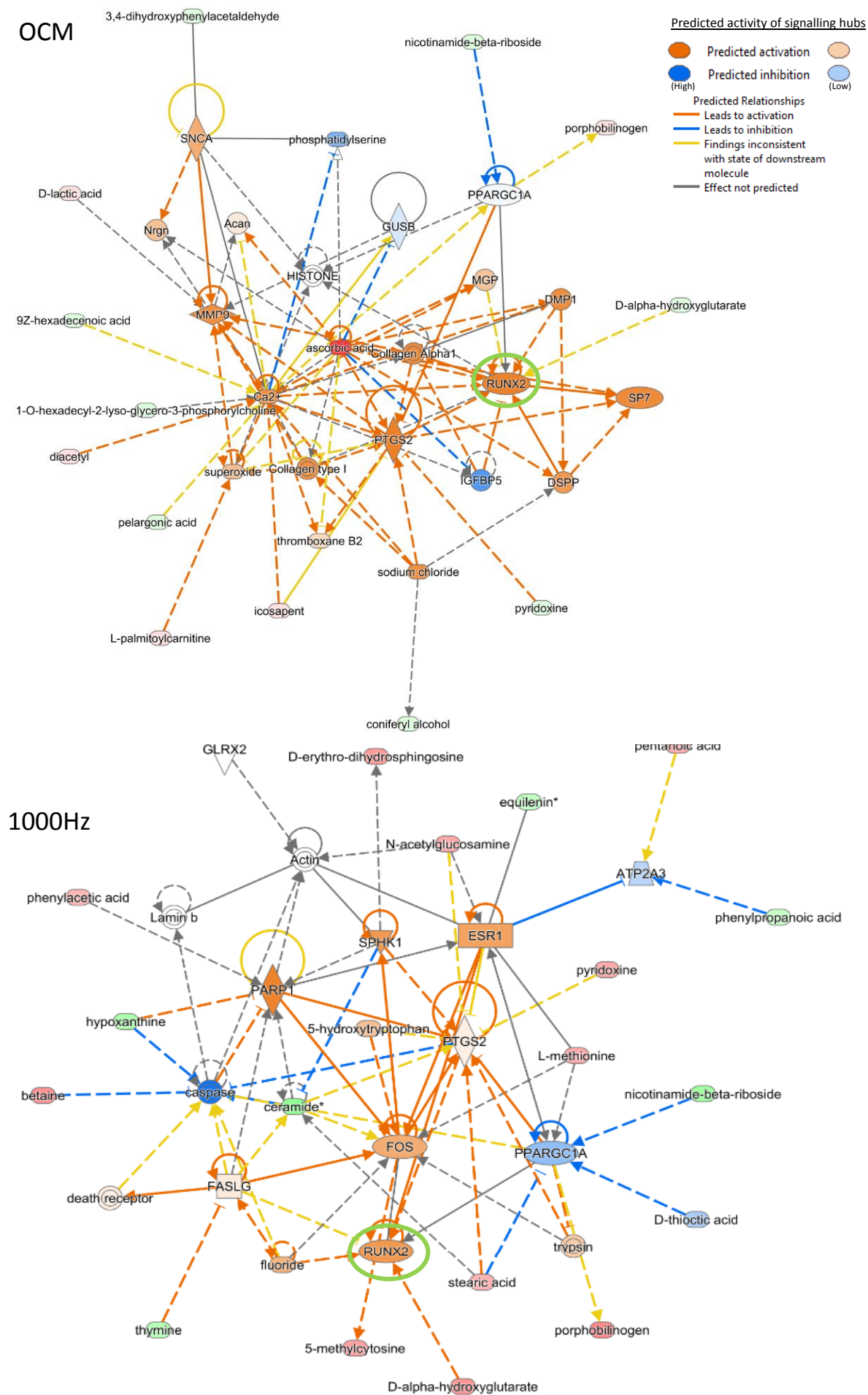
signalling hubs related to FAK and ERK pathway in mESCs following 7 day OCM stimulation. Orange indicates predicted activation of hub, blue indicates predicted inhibition.

IPA analysis also predicted ERK signalling hub activation in both OCM (figures 4.13), however to a lesser extent following 1000Hz (or OCM+1000Hz seen in figure 7.5). RUNX2 signalling sub was highlighted as activated following all culture conditions (Fig. 4.15), confirming downstream effects on osteogenesis. However only OCM+1000Hz revealed increase of bone gamma-carboxyglutamic acid-containing protein (BGLAP), considered a later stage osteogenic marker thus suggesting strong osteogenic induction following OCM+1000Hz stimulation. These results further imply the combination of OCM and nanovibration can improve osteogenic outcomes, consistent with differentiation studies in chapter 3.

Also present in networks highlighted by IPA were c-Jun N-terminal kinase (Jnk1/2) and low-density lipoprotein (LDL) signalling hubs, previously identified as potential effectors of multipotency in MSCs [190]. Modulation of this hubs by nanovibration (Fig 4.14) suggests they also play a role in differentiation of mESCs.

In mPCs focal adhesion kinase signalling was not highlighted by IPA analysis of OCM, 1000Hz or OCM+1000Hz suggesting it is not a major pathway affected in calvarial osteoblasts at this timepoint. This further implies FAK as a regulator of stem cell commitment to osteogenesis at an earlier stage of development, as observed in mESC results. ERK signalling hub was highlighted in mPC analysis but only upregulated in OCM+1000Hz and not by either OCM or 1000Hz alone. This may be connected to downstream osteogenic signalling in agreement with synergistic effect of OCM+1000Hz on mPC behaviour observed in chapter 3 but warrants further investigation.





OCM+1000Hz

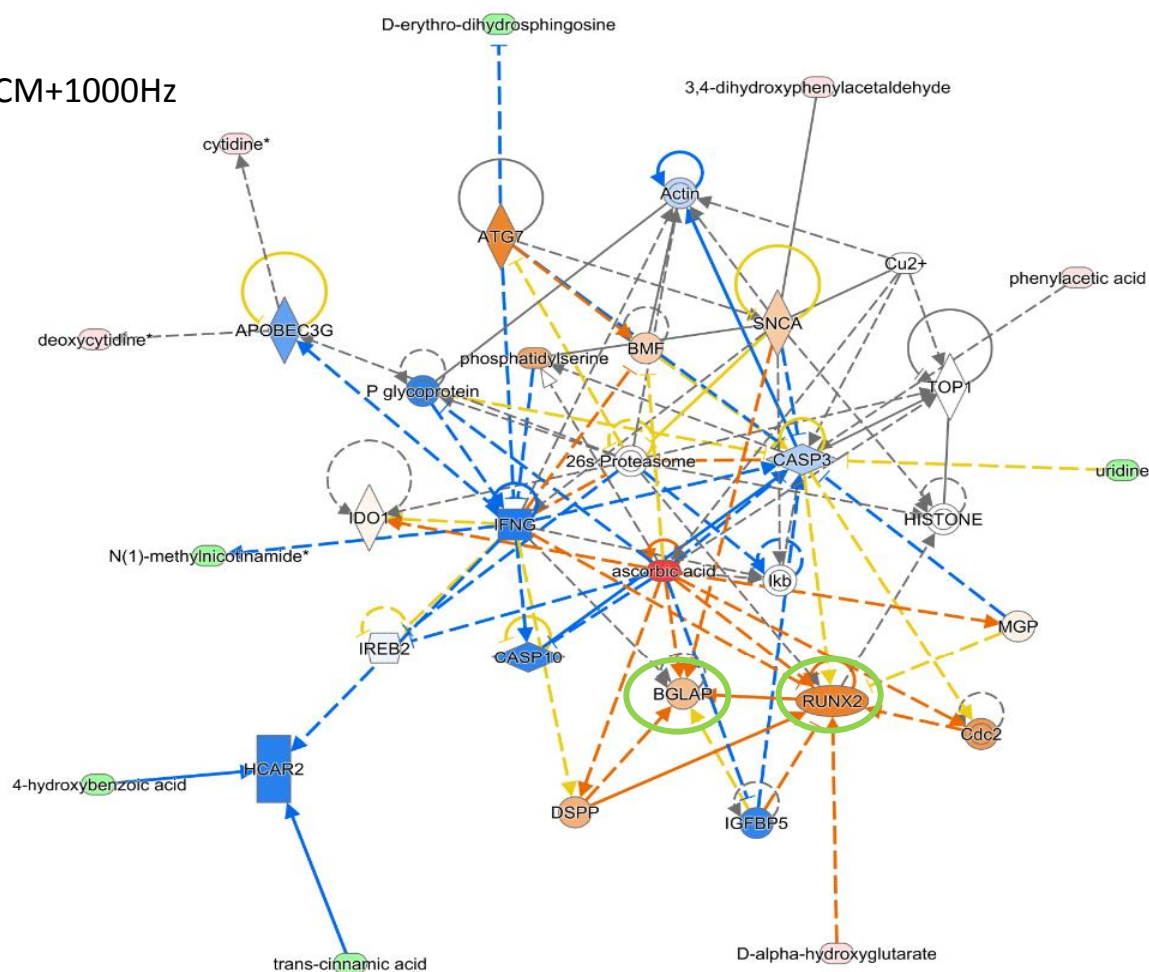


**Figure 4.15 Predictive activity of RUNX2 signalling hubs in mESCs**

IPA predictive analysis of signalling hubs related to RUNX2 pathway (highlighted by green circles) in mESCs following 7 day OCM or OCM+1000Hz stimulation, relative to control. Orange indicates predicted activation of hub, blue indicates predicted inhibition. BGLAP highlighted.

#### 4.4.5 1000Hz stimulation of 3D cell structures

1000Hz stimulation of 3D aggregates was studied in attempt to observe effects of nanovibration in a model more relevant to native cellular environment. Individual mESC cell aggregates were placed into wells and stimulated by 1000Hz vibration or by control (growth medium only). Cell/aggregate morphology was then observed as well as RUNX2 expression to assess osteogenesis of 3D structures.

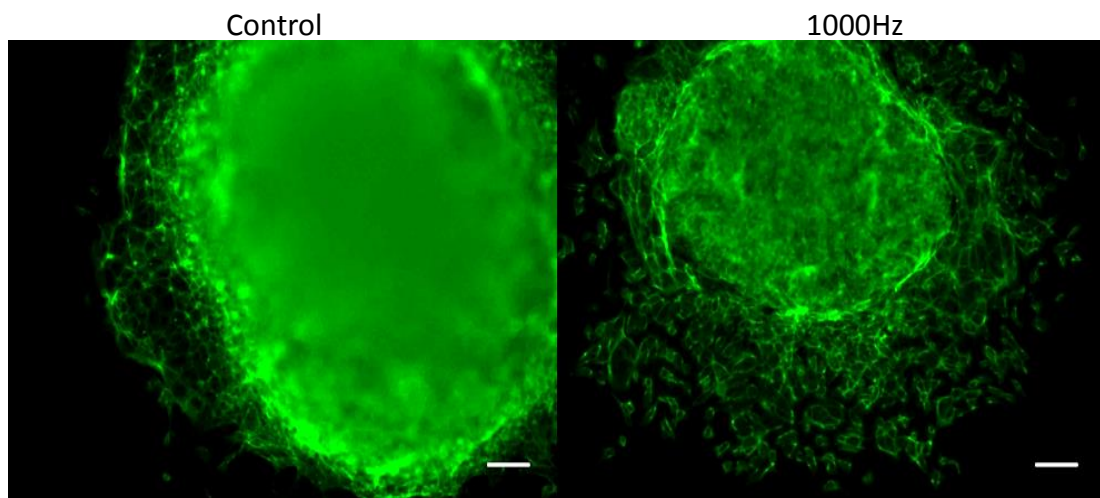


**Figure 4.16 1000Hz stimulation of mESC aggregates during attachment**

mESC aggregates stimulated during attachment to gelatin coated wells over 24 hours. Single aggregates pipetted into wells and allow to attach under normal cell culture conditions (control) or with continuous 1000Hz nanovibration stimulation. Typical images of actin cytoskeletal staining, scale bar = 80 $\mu$ m.

Visualisation of mESC aggregates as they attach to cell culture surface showed that after 24 hours aggregates had clear round morphology, with a small outer region of connected cells spreading out from the aggregate (Fig. 4.16). Stimulation of similar aggregates by 1000Hz nanovibration resulted in similar rounded cores however with noticeably larger area of cells spreading out from the edges of the aggregate (Fig. 4.16). 1000Hz stimulated aggregates also showed separate cells migrating away from the aggregates, not observed under control conditions.

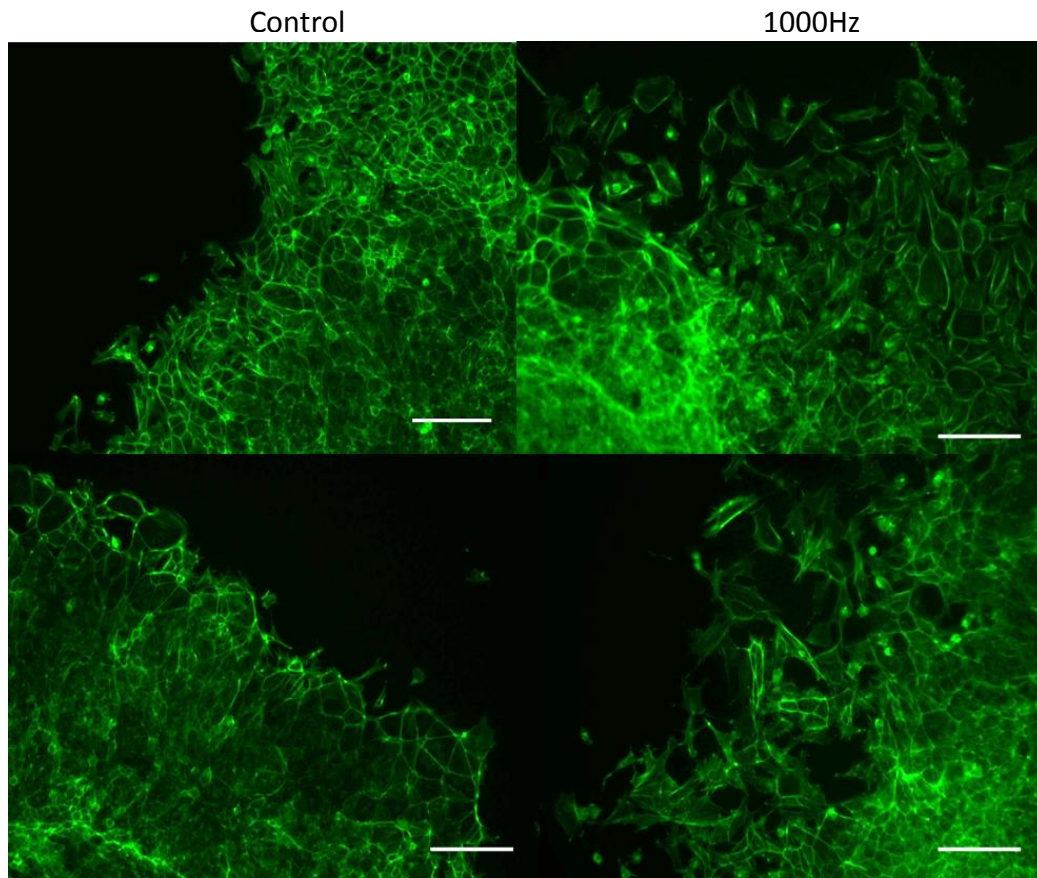


**Figure 4.17 1000Hz stimulation of mESC aggregates**

mESC aggregates allowed to settle and attach to gelatin coated wells for 48hours. Aggregates were then stimulated by 1000Hz nanovibration or left under normal culture conditions (control) for 24 hours. Typical images of actin cytoskeletal staining, scale bar = 80μm.

Allowing aggregates to attach prior to 1000Hz stimulation yielded similar results; as seen in figure 4.17 aggregates stimulated by nanovibration exhibited noticeably more migration of individual cells away from the outer edges of the aggregate. These results imply nanovibration promotes greater interaction of cells with the culture surface resulting in more spreading, also observed during 2D cultures earlier in this chapter and previous studies [110]. This effect may be due to nanovibration promoting maturation of focal adhesion complexes (as previously discussed) which are the anchor points used for cell

migration. The effects of nanovibration on cell migration will be studied in more depth in chapter 5.

Staining for RUNX2 following 1000Hz of mESC aggregates stimulation revealed some differences to the control conditions. RUNX2 staining appeared to be more intense in areas around the periphery of cell aggregates in 1000Hz stimulated samples compared to unstimulated controls (Fig. 4.18). This result suggests 1000Hz has a positive effect on RUNX2 (and therefore osteogenesis) in cellular aggregates as well as effects previously observed in 2D differentiation studies. However at this timepoint RUNX2 staining is localised mainly to cells at aggregate edges which have begun to migrate away from the aggregate and thus the nanovibrational effects are likely to be influencing cells attached in 2D. Further studies to characterise RUNX2 expression of cells throughout the aggregates should be carried out.

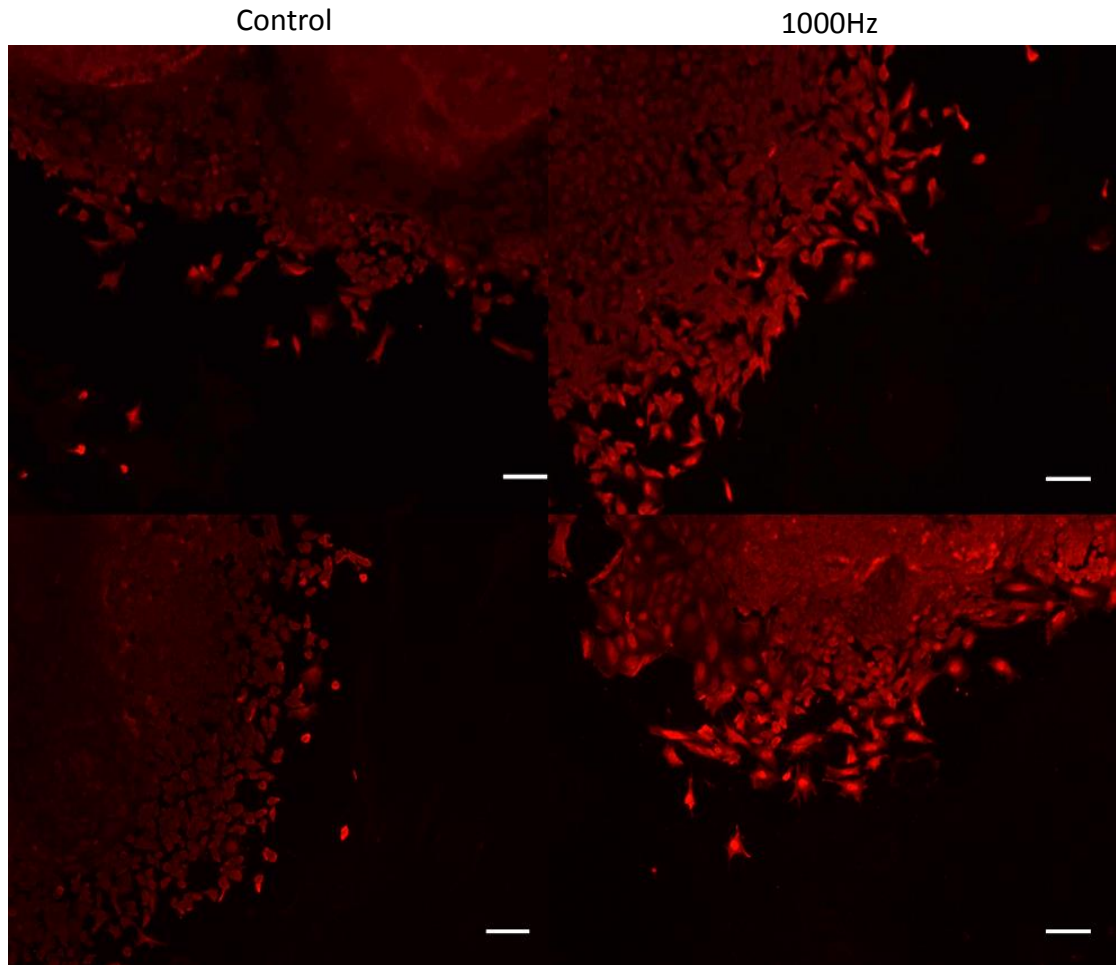


**Figure 4.18 RUNX2 staining of mESC aggregates**

Immunocytochemical staining for RUNX2 following mESC aggregate culture for 4 days by 1000Hz stimulation or unstimulated control. As per differentiation studies, mESC aggregates were allowed to attach overnight prior to osteogenic stimulation. Scale bar = 80 $\mu$ m.

3D aggregate studies appear to corroborate previous 2D culture resulting; suggesting nanovibrational stimulation at 1000Hz influences cell spreading and morphology, as well as promoting focal adhesion activity and subsequently encouraging osteogenesis of mESCs by activation of RUNX2. However it is unclear whether these effects are as a result of changes to cell behaviour within the 3D structure or whether observed differences are limited to cells that have already spread from the aggregate and are experiencing nanovibrational effects in 2D as per work discussed earlier in this chapter. This warrants further investigation and could be assessed by preparing cross sectional slices of fixed aggregates and assessing staining of

osteogenic markers throughout, or by gene level assessment of osteogenic markers. Furthermore, the effects of nanovibration on 3D structures should be compared to effects of osteogenic culture medium in 3D in order to identify mechanisms specific to nanovibration.

## **4.5 Conclusions**

Short term stimulation of both mESCs and mPCs (up to 4 days) revealed obvious changes in cell morphology and spreading as well as focal adhesion size and distribution. It appears these changes are associated with upregulation of FAK activity, particularly in mESC cultures. This highlights the importance of focal adhesion signalling in stem cell differentiation as previously observed [127] [190], whilst downstream effects of ERK1/2 activation were implied however not confirmed by statistical tests in this study.

The FAK signalling hub was also highlighted during metabolomics analysis of mESCs, whilst not present in mPC results suggesting its significance to stem cell differentiation specifically. IPA analysis revealed a range of factors related to FAK and MAP/ERK pathways affected by mESC nanovibration, implicating downstream activation of growth hormone and other pathways related to bone development [202] [203]. Whilst osteogenic culture medium also appeared to influence MAPK/ERK signalling hub, these effects were limited to ascorbic acid mediation of ALP activity and collagen synthesis, not a wide range of associated metabolites as shown to be influenced by nanovibration. RUNX2 activation was confirmed in all conditions confirming downstream effects on osteogenesis.

Nanovibration was also implicated in other signalling hubs such as LDL and Jnk, previously identified and mediator of MSC multipotency by Tsimbouri *et al.* (2012). These signalling hubs may be useful in characterisation of multipotency in stem cells and monitoring differentiation, however further studies should be carried out to confirm their involvement in mESC differentiation specifically. Nanovibration effects on energy production and RNA

translation metabolomic pathways were also observed, possibly connected to downstream differentiation events.

This work also briefly shows the effect of nanovibration on 3D cell aggregates, in which 1000Hz stimulation appears to promote aggregate spreading and elevation of key osteogenic marker RUNX2, however this 3D model requires further investigation.

These results suggest nanovibration promotes osteogenesis by controlling cell morphology and adhesion, whilst directing the metabolite population through multipotency regulators and promoting osteogenesis via key signalling hubs including focal adhesion kinase. These mechanisms may contribute to better *in vitro* osteogenic outcomes as observed in chapter 3. These results also further emphasise the differences in osteogenic outcomes between cell sources; with marked difference in differentiated ESCs compared to calvarial osteoblast cultures.

## **Chapter 5 - Effects of nanovibration stimulation on chemotactic behavior of osteoblasts**

### **5.1 Introduction**

#### **5.1.1 Overview**

In order for regenerative therapies to be successful, the cells used need to mimic osteoblast activity to promote repair *in vivo*. As described in chapter 1, recruitment of osteoblasts (and their progenitors) is a key part of bone remodeling; however this behavior of osteoblasts is less studied *in vitro*.

Platelet derived growth factor BB (PDGF-BB) has been identified as a potent chemoattractant for osteoblast cells [139]. It has also been shown that the response of primary calvarial osteoblasts to PDGF-BB differs between populations extracted at different times of calvarial development [144], suggesting that level of chemotactic response is associated to the development state of osteoblasts. To further explore this, this chapter in part studies the chemotactic response of osteoblasts at various stages of *in vitro* differentiation. However as shown in chapter 3, there are many factors which contribute to the differentiation of osteoblasts *in vitro*. For this reason this chapter also studies the influence of nanoscale mechanotransduction on the development of chemotactic behaviour.

#### **5.1.2 Mechanics of cell migration**

Cell migration in response to a migratory stimulus is facilitated by extension of cell protrusions in direction of migration. These protrusions can be large lamellipodia or smaller filipodia, but are typically driven by the restructuring and polymerisation of actin filaments within the cell [204]. The tensegrity model theorises that alignment of actin filaments also generates tension within the cell (as discussed in section 1.4) to allow force to be transmitted and thus to facilitate migration. Integrins at the face of protrusions attach to ECM molecules

creating traction points for cell movement which can detach at the rear of the cell to allow directional movement [205].

As discussed in chapter 4, focal adhesion (FA) activity is associated to cell behaviour and differentiation. The number and distribution of FAs is linked to stem cell fate [172], with mature osteoblasts having larger 'mature' FAs compared to MSCs [190] [206]. Since FAs act as anchor points to the cell matrix and allow migration by applied tension, changes in FA number and morphology during osteogenesis also has implications on osteoblast motility [182]. This would suggest changes in FA distribution in response to nanovibration observed in the previous chapter would also influence migratory behaviour.

### 5.1.3 *In vitro* chemotaxis methods

The Boyden chamber assay has been used to study migratory behaviours of many cell types *in vitro* [207], relying upon the migration of cells through a porous membrane towards a reservoir containing a migratory stimulus, as seen in figure 3. During the Boyden assay cells are seeded into the upper compartment, which migrate through the membrane towards the chemoattractant however remain attached to the underside of the membrane [208]. Migrated cells can then be counted as an indicator of the relative amount of cell migration occurring.

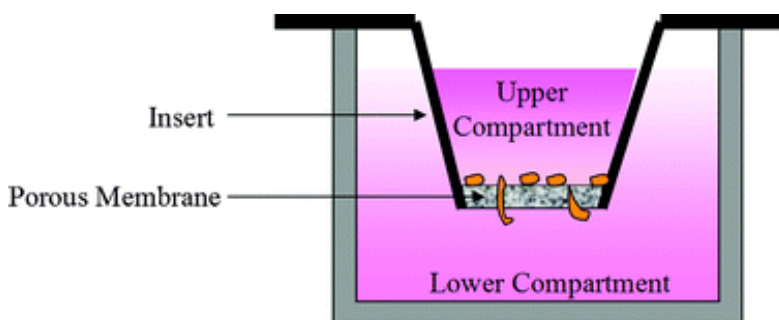


**Figure 5.1 Boyden chamber**

Schematic of Boyden assay set up [207], cells pictured in orange can be seen migrating through the porous membrane.

The Boyden is a simple, quantitative measure of chemotaxis, however, gradients in this model can be unstable due to fluid level imbalances between the two chambers [209]. Whilst chemotaxis is observed, the gradient is also hard to quantify and the assay does not allow direct, real-time observation of cells. Other multi-chamber assays such as Dunn chamber have been described, in which cell migrate over a 'bridge' between channels and suffer the same drawbacks. A number of migration studies based of adherence to 2D surfaces have also been developed, including 2D chemotaxis slides (Ibidi). As seen in figure 5.2, the Ibidi chemotaxis slide relies on the adherence of cells to a central chamber between two fluidic reservoirs. A chemoattractant is added to one reservoir in order to create a gradient to which cells respond. Cell migration can then be observed on time using time-lapse microscopy, allowing visualisation of cells during migration.

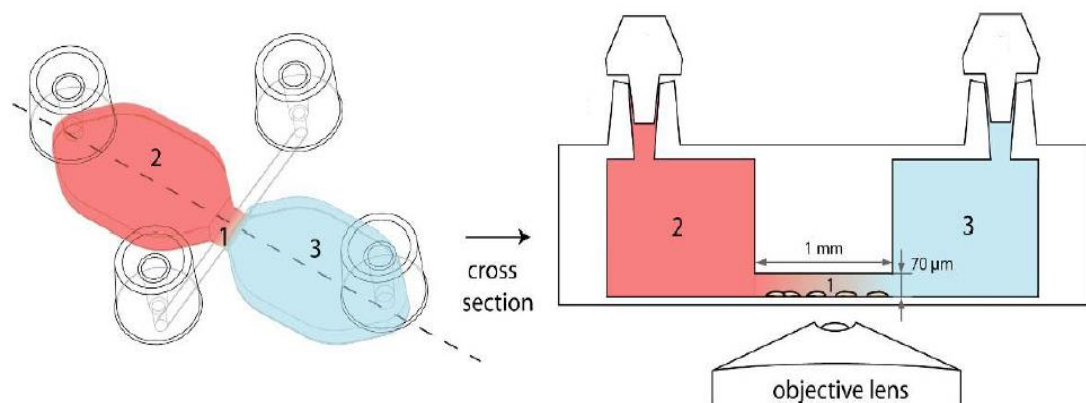
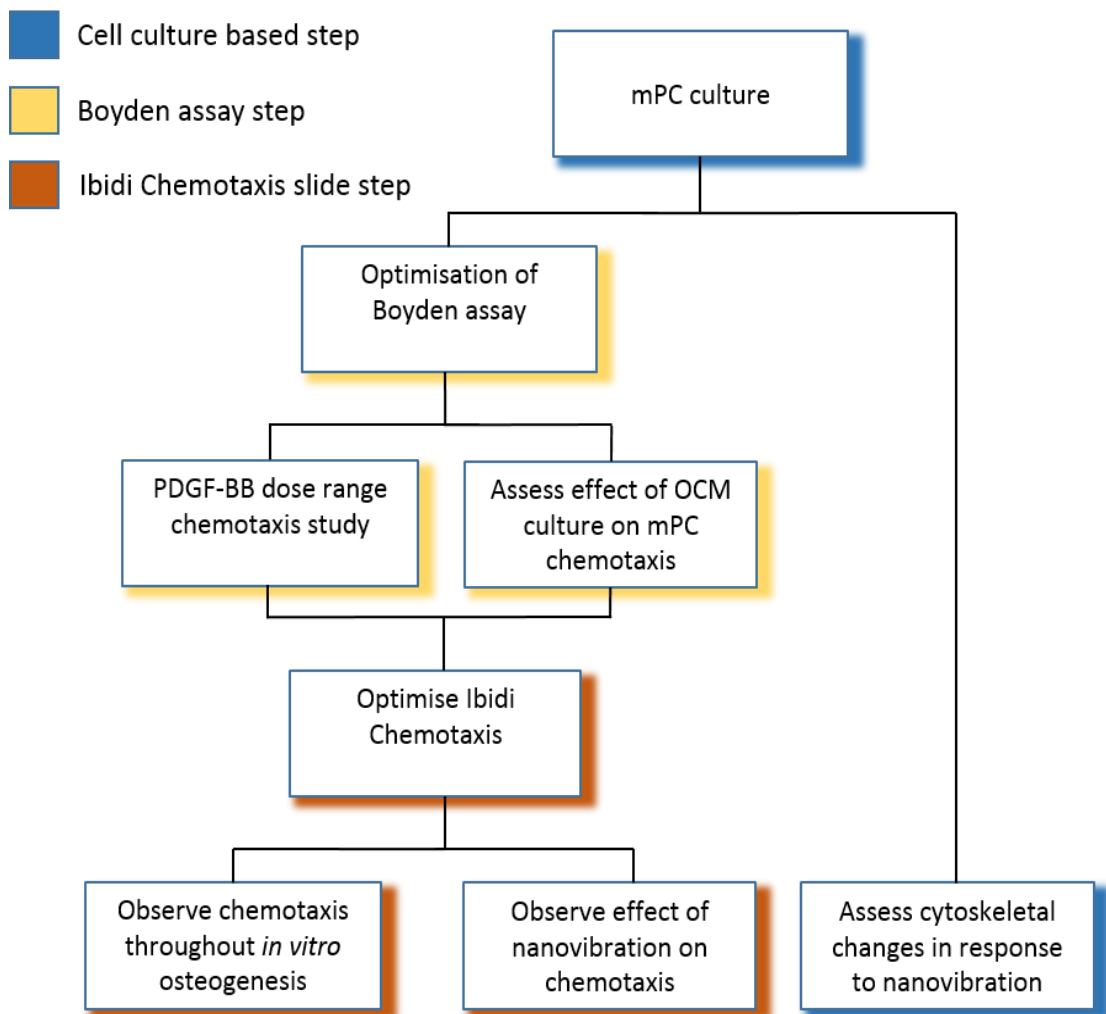


**Figure 5.2 Ibidi Chemotaxis Slide layout**

Schematic of 2D chemotaxis slides. Cells seeded into central chamber (1) between two reservoirs (2 and 3).

## 5.2 Objectives and experimental design

The overall aim of this chapter was to characterize the ability of osteoblasts to migrate towards a chemotactic stimulus, considering differentiation method (including nanovibration stimulation) and time in culture as factors that would affect this. The mechanisms by which migration occurs would also be observed. To this end the chapter follows the experimental plan depicted below.



## **5.3 Methods**

### **5.3.1 mPC culture**

For chemotaxis experiments, mouse primary calvarial cells were selected due to their differentiation profile observed in chapter 3. In their extracted state they display typical markers of early stage osteoblast cell type however can be induced to form mature mineralised osteoblasts under osteogenic conditions, allowing us to explore chemotactic behaviour throughout differentiation. mPCs also readily adhere to culture substrates with no need for additional coating, making them easily applicable to chemotaxis protocols. mPCs were cultured as per sections 2.2.1.i selecting cells at passage 3 in all cases (in some cases osteogenically stimulated as per section 2.2.2) until seeding for chemotaxis experiments described below.

### **5.3.2 Chemotaxis assays**

#### **5.3.2.i Boyden Chamber Assay**

A Boyden chamber style assay was used to assess chemotactic response of mouse primary calvarial cells towards PDGF-BB. This method was used in order to provide a high-throughput, well-established method to assess chemotaxis in response to a range of PDGF-BB concentrations. Hanging cell culture inserts (MCEP24H48, Millipore) were placed into 24 well plates, and cells seeded into the upper chamber (inside the insert) in growth medium. Cells were allowed to settle/ adhere for 2 hours before hanging inserts were moved into an adjacent well containing culture media with/without the relevant chemoattractant. Cells were then allowed to migrate for up to 24 hours. Hanging inserts were then removed and washed twice in PBS and cells were removed from the upper chamber via wiping with cotton buds. Cells that had migrated through the porous membrane and adhered to the underside of the membrane were then fixed in formalin for 15 minutes. Cells were then stained in Hoechst nuclear stain (ThermoFisher) for 30 minutes, and the number of cells migrated onto

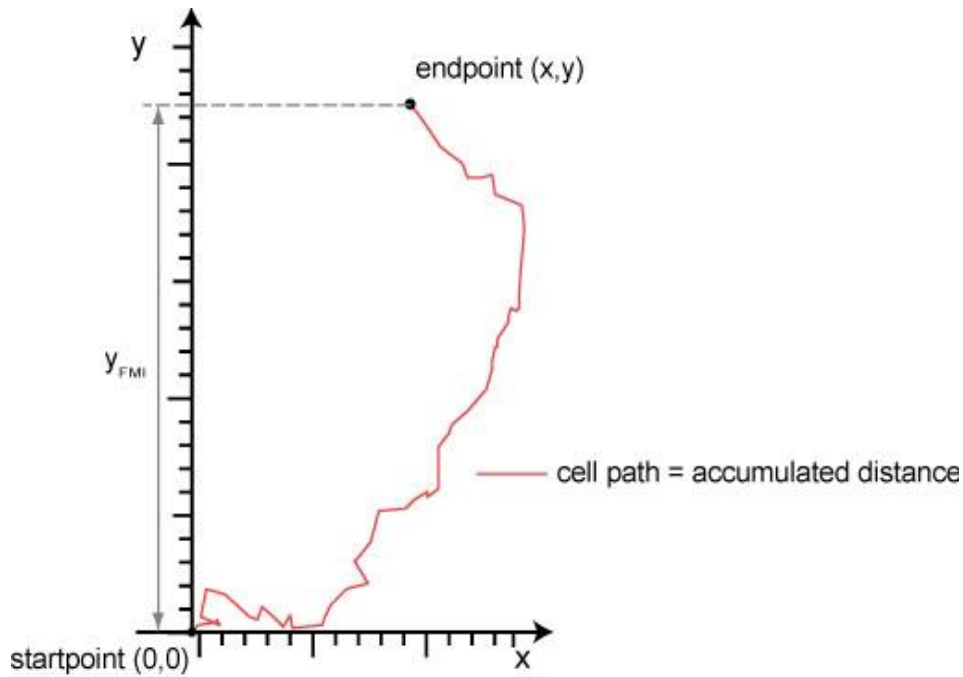
the underside of the membrane was counted. Chemotactic index (CI) was calculated as the number of cells migrated in experimental sample divided by the number of cells migrated in the control sample. Chemoattractants employed was PDGF-BB (R&D systems).

#### 5.3.2.ii Chemotaxis slides

Chemotaxis slides (Ibidi) were used to evaluate differences in migratory behaviour between different osteogenic conditions. Cells were trypsinized and counted, then resuspended to produce a solution of  $1.5 \times 10^6$  cell/ml. 6  $\mu$ l of this suspension was then added into the central channel (Fig. 5.2, position 1) by pipetting onto one filling port and aspirating 6  $\mu$ l from the other port to draw the cell suspension into the chamber. Cells were then allowed to adhere at 37°C for 4 hours. Reservoirs (Fig. 5.2, positions 2 and 3) were then filled with 80  $\mu$ l of mPC growth media of each side, before adding 18  $\mu$ l of 25nM PDGF-BB solution to one side. This concentration yields 10nM PDGF-BB within in the chamber, generating a gradient across the cell chamber suitable for mPC chemotactic response. In control samples both fluid chambers contained only cell culture medium.

#### 5.3.2.iii Cell tracking and analysis

Movement of cells within the central chamber of the Ibidi chemotaxis slide was observed using phase contrast time lapse microscopy with a Leica DMRBE inverted microscope (Leica, UK). Volocity software (PerkinElmer, UK) was used to capture images every 30 minutes for 24hours. Movement of individual cells were tracked using MtrackJ plugin in ImageJ, producing plots of individual cell paths. Cell paths were then inputted into the 'Chemotaxis tool' ImageJ Plugin (Ibidi) for migration analysis. Rose plots were generated which display overall magnitude and direction of migration, whilst forward migration index (FMI) was also calculated as per figure 5.3.



#### FMI (Forward Migration Index):

$$x\ FMI = \frac{x_{FMI}}{\text{accumulated distance}} \quad y\ FMI = \frac{y_{FMI}}{\text{accumulated distance}}$$

**Figure 5.3 Forward migration index calculation**

Calculation of forward migration index (FMI). Diagram shows a typical cell path, equations describes how xFMI and yFMI values are calculated from accumulated distance and  $X_{FMI}/Y_{FMI}$  values.

#### 5.3.3 Cytoskeletal staining

Actin cytoskeleton was visualised by staining with Alexa Fluor 488 Phalloidin (Thermo, A12379). Cells fixed in 3.7% paraformaldehyde were permeabilised using triton X-100 for 15 minutes, washed in PBS then stained in 165nM Phalloidin for 20 minutes. Cell nuclei were also stained with 1 $\mu$ g/ml Hoechst (Sigma).

## **5.4 Results and discussion**

### **5.4.1 Boyden assay chemotaxis**

Boyden assay was used to confirm chemotactic response to PDGF-BB. To test suitability of cell culture inserts for Boyden assay, mPCs were allowed to attach to membranes before being removed by scraping topside of membrane with sterilised cotton buds. As seen in figure 4, mPCs were able to attach to the membrane and were also removed by wiping the upper side of the membrane. This allows removal of non-migrated cells during Boyden assay.

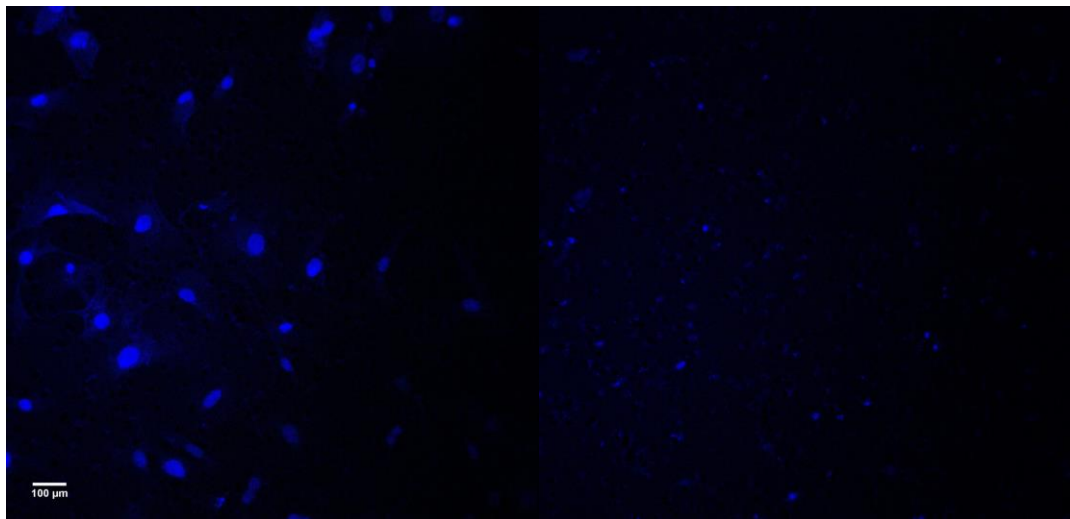


**Figure 5.4 Optimisation of Boyden assay**

Images of transwell membranes during mPC Boyden assay optimisation; cells attached (left) and cells wiped from membrane (right). Scale = 100μm.

As per manufacturer's instructions (Chemotaxis guide, Millipore) transwell cell culture inserts with 8μm pore size were selected for mPC experiments. Time required for cells to migrate through membranes during Boyden assay varies depending upon the cell type and chemoattractant [208], experiments typically running for 4-24 hours for osteoblast-like cells [139] [142] [147] [210]. Since mPCs are relatively slow moving cells in culture, 24 hours was selected for Boyden assay experiment in order to give sufficient time for migration to occur. Chemotaxis of mPCs was then tested using a range of PDGF-BB concentrations, choosing an

intermediate value of 10nM which is known to cause chemotaxis [211] [212]. As seen in figure 5.5, 1ng/ml produced chemotactic index of  $1.3 \pm 0.09$ , not significantly higher than control samples suggested this concentration is too low to promote chemotaxis in mPCs. 10nM PDGF-BB however was sufficient to cause mPC migration, with chemotactic index of  $2.27 \pm 0.2$ , statistically significant difference to controls. Similar levels of chemotaxis observed at 100ng/ml ( $2.58 \pm 0.13$ ). For this reason, 10ng/ml PDGF-BB was deemed sufficient to promote chemotaxis in mPCs and chosen for all subsequent chemotaxis experiments.

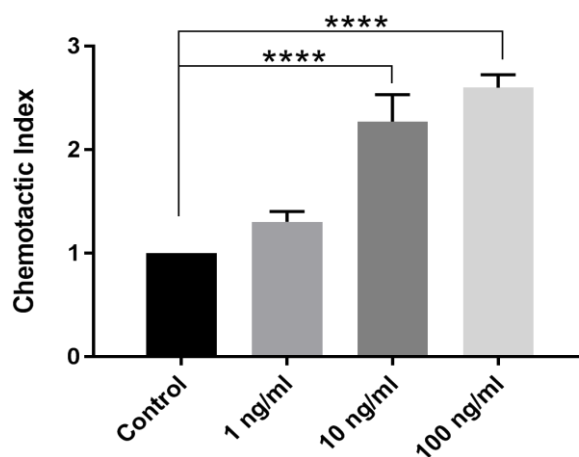


**Figure 5.5 Effect of PDGF-BB concentration on mPC chemotactic index**

Chemotaxis index of mPCs in response to PDGF-BB, 24 hour Boyden chamber assay. Error bars show standard error of the mean, n=6. \*\*\*\* =  $p < 0.0001$ .

The effect of osteogenic culture on chemotaxis was initially assessed by Boyden assay, by culturing mPCs in OCM for up to 7 days and subsequently assessing chemotactic response to 10nM PDGF-BB. As seen in figure 5.6, nascent (0 days) mPCs and those cultured in OCM for 3 days showed a chemotactic response, with chemotactic index of  $2.7 \pm 0.23$  and  $3.1 \pm 0.16$  respectively. Culture in OCM for 7 days resulted in an increase in response to PDGF-BB, with chemotactic index of  $5.1 \pm 0.18$ . These results suggest 3 days culture in OCM is not enough to significantly alter mPC chemotactic response to PDGF-BB, however 7 days of OCM culture

primes cells to migration towards PDGF-BB. It appears osteogenic culture promotes cell migration, the mechanism by which this happens is discussed later in this chapter.

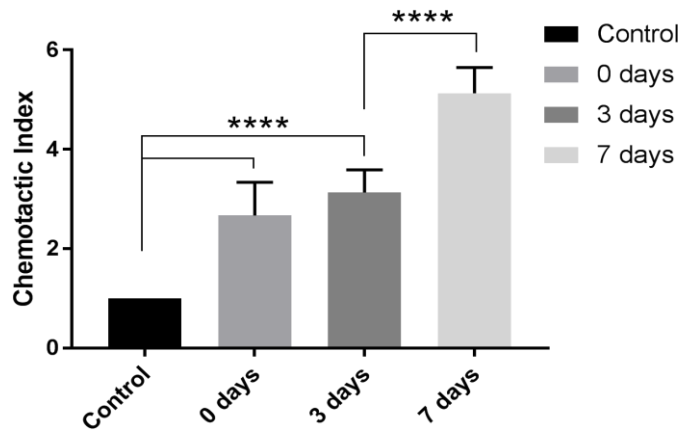


**Figure 5.6 Chemotactic index of mPCs following osteogenic culture**

Chemotactic index of mPCs towards PDGF-BB during osteogenic differentiation. mPCs cultured in osteogenic medium for up to 7 days, 0 day mPCs were cultured in growth medium only, control not exposed to PDGF-BB. Error bars show standard error of the mean,  $n=6$ . \*\*\*\* =  $p < 0.0004$ .

#### 5.4.2 Chemotaxis by Ibidi slides

Boyden assay experiments showed the chemotactic response of mPCs to PDGF-BB, and confirmed that culture in osteogenic medium affects their chemotactic behaviour. In order to assess cell migration in more detail, Ibidi chemotaxis slides were used. Ibidi chemotaxis slides allow the application of a stable chemokine gradient to cells adhered cells, and the live imaging of the cellular response to the imposed gradient.

##### 5.4.2.i Ibidi slide optimization

To confirm mPCs were migratory in the Ibidi 2D slide assay, 7-day OCM mPCs were tested in response to 10nM PDGF-BB due to their strong response under these conditions during Boyden assay studies. Tracking of individual cell paths and plots using Ibidi chemotaxis tool generated tracks that display movement of each cell over the 24 hour study, with net

movement towards stimulus (left to right) depicted in red, and net movement away from stimulus depicted in black. mPCs cultured in osteogenic medium for 7 days showed a trend of movement towards stimulus, with 17 out of 20 tracked cells showing a net movement from left to right and a final centre of mass (average final cell position) that was 90 $\mu$ m to the right of the starting position (Figure 5.7A). Conversely, when no PDGF-BB was added mPCs showed some movement however in no particular direction (Figure 5.7B) and final centre of mass remained close to the starting point. This non-directional cell movement can be attributed to normal movement of mPCs within culture.

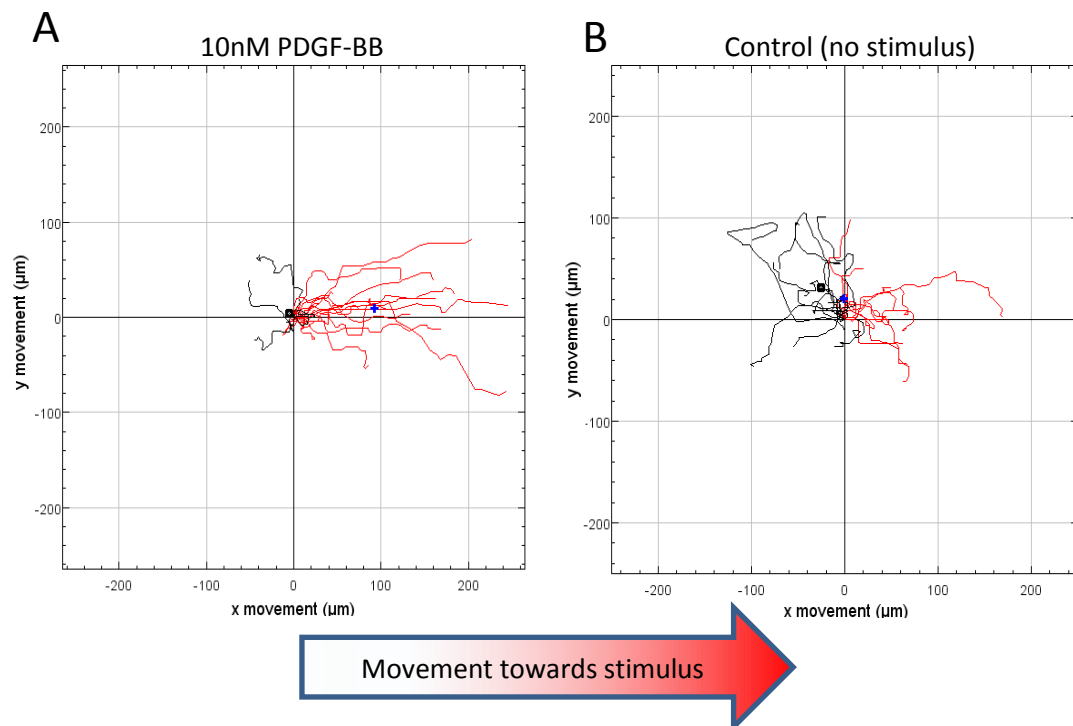


**Figure 5.7 Initial study of mPC chemotaxis using 2D slides**

Migration of mouse primary calvarial cells (mPCs) observed using 2D chemotaxis slides (Ibidi). mPC migration tested after 0 and 7 days of OCM culture, using 10nM PDGF-BB over 24 hours. Individual cells represented by one track; red lines indicate net movement towards stimulus, black lines indicate net movement away from stimulus, n=20 tracks shown. + indicates final centre of mass of cells.

#### 5.4.2.ii Effect of osteogenic culture on mPC chemotaxis

So far in this chapter it has been shown that chemotactic behaviour of mPCs changes following stimulation with osteogenic culture medium for 7 days. Since it was also shown in chapter 3 that mPC differentiation is influenced by nanovibrational stimulation, it might follow that the development of chemotactic behaviour would also differ following nanovibration (as compared to osteogenic medium). To study this mPCs were stimulated in OCM, 1000Hz nanovibration or by a combination of the two techniques and subsequently assessed for chemotactic response to PDGF-BB.

Following culture for up to 21 days, mPCs were seeded into Ibidi chemotaxis slides and cell migration tracked for 24 hours. The Ibidi chemotaxis tool was then used to generate Rose plots that display the direction and magnitude of cell movement. As seen in figure 5.8A, Rose plots for negative control sample (where no PDGF-BB was added), mPCs showed some migration in all directions, with no obvious directionality of movement. The same is said for nascent mPCs (Fig. 5.8B) which showed cell migration but in not distinct direction. Following OCM culture mPCs showed clear response to PDGF-BB stimulus, as indicated by movement from left to right at both 7 and 14 days. 1000Hz stimulated mPCs showed no directional movement after 7 days, however strong migration towards PDGF-BB after 14 days of culture. Interestingly, when 1000Hz and OCM were combined, mPCs only migrated towards PDGF-BB after 14 days of culture, suggested that nanokicking is the more influential factor in controlling the development chemotactic response to PDGF-BB. This could be due to the delay in overall osteoblast development shown in 1000Hz stimulation as observed in chapter 3, meaning that chemotactic ability towards PDGF-BB was also delayed.

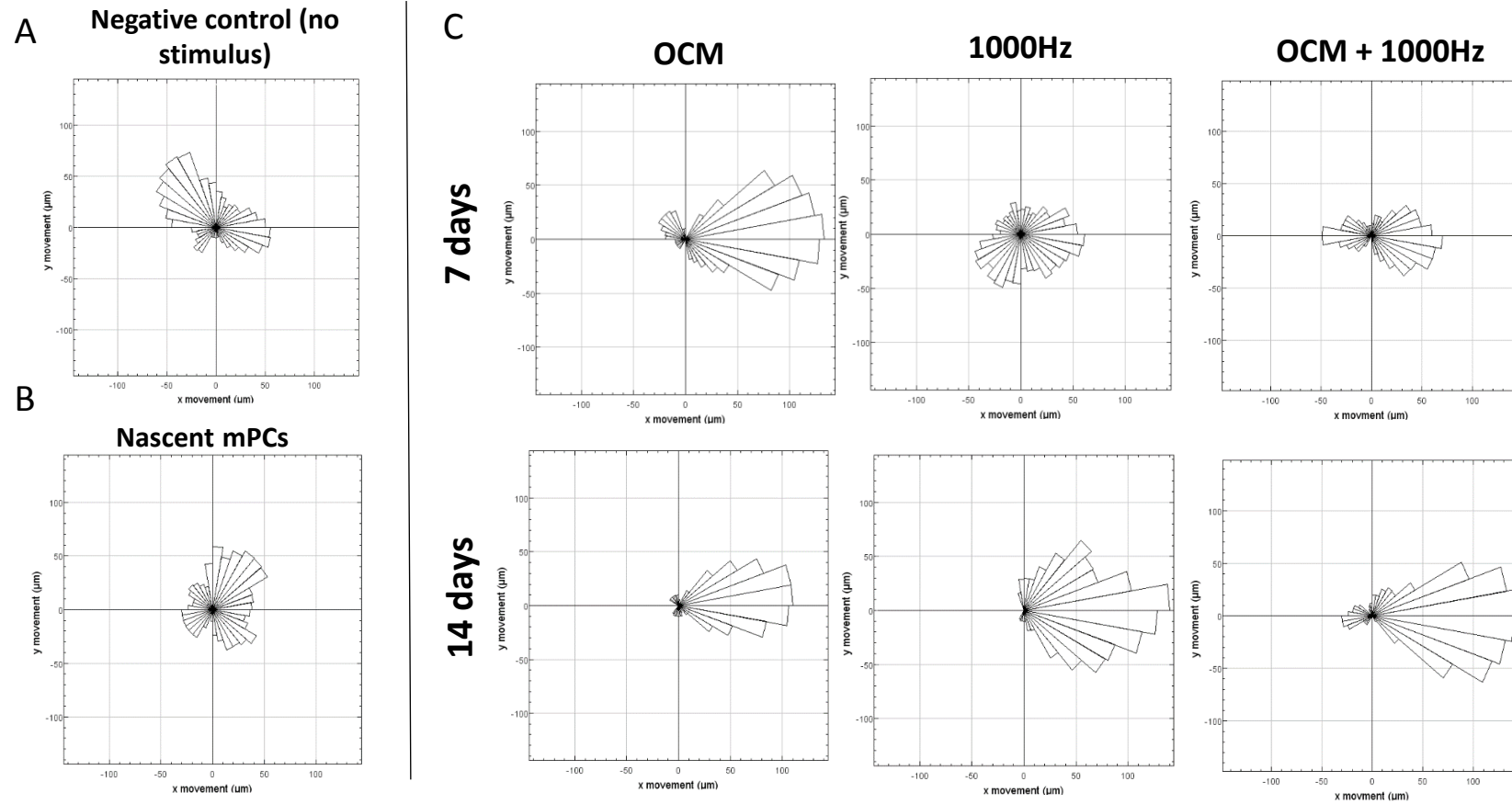


**Figure 5.8 Chemotaxis rose plots of mPCs following osteogenic culture**

Rose plots indicating direction and magnitude of migration of mouse primary calvarial cells (mPCs) towards PDGF-BB. **A:** Migration of control sample (no chemoattractant). **B:** Migration nascent (undifferentiated) mPCs in response to PDGF-BB. **C:** Migration of mPCs osteogenically stimulated for up to 14 days either by OCM, 1000Hz or OCM+1000Hz. Movement from left to right is in direction of stimulus, n=30.

xFMI was calculated to give a quantitative value for directionality of cell migration, with higher values indicating a more direct movement in the x direction (a value of 1 would indicate movement from left to right in a straight line). xFMI confirmed observations of Rose plots; 7 and 14 day OCM mPCs had xFMI values of 0.37 and 0.42 respectively, significantly higher than nascent mPCs (xFMI of 0.06). At 14 days xFMI values were 0.44 and 0.45 for 1000Hz and OCM+1000Hz mPCs respectively, similar to OCM results. No samples showed any directional movement after 21 days of osteogenic stimulation. Taken together, Rose plots and xFMI values suggests that developing osteoblasts become more responsive to PDGF-BB as they become more differentiated *in vitro* up to 14 days, however chemotaxis decreased at later differentiation times. This is to be expected since osteoblasts form extensive extracellular matrix and mineralised bone nodules at later stages of *in vitro* osteogenesis, similar to osteocytes which are stationary cells embedded in bone matrix *in vivo* [213].

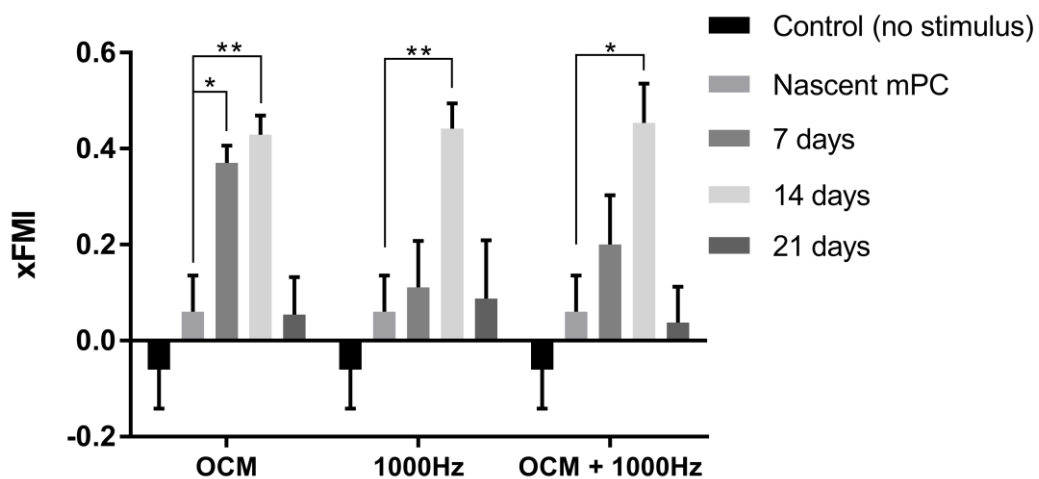


**Figure 5.9 Forward migration index of mPCs**

Forward migration index (xFMI) of mPCs in response PDGF-BB at various time points of osteogenic culture. Error bars show standard error of the mean, n=30. \* =  $p < 0.02$ , \*\* =  $p < 0.001$ .

### 5.4.3 Morphological changes in response to nanovibration

During chapter 4 the influence of nanovibration on actin cytoskeletal structure was observed, however those studies only observed up to 4 days of stimulation. To observe changes in cytoskeleton structure relevant to chemotaxis studies in this chapter, mPCs were cultured under osteogenic conditions for up to 21 days and stained with phalloidin.

As seen in figure 5.10, stimulation by OCM, 1000Hz and OCM+1000Hz caused increased intensity of staining for actin compared to controls (growth medium only), particularly at 7 and 14 days. In agreement with section 4.4.1 most actin staining was observed under 1000Hz stimulation, further implication nanovibration in mechanosensing behaviour. Staining also revealed noticeable alignment of actin fibres within mPCs stimulated by OCM, 1000Hz and OCM+1000Hz, not apparent under control conditions. Alignment of elongated fibres may allow for tension to be applied to cells during migration, as theorised by tensegrity model discussed previously [214]. The lack of alignment observed at 21 days of culture coupled with lack of chemotactic activity observed corroborates this theory.

Intensely stained areas resembling grouping of actin filaments around the edge of individual cells was also observed at 7 and 14 days in the case of OCM cultures, and at 14 days in the cases of 1000Hz and OCM+1000Hz. This staining coincides with increased chemotactic activity observed in response to PDGF-BB and suggests organisation of actin into larger bundles supports migratory behaviour.

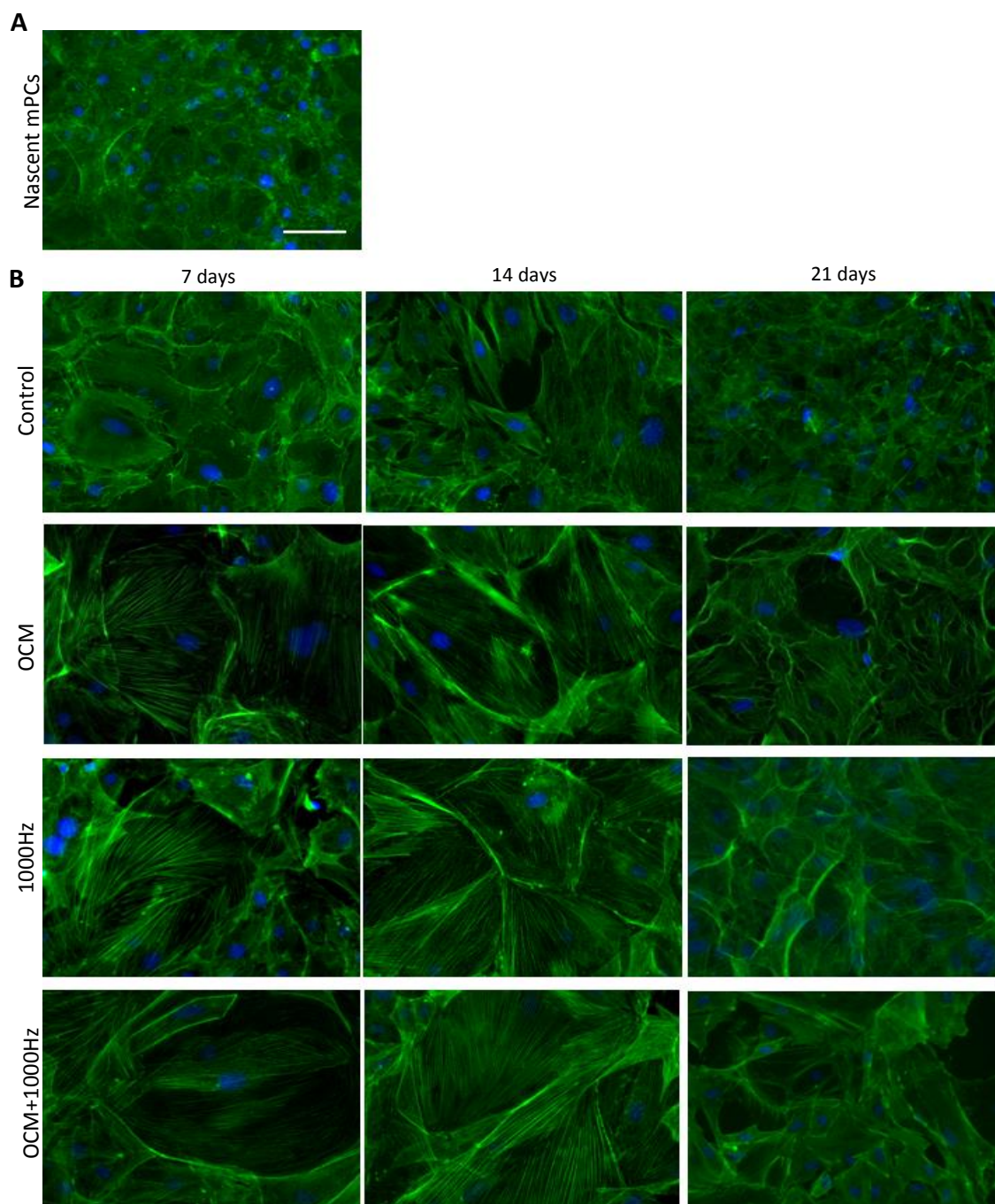


**Figure 5.10 Cytoskeletal structure of mPCs during chemotaxis studies**

Actin cytoskeletal staining (green) of mPCs stimulated by control, OCM, 1000Hz or OCM+1000Hz for up to 21 days alongside Ibidi chemotaxis studies. Co-stained for cell nucleus (blue). Scale = 200 $\mu$ m.

## **5.5 Conclusions**

The aim of this chapter was to explore the chemotactic behaviour of osteoblasts throughout the *in vitro* differentiation process, whilst comparing the effects of osteogenic culture medium and nanovibration on migratory behaviour. As a known chemoattractant for osteoblasts[142], PDGF-BB was selected and response of mPCs was confirmed initially by Boyden assay. mPC migration increased after 7 and 14 days in osteogenic medium however decreased again at 21 days, suggesting there is a 'window' during osteoblast development during which chemotactic response to PDGF-BB is highest. Differentiation by 1000Hz nanovibration also seems to promote chemotactic behaviour, however similarly to osteogenesis results in chapter 3 these effects were delayed compared to OCM-induced responses, with migratory behaviour only significantly higher after 14 days of 1000Hz culture. The combination of OCM and 1000Hz also produced this delayed effect suggesting that whilst OCM promotes migration, nanovibration has an overriding influence on the development of migratory response to PDGF-BB.

Differences in chemotactic activity are to be expected since OCM and nanovibration appear to promote osteoblast development via different mechanisms as explored in chapter 4; in particular the implication of focal adhesion kinase in nanovibrational osteogenesis. FAK activity has been implicated in cell motility [115], elevated expression is observed in highly malignant tumours [215], related to increased formation of invadopodia in invasive cell types [216] [217]. The influence of nanovibration on FAK activity and its role in motility may account for the dominating influence of nanovibration on cell migration observed in this chapter.

This work presents an important finding in that nanoscale mechanotransduction influences migratory behaviour of developing osteoblasts, and implicates cytoskeletal tension as an important mechanism by which this happens. However further work to characterise

mechanism of PDGF-BB chemotaxis in osteoblasts should be considered. Sanchez-Fernandez et al. (2008) have previously shown that expression of the PDGF-BB receptor PDGFR- $\beta$  decreases during osteoblastogenesis, which may account for decreased migration observed at later timepoints in this work. However this also raises the question of the relationship between different osteogenic techniques (in particular nanovibration) and the levels of PDGFR-  $\beta$  receptor, which should be studied in the future.

It should also be noted that this work studies chemotaxis in a 2D model, and more representative 3D models of cell migration can be employed to better understand osteoblast migration. The effective chemotactic gradient has also not been characterised in this work, therefore a model which allows more accurate presentation of PDGF-BB gradient should be considered. 3D gradients of tethered PDGF-BB have been described using a poly(ethylene glycol) hydrogel to recreate the perivascular niche, with alteration to MSC spreading and migration in response [218]. A similar model could be employed to further study migration of mESCs or mPCS *in vitro*.

Finally this chapter only explores the migratory behaviour of mPCs in response to PDGF-BB. Given the differences in osteogenic behaviour observed between cell types observed in previous chapters, the chemotactic study of differentiating mESCs would make an interesting comparison.

## Chapter 6 – Final conclusions and outlook

### **6.1 Conclusions**

In recent years the field of tissue engineering and regenerative medicine has grown rapidly [219], however in reality this has translated to very few clinical successes. It is clear that greater understanding of cell biology is required in order to design regenerative therapies and products capable of delivering outcomes that the field promises. In the case of bone therapies, the plethora of factors effecting bone biology need to be addressed sufficiently before a regenerative therapy can be achieved [71] [220]. The clinical use of BMP-2 and subsequent problems [132] highlight the danger of translating a regenerative medicine product to the market without fully understanding all the possible mechanisms of action. Developing a cell-based therapy (and stem cells in particular) presents an even greater challenge, with associated risk of differentiation to undesired phenotype and the potential for uncontrolled proliferation [9] [221].

This thesis aims to further characterise osteoblast behaviour *in vitro*, using both primary calvarial osteoblasts and embryonic stem cell-derived osteoblasts. Chapter 3 highlights the differences in osteogenic behaviour observed between these two cell types, in particular the extent of mineralisation. This is likely to be in part due to the mechanisms of bone formation; embryonic stem cells appear to differentiate *in vitro* by endochondral ossification whilst calvarial osteoblasts appear to exhibit intramembranous ossification, as is to be expected [46]. Variation was also observed in osteoblast activity and phenotypic marker expression. These differences in *in vitro* characteristics have important implications on the use of stem cell-derived osteoblasts in regenerative therapies, and should be considered when making comparisons during *in vitro* differentiation studies.

This work also observes effects of nanoscale mechanotransduction on osteogenesis as compared to conventional medium supplementation, revealing influence over osteoblast morphology, mineralisation, expression of bone-specific proteins and alkaline phosphatase activity; all factors which will affect successful integration of a regenerative bone therapy. Importantly, this work also shows for the first time osteogenesis of mouse embryonic stem cells by use of nanovibrational stimulation, without the need for traditional osteogenic supplements, as previously shown in human MSCs [111]. Whilst mineralisation of osteoblasts cultures by the 1000Hz stimulation was delayed compared to conventional osteogenic medium, osteogenic markers RUNX2, osteocalcin and alkaline phosphatase activity confirm commitment to the osteoblast phenotype. Furthermore nanovibration appears to increase amount of phosphate species incorporated into the forming bone matrix, resulting in a mineral composition more similar to native bone tissue compared to conventional osteogenic methods. The combination of nanovibration with 1000Hz stimulation also had positive effects on ALP activity. This highlights the importance of nanoscale mechanical stimulation, and the efficacy of combination protocols in achieving robust osteogenesis of embryonic stem cells *in vitro*. The nanovibrational bioreactor presents a clean, reliable tool for stem cell differentiation *in vitro* that has the potential for upscale for use in regenerative medicine therapies.

As observed in chapter 4, comparison of the mechanisms of osteogenesis by nanovibration and osteogenic supplementation suggested a nanoscale mechanotransduction effect induced by 1000Hz stimulation, promoting formation of focal adhesion complexes and altering cytoskeletal morphology. Focal adhesion kinase activity was strongly linked to ESC differentiation in response to nanovibration whilst not strongly regulated in calvarial osteoblasts suggesting this signalling pathway plays a key role in stem cell response to nanoscale stimulation at early stages of development. Furthermore, OCM osteogenesis appeared to be mostly linked to ascorbic acid directed signalling whilst the addition of

nanovibration promote changes in a wider range of metabolites associates to MAPK/ERK signalling pathways.

Nanovibration is highly influential over cell behaviour, as shown by changes in RNA translation and energy metabolism related pathways observed during metabolomics analysis [109]. Predictive analysis revealed downstream RUNX2 activation in all osteogenic conditions however, only OCM+1000Hz revealed influence on BGLAP signalling, further evidence to support the efficacy of a combination protocol for osteogenesis.

The differences between cell types observed in this work help to dissect stem cell differentiation mechanisms but also have implications on the source of cells chosen for regenerative bone therapies as other researchers have previously discussed [163]. Alongside this, the importance of nanoscale mechanical stimulation in development of the osteoblast phenotype has been highlighted, in agreement with previous studies surrounding the effect of nanoscale cues on differentiation [123] [127].

Recruitment of osteoblasts and their precursors to a repair site is a key part of bone remodelling [58] [59] and is pivotal to the success of regenerative bone therapies, yet it is under explored compared to other aspects of bone biology. In chapter 5 the effects of nanoscale vibration of migratory behaviour of osteoblasts is explored, revealing a delayed development of chemotactic behaviour in 1000Hz stimulated cell compared to conventional osteogenic medium. Interestingly in the combination of 1000Hz and OCM migratory ability was still delayed, highlighting the dominating influence of nanovibration on osteoblast development. The results also corroborate changes in cytoskeleton structure in response to nanovibration observed in chapter 4, which are likely to be a key factor in migratory alterations [117].

It is hoped that the research in this thesis has emphasised the gap that remains between stem cell-derived osteoblasts and their native counterparts, but also that the importance of

nanovibrational stimulation in osteogenesis and its potential to help close this gap has been demonstrated. This information will further the understanding of ESC differentiation and aid the future development of repair strategies for bone.

## **6.2 Outlook and future developments**

The work of thesis has potential translational applications in helping to develop more robust osteogenic differentiation protocols and thus improving efficacy of stem-cell-derived osteoblasts protocols for bone repair therapies. Further understanding mechanisms of ESC differentiation can also benefit stem cell research as a whole, helping dissect important factors affecting stem cell development and commitment. In particular the importance of focal adhesion dynamics in determining cell behaviour and cell fate is reinforced by this work. This information can also be applied to *in vitro* models of stem cell and bone biology for the use in drug screening, with the aim of generating representative cellular models able to replace animal models [14] [222].

However to achieve these goals, several aspects of this research highlighted in this thesis require further study. Whilst cell source-dependent differences in osteoblast development was observed in chapter 3, many research avenues still remain unexplored. The majority of this work focused on protein level assessment and mature osteoblast mineral and functions, whilst the gene level factors promoting phenotypic change were relatively unexplored. qPCR revealed RUNX2 expression in agreement with immunocytochemistry staining however analysis of other genes (for example *Msx-2*, *Osterix*, *Osteopontin*, *Osteocalcin*) could characterise in more detail gene level changes that account for differences between mESC-derived osteoblasts and calvarial osteoblasts [33] [34]. The effects of 1000Hz nanovibration on osteogenesis were also observed, and further qPCR analysis could help to understand mechanisms behind this.

The nature of the cell populations used for osteogenesis studies should also be considered. Isolated mPC population is likely to be mixed, with a small number of fibroblasts, haematopoietic cells, and other non-osteoblast lineage cells which may detrimentally influence *in vitro* osteogenesis. A mixed population of cells will also be present following aggregation of mESCs during first step of osteogenesis protocol. This issue could be partly overcome by the sorting of cells prior to osteogenic experiments. Sidney *et al.* described sorting of mESCs based on cadherin-11 expression, improving osteogenic efficiency of ESC population [168].

Nanovibration parameters of 1000Hz frequency and 30nm amplitude used in this work were based on positive osteogenic outcomes from previous work [111] [223], however these studies focused on mesenchymal stem cells. ESC and mPC osteogenesis may benefit from optimizing frequency and amplitude. Furthermore, the variation of measured displacement between wells in cell culture plates is likely to influence the efficacy of mechanotransduction effects and should be minimized by further bioreactor calibration.

Mechanisms of nanovibrational implied in chapter 4 should be further explored. For example changes in focal adhesion size and distribution could be characterized by measuring focal adhesion length, whilst actin alignment could also be quantified [190] [206]. The implicated FAK signaling hub could also be further studied by inhibition to block this pathway and thus confirm its role in osteogenesis of ESCs. Metabolomics analysis of mESC and mPCs in this work focused mainly on metabolites relevant to osteogenesis at 7 days of *in vitro* differentiation however analysis at later timepoints should be carried out to study metabolite population changes at later stages of osteoblast development in line with differentiation studies in chapter 3.

Chemotaxis experiments in chapter 5 were based on a 2D assay and may not be representative of cell migration within a 3D extracellular matrix in native tissue, thus a 3D

model should be considered as discussed earlier. Biomaterials approach could also be used to generate a more robust chemoattractant gradients with PDGF-BB molecules tethered to a hydrogel structure that allows migration [224] [225] [226]. Chemotactic gradient could then be more accurately analysed by Time-of-flight secondary ion mass spectrometry (ToF-SIMS) [227] or Attenuated total reflection-Fourier transform infrared (ATR-FTIR) imaging [228]. The use of holographic optical tweezers to position growth factor release sources in relation to cells has also been described [229], and may be useful in creating an accurate micron-scale model for chemotaxis experiments.

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## Chapter 7 - Appendix

### 7.1 - Serum batch testing

#### Methods

Serum batch testing was performed to select foetal bovine serum (FBS) most permissive of osteogenesis in both mPCs and osteo-mESCs. For purpose of serum testing, osteogenesis was assessed using alizarin red staining to assess mineralisation.

Batches of sera from a range of suppliers were tested. Cells were seeded into 6 well plates at 10,000 cells/cm and cultured in media containing one of the sera to be tested, both with and without osteogenic supplements (50 µg/mL L-ascorbic acid, 50 mM β-glycerophosphate and 10 µM dexamethasone).

| Serum | Supplier | Product Code | Lot number |
|-------|----------|--------------|------------|
| A     | Sigma    | F9665        | BCBS1626V  |
| B     | Sigma    | F9665        | 075M3363   |
| C     | BioSera  | FB-1001/50   | 013BS410   |

**Table 7.1 Serum for batch testing**

As seen in figure 7.1, all three sera allowed mineralisation in both cell types. However in the case of mPCs, serum A allowed more mineralisation both with and without osteogenic supplements compared to sera B or C. For this reason Serum A was chosen for all cell culture.

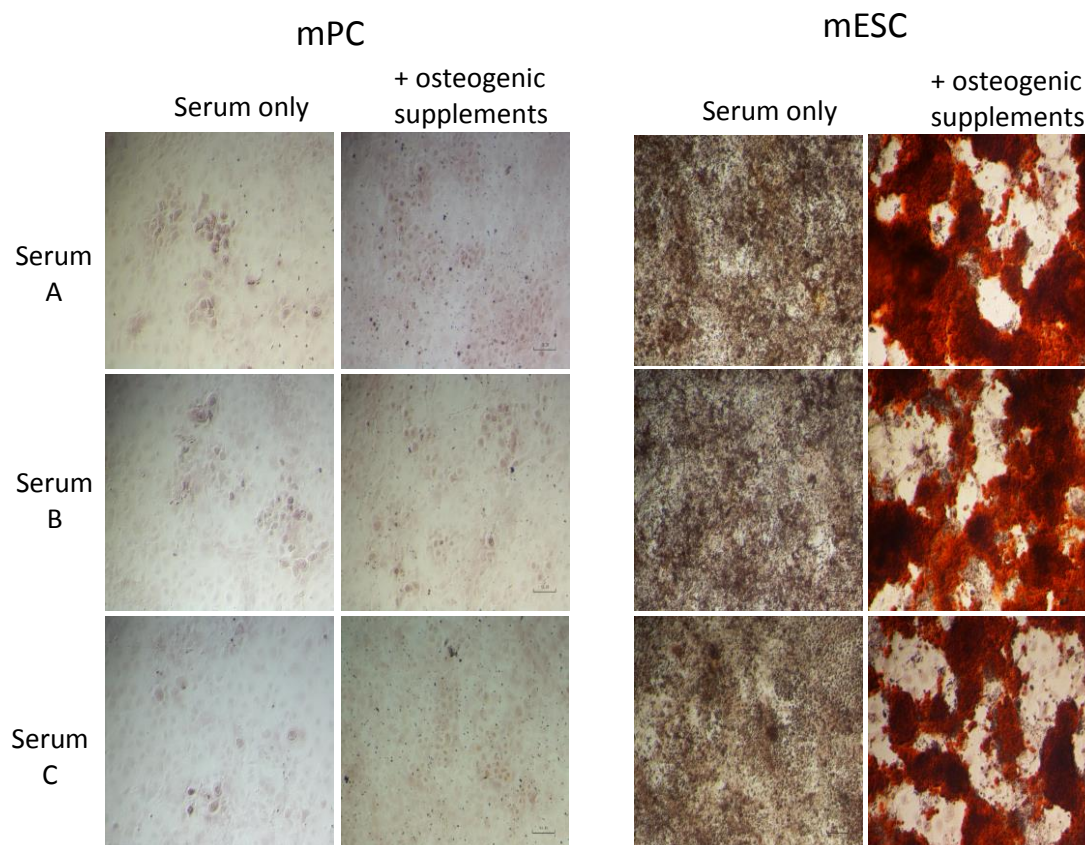


**Figure 7.1 Mineralisation in batch tested sera**

Alizarin red staining of osteogenic cultures after 21 days.

## **7.2 Alkaline phosphatase standard curve**

During alkaline phosphatase assay relative fluorescence values (RFU) were calculated by subtracting blank (pNPP only) absorbance from experimental absorbance values. ALP concentration was calculated from RFU using the standard curve (Figure 7.2) generated by serial dilution of alkaline phosphatase standard from calf intestine (AnaSpec) in deionised water.

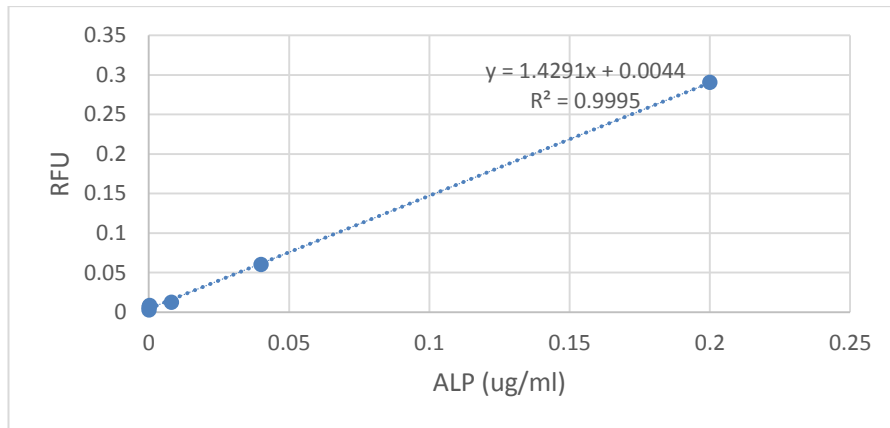


**Figure 7.2 Alkaline phosphatase standard curve**

Standard curve; RFU values of calf intestine alkaline phosphatase standard. Diluted values ranging from 0.0000128 to 0.2 µg/ml.

### **7.3 Mineralisation in glass-bottomed culture dishes**

For the purpose of SEM imaging, cells were cultured and on CellView glass-bottomed dishes (Greiner Bio-One). Prior to this, the ability of mPCs to form mineralised osteoblast tissue was assessed, as shown in figure 7.3.

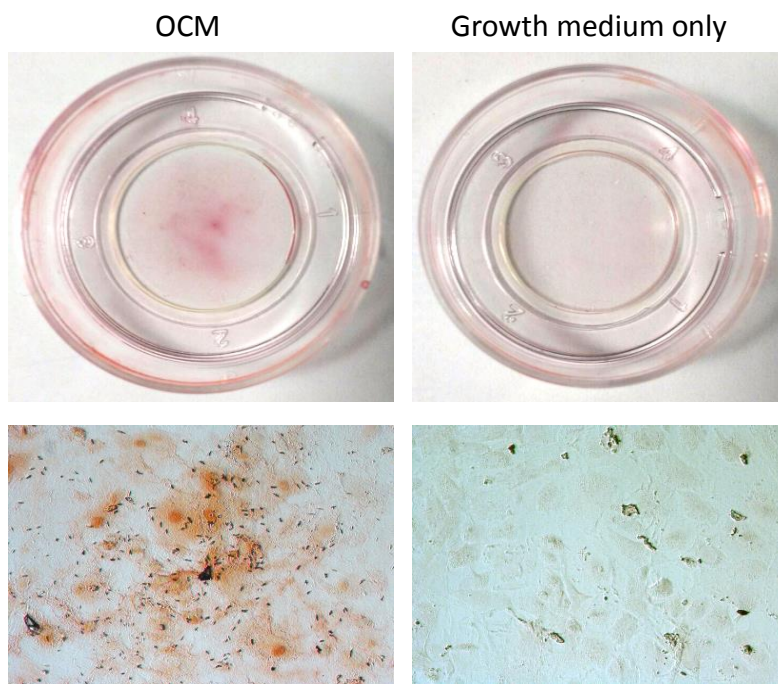


**Figure 7.3 Mineralisation on CellView dishes**

Mineralisation of mPCs in CellView wells assessed by alizarin red staining.

## 7.4 Complete Raman spectra

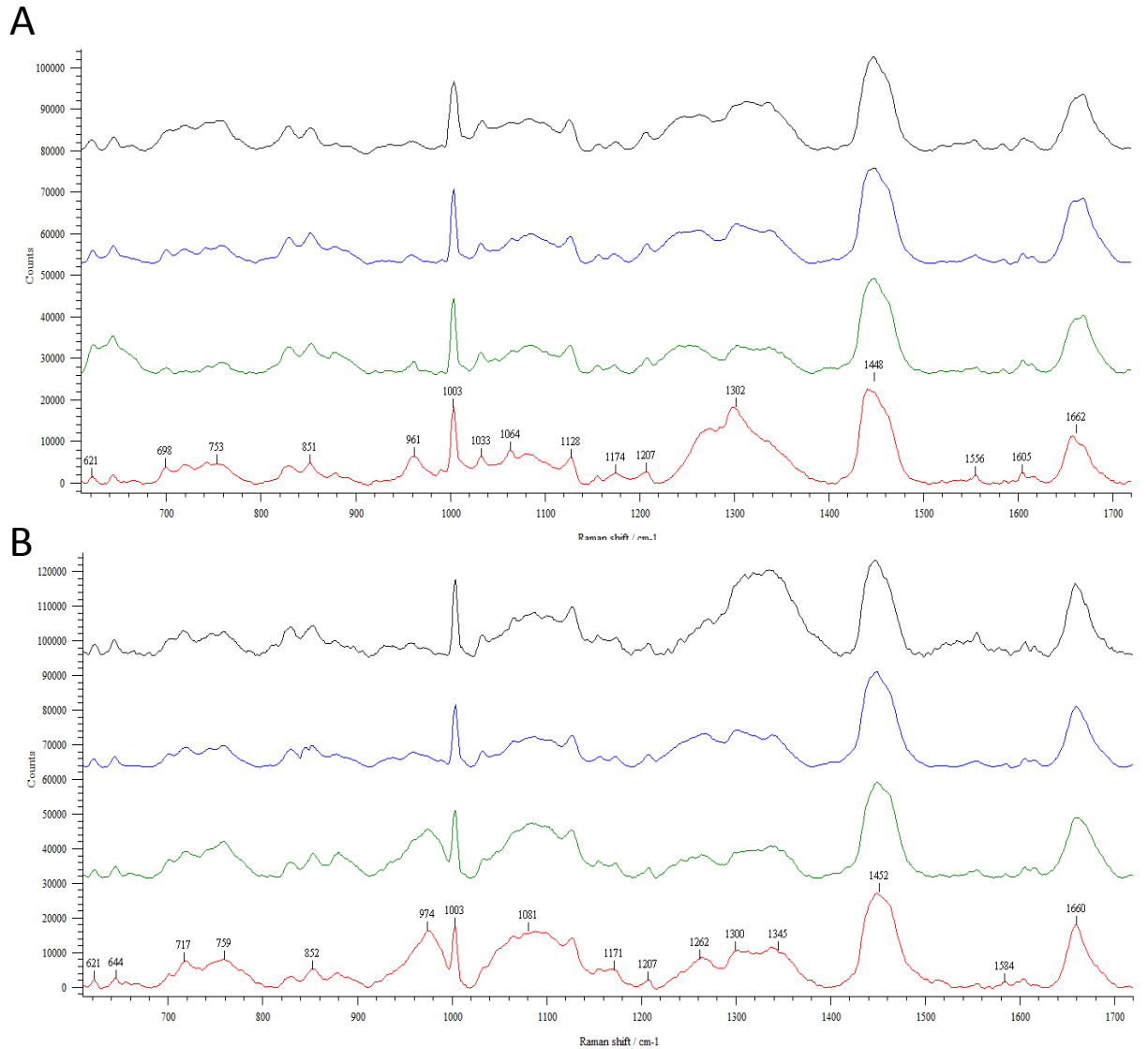


**Figure 7.4 Full Raman spectra**

Full Raman spectra of mESC (A) and mPC (B) following control culture (black) or stimulation by OCM (blue), 1000Hz (green), or OCM+1000Hz (red).

## **7.5 Additional metabolomics data**

### Predicted activity of signalling hubs



**Figure 7.5 Predictive activation/inhibition of FAK/ERK signalling hubs in response to OCM+1000Hz**

IPA predictive analysis of signalling hubs related to FAK and ERK pathway in mESCs following 7 day OCM stimulation. Orange indicates predicted activation of hub, blue indicates predicted inhibition.

## 7.6 Nanokick calibration data

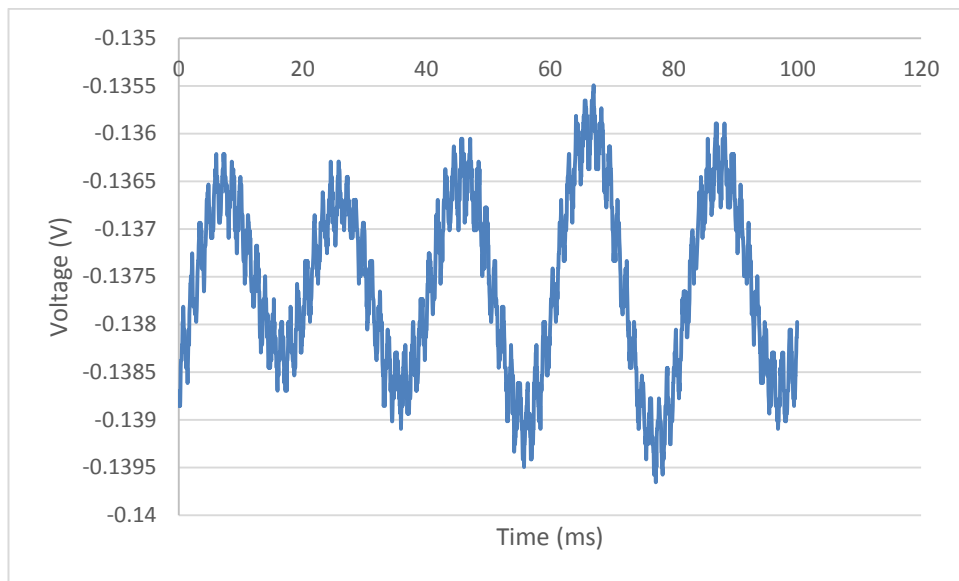


**Figure 7.6 Time series of interferometer voltage data**

Voltage readings during interferometer measurements of Nanokick bioreactor displacement as a function of time.

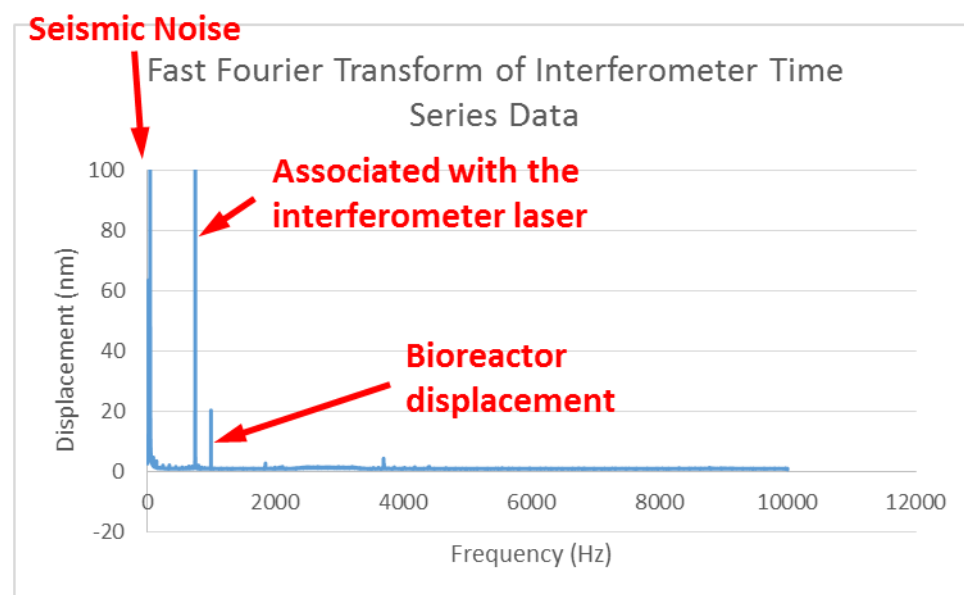


**Figure 7.7 Fast Fourier Transformation (FFT)**

FFT of interferometer time series data revealing displacements associated to bioreactor, seismic noise, and noise related to interferometer.