Evaluating the effects of acoustic stimulation on fibroblast cell migration and muscle fiber formation

Taybia Mohammed

A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy (PhD)

December 2016

Abstract

Cells are known to interact and respond to external mechanical cues and recent work has shown that application of mechanical stimulation, delivered via acoustic vibration, can be used to control complex cell behaviours. Much of the work in this area has focused on the effects of mechanical stimulation upon bone cells. Very little has been done to investigate the effects of mechanical stimulation on other cell types. Therefore, could mechanical stimulation be used to accelerate the wound healing process? Could mechanical stimulation be used to encourage muscle cell growth? The data generated in this thesis will be completely new knowledge which will help us develop our understanding of mechanotransduction and help us develop new approaches and devices to address medical challenges such as accelerating wound healing process, increasing muscle mass in diseases associated with muscle degeneration and aging.

The aim of this research was to investigate the effects of mechanical stimulation upon the migratory and morphological properties of three different fibroblast cells and muscle cells namely; human lung fibroblast cells (LL24), subcutaneous areolar/adipose mouse fibroblast cells (L929), Human dermal fibroblast cells (HDF) and C2C12P12 muscle cells. Using a speaker-based system, the effects of mechanical stimulation (0-1600Hz for 5 and 20 minutes) on wound/scratch assays, the mean cell migration distance (µm), actin and vinculin organisation, cell viability, muscle fibre diameters and the effects of temperature were investigated. The results show that 100Hz acoustic vibration can enhance cell migration, wound healing in LL24 and L929 cells and possible increase muscle fibre diameters although there was no significance, the trends were repeatable. Mechanical stimulation was also found to promote changes to the morphology of LL24 and L929 cell lines, particularly the formation of lamellipodia and filopodia. Overall lamellipodia was the most prominent actin structure displayed by the lung cell (LL24), whereas filopodia was the most prominent actin feature displayed by the fibroblast derived from subcutaneous areolar/adipose tissue. Vinculin appeared to be more punctate in L929 cells compared to LL24 cells. Mechanical stimulation at all the frequencies used here was found not to affect cell viability. However, as the frequency increased, the temperature also increased. These results suggest that low-frequency acoustic vibration may be used as

a tool to manipulate the mechano-sensitivity of cells to control cell migration and to enhance the diameters of muscle fibres.

Acknowledgements

Firstly, I would like to start off saying a great big thank you to a wonderful supervisor Dr. Mark Murphy for all his guidance and support through this thesis. I would also like to thank Dr Francis Lilley for being my supervisor and proof reading my thesis. Additionally, I would like to thank Professor David R. Burton for being a wonderful third supervisor.

I would also would like to thank Prof Ahmed Al-Shamm'a for being the best Dean ever for supporting me financially and academically through hardship. I would also like to thank Ms. Helen Pottle, Ms Elizabeth Hoare and Ms. Tricia Waterson for guiding me and being the best people to talk to. I would also like to thank Dr Andre Batako for building my confidence, Dr Mike Morgan for believing in me, Dr Martin Sharp and Dr Paul French for career guidance, Dr Frederick Bezombes for being a great friend, Dr Tahsin Opoz for encouraging me for to apply for grants, Dr David Mcllroy for being a superb friend, Mr Pete Moran for listening to me. Everyone else at LJMU that I know including Elaine (receptionist) and all the workers at Starbucks.

I would like to thank my family starting from my mum Nahid, my step dad Irfan, brothers Bilal and Imran, my little sister Aqsah for all the motivation and my aunt Sajida for her love and support. I would also like to thank my best friend Sabah Hafeez for supporting me through tough times. My friends Kankgana, Saiqa, Ruqaiya, Rosie, Jess and Handi, for listening to me moan about stress.

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Table of contents

List of figures

Figure 4.51 Mean muscle fibre diameter (µm) versus frequency (Hz) for 20 minutes every 24 hours for 4 days. Error bars represent standard deviation. * denotes Significance (p<0.05). **123**

Abbreviations

ºC	Degrees Celsius
µm	Micrometres
α	Alpha
β	Beta
ATP	Adenosine triphosphate
BEAS-2B cell line	Human bronchial epithelial *cell* line
BFR	Blood flow Restriction
BMSCs cells	Bone marrow stromal
BMC	Bone mineral content
BMD	Bone mineral density
C2C12P12	Mouse myoblast cell line
CMAC	Cell-matrix adhesion complexes
Ctrl	Control
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELF	Electromagnetic field
EMG	Electromyograph
ER	Estrogenic receptors
F actin	Filamentous actin
FBS	Fetal Bovine Serum
g	Gram
G actin	Globular actin
GTPases	Guanosine-5'-triphosphate
HDF	Human dermal fibroblasts
HFV	High frequency vibration

Hz	Hertz
Hr	Hours
kHz	KiloHertz
KL2	Krüppel-like Factor 2
LL24	Human lung fibroblast cells
L929	Areolar mouse cells
LIV	Low intensity vibration
LMHFV	Low magnitude high frequency vibration
MAPK	Mitogen-activated protein kinase
MRFs	Myogenic regulatory factors
MPC	Mesenchymal progenitor cells
MSC	Mesenchymal stem cells
MTOC	Microtubule-organizing centre
nm	Nanometres
Ov	Ovariectomized
Pa	Pascals
PBS	Phosphate Buffer saline
PC12	Pheochromocytoma cells
PGE2	Prostaglandin E2
PTH	Teriparatide
SR	Strontium ranelate
VV	Vertical vibration
WBV	Whole body vibration

Chapter 1

1.0 Background

Cells are the fundamental unit of all living organisms, are capable of independent existence and can carry out all the functions which are necessary for a living being. Through the study of cell biology, we have been able to develop a very detailed understanding of cell structure and function which has enabled us to develop deeper insights into tissues and organisms as well as disease. In general, there are different type of organisms, some organisms are composed of only one cell, and others are structured into groups with large numbers of cells. However, cell biology focusses mainly on the structure and function, from general properties shared by all cells, to the individual properties, that have particular functions to specialized cells.

Cells and tissues, by their very nature, have evolved to sense their physical surroundings by converting mechanical forces and distortions into biochemical signals through the activation of diverse intracellular signalling pathways. This is known as mechanotransduction (Ingber *et al.,* 2006) and involves mechanosensitive feedback controls that help regulate important cellular functions such as development, homeostasis, apoptosis, differentiation and proliferation. Very little research has been carried out to understand the effects of mechanical stimulus on cell/tissue behaviour. However, we do know that changes in a cell's/tissue's ability to respond to forces are associated with certain disease states including; muscular dystrophies, cardiomyopathies, cancer progression and metastasis (Wang *et al.,* 2013; Ingber *et al.,* 2006).

Whole body vibration (WBV) in patients suffering from a range of conditions has been shown to have therapeutic benefits. For example, in a study carried out on patients who have Multiple Sclerosis (MS) it was found that vibration of 40 and 50Hz (delivered through a vibrating platform) in combination with exercise, helped to

improve muscle strength, functional ability and general wellbeing (Schuhfried *et al.,* 2005). While in a study by Weinheimer-Haus *et al.,* using diabetic mice it was found that at 45 Hz increased angiogenesis and resulted in a faster wound closure compared to those mice that were not exposed to vibration (Weinheimer-Haus *et al.,* 2014).

At the cellular level recent studies have shown mechanical stimulus may be used to help the formation of new bone. Bone tissue adapts to its functional environment by optimizing its morphology for mechanical demand. Among the mechanosensitive cells that recognize and respond to forces in the skeleton are osteocytes, osteoblasts, and mesenchymal progenitor cells (MPCs). The ability to use mechanical signals to improve bone health is an attractive solution to conditions associated with age-related bone loss.

Examples of work in this area include that of Wang *et al*., who looked at mechanically-regulated protein secretion by MPCs. Using a dynamic motion platform, the workers applied low-magnitude stimulation (32-37 Hz) to MPCs for 10 and 20 minutes, respectively and found that secreted proteins (or vibe-proteins), which have the capacity to promote bone formation, were up-regulated. The authors suggest that by virtue of their secretory status, some vibe proteins may be candidates for pre-clinical development as anabolic agents for the treatment of osteoporosis (Wang *et al.,* 2013). In another study Kulkarni *et al*., used a modular piezoelectric actuator to vibrate cells at 4Hz for 1 hour, on 3 consecutive days and found that bone reabsorption (breakdown of bone) was inhibited (Kulkarni *et al*., 2013). Whilst in a similar study Wu *et al.,* found that LMHF vibrations (45Hz for 15 min per day) could also be used produce an anabolic effect on bone through inhibition of osteoclast differentiation (Wu *et al.,* 2012). In contrast, Kim *et al,* found that exposure of human mesenchymal stromal cells (hMSC) to vibrations (30-40Hz) resulted in an increase in proliferation. The authors suggest that vibrations could enable the osteogenic process (Kim *et al.,* 2012).

Such studies suggest that mechanical stimulation may have great potential as a novel therapeutic treatment for medical intervention for a range of conditions. This work is concerned with investigating the effects of the effects of mechanical stimulation on fibroblast cell migration and muscle fibres. Fibroblast cells play an important role in wound healing and we hypothesised that mechanical stimulation may be used to

enhance the wound healing process. Whole body vibration devices are currently being used in gyms and clinical settings using vibrational devices to reduce pain or to lose weight, therefore we also hypothesise that stimulation does increase muscle fibre formation.

1.1 Basic cell structure

The starting point of cell biology was originally in the 1830s however scientists had been previously using microscopes for centuries but had no idea what they were looking at. In 1665, an English philosopher, Robert Hooke initially observed plant-cell walls in cork slices. This was followed by another Dutch tradesman and scientist, Antonie van Leeuwenhoek, who handcrafted his own microscopes and observed microorganisms which were originally referred to as *animalcules*. In the 1830s, two scientists and colleagues, named Schleiden, who looked at plant cells, and Schwann, who looked at first animal cells, showed the first clear definition of the cells. From this early work a cell theory was formed, which suggested that all living creatures, both simple and complex, are made out of one or more cells, and that the cell is the structural and functional unit of life (Mazzarello 1999). Throughout the nineteenth and twentieth centuries, microscopes improved and scientists were able to see more detail within cells. For example, the microscope handcrafted by van Leeuwenhoek enabled greater magnification than before. Today, there are a range of microscopes that can be used to magnify specimens to reveal the shapes and scale of organelles in micrometres such as the electron microscope, atomic force microscope and confocal microscope. The development of these imaging techniques has significantly advanced our understanding of cell structure and has led us to an understanding of the complexity of cells.

There are many different branches in cell biology for example cellular biochemistry; which has enabled us to study the biochemical mechanisms that allows cellular metabolism. Another branch is genomics, and its interconnection with the proteins releasing genetic information from the nucleus to the cell cytoplasm or proteomics. Cell biology has also branched off to study the structure of subcellular compartments, cell communication and signalling, cell crawling and the cell cycle. This is where the rotation of phases beginning and ending with cell division and different periods of growth and DNA replication occur.

Cells have their own anatomy (Figure 1.1). The boundary of cells is known as a membrane. The large body in the middle is known as the nucleus. The rest of the interior of the cell is filled with a transparent substance called cytoplasm. The cells have an outer membrane called the plasma membrane and the membrane that

surrounds the organelles inside the cell is called the internal membrane. A eukaryotic cell (cell with a nucleus) has many organelles such as nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes and cytosol.

Cytosol is a water based gel rather than a liquid where many chemical reactions occur. The cytosol is supported by a network of long thin filaments of protein. These filaments, known as the cytoskeleton, are anchored at one end of the plasma membrane and also to the nucleus and allow a direct communication between the intracellular and extracellular environment (Bruce Alberts, 2013).
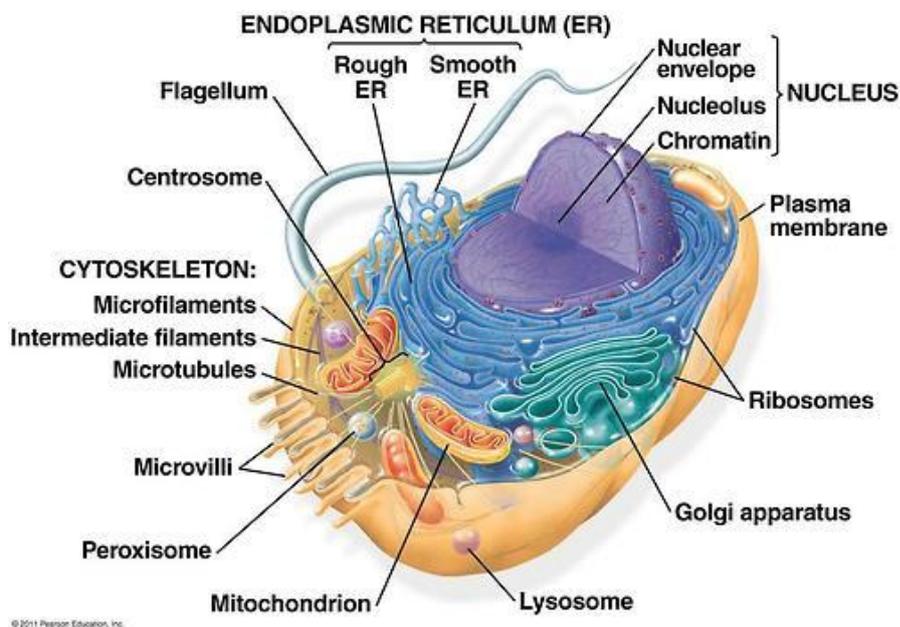
Figure 1.1 Detail image of a cell. Reproduced with permission from Pearson education, 2011.

1.2 Cytoskeleton

The cytoskeleton is made up of a network of interconnecting proteins which function together to provide the cell with structural, functional and mechanical integrity. Due to a significant research effort and advancement in scientific methods/techniques we now have a detailed understanding of the complexity of the cytoskeleton and its associated proteins. The cytoskeleton has many roles; firstly, it provides a structural framework for the cell, that helps determines cell shape and the organization of the

cytoplasm. To add to the structural role, the cytoskeleton is responsible for cell movements and also the internal transport of organelles and other structures (such as mitotic chromosomes) throughout the cytoplasm. Given the importance of the cytoskeleton and its many roles, it is no surprise that many pathological conditions have been associated with a disorganised cytoskeleton for example; muscular dystrophy, cancer, pulmonary fibrosis and cardiovascular diseases (Ramaekers FC, 2004). Therefore, understanding more about this complex structure is important in helping us understand certain pathological disorders.

The cytoskeleton is made of three types of filaments which are the microtubules, intermediate filaments and actin filaments (Figure 1.2). Microtubules are composed of a protein called tubulin and have a diameter of about 25 nanometres (nm). Intermediate filaments are constructed from a large number of different proteins depending on which tissue they originate in (e.g. keratin in epithelial cells and vimentin in fibroblast cells) and have a diameter of approximately 10 nm. Actin filaments are made of a protein called actin with a diameter of about 6 nm. These three protein filaments are essential as they form an organised network which helps cells maintain structural and mechanical integrity of the cell while also allowing direct links between the inside of the cell and the outside environment. This is achieved through cytoskeletal links between the cell membrane, nucleus and extracellular matrix as wells as with neighbouring cells (Bruce Alberts, 2013).
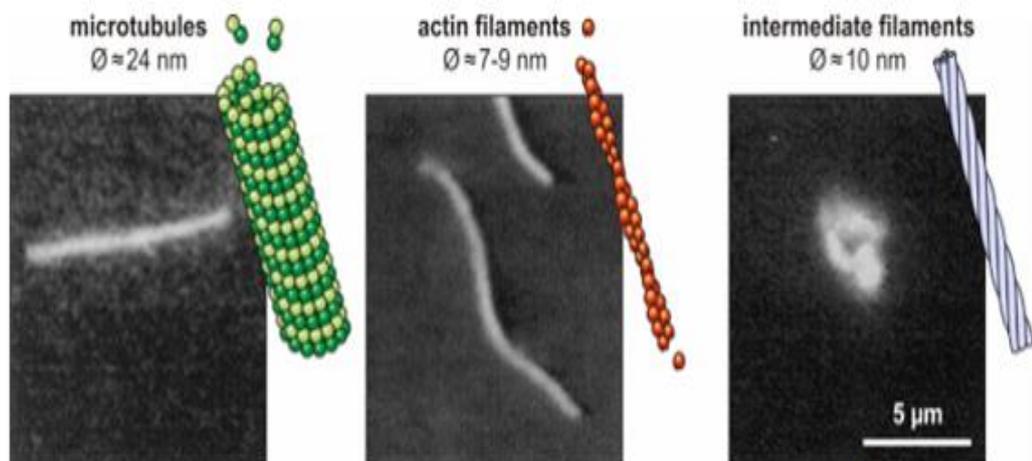
Figure 1.2 Three major proteins that make up cytoskeleton (Käs *et al.,* 2009).

1.2.1 Microtubules

Microtubules are present in all eukaryotic cells and play important role in a variety of cellular processes. A key characteristic of microtubules is their dynamic nature, and microtubules dynamically alter their organization in response to the needs of the cell. Microtubules are made of tubulin. The tubulin molecule is composed of a polymer of α- and β-tubulin dimers. The tubulin dimers assemble in a head-to-tail manner to form linear polymers called protofilaments. These protofilaments combine together to form the hollow structure of microtubules. The dimers at both ends of the filament are constantly adding and subtracting, leading to one end that exposes β-tubulin (the plus end) growing rapidly and other end exposing α-tubulin (the minus end). The minus ends of microtubules are anchored to structures called microtubule organizing centres (MTOCs), these are found close to the nucleus and are known as centrosomes (Horio and Kurata, 2014).
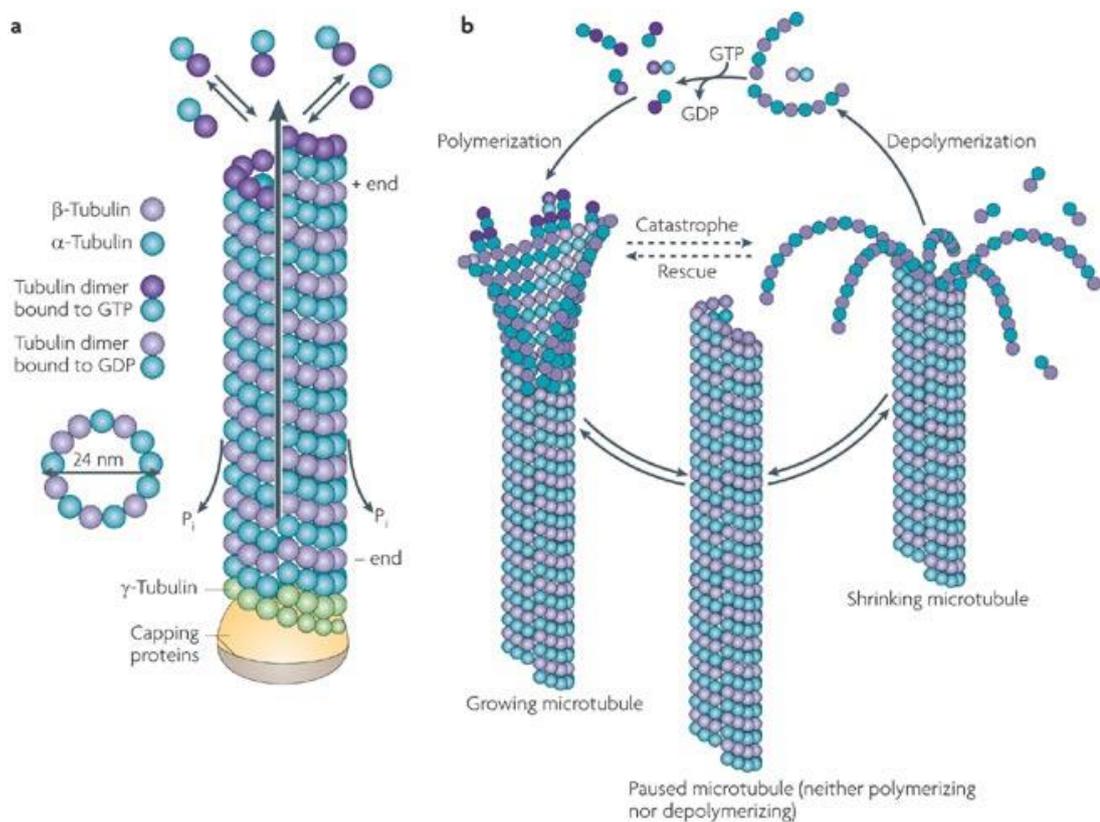
Figure 1.3 Microtubules are composed of alpha- and beta-tubulin subunits that accumulate into linear protofilaments. Protofilaments contain 10 to 15 microtubule (13 in mammalian cells) that wind together to form a 24 nm wide hollow cylinder. Microtubules can rapidly grow via polymerization or shrink via depolymerisation in size (Conde and Cáceres, 2010) Nature Reviews. All rights reserved.

1.2.2 Intermediate filaments

Intermediate filaments are a primary component of the cytoskeleton, although they are not found in all eukaryotes, and are absent in fungi and plant. Intermediate filaments are strong, rope like structures (Figure1.4). In epidermal cells and the axons of neurons, intermediate filaments are at least 10 times more abundant than microfilaments or microtubules. In immunofluorescence micrographs, we can see a network of intermediate filaments filling the entire cytosol of a cell in a pattern usually distinct from that of microfilaments but similar to that of microtubules (Lodish, 2000).

The protein subunits of some intermediate filaments are associated with either specific cell type or are distributed widely. For example, intermediate filaments such as neurofilaments, are only found on neurons, whereas vimentin filaments are found in a broad range of cell types such as mesenchymal and connective tissue cells (Ivaska, 2007).

Despite the wide number of intermediate filaments, all the members of this family share the same structural organization. The structure of intermediate filaments is important for self-assembly. In fact, unlike the other cytoskeleton components, intermediate filament monomers do not have any enzymatic activity, thus their assembly is based on the association of several monomers. In particular, two -helical rods associate in parallel creating a dimer, which, in turn, associates with another dimer in an antiparallel manner, giving rise to a tetramer (Margiotta and Bucci 2016) (see Figure 1.4).
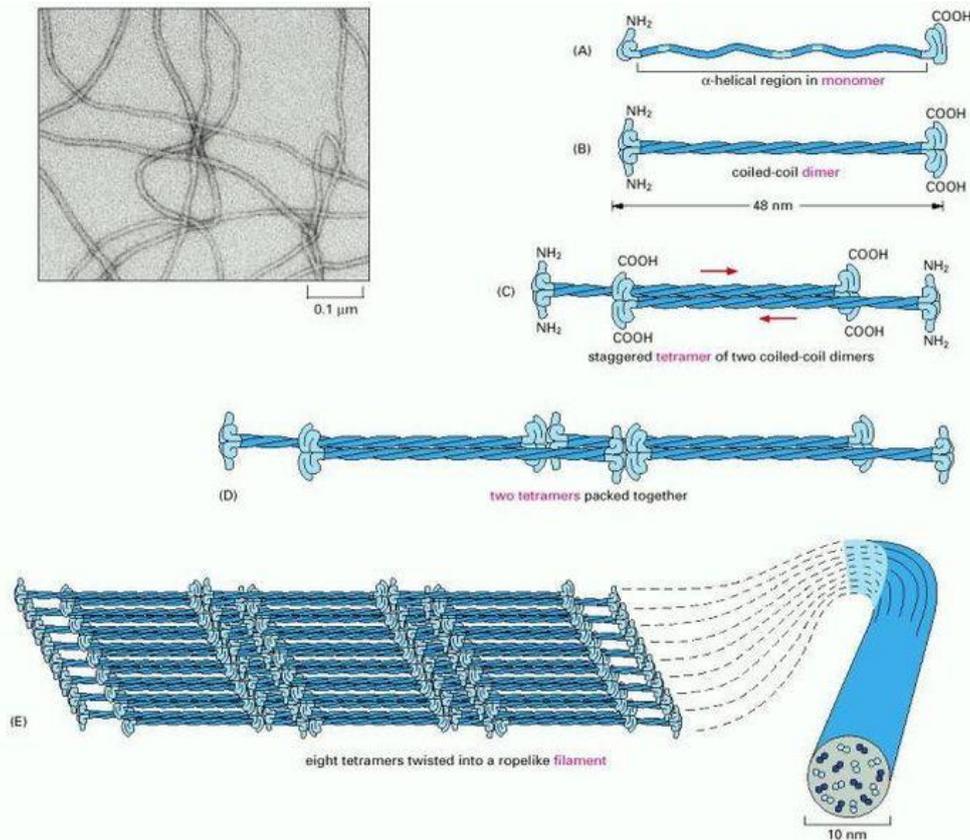
Figure 1.4 Structure of intermediate filaments (Alberts *el al.,* 2002).

1.2.3 Actin filaments

In eukaryotic cells, actin is the abundant protein, beneath the plasma membrane, to form a network that provides mechanical support, determines cell shape, and allows movement of the cell surface, allowing cells to migrate, engulf particles, and divide. Actin is rich in cells and the actin filaments are made up of identical actin proteins arranged in a long helical chain. Each actin monomer named globular actin (G actin), has binding sites that allow head-to-tail interactions with two other actin monomers, resulting in the actin monomers to polymerize to form filaments (F actin). Due to all the actin monomers being oriented in the same direction, actin filaments have a distinct polarity and their ends, called the plus and minus ends, are distinguishable from one another. This polarity is important both in their assembly and in establishing a unique direction of myosin movement relative to actin (Pollard and Cooper, 2009). Using an electron microscope and viewing the negatively stained actin fibres, it can be seen that F-actin is composed of two chains of actin monomers that turn gradually around each

other to form a right-handed helix (Dominguez and Holmes, 2011) (Figure 1.5). The structure actually appears like two slowly turning right-handed chains.

Actin filaments are important to establish cell polarity and cohesion among epithelial cells. For example, the integrity of epithelial cell layers is maintained by a belt of actin filaments (i.e. adhesion belt). This belt links the cytoskeleton of adjacent cells. Also, certain cells use actin filament rigidity to sense vibrations, such as those found bundled on the surface of hair cells in the inner ear, which tilt in response to sound and the individual actin filaments are continuously remodelled and replaced once every 48 hours (Pollard and Cooper 2009).

Actin filaments are important in cell motility and are found in membrane protrusions such as filopodia (microspikes) and lamellipodia (thin sheet or foot) rapidly assemble and disassemble. These cellular structures are essential in cell migration and are found at the leading edge of a moving cell. The actin filaments allow the cell to sense its microenvironment. Additionally, actin-associated myosin motor proteins use ATP hydrolysis to exert forces against the stress fibres during muscle contraction; the energy of hydrolysis can also be converted to tensile forces at the trailing cell edge to promote edge retraction in motile cells (Pollard and Cooper 2009).

Minus end

Plus end
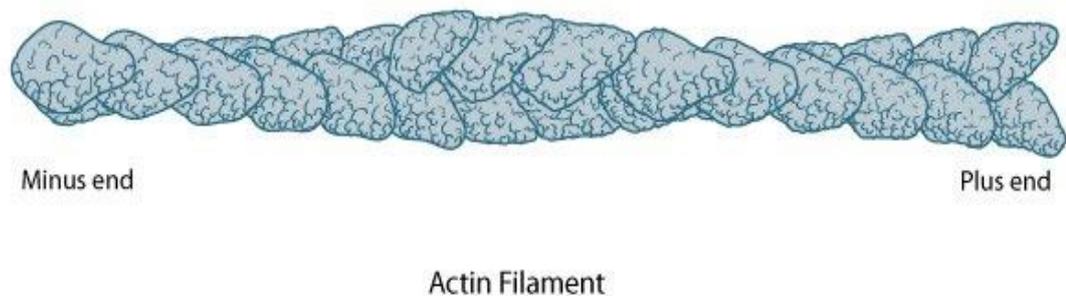
Actin Filament

Figure 1.5 Structure of an actin filament showing the barbed (or plus) and pointed (or minus) ends. MBInfo contributors. Structure of an actin filament showing the barbed (or plus) and pointed (or minus) ends. In MBInfo Wiki, Retrieved 10/21/2014 from http://mbinfo.mbi.nus.edu.sg/figure/1379651820893/.

1.3 Role of actin filaments in cell migration

Many cells have actin filaments under the cell cortex. This is the meshwork of membrane-associated proteins which supports and strengthens the plasma membrane. This network allows the cells to hold and move e.g. the brush border of microvilli (Dominguez and Holmes, 2011). Cell migration is made up of a complex network of signal transduction pathways which include lipid second messengers, small GTPases, kinases, cytoskeleton-modifying proteins, and motor proteins. When the cell is ready to migrate or crawl the cell uses its cytoskeletal filaments as the main source for cell movement. Some cell movement involves pinching off the cell membrane which is also known as cytokinesis. Cytokinesis contains capacity of actin filament networks. The networks are extremely dynamic and rapidly form and disassemble which underlies the crawling behaviour of cells. Cell crawling/migration involves a cycle of movements, which can be viewed in three stages. First, protrusions such as pseudopodia, lamellipodia, or microspikes are extended from the leading edge of the cell. Second, the extensions must attach to the substratum across which the cell is migrating. Finally, the trailing edge of the cell must detach from the substratum and retract into the cell body.

The extension of the leading edge involves polymerization and crosslinking of actin filaments. The regulated turnover of actin filaments, leads to the extension of processes such as filopodia and lamellipodia at the leading edge of the cell. Myosin may also participate in the extension of processes at the leading edge. Next, protrusions from the leading edge must attach to the substratum in order to function in cell locomotion. For slow-moving cells, attachment involves the formation of focal adhesions, such as fibroblasts. Whereas fast moving cells, form more diffuse contacts with the substratum e.g. white blood cells.

The last stage involves retraction of the trailing edge. The attachments between the trailing edge and the substratum are broken, and the cell recoils into the cell body. This requires a tension between the front and rear of the cell, resulting in contractile force that pulls the rear of the cell forward (Cooper, 2000) (Figure 1.6).
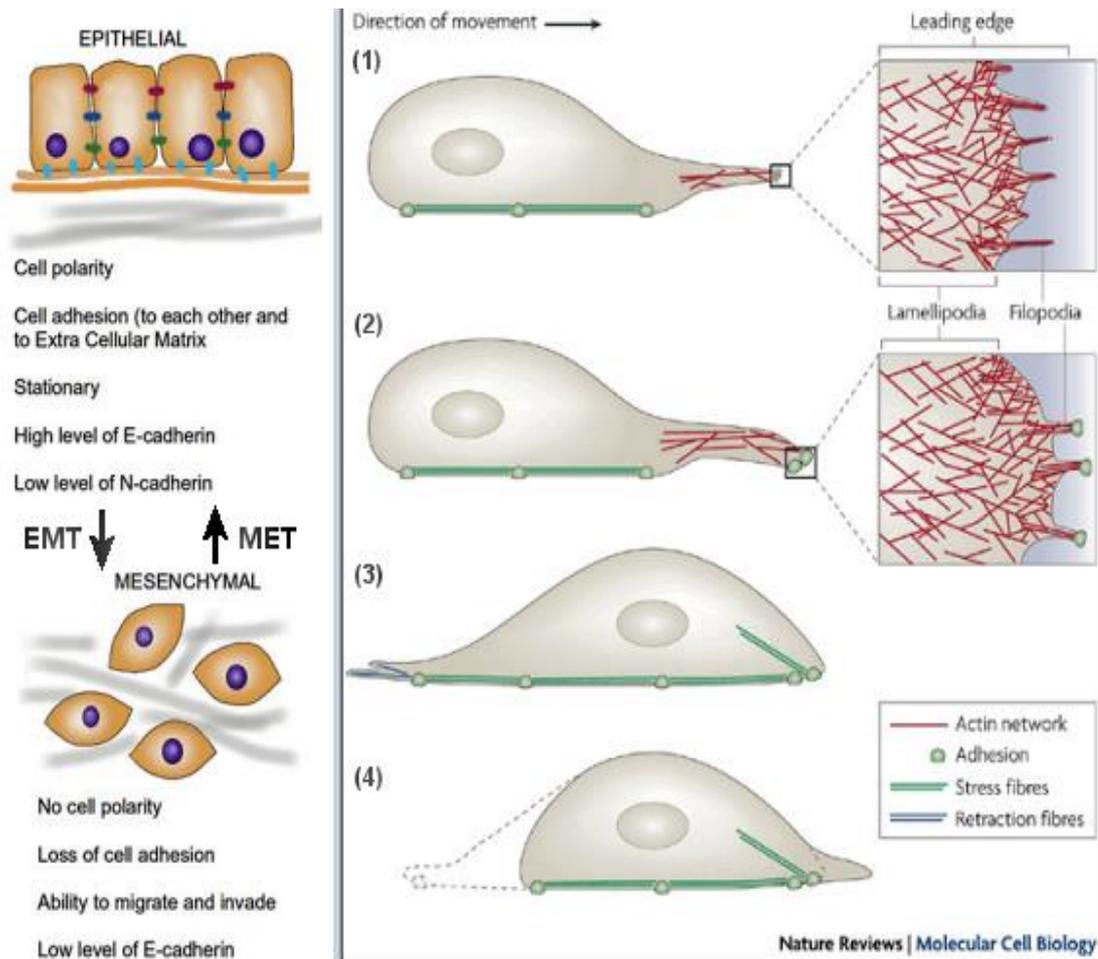
Figure 1.6 Cell crawling/migration is dependent on different actin filament structures. (A) Protrusion of the cell's leading edge, which is composed of structures called lamellipodia and filopodia. (B) The membrane sticks to the surface at the leading edge. (C) The nucleus and the cell body are pushed forward through contraction forces mediated by stress fibres. (D) The retraction fibres pull the rear of the cell forward © 2008 Nature Publishing Group (Mattila *et al.,* 2008).

However, cell migration can be altered when cells respond to mechanical stress (Vogel, 2006). Cells require mechanical stress to adapt to their physical surroundings which is important for homeostasis, tissue architecture and muscle regeneration. Therefore all cells and organisms have the ability to sense mechanical stress (Ingber, 2006). Multicellular organisms require physical forces to hold themselves together. The existence of forces can act directly on molecules or cells to trigger a biological response. The ability to change biological outcomes through mechanical forces is known as mechanotransduction (Ewa K. Paluch, 2015).

1.4 The role of mechantranduction

Mechanotransduction, the modulation of biological chances by physical forces, has been found in all biological realms and with a diverse set of mechanisms. Some of these mechanisms involves mechanosensitive channels that allow physical stimuli on or across membranes to control the flow of molecules across these membranes: flow that can in turn release osmotic pressure or trigger another signalling pathway. For example, the role of the cytoskeleton in responding to physical cues such as the rigidity of its environment is one of the most studied examples of mechanotransduction.

The cytoskeleton can easily exert force on its surroundings and sense the mechanics of cells or substrates around it. The physical forces play a direct role in important biological choices such as cell differentiation and motility. The physical tension of the plasma membrane can also play a role as an orchestrator of many cellular events. However, in the cytoskeleton actin at focal adhesion points can at least partially recapitulated by artificially exerting forces on other locations of the cells (Ewa.K Paluch, 2015)

All cells of an organism are subject to mechanical stresses generated by either compression, shear stress or tension. When cells respond to the mechanical stress they alter their shape, function and behaviours including proliferation, differentiation, apoptosis and migration (Vogel, 2006). However, the exact mechanisms how cells sense and react to the mechanical signals are still largely unknown. However, it is known that the cytoskeleton and associated molecules are important regulators of mechanotransduction. In general, cells can interact with their environment by cell adhesion molecules, tight junctions and cell surface receptors. These molecules send signals to transmit the changes from the environment to the inside of a cell.

These signals are then translated into chemical signals. This is carried out by activation of signalling cascades or release of intracellular Ca^{2+} leading to activation of ion channels in the plasma membrane (Alberts *et al.,* 2002).
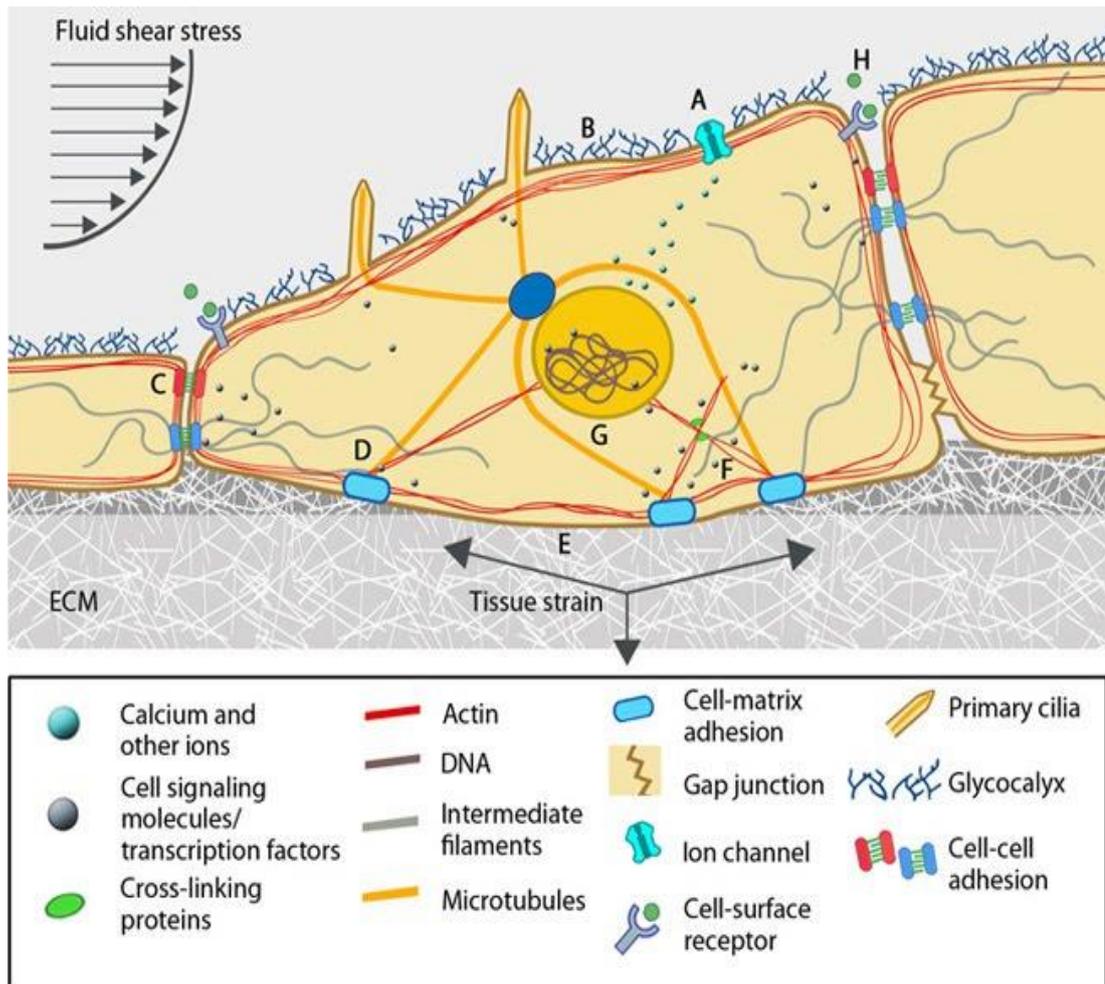
Figure 1.7 Several biological components have been proposed to act as cellular mechanosensors and most of these components can be found in many different cell types. These include: (A) The mechanosensitve ion channels (B) The glycocalyx (C) Cell-cell junctional receptors. (D) Cell-matrix focal adhesions (E) Force-induced unfolding of extracellular matrix proteins (F) Intracellular strain can induce conformational changes in cytoskeletal elements (G) The nucleus (H) Intracellular deformations. MBInfo contributors. Overview of mechanotransduction in a cell. In MBInfo Wiki, Retrieved 10/21/2014 from http://mbinfo.mbi.nus.edu.sg/figure/1384244676112/.

1.4.1 The mechanosensitve ion channels

The mechanosensitve ion channels in the plasma membrane open in response to lipid fluidity allowing influx of calcium and other ions. Additionally, changes in G-protein coupled receptors can induce intracellular signals

1.4.2 The glycocalyx,

Glycoclayx is a glycoprotein-polysaccharide covering that surrounds the cell membranes, that can facilitate mechanotransduction signalling in response to fluid shear stress (Cruz-chu *et al.,* 2014).

1.4.3 Cell-cell junctional receptors.

There are four main types: selectins, cadherins, integrins, and the immunoglobulin superfamily. Selectins are cell adhesion molecules that play an important role in the initiation of inflammatory processes. Together with an alpha-beta catenin complex, the cadherin can bind to the microfilaments of the cytoskeleton of the cell. This allows for homophilic cell–cell adhesion. The β-catenin–α-catenin linked complex at the adherens junctions allows for the formation of a dynamic link to the actin cytoskeleton. Integrins act as adhesion receptors, transporting signals across the plasma membrane in multiple directions. These molecules are an invaluable part of cellular communication, as a single ligand can be used for many integrins. Immunoglobulin superfamily are a group of calcium independent proteins capable of homophilic and heterophilic adhesion. Cell adhesion is a vital component of the body. Loss of this adhesion effects cell structure, cellular functioning and communication with other cells and the extracellular matrix and can lead to severe health issues and diseases (Eric Lambie, 2004).

1.4.4 Cell-matrix focal adhesions

Cell-matrix interactions are mediated by adhesion receptors and lead to the formation of multi-protein adhesion structures that interact with the actin cytoskeleton at the cell interior; collectively, they are called cell-matrix adhesion complexes (CMACs). These adhesions act as vital information processing centers that enable cells to sense numerous extracellular signals that convey information about the chemistry, geometry, and physical properties of the ECM. The substrate type or chemical composition, its rigidity, and the surface topography influence force-induced events through CMACs, and mechanosensitive cells transmit this information through subsequent mechanotransduction pathways and signaling cascades to influence

diverse processes such as the cell shape, polarity, fate, motility and deposition and/or restructuring of ECM components (Lodish, 2000).

1.4.5 Force-induced unfolding of extracellular matrix proteins

Force-induced conformational changes in the ECM components can sensitize the receptors and initiate mechanotransduction signals e.g. fibronectin which can initiate mechanotransduction signalling outside the cell (Smith *et al.,* 2007).

1.4.6 Intracellular strain

Intracellular strain can induce conformational changes in cytoskeletal elements such as filaments, cross linkers or motor proteins, thereby changing binding affinities to specific molecules and activating signalling pathways (Gilchrist *et al.,* 2007).

1.4.7 The nucleus

The nucleus is proposed to sense the mechanical signals and accordingly modulate the transcription machinery by changing chromosome positions, chromatin arrangements and transport of molecules across the nuclear membrane (Gilchrist *et al.,* 2007).

1.4.8 Intracellular deformations

It is to be noted that changes in inter-cellular space is cell-type specific. This can influence concentration of signalling molecules/ proteins that bind cell-surface receptors (Gilchrist *et al.,* 2007).

Compression of the intercellular space can alter the effective concentration of autocrine and paracrine signalling molecules. Additionally, changes in G-protein coupled receptors, lipid fluidity, and even mitochondrial activity have been proposed as mechanosensors. Generally, almost all cells respond to mechanical stimulation with adaptive changes in cell function. These changes include short term responses such as increases (or decreases) in intracellular tension, adhesion, spreading or migration, as well as long term effects (e.g., protein synthesis/secretion, structural reorganization,

proliferation, viability) often mediated through multiple, overlapping and cross talking signalling pathways (Ewa K. Paluch, 2015).

One of the key features of environmental sensing is the mechanical crosstalk between integrins and the actin-cytoskeleton. However, other molecules inside and outside a cell can contribute to mechanosensation (Ewa K. Paluch, 2015).

Ingber investigated mechanotransduction on cells and suggested that not enough research is carried out to understand these different molecules function in living cells, tissues, and organs to produce the organisation of cellular behaviours required for mechanosensation, embryogenesis, and physiological control. Recent work shows that organ, tissue, and cell anatomy are important for mechanotransduction as individual mechanosensitive proteins and that our bodies consist of networks starting from the macroscale to the nanoscale in order to focus stresses on specific mechanotransducer molecules. There is also isometric tension (prestress) at all levels of networks in order for the mechanochemical transduction mechanisms to proceed at once to produce a response (Ingber, 2006).

The internal forces that determine cell adhesion and control cell shape are not shown in cultured cells and in cells within living tissues as they are isometric which is balanced by opposing forces due to the rigidity of the adhesion substrate (e.g., culture dish or ECM), and to the pulling forces of neighbouring cells. Prestress can be seen by culturing cells on substrates, such as silicon rubber, that fold up into compression creases when the cells exert forces on their basal ECM adhesions. These forces are important because the physical forces acting at the organ level can influence the function of cells within living tissues. Some examples are compression on bone, tension on muscle, pressure on lung, and fluid shear on blood vessels (Ingber, 2009).

Ingber also showed that microtubules can bear compression in living cells, and that they resist inward-directed forces generated by the surrounding contractile actin cytoskeleton. The cells anchor to the ECM substrate through multiple small discrete tethering sites, known as "focal adhesions". This is where cells cluster together their integrin receptors, which mediate cell surface binding to various ECM proteins. The cytoplasmic tails of integrins bind to molecules, such as talin, vinculin, paxillin, and α-actinin, which forms the backbone of the focal adhesion resulting in a molecular bridge linking ECM and integrins to the internal contractile cytoskeleton. These focal

adhesions form at the distal ends of each stress fibre, which shortens when their internal actomyosin filaments slide and creating tension; this results in an increase in cytoskeletal prestress and flattening in a cell adherent to a rigid substrate (that can resist filament contraction), whereas cells round and form compression wrinkles as these adhesions are pulled closer to each other when the cell is cultured on a flexible substrate. Hence, the cell's ECM adhesions also can work in a corresponding way with microtubules to resist inward-directed cell tensional forces, and forces can be shuttled back and forth between ECM, microtubules, and the actin cytoskeleton in living cells (Ingber, 2009).

Defects in cellular mechanotransduction are often through mutations which result in various human diseases. Changes in the cellular physical environment could provoke pathological consequences, even when the cellular mechanotransduction processes function properly. An example can be fluid shear stress at bifurcations triggering vascular remodelling resulting in atherosclerosis. In these cases, it is the abnormal mechanical stress at the cellular level rather than normal mechanotransduction signalling (Ingber, 2006).

1.5 The effects of force on cells and tissue

For the maintenance of organ development mechano-response is important, especially in mechanically stressed tissues such as bones, muscles, cartilage, and blood vessels (Bakker, 2003). Long bed rest can lead to loss of bone mass and muscle atrophy (Flück M, 2003). However, it has also been seen that after fractures the stress fibers become realigned. Mechanical forces in bones use the lacunae-canalicular network. This involves small cavities (known as lacunae) within layers of bone matrix. The lacunae, are scattered at intervals, harbor osteocytes and are connected through canaliculi. When the muscle contracts, the gravity and compression leads the interstitial fluid to enter the lacunae-canalicular network causing deformations of the bone. This results in localized bone remodelling through mechanotransduction signalling (Burger, 1999).

Cells of the cardio vasculature have two types of mechanical forces exerted on them. One is sheer stress caused by friction of blood against the vessels and the other is

cyclic tensile stress, caused by blood pressure. When the blood flows, shear stress causes the artery to mature leading to endothelial filamentous cytoskeletal networks to elongate and align with the direction of flow. The pressure and shear stress from the flowing blood in the cardiovascular system influence the physiology and morphology of the heart. In developing zebrafish heart, changes in the flow patterns have led to an abnormal third chamber with defective valves which is similar to some congenital heart diseases (Stefan Hammerschmidt1, 2007) (Figure 1.8).
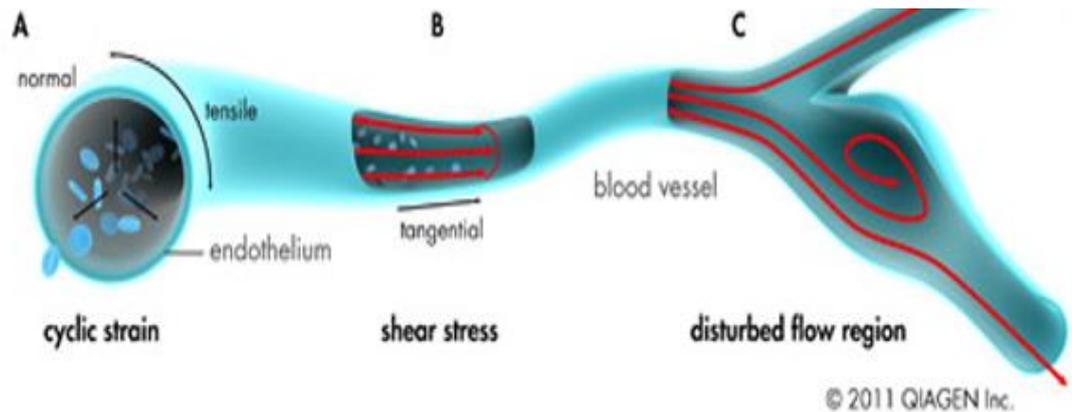
Figure 1.8 Forces affecting endothelial cells. Endothelial cells are exposed to two major forces: cyclic strain (A) and fluid shear stress (B). (C) ECs in areas of high curvature or downstream of bifurcations (e.g. carotid sinus, coronary bifurcation) are exposed to disturbed shear stress (oscillatory with a low time-average shear stress) (Conway and Schwartz, 2011). The image reproduced with the kind permission of QIAGEN'

The ability of cells to respond to changes in their physical environment is critical in the development and maintenance of tissues that are exposed to varying mechanical stress (for example, muscle and bone), but also in physiological processes which affect the entire organism (for example, control of blood pressure and blood flow). On the cellular level, mechanotransduction can modulate diverse functions such as protein synthesis, secretion, adhesion, migration, proliferation, viability and apoptosis.

1.6 The effects of whole body vibration (WBV) in human and animal studies.

Cells are mechanosensitive and some work has been carried out to try and exploit these mechanosensitive properties of cells. Starting off with recent work has shown that mechanical stimulation at the whole organism level can have therapeutic benefits, while at a cellular level it can be used to control stem cell differentiation. For example, whole body vibration (WBV), which is being increasingly used in a clinical setting, has been shown to have therapeutic benefits. In a study carried out on patients with Multiple Sclerosis, it was found that mechanical stimulation at frequencies between 40 and 50 Hz (delivered through a vibrating platform) in combination with exercise, helped to improve patient muscle strength, functional ability and general wellbeing (Schuhfried *et al.,* 2005).

In contrast to MS, another study has looked at the rehabilitation of hand function in persons with severe impairment following stroke. A study by Cordo *et al.,* investigated whether an involvement of robot-assisted movement and muscle vibration could aid to reduce the impairment and enable the hand-opening to a greater extent. The method involved selecting forty-three participants with severe hand impairment due to chronic stroke were randomly allocated in to 1 of 2 treatment groups receiving assisted movement and muscle vibration. Every participant received 30 minutes of vibration per session directed at the impaired hand over 10 to 12 weeks. The results showed that 28 of 43 participants had no baseline finger extension; the rest had an average of 23 ± 26 mm extension in the most active finger. Assisted movement and muscle vibration were associated with a significant increase in all outcome measures across both treatment groups. Assisted movement and muscle vibration, appears to reduce upper limb impairment, improve volitional activation of the hand muscles, and restore a modicum of hand function in some persons with severe hand impairment due to chronic stroke (Cordo *et al.,* 2013).

Another study used whole body vibration on young men, a study by Aquayo *et al.,* suggested that whole-body vibration (WBV) is insufficient to expand satellite cell (small multipotent cells with virtually no cytoplasm found in mature muscle) numbers 24hr post exercise, whereas WBV in combination with blood flow restriction (BFR)

is sufficient. The study tested twenty-five young men who were randomly split into three groups: WBV, BFR exercise or WBVBFR. Exercises were performed in two or three sessions a week and the WBV group were asked to support a static half squat position with knee angle of 135° standing on a vibration plate with vibrational frequency of 30Hz. Satellite cell numbers were determined from muscle biopsies of the *vastus lateralis* muscle (a muscle in the thigh) using immunohistochemistry. Satellite cell quantity and frequency increased only in the WBVBFR group. Similar results were obtained for the quantity and frequency of myogenin-positive. The study concluded that modification of WBV by superimposition of BFR induced activation and differentiation of satellite cells in young men, which had not been observed with WBV or BFR alone (Aquayo *et al.,* 2016).

Interestingly another study used WBV on severely burned children, whose burn resulted in significant disability with loss of bone mass, muscle mass, and strength. A study by Edionwe *et al.,* 2016 looked at the effects of exercise combined with whole-body vibration (WBV) on bone mass, lean mass (LM), and muscle strength in children recovering from burns. The method includes nineteen burned children (≥30% total body surface area [TBSA] burns) were randomly assigned to a 6-week exercise regimen either alone (EX; n=10) or in combination with a 6-week WBV training regimen (EX+WBV; n=9). The WBV was performed for 5 days/week on a vibrating platform. Dual-energy X-ray absorptiometry quantified bone mineral content (BMC), bone mineral density (BMD), and LM; knee extension strength was assessed using isokinetic dynamometry before and after training. Participants were similar in age, height, weight, TBSA burned, and length of hospitalization. The results show that the whole-body LM increased in the EX group and an increase in the EX+WBV group. On the other hand, there were decreases in leg BMC for both groups, and in leg BMD for only the EX group. Truncal BMC decreased in only the EX group, while BMD decreased in both groups. Leg strength increased over time in the EX group and the EX+WBV group. Exercise in combination with WBV may help attenuate regional bone loss in children recovering from burns (Edionwe *et al.,* 2016).

After applying WBV on humans, many researchers have carried out investigations using animals such as rats and mice. A study by Komrakova *et al.,* looked at the combined therapy of teriparatide (PTH) or strontium ranelate (SR) with whole-body

vibration (WBV). Teriparatide is a recombinant form of parathyroid hormone and is an effective anabolic (i.e., bone growing) agent used in the treatment of some forms of osteoporosis. Strontium ranelate is a drug that increases deposition of new bone by osteoblasts and reduces the resorption of bone by osteoclasts. The treatment of drugs and vibration was used on bone healing and muscle properties in an osteopenic rat model. Seventy-two rats were bilaterally ovariectomized (Ovx), and 12 rats were left intact (Non-Ovx). PTH (40 µg/kg BW sc. 5×/week) and SR (613 mg/kg BW in food daily) were applied on the day of ovariectomy, vibration treatments 5 days later (vertical, 70 Hz, 0.5 mm, 2×/day for 15 min) for up to 6 weeks. The rats with WBV + SR group, the callus density, trabecular number, decreased compared to SR alone. However, with WBV + PTH group, the cortical and callus widths, biomechanical properties, were increased; the cortical and callus densities were decreased compared to PTH alone. The results showed that vibration alone did not change the bone parameters; however, PTH possessed a stronger effect than SR therapy (Komrakova *et al.,* 2016).

Another study using whole body vibration in rats by Chow *et al.,* looked at ovariectomy in rats but at the estrogen receptor (ER) using whole-body vibration. The estrogen receptor (ER) is involved in mechanical signal transduction in bone metabolism. Ovariectomy-induced osteoporotic SD rats had whole-body vibration treatment and ICI for ER antagonization by ICI 182,780. The vibration was able to enhance ER expression in ovariectomy-induced osteoporotic fracture healing. ER was essential in mechanical signal transduction and enhancement in callus formation effects during osteoporotic fracture healing enhanced by vibration. The enhancement of ER-α expression by mechanical stimulation was not likely to be related to the increased expression in osteoblastic cells but rather to the systemic enhancement in recruitment of ER-expressing progenitor cells through increased blood flow and neo-angiogenesis (Chow *et al.,* 2016).

A similar study by Matsumoto *et al.,* investigated the effects of whole body vibration on bone repair using a noise like whole body vibration. The mice were ovariectomized and divided into four groups (n = 12 each): a control group, and groups treated with parathyroid hormone of 30 µg/kg/day, noise-like whole-body vibration (45-100 Hz for

20 min/day), and both. The results show that the combined treatment resulted in regeneration of bone with high indentation modulus and hardness, suggesting the therapeutic potential of the combined use of noise-like whole-body vibration and intermittent administration of parathyroid hormone for enhancing osteoporotic bone healing (Matsumoto *et al.,* 2016).

Wei *et al.,* looked at low-magnitude high-frequency vibration (LMHFV) in osteoporotic fracture healing. They hypothesised that LMHFV promoted osteoporotic fracture healing by enhancing mesenchymal stem cell (MSC) migration through the stromal derived factor 1 (SDF-1) pathway. In total 152 ovariectomised rats received closed femoral fracture in groups of vibration+MSC, for 20 min per day for 5 days a week, vibration+MSC+AMD3100 (VMAG; AMD, a CXCR4 inhibitor) (1 mg/kg/d, intraperitoneal), MSC (MG) (1×106 MSC, intracardiac) or control (CG) for a treatment duration of 2, 4 or 8 weeks. The MSC migration was evaluated by ex-vivo green fluorescent protein signal in the callus; and fracture healing was examined by weekly radiographs. At week-2 and week-4, ex-vivo callus GFP intensity of VMG was significantly higher than other groups. From week-2 to week-3, both callus width and callus area in VMG were significantly larger; and from week-7 to week-8, smaller than other groups. At week-8, high-density bone volume fraction, bone volume fraction, bone mineral density and stiffness in VMG were significantly higher than other 3 groups. This showed that LMHFV promoted MSC migration and fracture healing in osteoporotic rats (Wei *et al.,* 2016).

Guo *et al.,* was interested in looking at fall-related injuries in elderly people. Previous studies show that the low-magnitude high-frequency vibration (LMHFV) was proven beneficial to the musculoskeletal system and recommended for sarcopenia treatment (treatment for the loss of skeletal muscle mass and strength as a result of ageing). Guo *et al.,* aimed to evaluate the effects of LMHFV on the loss of skeletal muscle mass and strength as a result of ageing. They used mice at month 6 and were randomized into control (Ctrl) and vibration (Vib) groups. The Vib group were given LMHFV (0.3 g, 20 min/day, 5 days/week) treatment at each month 0, 1, 2, 3, and 4 post-treatments the muscle mass, structure, and function were assessed. The results showed that, the mice in the Vib group had greater muscle strength. The contractibility was significantly

improved by LMHFV. No significant difference of muscle mass was observed. In conclusion the LMHFV significantly improved the structural and functional outcomes of the skeletal muscle (Guo *et al.,* 2016).

Another study looked at the effects of WBV low-magnitude high-frequency vibration (LMHFV). The investigation was to see the effects of LMHFV on fracture healing in aged osteoporotic mice. Female C57BL/6NCrl (mice) (*n*=96) were either ovariectomised (OVX) or sham operated (non-OVX) at age 41 weeks. After 49 weeks, all mice received a femur osteotomy. Next, the mice received whole-body vibrations of 20 minutes/day for 10 and 21 days at a frequency of 45 Hz. The results from the study shows that the LMHFV disturbed fracture healing in aged non-OVX mice, with significantly reduced flexural rigidity and bone formation in the callus. However, in OVX mice, LMHFV significantly improved callus and increased flexural rigidity and bone formation (Wehrle *et al.,* 2015).

In another study Butezloff *et al.,* 2015, investigated the effect of vibration therapy on the fractured femurs and the bone quality of intact femurs in ovariectomized rats. They randomly selected fifty-six rats aged seven weeks and were divided into four groups: control with femoral fracture, ovariectomized with femoral fracture, control with femoral fracture plus vibration therapy and ovariectomized with femoral fracture plus vibration therapy. After three months of surgery, a complete fracture was produced. Next, three days later, the vibrational therapy groups underwent vibration therapy of 60 Hz for 20 minutes, three times per week for 14 or 28 days. The results showed that the ovariectomized rats demonstrated a substantial loss of bone mass and severe impairment in bone microarchitecture, both in the non-fractured femur and the bone callus. However, the whole-body vibration therapy amended the bone and fracture callus parameters in the osteoporotic bone. The therapy improved bone quality and the quality of the fracture bone callus in ovariectomized rats (Butezloff *et al.,* 2015).

A study by Weinheimer-Haus *et al.,* found that low intensity mechanical stimulation (45 Hz) significantly increased the wound closure rate in diabetic mice, compared to those mice that were not exposed to mechanical stimulation (Weinheimer-Haus et al., 2014. Similarly, Wang et al., used a dynamic motion platform to apply mechanical

stimulations (32–37 Hz) to mice and found that expression of the mechanical stimulation-induced protein R-Spondin, which has the capacity to promote bone formation, was enhanced. The authors suggest that some 'vibe proteins' may be candidates for pre-clinical development as anabolic agents for treatments of osteoporosis (Wang *et al.,* 2013).

After identifying some literature review on human and animal studies using mechanical stimulation, it was interesting to see if any previous studies have been carried out using mechanical stimulation on fibroblast cells or even on muscle fiber formation as this work is concerned with investigating the effects of mechanical stimulation on fibroblast cell migration and muscle fibres.

1.7 Effects of mechanical stimulation on tissue and cells

Previous studies have mentioned that mechanical stimulation can result in nerve and muscle damage and also induces a change in muscle reflex or bone growth. It can also cause damage to the arterial endothelial cells, the smooth muscle and the central nervous system (Puig *et al.,* 2005) However, there are several studies that have looked at how mechanical stimulation can promote bone formation and prevent osteophorosis. For example, a study carried out by Wang *et al.,* looked at mechanically-regulated protein secretion by MPCs. Using a dynamic motion platform, the workers applied low-magnitude stimulation (32-37 Hz) to MPCs for 10 and 20 minutes, respectively and found that secreted proteins (or vibe-proteins), which have the capacity to promote bone formation, were up-regulated. The authors suggest that by virtue of their secretory status, some vibe proteins may be candidates for pre-clinical development as anabolic agents for the treatment of osteoporosis (Wang *et al.,* 2013).

In another study Kulkarni *et al.,* used a modular piezoelectric actuator to vibrate murine osteoclastic-like cell line RAW264.7 cells at 4Hz for 1 hour, on 3 consecutive days and found that bone reabsorption (breakdown of bone) was inhibited (Kulkarni *et al.,* 2013). Whilst in a similar study Wu *et al.,* found that low-magnitude high-

frequency vibration (LMHF) for 45Hz for 15 min per day using a vibrating platform to hold the culture dishes could also be used produce an anabolic effect on bone through inhibition of osteoclast differentiation (Wu *et al.,* 2012). In contrast Kim *et al.,* found that human exposure of mesenchymal stromal cells (hMSC) to low-magnitude high-frequency (LMHF) vibrations (30-40Hz) resulted in an increase in proliferation. The authors suggest that LMHF could enable the osteogenic process (Kim *et al.,* 2012).

A similar bone cell study by Bacabac *et al.,* looked at the effects of low-amplitude; high-frequency loading stimulation on bone growth by enhancing bone formation and inhibiting disuse osteoporosis, disuse osteoporosis localized or generalized bone loss resulting from reduction of mechanical stress on bones. They investigated bone cell responses to vibration stress at a wide frequency range (5–100 Hz). They looked at different enzymes (NO and prostaglandin E2 (PGE2) release, and COX-2 mRNA expression) as parameters for bone cell response, since these molecules regulate bone adaptation to mechanical loading. The results showed that the NO release positively correlated whereas PGE2 release negatively correlated to the acceleration rate of the vibration stress. The COX-2 mRNA expression showed increase in a frequency-dependent manner, which relates to increase NO release at high frequencies. However, the negatively correlated release of NO and PGE2 suggests that these signalling molecules play different roles in bone adaptation to high-frequency loading (Bacabac *et al.,* 2006).

Another bone cell study by Pre *et al.,* looked into investigating whether differentiation of adult stem cells toward bone tissue is encouraged by high-frequency vibration (HFV). They used bone marrow stromal cells (BMSCs) and stimulated them at 30 Hz for 45 minutes a day for 21 or 40 days. The results showed that the amount of calcium was almost double in the treated samples; the expression of genes involved in differentiation was increased and protein content was higher for all osteogenic proteins. Also, the proliferation stimulated BMSCs with stimulation have a decreased growth rate in comparison with controls, (Pre *et al,* 2013).

In contrast to bone cells, another study used stimulation on human mesenchymal stem cell MSCs. Uzer *et al.,* developed an in vitro model in which they used parameters

including acceleration magnitude and fluid shear which can be controlled independently during vibrations. Adipose derived human mesenchymal stem cell MSCs were subjected to vibration frequencies and acceleration magnitudes that induced fluid shear stress ranging from 0.04Pa to 5Pa. Vibrations were applied at 0.15g, 1g, and 2g using frequencies of both 30Hz and 100Hz. After 14 days and under low fluid shear conditions associated with 100Hz oscillations, mineralization was greater in all vibrated groups compared to the controls. Greater levels of fluid shear produced by 30Hz vibrations enhanced mineralization only in the 2g group. Over 3 days, vibrations led to the greatest increase in total cell number with the frequency/acceleration combination that induced the smallest level of fluid shear (Uzer *et al.,* 2013).

Compare to MSC cells another study used endothelial cells. Curtis *et al.,* investigated nanometric movements of the substrate on which endothelial cells were growing. The movements were driven by a periodic sinusoidal wave using piezo actuators, at frequencies ranging between 1-50 Hz. It was found that repeated mechanical stimulation of the endothelial capillary cells led to the appearance of Krüppel-like Factor 2 (KLF2) and endothelin-1 expression factors. Curtis also suggested that effective stimulation in this mode has to be coherent in time and space and if this is altered the gene expression of KLF2 and endothelin-1 is lost (Curtis *et al.,* 2013).

In contrast to 50Hz, another used 45 Hz stimulation on murine monocyte cell line. Wu *et al.,* investigated low-magnitude high-frequency vibration (LMHFV) on osteoclast differentiation in vitro. However, they used RAW264.7 cells in the presence of receptor activator of nuclear factor-kappaB ligand (RANKL) which were treated with or without LMHFV at 45 Hz for 15 min day. They found that LMHFV significantly decreased the number of RANKL-induced TRAP-positive multi nucleated cells. The results suggest that LMHFV can inhibit the RANKL-induced osteoclast differentiation of RAW264.7 cells, which give some new insight into the anabolic effects of LMHFV on bone (Wu *et al.,* 2012).

In another study, 10Khz was used in a study by Ito *et al.,* who was interested to investigate the effects of vibration on differentiation of rat pheochromocytoma (PC12) cells. A nano-vibration system consists of a vibrator plate on which a multi-well

culture plate was placed which was designed to produce nanometer-scale vibrations. The frequency and amplitude of the nano-vibrations were monitored by a capacitance displacement sensor connected to an oscilloscope. The PC12 cells were exposed to vibration at 10 kHz. The results from the study showed that vibration promoted differentiation of PC12 cells (Ito *et al.,* 2011).

An interesting study was carried out using a speaker device to generate stimulation. Puig *et al.,* was interested in snoring-induced airway inflammation and to assess whether a vibration stimulus induced inflammation in airway epithelial cells. Human bronchial epithelial cells (BEAS-2B cell line) were used and vibrated on vibrating platform using a speaker at 60 Hz, ± 0.3 mm) for time periods of 6, 12, and 24 hours. The results showed that mechanical vibration simulating snoring triggered an inflammatory cascade, as reflected by the increase in IL-8 release mediated by MAPK pathway (Puig *et al.,* 2005).

In contrast to epithelial cells, another study used muscles cells as C2C12P12 was used, this cell line is safe to use as previous study by Wang *et al.,* looked at the effect of vertical vibration (VV) treatment with frequencies of 5, 8, or 10 Hz on the expression of extracellular matrix proteins and myogenic regulatory factors (MRFs) as well as myotube formation in C2C12P12 myoblasts. They showed that VV stimulation is safe and effective at stimulating myogenesis in C2C12P12 myoblasts and that VV treatment at frequencies of 8 to 10 Hz can stimulate the expression of ECM proteins and MRFs in myoblasts and, in turn, increase myotube formation Wang *et al.,* 2010.

As can be seen from above, much of the work in this area has focused on the effects of mechanical stimulation upon bone cells, as a means to encourage new bone growth or inhibit bone reabsorption. Very little has been done to investigate the effects of mechanical stimulation on other cell types. For example, many conditions which are associated with loss of bone mass are also associated with loss of muscle mass (Andreoli 2001). Therefore, could mechanical stimulation be used to encourage muscle cell growth? Could mechanical stimulation be used to accelerate the wound healing process, for example through increasing fibroblast proliferation? The data

generated during this project will be completely new knowledge which will help us develop our understanding of mechanotransduction and help us develop new approaches and devices to address medical challenges such as accelerating wound healing process, increasing muscle mass in diseases associated with muscle degeneration and aging.

Chapter 2

2.0 Experimental Design

It is known that cells respond to their external physical environment through a process known as mechanotransduction, whereby physical cues acting upon the cell are converted to a biochemical response. Understanding how cells respond to external forces is a growing area of research known as 'mechanomics'. The aim of this research theme is to understand more about how cells respond to force (the cell mechanome) and how this changes with certain disease states e.g. cancer.

As cells are mechanosensitive then it raises the possibility of exploiting this fundamental behaviour to our advantage, for example in biomedical applications. Recent work has shown that mechanical stimulation of bone progenitor cells (stem cells), in a frequency dependent manner, results in differentiation into bone cells i.e. growth of new bone (Wang *et al.,* 2013, Wu *et al.,* 2012). Such findings present potential novel applications for therapies that exploit the mechanical response of cells and tissues to force. However, we must first try to understand more about how cells respond to force at both the macro and sub-cellular scale. Given that recent work has shown the potential of exploiting the use of physical stimulation to control cell behaviour i.e. differentiation, this thesis is concerned with investigating the effects of mechanical stimulation on fibroblast cell migration and muscle fiber formation. Many conditions are associated with poor wound healing and loss of muscle mass. Therefore, if we can exploit the mechanosensitive properties of cells to aid wound healing and muscle growth, then it would open up new avenues of research and possibly new treatments in this area.

2.1 Cell models and rationale

Fibroblast cells were studied as they play an important role in wound healing. Given that fibroblast cells have been shown to be mechanosensitive (Turner N.A and Potter, 2013) and respond to mechanical signals generated within the extracellular matrix (Chiquet *et al.,* 2009), it was hypothesised that mechanical stimulation to the surface on which the cells attach would lead to changes in the migratory properties of the cells. Such data will be completely new knowledge and will develop our understanding of the mechanosensitive response of fibroblast cells. This may in the future help us develop new approaches and devices to address significant medical challenges, such as accelerating the wound healing process in conditions associated with poor wound healing.

The work described in this thesis also focussed on the effects of mechanical stimulation on muscle fibre formation. For this work myocytes were used as these cells fuse to form myotubes (muscle fibres). Given that muscle fibre formation is somewhat dependent on physical stimulation of muscle tissue (i.e. exercise), it was hypothesised that mechanical stimulation of muscle cells/fibres *in vitro* would affect muscle fibre formation and size.

Many age and disease-related conditions are associated with a loss of muscle mass (e.g. Muscular Dystrophy, Muscular Atrophy). Therefore, if mechanical stimulation is shown to enhance muscle fibre formation, then it may offer potential therapeutic benefits for conditions associated with loss of muscle mass.

2.2 Mechanisms for mechanical stimulation of cells

To mechanically stimulate cells a system was developed that can be used to deliver mechanical stimulation using acoustic vibration to cells in culture. Previous studies have used variety of systems to mechanically stimulate cells. For example, Wang *et al.,* 2013 used a motion platform to apply low-magnitude stimulation (32-37 Hz) to mesenchymal progenitor cells (MPCs) for 10 and 20 minutes. Whereas Ito *et al.,* used a nano-vibration system to deliver nanometre scale vibrations to rat pheochromocytoma (PC12) cells (Ito *et al.,* 2011). Many studies reported in the literature have employed a speaker-based device to stimulate cells. For example, Puig

et al., used a speaker-based system to stimulate human bronchial epithelial cells (BEAS-2B cell line) and results showed that the effect of vibration was assessed by comparing cell proliferation and release of interleukin-8 (IL-8; measured by enzyme-linked immunosorbent assay) in cells subjected to the vibratory stimulus (both when treated and untreated with MAPK inhibitors) and in controls. There was no significant increase over 24hours cell proliferation however the concentration of IL-8 in the supernatant was increased after 12hours and 24 hours of vibration. The inhibition of the p38, MEK1/2, and JNK MAPK pathways significantly reduced the overexpression of IL-8 resulting from the vibration stimulus (Puig *et al.,*2005). While Kim *et al,* used a custom-made vibration platform attached to a shaker, to stimulate human mesenchymal stromal cells (hMSC) to low-magnitude high-frequency (LMHF) vibrations (30-40Hz) resulting in an increase in proliferation (Kim *et al.,* 2012).

Given the success of using a speaker-based device for differentiating stem cells, the work described throughout this thesis also employed a speaker-based system to mechanically stimulate cells in culture. This system was designed to deliver acoustic vibration, via a sinusoidal waveform, at frequencies ranging between 100-1600Hz to the surface upon which the cells attach and grow. A Mylar speaker was used as it is suitable for use in moist environments, such as the cell culture incubator. This system is described in Chapter 3.0 Materials & Methods.

2.3 The effects of mechanical stimulation on single and collective cell migration

As previously mentioned cells have been shown to be mechanosensitive. Therefore, part of the work described within this thesis was concerned with determining the effects of mechanical stimulation upon cell migration. The use of acoustic vibration as a physical stimulus has been reported within the literature, where it has been shown that low-frequency-low-amplitude mechanical stimulation results in bone stem cell differentiation. Such work has focussed on frequencies ranging between 45-100Hz with most of the reported work focussing on vibration frequencies of <100Hz. Therefore, this research focussed on using similar frequencies to those reported in the literature and which have been shown to be effective in altering the behaviour of cells,

whilst also using higher frequencies than those generally reported in the literature (i.e. >100Hz). These specific frequencies used here were 0, 100, 200, 400, 800 and 1600 Hz (see Chapter 4.0 for more detail on system).

Stimulation time is also an important factor. Although we know that cells and tissues *in vivo* have evolved to respond to mechanical stimulation we know very little about the frequency or exposure time of forces that are required to elicit a cellular response. Previous studies have exposed cells to mechanical stimulation for relatively short periods of time. For example, Kulkarni *et al.,* exposed RAW264.7 (a murine osteoclastic-like cell line) cells to mechanical stimulation for 1 hr, while Pre *et al.,* exposed bone marrow stromal cells (BMSCs) to mechanical stimulation for 45 minutes and Song Hui *et al.,* exposed murine monocyte cell line RAW264.7 cells for 15 minutes (Kulkarni et al., 2013; Pre *et al.,* 2013, Wu *et al.,* 2012). All of these studies showed that the exposure time was enough to induce a mechanosensitive response hence, the work described in this thesis was decided that exposure times of 5 and 20 minutes would be used.

To my knowledge at the time of writing this thesis nothing had been done to investigate the effects of mechanical stimulation on cell migration/wound healing *in vitro*. As mentioned for this study fibroblast cells were used as these cells play an important role in wound healing and are known to respond to mechanical signals generated within the extracellular matrix (Schwartz, 2010). Also, given that cell density can affect cell behaviour, then the effects of mechanical stimulation were investigated for both single cell migration (i.e. cells not at confluence) and collective cell migration (i.e. cells at confluence migrating collectively into the wound area). For single cell migration, time-lapse video sequences were captured over a 4-hour period post stimulation. These video sequences were then analysed offline using ImageJ so as to determine whether mechanical stimulation was enhancing or reducing the mean cell migration distance (µm) of the exposed population.

During wound healing, cells at the wound's edge, migrate into the wound area in a collective manner. It was therefore important to investigate if mechanical stimulation affects collective cell migration. For this purpose, a standard wound healing (scratch assay) was employed. This involved growing cells to confluence and artificially

wounding the monolayer to create a void (or wound). Cells would then be stimulated and the wound area imaged on an hourly basis to determine by how much the wound area was reducing over time. This would allow one to determine if mechanical stimulation may influence wound healing through enhanced cell migration into the wound or decreased cell migration into the wound. Such novel data would provide an indication as to whether mechanical stimulation may have potential as a novel therapeutic treatment for promoting wound healing.

2.4 The effects of intermittent mechanical stimulation on single cell migration and collective cell migration

Early results obtained during this investigation suggested that the effects of a single stimulus (e.g. 5 min) to cells may be time dependent and that the effect may wear off over time. Therefore, it was decided that some experiments would be repeated in order to determine if the effects of intermittent mechanical stimulation (e.g. 5 minute or 20 minutes of mechanical stimulation was applied every hour for 4 hours) would affect single cell migration and collective cell migration differently, compared to when using one stimulation (i.e. 5 min at the start). For this, only 100 and 1600Hz were used, along with a control (0Hz).

2.5 The effects of mechanical stimulation on cell viability

Given that this work focussed on applying physical stimulation to cells, it was important to determine if there were any changes to cell viability following stimulation. For this, two established cell viability assays were used namely; the trypan blue dye exclusion assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT cell viability assay. The trypan blue assay works on the basis that healthy, viable cells will exclude the blue dye from entering the cell. In contrast, if the cell membrane has been compromised, as a result of mechanical stimulation, then the blue dye can freely pass into the cells and cells will appear blue when examined microscopically. This assay thus allows the detection/quantification of cell viability. To corroborate the trypan blue assay results the MTT cell viability assay was employed. This assay measures the metabolic capability of the cells (specifically mitochondrial activity). Healthy, viable cells will reduce the yellow MTT solution to

a purple product called formazan. The amount of formazan produced is directly proportional to the number of viable cells. The results will show if using mechanical stimulation at the given frequencies will have if any effect on cell viability.

2.6 The effects of mechanical stimulation on cell proliferation

Previous work has shown that low magnitude mechanical signals (LMMS) can promote proliferation of mesenchymal stem cells following vibration at 90Hz for 15min per day (Luu *et al.,* 2009). In order to determine if mechanical stimulation had any effect on fibroblast cell proliferation a cell proliferation assay was carried out following mechanical stimulation (0-1600Hz). This involved quantifying cell numbers every 24hr over a 72hr period. Enhanced cell proliferation and biosynthesis are thought to mediate improved wound repair. Therefore, if the data was to show that acoustic vibration does enhance cell proliferation then it may lead to novel wound care treatments/technologies.

2.7 The effects of mechanical stimulation on actin organisation

The actin cytoskeleton is important in regulating cell migration. When a cell is ready to/or is migrating, there is a reorganisation of the actin cytoskeleton to facilitate migration (Mejillano *et al.,* 2004). To test if mechanical stimulation has any effect on the organisation of actin in the cytoskeleton, the cells were stimulated, chemically fixed and the F-actin labelled with phalloidin. Images were then taken using confocal microscopy, to demonstrate any changes in actin organisation. The work was particularly interested in quantifying the formation of key actin structures/organisations associated with cell migration namely, lamellipodia/membrane ruffling and filopodia/micro-spikes. Such experiments would support the time-lapse migration studies and provide some insight into the underlying mechanisms associated with any changes to the migratory properties of the cells.

2.8 The effects of mechanical stimulation on vinculin formation in cells

Vinculin is a membrane-cytoskeletal protein, which forms links with the actin cytoskeleton and aids cell adhesion. Vinculin has been shown to be important in converting actin-based cell protrusion into persistent cell shape change and migration (Thievessen *et al.,* 2015; Humphries *et al.,* 2007). In order to investigate, if mechanical stimulation has any effect on vinculin formation cells were stained using a rabbit monoclonal antibody directed against vinculin. Images were captured using confocal microscopy to demonstrate whether mechanical stimulation alters the distribution/levels of vinculin expression. These experiments were designed to support the time-lapse studies and actin staining. For example, vinculin controls focal adhesion formation by direct interactions with actin. In turn focal adhesions regulate cell migration. For example, it has been reported that larger focal adhesion complex sizes result in a greater cell migration rate (Kim and Wirtz 2013). Therefore, any changes in the apparent vinculin distribution and levels may be used to explain any changes in cell migration.

2.9 The effects of vibration amplitude on single cell migration

Given that the original system used to mechanically stimulate cells only controls vibration frequency it was important to assess the effects of vibration amplitude on cell migration. This would allow one to decouple the effects of frequency and amplitude on cell migration and structure. Therefore, the system was further developed to contain a variable resistor which allowed displacement of amplitude to be controlled. To investigate the effects of amplitude on single cell migration, the cells were stimulated at 100Hz with a vibration amplitude of either 100% or 50% (i.e. original amplitude reduced by half). As early results had shown that cell migration was enhanced at 100Hz this frequency was chosen for these new experiments. Once stimulated single cell migration time-lapse video sequences were captured over a 2-hour period, post stimulation. The outcome of this experiment would demonstrate the effects of frequency and amplitude of vibration on cell migration. The results of which would allow us to better understand the response of cells to force while also allowing improved system development moving forward.

2.10 The effects of mechanical stimulation on temperature

Given that the vibration of the speaker is generating energy it was important to determine if this energy was altering the temperature of the cell culture environment. Cells are sensitive to fluctuation in temperature therefore any changes in temperature may affect the behaviour of the cells. Therefore, temperature was recorded at the surface of the cell culture dish to which the cells attach using a thermocouple. The dish (containing pre-warmed medium) was placed on the speaker, which was positioned in the incubator (37°C, 5% CO_2). The speaker was set to vibrate at either 100, 200, 400, 800, 1600Hz for 20 minutes. The temperature at the surface of the dish was recorded automatically at 1-minute intervals.

2.11 The effects of mechanical stimulation on muscle fibres formation

In general, many people use vibrational devices at fitness centres and in clinical settings for weight loss or for reducing swelling in joints such as ankles and knees. Whole body vibration (WBV) is also used in patients suffering from a range of conditions and has been shown to have therapeutic benefits. For example, in a study carried out on patients who have Multiple Sclerosis it was found that vibration of 40 and 50Hz (delivered through a vibrating platform) in combination with exercise, helped to improve muscle strength, functional ability and general wellbeing (Schuhfried *et al.,* 2005). However, little is known about how mechanical stimulation affects muscle cells at the cellular and sub-cellular level.

In order to assess the effects of mechanical stimulation on muscle fibre formation and size the cells were stimulated for 5 minutes or 20 minutes every day for 4 days with images taken on day 4. The level of muscle fibres formed (i.e. more or less formed compared to the control) was observed along with the mean muscle fibre diameter. This data would provide completely new knowledge. If it is shown that mechanical stimulation, using acoustic methods, does alter muscle fibre formation and size, then it would open up new avenues of research/treatments that focus on the effects of physical stimulation on muscle cells both *in vivo* and *in vitro*.

2.12 Aims and Objectives

The aims of this research are to:

1. System Development: To develop and calibrate a speaker-based system that can mechanically deliver acoustic vibration to cells *in vitro*.

2. Cell Migration: To determine whether mechanical stimulation affects the migratory properties of fibroblast cells migration.

3. Cell Morphology: To determine whether mechanical stimulation results in characteristic changes to the morphological and structural properties of fibroblast cells associated with cell migration.

4. Muscle Fibre Formation: To determine if mechanical stimulation affects muscle fibre formation

The above aims will be achieved through the following objectives:

1. System Development: A speaker-based system was designed and built using an Arduino board and Mylar speaker. It was calibrated using laser vibrometry to determine a stable working frequency range and also amplitude of displacement (μm).

2. Cell migration: Human lung fibroblast cells (LL24), fibroblasts derived from the areolar/adipose tissue of a mouse (L929) and primary human dermal fibroblast cells (HDF) were mechanically stimulated at a range of frequencies and time-lapse imaging used to quantify cell migration. This would provide new knowledge as to whether mechanical stimulation would enhance or reduce cell migration in a frequency-dependent manner.

3. Cell Morphology: The use of laser scanning confocal microscopy to image structures associated with cell adhesion and migration i.e. actin and vinculin, would allow one to qualify and quantify whether mechanical stimulation results in changes to cellular morphology and structure, particularly macro-scale morphological changes associated with cell migration, i.e. lamellipodia and filopodia formation. This would

provide indications to the underlying mechanisms associated with any changes in the migratory properties of cells.

4. Muscle Fibre formation: The use of confocal microscopy would allow for quantification of changes to muscle fibre formation and size, particularly changes to mean muscle fibre diameter (µm) response to mechanical stimulation.

Chapter 3

3.0 Materials and Methods

3.1 Materials & Equipment

The following lists the materials and equipment used throughout this research project:

Appleton Woods, UK

Sterile surgical pipettes (5 and 10ml); universal tubes (25ml), 30mm circle coverslips glass microscope slides (75x25x1.1mm), cell culture flasks (75cm²), Cryogenic Vials 2.0ml.

Sigma- Aldrich UK

Phosphate Buffered Saline tablets (PBS), Dulbecco's Modified Eagles Medium (DMEM) with 2mM L-glutamin (In-vitrogen, D6429), Foetal Bovine Serum (0804), Trypsin-EDTA solution (0.05% trypsin with 0.02% EDTA- ethyenediamin), Ethanol, Dimethyl sulphoxide (DMSO), Gel Mount aqueous mounting medium, Trypan Blue solution (0.4%, w/v), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Triton X-100, methanol, Bovine Serum Albumin (BSA), Porcine skin gelatin, Horse Serum (H1138).

Corning

35mm dishes, 96 well plate

Cytoskeleton Inc., USA

Phalloidin staining kit.

Abcam, UK

Rabbit monoclonal anti-Vinculin antibody (Alexa Flour488)

3.1.2 Major equipment used in this thesis

Following is a list of equipment used to carry out the research:

- Zeiss Laser Scanning Confocal Microscope, 510 META.
- Clariostar Plate reader, BMG labtech
- Zeiss Axiovert light microscope
- Custom designed and built speaker-based vibration system
- Image J software

3.2 Methods

3.2.1 Cell lines used in this study

All cell lines used in this study were obtained from European Collection of Animal Cell Cultures (ECACC Porton Down, Salisbury, UK) except where stated otherwise. Four different cell lines were used:

- LL24 (human lung fibroblast cells)-adherent cell line from 5-year-old male.
- L929 (areolar adipose tissue from mice)
- HDF (human dermal fibroblast primary cells from adult foreskin)
- C2C12P12p12 (mice muscle cells) a kind gift from Professor Claire Stewart, Sports and Exercise Sciences, Liverpool John Moores, Byrom Street, Liverpool, L3 3AF.

3.2.2 Acoustic vibration system

A speaker-based device was developed using a 0.2W super-thin, waterproof Mylar speaker (45mm) and an Arduino board for control. This system enables vibration to be applied to the underside of the cell culture dish to which the cells adhere, at frequencies ranging between 100-1600Hz (see Figure 3.1).

Figure 3.1 Speaker-based device which was used in all experiments to mechanically stimulate cells at frequencies ranging between100Hz and 1600Hz.

3.2.3 Calibration of acoustic vibration system

To calibrate the frequency and amplitude generated by the speaker a laser vibrometer (Polytech Ltd.) was used. Briefly, a cell culture dish (35mm) containing cell culture growth medium was rested upon the speaker (40mm diameter). The laser spot from the vibrometer was focused, through a ×10 microscope objective lens, onto the inner bottom surface of the dish (the surface to which the cells would adhere). Next, the speaker was set to vibrate at either; 100, 200, 400, 800 or 1600Hz. Vibration frequency (Hz) and amplitude of displacement (µm) were obtained though measurement of the displacement of the laser spot, which is software driven in the Polytech system (see Figure 3.2 for vibrometer set-up).
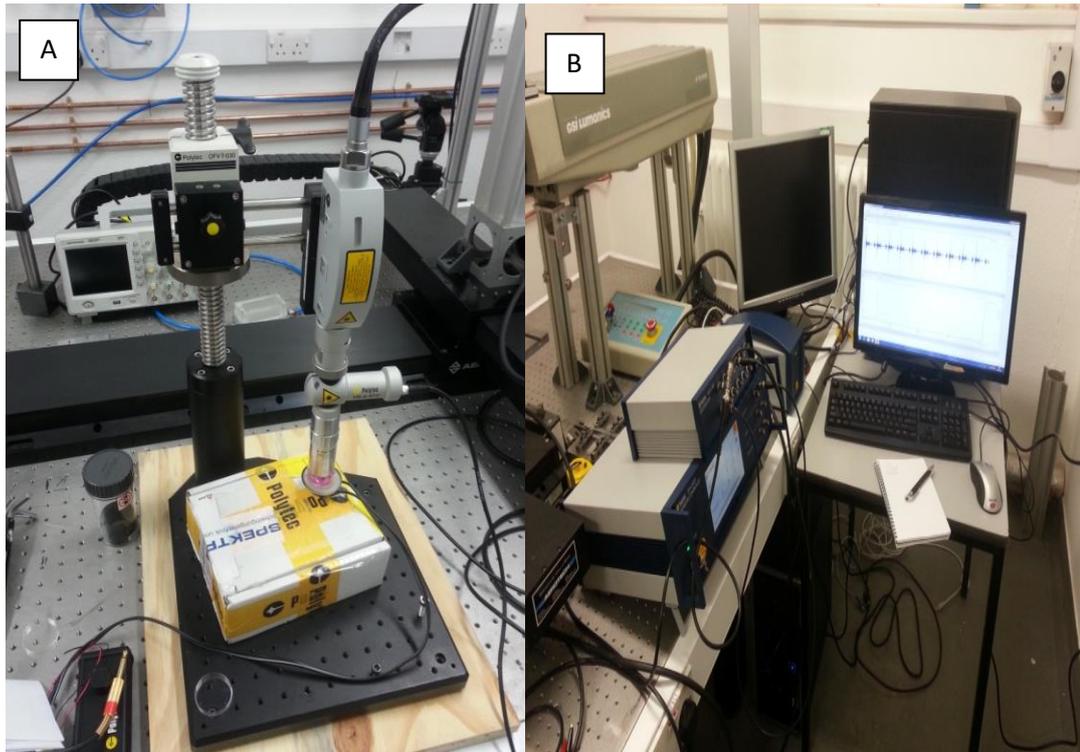
Figure 3.2 A Shows the laser positioned above a 35mm cell culture dish (containing cell culture medium) which is placed on top of the speaker. B Data capture system.

3.2.4 Cell models and routine cell culture

As fibroblast cells play an important role in wound healing this work focussed primarily on investigating the migratory response of fibroblast cells following mechanical stimulation. Also, given that there is evidence to suggest that fibroblast cells from different tissues may be genetically different, fibroblast cells from different tissue types were studied. For this study human lung fibroblast cells (LL24), a fibroblast cell line derived from the adipose tissue of a mouse (L929) and primary human dermal fibroblast cells (HDF) were used. These cells are commercially available from the European Collection of Animal Cell Cultures (ECACC) and are well characterised. There are no ethical issues or special requirements needed to work with these cells. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM-D6429) supplemented with 2mM L-glutamin10% (v/v) foetal bovine serum (FBS-0804) (Sigma Aldrich, UK) and 1% (v/v) penicillin and streptomycin in T75 cell culture flasks, at 37°C in a humidified atmosphere with 95% air and 5% CO_2.

To culture mice muscle cells (C2C12P12) all cell culture vessels were first coated with 0.2 % gelatin (Sigma Aldrich, UK) which was made using sterile PBS. Using gelatin

with these cells has been shown to promote attachment. T75 flasks were coated with 2ml of gelatin solution, whereas 35mm dishes were coated with 100µl of gelatin solution. Briefly, the gelatin solution was added to the surface of the vessels and left for 20 minutes at room temperature. After 20 minutes the gelatin was removed.

For routine cell culture the C2C12P12 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM L-glutamin20% (v/v) foetal bovine serum (FBS) (Sigma Aldrich, UK) and 1% (v/v) penicillin and streptomycin in T75 cell culture flasks at 37˚C in a humidified atmosphere with 95% air and 5% CO_2.

In order to promote the formation of muscle fibres (myotubes) the cells were cultured as described above and left for 24 hours. After 24 hours the medium was changed to contain 2% (v/v) of horse serum. The cells were subsequently left for 72hours at 37˚C, 95% air and 5% CO_2. This has been shown to be sufficient time for the cells to fuse into myotubes.

3.2.5 Cryopreservation of cells

When any cell lines were not being used they were stored in liquid nitrogen. To prepare the cells for cryopreservation the cells were detached from the flasks using trypsin-EDTA. Briefly, the cell culture medium was removed and the cells washed with 5ml of sterile phosphate buffered saline (PBS). Next, 2ml of 1× trypsin-EDTA solution (Sigma Aldrich, UK) was added to the flask, which was incubated for 5 minutes at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. Detachment of cells from the flask was determined microscopically. After detachment the cells were centrifuged for 5 minutes at 280×g, then resuspended in freezing mixture which consisted of; 70% DMEM, 20% FBS and 10% DMSO. Next, cells were dispensed into cryogenic vials at a cell density of $5x10^6$ cells/ml and stored at -70ºC freezer for 24 hours. After 24 hours the cells were transferred into liquid nitrogen for long term storage.

To resuscitate the cells from cryopreservation the cells were defrosted at 37ºC and then transferred to 10ml of pre-warmed DMEM. Next, the cells were centrifuged for

5 minutes at 280×g in order to remove any DMSO and the resulting pellet resuspended in fresh DMEM.

3.2.6 Laser scanning fluorescence confocal microscopy

All images obtained for this study were from a Zeiss 510 Meta laser scanning microscope, mounted on an Axiovert 200M BP computer-controlled inverted microscope. This microscope is equipped with the following laser lines; blue diode 405nm, Argon ion 458, 477, 488 and 514nm, He-Ne 543nm. For cell migration studies, Differential Interference Contrast (DIC) microscopy was carried out using the He-Ne 543nm laser. For imaging actin, a wavelength of 543nm was used. For imaging vinculin, the Argon ion laser was used with an excitation wavelength of 488nm.

3.2.7 The effects of mechanical stimulation on single cell migration

Cells were seeded into 35mm cell culture dishes at a density of 2×10^4cells/cm^2 and left to attach to the dishes for 24 hours at 37°C in a humidified atmosphere, containing 95% air and 5% CO_2. After 24 hours the dish was removed from the incubator and mechanically stimulated for 5 minutes at either 0 (control), 100, 200, 400, 800, or 1600Hz. Next, the dish was immediately placed into the environmental chamber (37°C humidified atmosphere with 95% air and 5% CO_2) of the laser scanning microscope and imaged every 5 minutes for 4 hours using DIC time-lapse microscopy with 543nm excitation and magnification of ×10 DIC.

Each experiment was repeated three times and ImageJ software was used to manually track the migration of single cells (n=30 cells from each frame in the time-lapse sequence) in each population for 4 hours so as to determine mean migration distance (µm).

3.2.8 The effects of mechanical stimulation on collective cell migration

To investigate the effects of mechanical stimulation on collective cell migration a wound healing assay (scratch assay) was used. This *in vitro* assay allows one to measure collective cell migration at the wound edge. These experiments were carried out in 35mm dishes. However, before seeding the cells into the dishes a black horizontal line with N (North) and S (South) were drawn on the underside of the

dishes. This was done as a reference so as to provide a marker that could be used to help reposition the dishes at the same location on the confocal microscope at each imaging time point (see Figure 3.3).

Next, 20,000 cells were seeded into cell culture dishes (35mm) and left for 24 hours to attach to the surface of the dishes. A sterile pipette tip was then used to create an artificial wound by scratching the surface of the cells through the center of the dish thus creating a void (scratch) in the cell layer. In total three scratches were made in each dish running perpendicular to the horizontal line (as indicated by the red lines shown in Figure 3.3). For each dish (treatment) six scratched areas were measured i.e. three North of the reference line and 3 South of the reference line.

Immediately after wounding, images were taken of the wound area and then the cells were mechanically stimulated at either; 100 or 1600Hz for 5 or 20 minutes. These frequencies and times were chosen as they represent a similar range to those reported within the literature. Also, laser vibrometry studies have shown the system to be stable between these frequencies (See Appendix 1.1). Immediately after stimulation the cells were put back into the incubator (37ºC in a humidified atmosphere, containing 95% air and 5% CO_2) and imaged every hour for 5 hours. Five hours was chosen to look at the immediate response of cells to stimulation and to also limit the chances of cell proliferation affecting the results. In total two dishes were prepared for each treatment and cells receiving no vibration (0Hz) were used as a control. Each experiment was repeated at least 3 times.
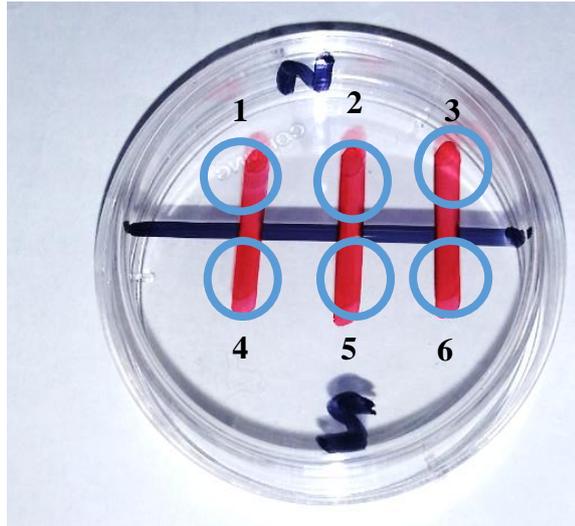
Figure 3.3 A 35mm cell culture dish. N and S represent North South of the black horizontal line. The line is used as a reference point to help reposition the dish on the microscope stage between imaging. The circles represent each scratch and are numbered accordingly (1-6).

3.2.9 Analysis of cell migration

ImageJ was used in order to analyze cell migration. This software package allows one to quantify the total migration distance (µm). ImageJ software is calibrated to read images from confocal microscope and to be used for image analysis. For collective cell migration (scratch assay) change in wound area (µm^2) over time was recorded by drawing around the edges of the wound and calculating change in wound area (µm^2) over time (see Figure 3.4).
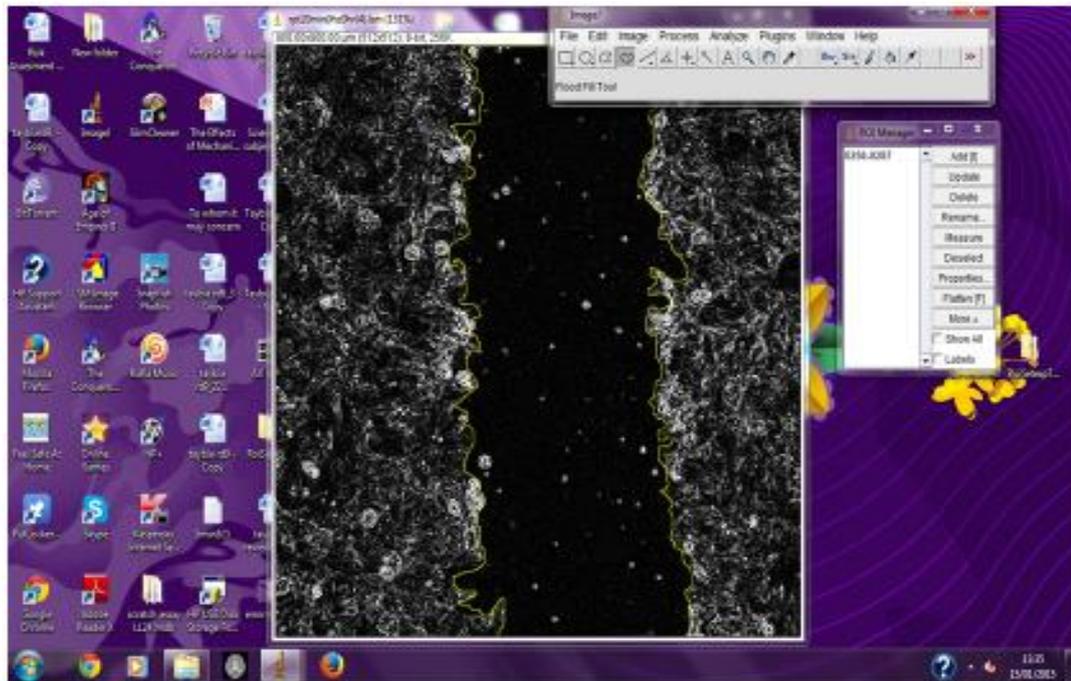
Figure 3.4 Using image J the area of a scratch is drawn (yellow lines) and measured.

For single cell migration the cell tracking feature within ImageJ was used to track individual cell migration over time (see Figure 3.5). In total 30 cells were tracked from each time-lapse video sequence and the mean migration distance (µm) value calculated.
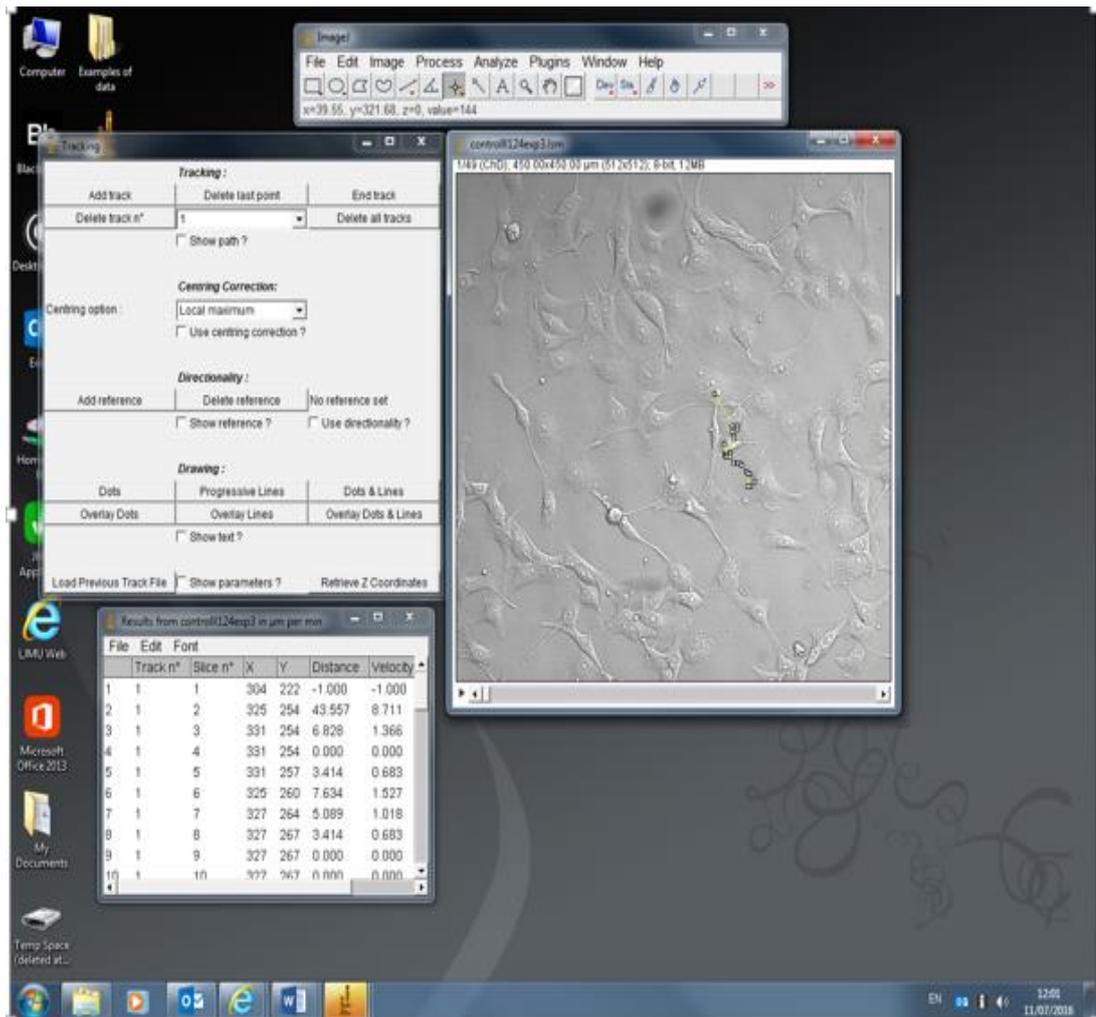
Figure 3.5 Using imageJ single cells were tracked (n=30 from each time-lapse sequence) over a four–hour period (yellow line represents the migration trail of an individual cell).

3.2.10 The effects of intermittent stimulation on single cell migration and collective cell migration

Initial results using one stimulation indicated that the response of cells to this stimulus may be time-dependent, whereby the effects of the stimulus may wear off over time (see results Section 4.0). Therefore, experiments were repeated to assess the effects of intermittent mechanical stimulation on an hourly basis. For these experiments only three frequencies were investigated which were; 0, 100 and 1600Hz. For single cell migration the cells were seeded into cell culture dishes (35mm) at a cell density of approximately 200,000 cells/dish and left to attach to the dish for 24 hours. Next, the cells were mechanically stimulated at either; 0, 100, 1600Hz for 5minutes and then placed into the environmental chamber of a confocal microscope for time-lapse

imaging. The dish was removed every hour, for four hours, from the confocal microscope and then mechanically stimulated for 5minutes in the incubator (37ºC, 95% air, 5% CO_2) before being placed back into the microscope environmental chamber to continue imaging. This experiment was repeated using 20 minutes of stimulation.

For the wound healing assays (collective cell migration) cells were seeded into the cell culture dishes (35mm) at a cell density of approximately 200,000 cells/dish and left to attach to the dish for 24 hours. Next, artificial wounds (scratches) were created using a sterile pipette and the dish was then placed on the speaker in the incubator to be stimulated for 5 minutes at 0, 100 or 1600Hz. The dish was then placed into the environmental chamber of the confocal microscope and images of each scratched area (6 in total) were taken (HeNe453nm laser and 20×DIC lens). This represented wound size at 0 hours. After 1 hour the dish was removed from the microscope chamber and the cells mechanically stimulated as described above before being put back on the microscope for further time-lapse imaging. This was repeated every hour over a 4-hour period. The experiment was repeated using 20 minutes stimulation. The images were analysed using ImageJ as described previously.

3.2.11 The effects of mechanical stimulation on actin organisation

Cells migration requires a reorganisation of the actin cytoskeleton. In order to investigate if mechanical stimulation has any effect on actin organisation, fluorescence microscopy was used to image actin architecture. Cells were seeded at a density of 20,000 cells per 35mm dish and left to attach for 24 hours. Next the cells were mechanically stimulated for 5 minutes or 20 minutes at 0, 100, 200, 400, 800 or 1600Hz. The cells were then washed (×1) in phosphate buffered saline solution (PBS) for 5 minutes and then fixed at room temperature for 10 minutes using 10% paraformaldehyde. Following fixation, the cells were washed with PBS and permeabilised for 20 minutes using 0.5% Triton-X 100. Finally, the dishes were washed (×2) with PBS and the actin filaments labelled by adding Rhodamin Phalloidin to the cells and incubating for 30 minutes at ambient temperature. After staining, the dishes were washed (×3) with PBS to eliminate any background staining. The cells were imaged using Zeiss 510 Meta laser scanning confocal microscope with an

excitation wavelength of 543nm and magnification of 40×/1.4 Oil DIC and 63×/1.4 Oil DIC. Images were analysed to determine changes to the morphology of cells. In total 200 cells from each sample (across ×10 images from each sample), were counted and the percentage of cells displaying prominent rounding, filopodia and lamellipodia were recorded and the data plotted in graphical form.

3.2.12 The effects of mechanical stimulation on vinculin formation in cells

Vinculin is a membrane-cytoskeletal protein, which is abundant in focal adhesion plaques and is known to form links with the actin cytoskeleton. To investigate the effects of mechanical stimulation on vinculin formation/distribution the cells were grown on a 35mm cover slip, at a density of 200,000 cells per coverslip and left to attach for 24 hours. Next, the coverslip was placed in the bottom of a 35mm cell culture dish which was mechanically stimulated for 5 minutes at 0, 100, 200, 400, 800 or 1600Hz. Coverslips were used in this instance, as for unknown reasons, staining cells growing directly on the surface of the dish did not yield a strong fluorescent signal. After stimulation, the cells were fluorescently tagged with the vinculin antibody according to the manufacturers' instructions. Briefly, the cells were chemically fixed in 100µl of ice-cold methanol for 10 minutes at room temperature. Next, the methanol was removed and cells were washed (×2) with PBS. The cells were then permeabilised for 5 minutes at room temperature using 0.5% Triton-X 100, before being washed (×2) with PBS. Next, 100µl of 1% of BSA was added to the cells, which were left to incubate for 1 hour. The cells were again washed (×2) with PBS and 100µl of rabbit monoclonal (1/100 dilution in PBS) was added to the cells, which were left to incubate for 24 hours at 4°C. After 24 hours, the cells were washed (×3) with PBS to reduce any background staining. Next, the coverslips were mounted onto a slide using gel mount aqueous medium (Sigma, UK). The cells were then imaged using a Zeiss 510 Meta laser scanning microscope with an excitation wavelength of 488nm and ×63 magnification.

3.2.13 The effects of mechanical stimulation on cell viability: MTT assay

To investigate the effects of mechanical stimulation on cell viability the MTT assay was used. This is a colorimetric assay that relates absorbance to viable cell number. The cells were seeded into 35mm cell culture dishes at 200,000/dish and left to attach for 24 hours at 37˚C in a humidified atmosphere, containing 95% air and 5% CO_2. Next, the cells were mechanically stimulated for 5 or 20 minutes at 0, 100, 200, 400, 800, 1600Hz and the MTT assay carried out. Briefly, 100µl of 10% of MTT solution (5mg/ml) was added to each dish and the cells left to incubate at 37°C in a humidified atmosphere, containing 95% air and 5% CO_2 for 2 hours (determined empirically for these cell lines). Next, 10% dimethyl sulfoxide (DMSO) was added to each dish in order to solubilize the resulting formazan crystals. The formazan solution was then added to wells of a 96-well plate and the absorbance measured at 540 nm using a 96-well plate reader. The experiment was carried out in duplicate and a DMSO control was used which was subtracted from the absorbance values. Each experiment was carried out three times to ensure repeatability.

3.2.14 The effects of mechanical stimulation on cell viability: Trypan blue assay

To corroborate the MTT cell viability assay results a trypan blue dye exclusion assay was carried out. This assay works on the basis that intact, viable cells will have an intact cell membrane and will exclude the trypan blue dye. If the cell membrane has been compromised, then the dye can freely pass across the cell membrane thus allowing the detection/quantification of cell viability using light microscopy.

First, the cells were seeded into the cell culture dishes (35mm) at a cell density of 20,000 cells/dish and left to attach to the dish for 24 hours. Next, the cells were exposed to mechanical stimulation (0, 100, 200, 400, 800, 1600 Hz) for 5 minutes or 20 minutes. The medium was then removed and the cells washed ($\times$2) with PBS. Next, 100µl of trypsin-EDTA was added to the cells which were subsequently placed into the incubator (37ºC in a humidified atmosphere, containing 95% air and 5% of CO_2) until the cells were detached (approximately 5 minutes). After the cells had detached

from the dish (observed microscopically), 2ml of PBS was added to the dish and then the cell suspension was transferred to a 15ml centrifuge tube. The cells were centrifuged at 280×g for 5 minutes. Next, the supernatant was removed and the pellet was resuspended in 100ul of fresh PBS. Next, 10µl of cell suspension was added to 10µl of Trypan blue and a viable cell count carried out using a haemocytometer and light microscope with a ×10 objective lens. This experiment was repeated three times with triplicate samples used for each experiment, so as to ensure reliability/accuracy.

3.2.15 The effects of mechanical stimulation on cell proliferation

In order to investigate if mechanical stimulation had any effect on cell proliferation, 20,000 cells were seeded into 35mm dishes (×3) and were left for 24 hours in an incubator (37ºC in a humidified atmosphere, containing 95% air and 5% CO_2). Next the three dishes were mechanically stimulated for either 5 or 20 minutes at each frequency 0, 100, 200, 400, 800 or 1600Hz and then put back in the incubator for 24, 48 and 72 hours. After 24 hours, one stimulated dish from each frequency was removed from the incubator. The medium was removed and trypsin was added to detach cells from the dish. Once detached, 100µl of PBS was added to the dish and the cell suspension was removed from the dish into a 15ml centrifuge tube. After centrifugation at 280×g for 5 minutes the resulting pellet was resuspended in 100µl of PBS. Next, 10µl of cell suspension was mixed with 10µl of trypan blue and a viable cell count conducted using a haemocytometer to determine total viable cell number after 24 hours. This protocol was repeated at both 48hours and 72hours, respectively.

3.2.16 The effects of amplitude on single cell migration

In order to investigate if amplitude was having any effect on single cell migration, the system was altered to control amplitude. This was achieved using a variable resistor with help from engineering colleagues. This enabled manual control of amplitude at a given vibration frequency. Cells were grown in 35mm dishes at a cell density of 200,000 cells/dish and left to attach to the dish for 24 hours. Next, the cells were mechanically stimulated at 100Hz and the amplitude was reduced to 50% (which is 4.5µm) and compared to cells that were stimulated at 100% amplitude (approx.~9.0

µm). A frequency of 100Hz was chosen as previous experiments have shown that this frequency promotes the greatest cells migration.

Once stimulated the dishes were placed onto the environmental chamber (37˚C humidified atmosphere with 95% air and 5% CO_2) of the laser scanning microscope and imaged every 5 minutes for 2 hours using DIC time-lapse microscopy with 543nm excitation. The time 2 hours was chosen because initial experiments have shown that the cell migration was greater over the first two hours when compared to the control. Each experiment was repeated twice and ImageJ software was used to manually track the migration of single cells (n=30 cells from each frame in the time-lapse sequence) in each population so as to determine mean migration distance (µm).

3.2.17 The effect of mechanical stimulation on temperature

In order to investigate whether mechanical stimulation resulted in changes in temperature at the apical surface of the dish, a thermocouple was used to measure the temperature. This is important as small changes in temperature may have an effect on cell physiology/behaviour. Briefly, the thermocouple was attached to the apical surface of a 35mm dish using adhesive tape (see Figure 3.6). Next, 2ml of pre-warmed (37°C) sterile medium was added to the dish, which was placed upon the speaker inside the incubator (37ºC in a humidified atmosphere, containing 95% air and 5% CO_2). The speaker was programmed to vibrate at either; 0, 100, 200, 400, 800 and 1600Hz and the temperature recorded every minute for 20 minutes. This experiment was repeated three times to ensure consistent results.

Figure 3.6 Thermocouple attached to apical surface of a 35mm dishes.

3.2.18 The effects of mechanical stimulation on muscle fibre formation

In order to investigate if mechanical stimulation has any effect on muscle fibre formation the muscle cells were mechanically stimulated and the muscle fibre diameters measured. For this work muscle cells (C2C12P12) were grown in DMEM supplemented with 2mM L-glutamine, 20% (v/v) foetal bovine serum (FBS) (Sigma Aldrich, UK) and 1% (v/v) penicillin and streptomycin at 37ºC in a humidified atmosphere, containing 95% air and 5% of CO_2, in gelatin-coated T75 flasks until 70-80% confluent. All experiments were carried out in 0.2% in gelatin coated cell culture dishes (35mm). Briefly, 200,000 cells were seeded into the dishes and left until 70-80% confluent (approximately 72hours). Next, the medium was removed and 2ml of DMEM containing 2% horse serum (promotes cell fusion) and 1% penicillin/streptomycin solution was added to the cells. The cells were then mechanically stimulated for 5 or 20 minutes at either; 0, 100 or 1600Hz. The cells were then incubated at 37ºC in a humidified atmosphere, containing 95% air and 5% CO_2 and mechanically stimulated daily for 4 days. Images were taken on the fifth day using a laser scanning confocal microscope with transmission DIC.

Using ImageJ, the images were analysed to determine mean fibre diameter (μm). In total 3 images per dish were taken. From each image ten fibres were randomly

selected. To obtain the mean fibre diameter, seven measurements were taken along each fibre (see Figure 3.7 for example of how measurements were taken).
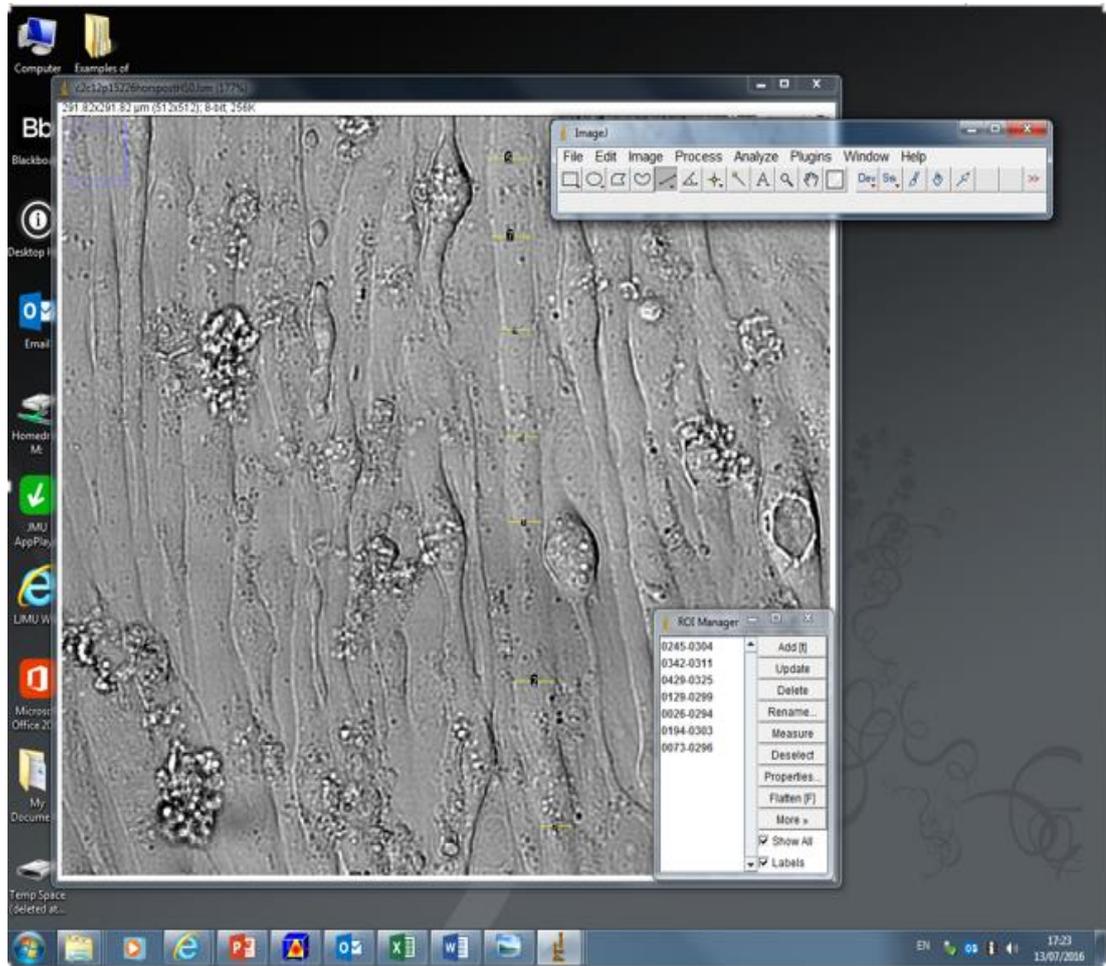
Figure 3.7 Using image J measurement tool seven measurements (yellow lines) were carried out along the length of each muscle fibre (myotube).

3.2.19 Statistical Analysis

Statistical analysis was carried out using Microsoft Excel. Where appropriate tests for significance, between the control and individual treatments, were done using an unpaired, two-tailed Student's *t*-test at 95% confidence limit.

Chapter 4

4.0 Results

4.1. Speaker vibration system & calibration

To assess the effects of mechanical stimulation upon fibroblast cell migration, a system was developed that can deliver low-frequency-low-amplitude acoustic mechanical stimulation, via a sinusoidal waveform, to cells growing in a cell culture dishes (Figure 4.1 left). This system allows the frequency of mechanical stimulation to be controlled via an Arduino controller. In order to determine if the vibration frequency was accurate and stable, a laser vibrometer was used to calibrate the system. It was found that below 100Hz and above 1600Hz the system was unstable (in terms of frequency), as laser vibrometry recorded multiple harmonics outside of this frequency range (See Appendix 2). Therefore, frequencies between 100 and 1600Hz were used to deliver stable, low-frequency-low-amplitude mechanical stimulations to cells in a continuous manner. Laser vibrometry also recorded amplitude of displacement (µm), which can be seen to decrease as frequency is increased (Figure 4.1 right).
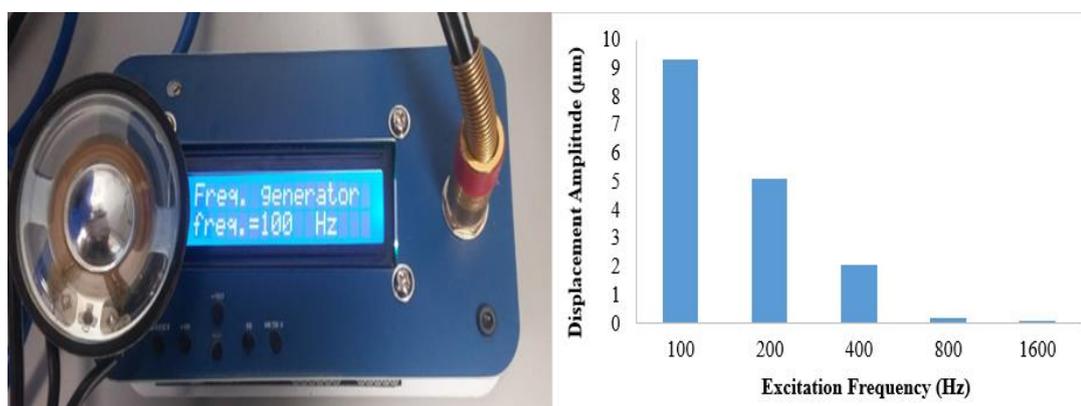
Figure 4.1 Speaker-based system (left) and calibration of frequency *versus* amplitude of displacement of the inside bottom surface of a 35mm cell culture dish to which the cells adhere (right).

4.2. The effect of mechanical stimulation on single cell migration

In order to investigate if mechanical stimulation has any effect on the migration of human lung (LL24), mouse alveolar (L929) and human dermal fibroblast cells (HDF) time-lapse microscopy imaging and subsequent cell tracking using ImageJ software was carried out following mechanical stimulation. Cells were mechanically stimulated using 100, 200, 400, 800, or 1600Hz for either 5 or 20 minutes and cell migration (n=30) was subsequently recorded over a 4-hour period. As can be seen from Figure 4.2, mechanical stimulation for 5 minutes at 100Hz increased the mean cell migration distance of the LL24 cells by approximately 1.8µm (10% increase) when compared to the control population (p<0.05). In contrast, mechanical stimulation for 5 minutes for all frequencies above 100Hz resulted in a decrease in the mean cell migration distance (µm), when compared to the control (p<0.05). This decrease can be seen to occur in a steady manner as frequency is increased (and amplitude decreased) resulting in a decrease in the mean migration distance (µm) of approximately 8.6µm (approximately 48%) at 1600Hz, compared to the control population. Overall the results obtained in figure 4.2 were significant (p<0.05).
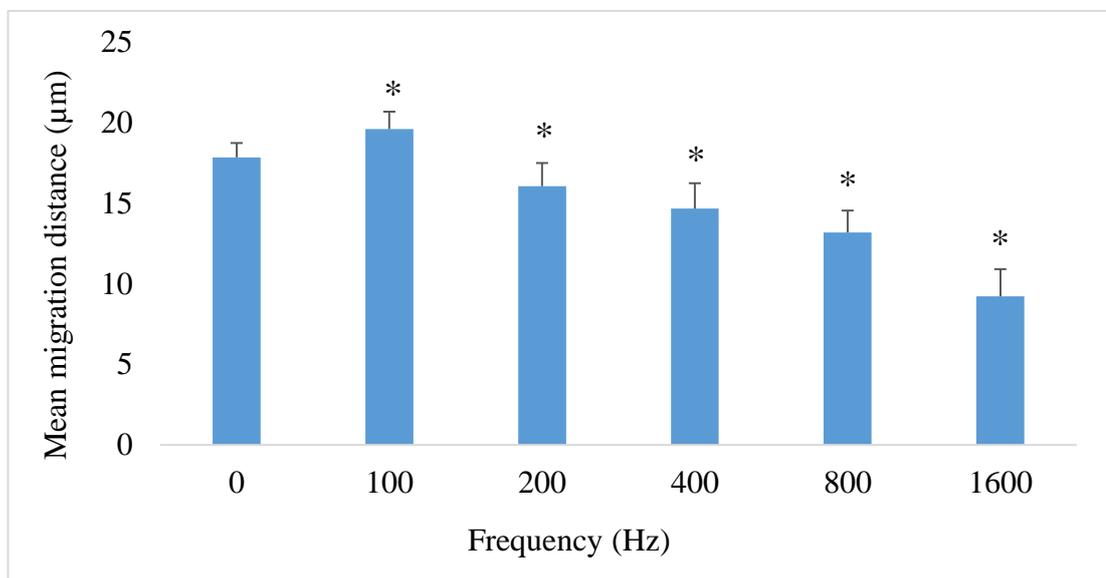
Figure 4.2 Mean migration distance (µm) versus frequency (Hz): LL24 cells were mechanically stimulated for 5 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation. * denotes Significance (p<0.05).

The results show a similar trend for the L929 (mouse alveolar) cells, in that mechanical stimulation at 100Hz resulted in a significant increase in the mean cell migration distance of approximately 1.7µm (9%) compared to the control population (p<0.05)

(Figure 4.3). Above 100Hz there was a steady decrease in the mean cell migration distance (µm) up to 400Hz (p>0.05). Stimulation at 800Hz resulted in an almost identical mean cell migration distance (µm) compared to the control population, while stimulation at 1600Hz resulted in a lower mean migration distance (µm) compared to the control, although this was not found to be statistically significant (p>0.05).
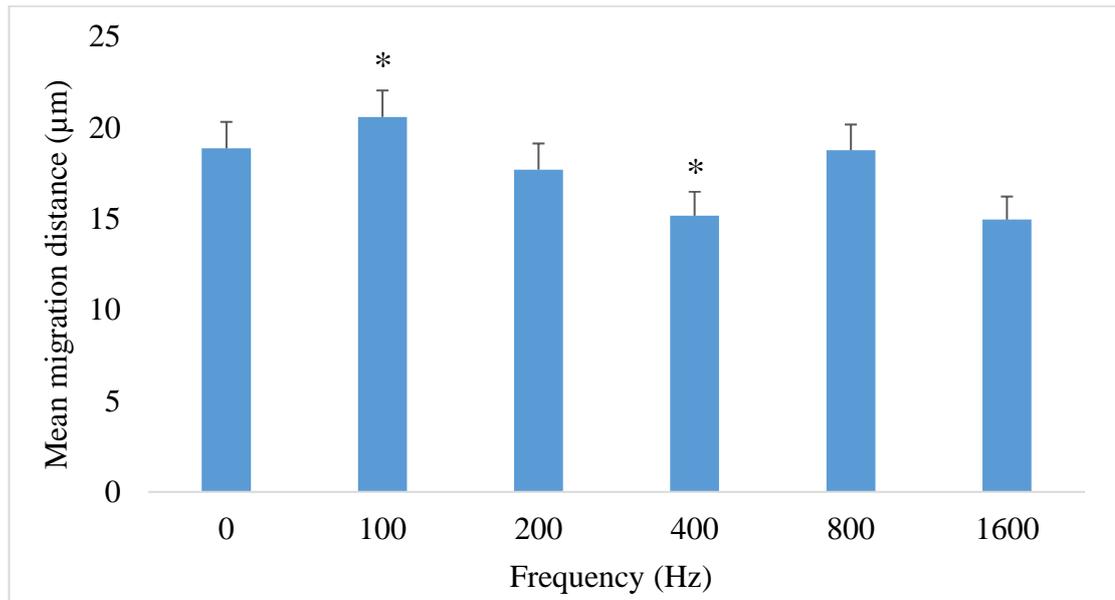
Figure 4.3 Mean migration distance (µm) versus frequency (Hz): L929 cells were mechanically stimulated for 5 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation. * denotes Significance (p<0.05).

These results following 5 minutes stimulation show that fibroblast cell migration can be controlled using acoustic vibration and that migration distance seems to be dependent upon vibration frequency. The trend observed was repeatable for both cell lines, particularly the increase in mean cell migration distance (µm) seen at 100Hz. On all occasions a steady decrease in mean cell migration was observed for the lung cells (LL24), with 1600Hz always resulting in lowest mean cell migration distance (µm). The results for the L929 cells somewhat mirrored those of the LL24 cells, however the trend did not always produce a steady decrease in mean cell migration.

After 20 minutes stimulation was applied the results were somewhat different compared to those for 5 minutes. As can be seen from Figure 4.4, for 100 and 200Hz stimulation, there was very little difference in the mean cell migration distance (µm) compared to the control. In contrast mechanical stimulation at 400Hz resulted in a

significant increase in the mean migration distance of approximately 2.1µm (10%) compared to the control (p<0.05). At both 800 and 1600Hz there was significant decline in the mean migration distance of approximately 1.5µm and 2.1µm (7 and 10%), respectively when compared to the control (p<0.05). Significance was only found from 400Hz to 1600Hz (p<0.05).
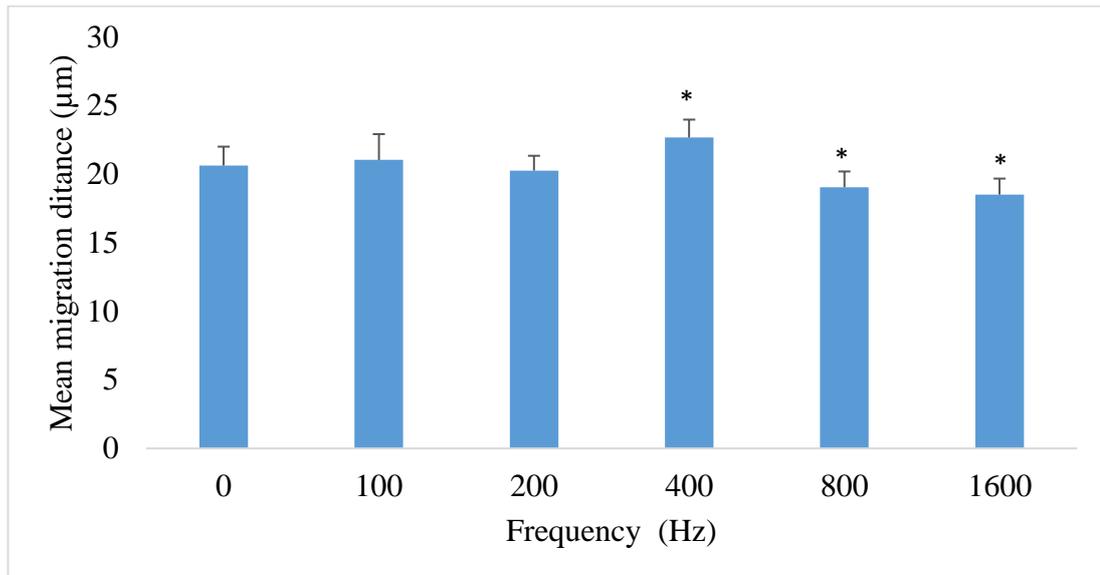
Figure 4.4 Mean migration distance (µm) versus frequency (Hz): LL24 cells were mechanically stimulated for 20 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation. * denotes Significance (p<0.05).

Results for the 20 minutes stimulation of L929 cells showed a similar trend to the LL24 cells (Figure 4.5). For example, stimulation at 100Hz resulted in mean migration distance (µm) which was similar to the control. For 200Hz there was a reduction in the mean cell migration distance of approximately 2.3µm (11%) compared to the control, however this was found not to be significant (p>0.05). Like with LL24 cells, stimulation at 400, 800 and 1600Hz all resulted in a lower mean migration distance (µm) compared to the control. However, this was also found not to be significant (p>0.05).
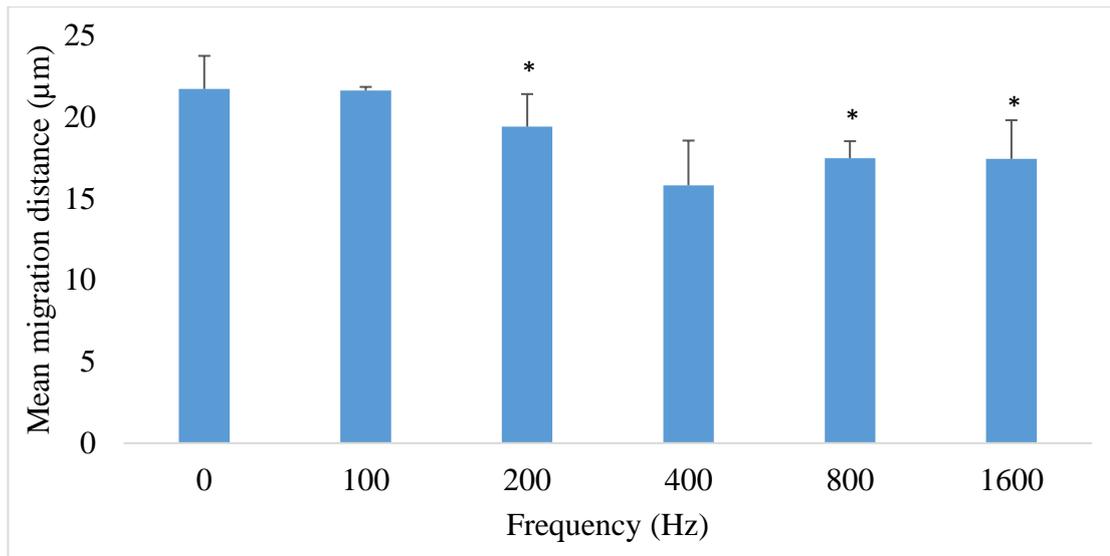
Figure 4.5 Mean migration distance (µm) versus frequency (Hz): L929 cells were mechanically stimulated for 5 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation. * denotes Significance (p<0.05).

The results obtained above were carried out on established cell lines. Therefore, in order to investigate if primary cells would also respond to mechanical stimulation, primary human dermal fibroblast cells (HDF) were investigated. These cells were also mechanically stimulated for 5 minutes and 20 minutes. From previous results, it was found that for both LL24 and L929 cells, that all frequencies above 100Hz resulted in a decrease in the mean cell migration (µm) distance when compared to control. Therefore, these frequencies were used to assess the effects of mechanical stimulation on HDF cell migration. As can be seen from Figure 4.6, when the HDF cells were stimulated at 100Hz for 5 minutes, there was an increased mean migration distance (µm) of approximately 2.9µm (14%) compared to the control. Stimulation at 1600Hz also resulted in an increase in the mean migration distance (µm) of approximately 2.2 µm (13%) compared to the control. However, this was found not to be significant (p>0.05).
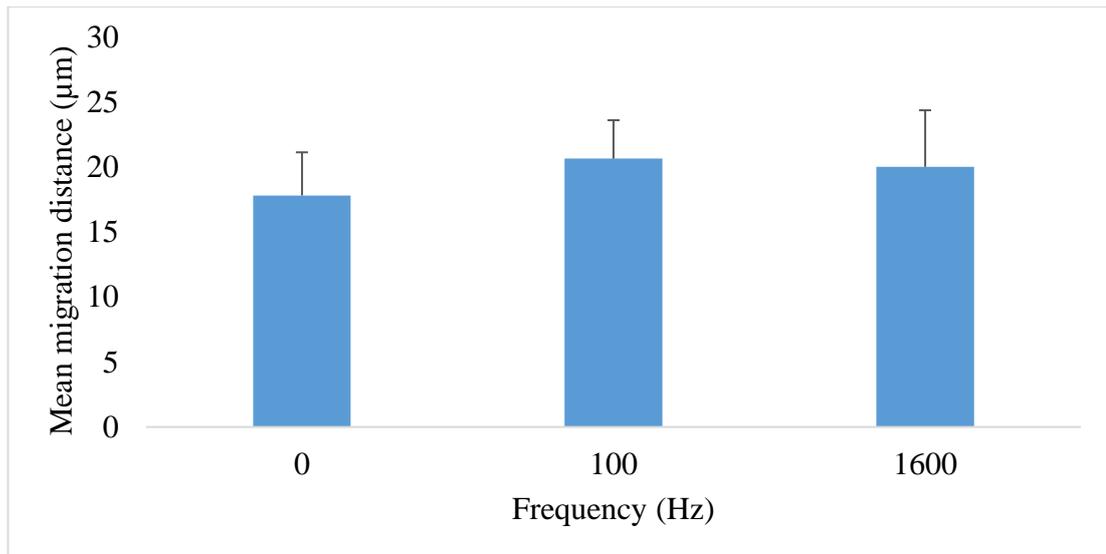
Figure 4.6 Mean migration distance (µm) versus frequency (Hz): HDF cells were mechanically stimulated for 5 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation.

Experiments were repeated using 20 minutes stimulation with the results displayed in Figure 4.7. The cells with no stimulation and the cells with 100Hz stimulation both showed very similar mean migration distance (µm). In contrast, when stimulated for 20 minutes at 1600Hz there was an overall decrease in the mean migration distance (µm) by approximately 2.6µm (16%) when compared to the control. No significance was found in Figure 4.7 ($p > 0.05$).

In summary, overall it was found that mechanical stimulation, delivered by acoustic vibration can result in an increase or decrease in cell migration in a frequency dependent manner. Stimulation for 5 minutes at 100Hz was found to be particularly effective at enhancing the migration of all fibroblast cells. In contrast, stimulation for 20 minutes at 400Hz was found to be effective with LL24 cells, no stimulation was effective with L929 cells and 100Hz was effective for HDF cells.
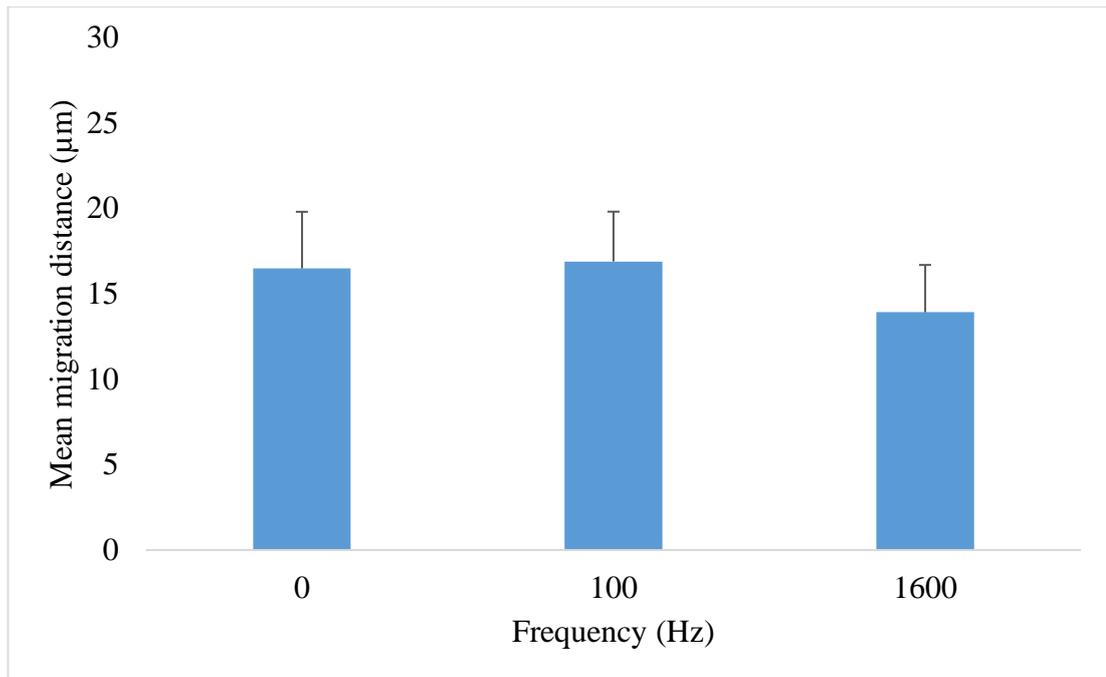
Figure 4.7 Mean migration distance (µm) versus frequency (Hz): HDF cells were mechanically stimulated for 20 minutes and cell migration (n=30) recorded over a 4-hour period. Error bars represent standard deviation.

4.3 The effect of mechanical stimulation on collective cell migration for all cells

During the wound healing process cells migrate as a 'sheet' or 'collectively' to close the wound. Therefore, in order to investigate if mechanical stimulation has any effect on collective fibroblast cell migration, a wound healing assay was carried out. A wound was created in cell monolayers and the cells were mechanically stimulated for 5 or 20 minutes at 100 or 1600Hz, respectively. These frequencies were chosen as they had been shown to alter the migratory properties of the cells during the single cell studies.

Cells were imaged every hour for 4 hours using confocal microscopy and images were analysed using ImageJ. This data is shown below in Figures 4.8 and 4. 9.
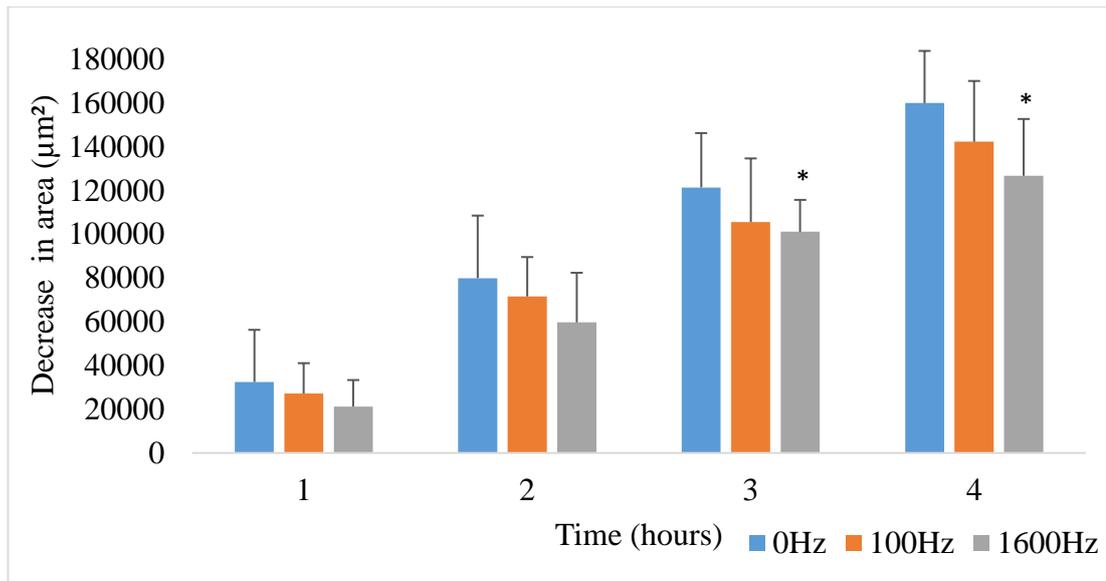
Figure 4.8 Decrease in area (μm^2) over 4-hour period, following mechanical stimulation of LL24 cells for 5 minutes. Error bars represent standard deviation. * denotes Significance ($p<0.05$).

As can be seen from Figure 4.34 there was a steady decrease in wound area (μm^2) over time. However, it was found that mechanical stimulation, at 100Hz for 5 minutes, seemed to reduce cell migration into the wound area. This was evident by comparing the control to the mechanically stimulated cells, whereby it can be seen that those cells that were not exposed to vibration, had an overall greater decrease in wound size over time. A similar effect is also seen when 1600Hz vibration was applied for 5 minutes. Again, there was a steady decrease in wound size over time for both the 1600Hz stimulated cells and control cells. However, mechanical stimulation at 1600Hz for 5 minutes was also found to significantly ($p<0.05$) reduce collective cell migration into the wound area over time compared to the control.

Cells were also exposed to acoustic vibration for 20 minutes (Figure 4.9). Here it was found that wound closure was steady over time for both the mechanically stimulated and control cells over the 4-hour period. However, the data also shows that applying 100Hz vibration for 20 minutes enhanced cell migration compared to the control. In contrast, applying 1600Hz vibration to the cells for 20 minutes resulted in a decrease in wound closure compared to the control. These experiments were repeated a number of times and each time it was found that 100Hz vibration for 20 minutes resulted in an enhancement in collective cell migration into the wound area compared to the control. However, although the trend displayed in Figure 4.9 was repeatable, statistical analysis

found this difference not to be statistically significant (p>0.05). When comparing the wound closure over time, it can be seen that for 20 minutes stimulation at 100Hz, the increase in cell migration (or wound closure) is more pronounced at 1 and 2hours post-stimulation when compared to the control. However, after 3hours the wound closure (decrease in wound size) for the control has almost 'caught up' to the 100Hz population. At 4hours post-stimulation the control population has a greater wound closure compared to the 100Hz sample. This general trend was found to be repeatable.
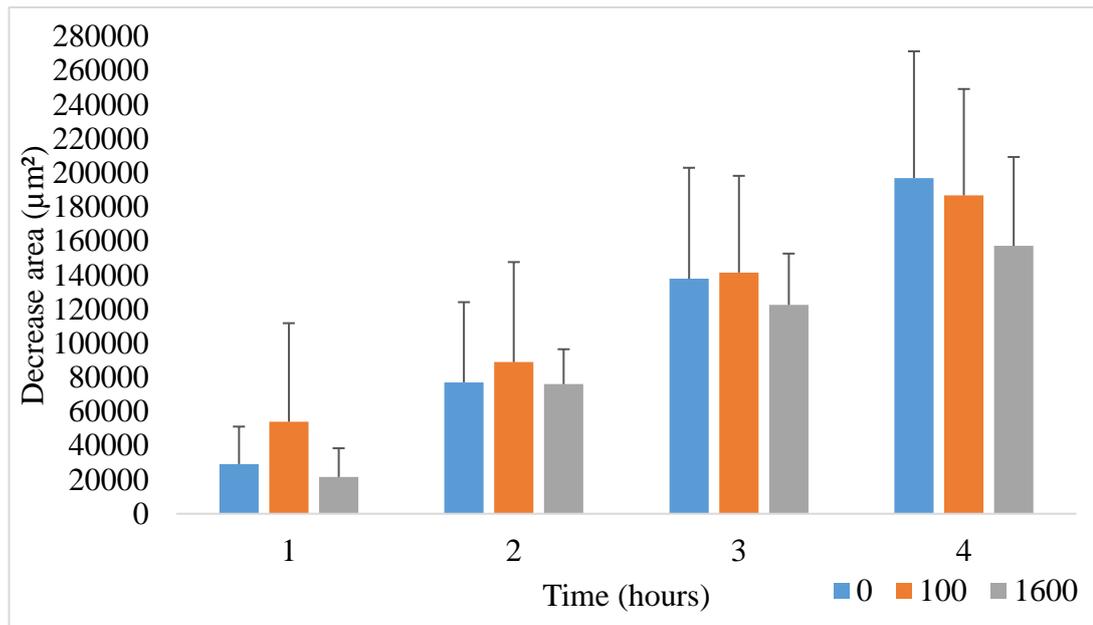
Figure 4.9 Decrease in area (μm^2) over 4-hour period, following mechanical stimulation of LL24 cells for 20 minutes. Error bars represent standard deviation. *denotes Significance (p<0.05).

As L929 cells are derived from a different tissue (i.e. areola/adipose) the effects of mechanical stimulation on collective cell migration of L929 cells was carried out. Figure 4.10 shows a decrease in area (μm^2) over time (4-hour period), when L929 cells were stimulated for 5 at 100 and 1600Hz, respectively. As can be seen from Figure 4.36, after the first hour there was increased wound closure following mechanical stimulation at 100 and 1600Hz compared to the control. After 2 hours the wound closure was similar for the control and 100Hz, while those cells stimulated at 1600Hz displayed the greatest wound closure. After 3 and 4 hours post-simulation the control population were found to have a greater wound closure compared to the 100Hz population while those cells stimulated at 1600Hz had the greatest wound closure at each time point and over the total 4-hour period. These results somewhat mimic those

for the LL24 cells at 100Hz for 5 minutes stimulation, in that the cells stimulated for 5 minutes at 100Hz displayed more migration (compared to the control) into the wound area over the first 2 hours with the control population 'catching up' (in terms of migration) after 3-4hours post stimulation. However, for the L929 cells it was found that 5 minutes mechanical simulation at 1600Hz induced the greatest wound closure. This is somewhat contrasting to the LL24 cells, which saw a decrease in wound closure following stimulation at 1600Hz for 5 minutes. Figure 4.11, shows the decrease in area (μm^2) over 4 hours for L929 cells when stimulated for 20 minutes. From Figure 4.11 the trend is very similar to the behaviour of the LL24 fibroblast cells, when also mechanically stimulated for 20 minutes. After the first hour the decrease in wound area was very similar in the both the control group and for cells that had received 100Hz stimulation. In contrast those cells stimulated at 1600Hz displayed the least migration into the wound area. However, after the second hour, those cells mechanically stimulated at 100Hz, displayed a greater decrease in wound area (μm^2) compared to the control. After 3 hours post-stimulation the control population had an almost identical wound area (μm^2) than those cells that had received 100Hz mechanical stimulation. While after 4 hours post stimulation the greatest wound closure was found to be for those cells stimulated at 100Hz. Overall, those cells stimulated at 1600Hz displayed the greatest wound closure which is consistent with the LL24 cells (20 minutes stimulation). However, although the trend was repeatable, statistical analysis found this difference not to be statistically significant ($p > 0.05$).
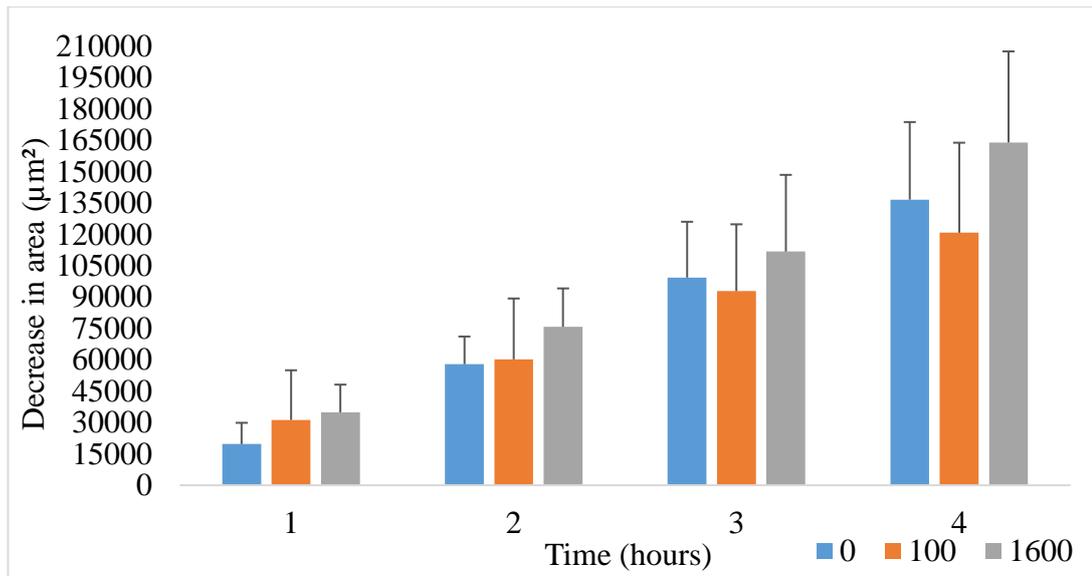
Figure 4.10 Decrease in area (μm^2) over 4 hour period, following mechanical stimulation of L929 cells for 5 minutes. Error bars represent standard deviation. * denotes Significance ($p<0.05$).
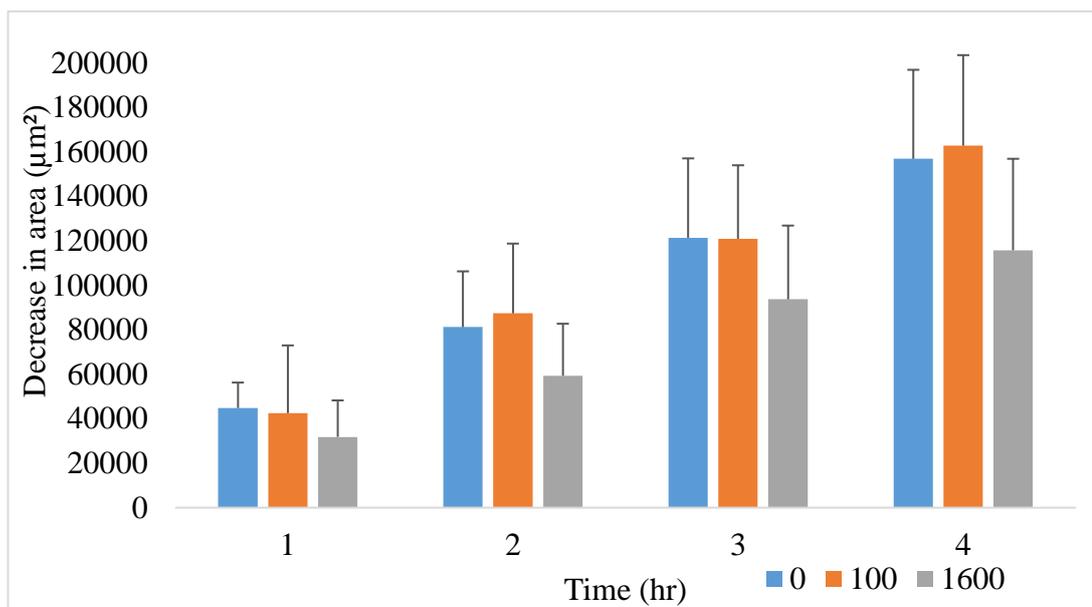
Figure 4.11 Decrease in area (μm^2) over 4-hour period, following mechanical stimulation of L929 cells for 20 minutes. Error bars represent standard deviation. *denotes significance ($p<0.05$).

For HDF cells the cells were mechanically stimulated for 5 and 20 minutes at 100 and 1600Hz (0Hz as control) and the data analysed as described previously. As can be seen from Figure 4.12 (5 minutes stimulation), after the first hour the decrease in wound area (μm^2) for the 100Hz population is slightly greater (approximately 3%) compared to the control population (approximately 3% greater). However, after 2, 3 and 4 hours

the decrease in area (μm^2) was greatest for the control population. Those cells that received 1600Hz were found to display the least wound closure. Additionally, mechanical stimulation at 1600Hz for 5 minutes was also found to significantly ($p<0.05$) reduce collective cell migration into the wound area during the third hour, when compared to the control. When the HDF cells were stimulated for 20 minutes (Figure 4.13) there was a similar trend in wound closure compared with the LL24 cells when also stimulated for 20 minutes. For example, over the first 3 hours those cells stimulated for 20 minutes displayed a greater degree of wound closure compared to the control. However, on the 4th hour the control population was found to display a greater degree of wound closure than the control. Overall, those cells that were stimulated at 1600Hz displayed the least wound closure at all-time points and this was found to be significantly different to the control both 3 and 4 hours post stimulation. Figure 4.13 also shows mechanical stimulation at 1600Hz for 20 minutes at hours 3 and 4 found to significantly ($p<0.05$) reduce collective cell migration into the wound area, compared to the control.
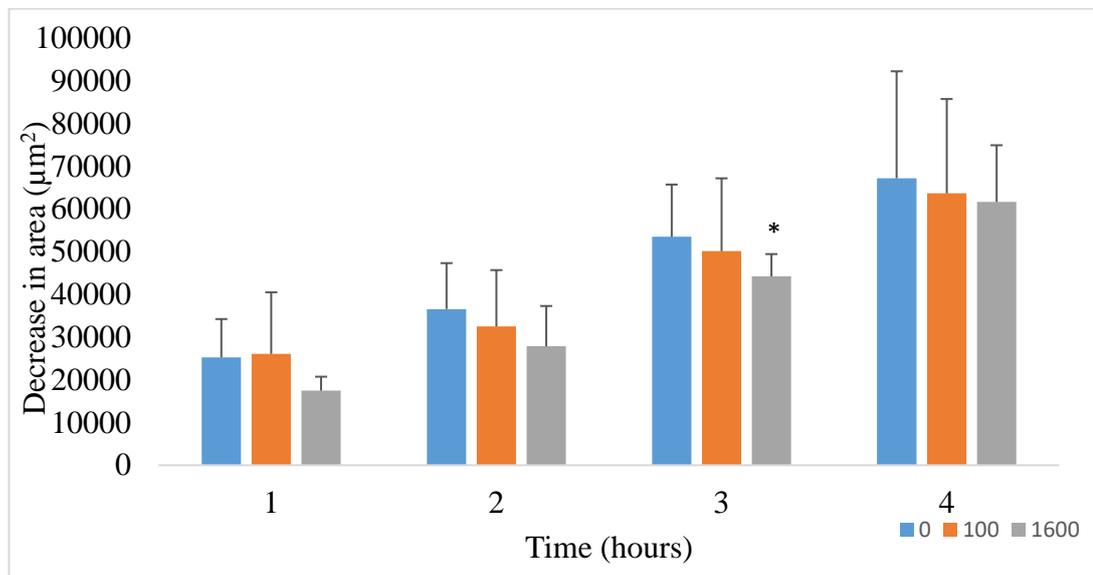
Figure 4.12 Decrease in area (μm^2) over 4-hour period, following mechanical stimulation of HDF cells for 5 minutes. Error bars represent standard deviation. *denotes Significance ($p<0.05$).
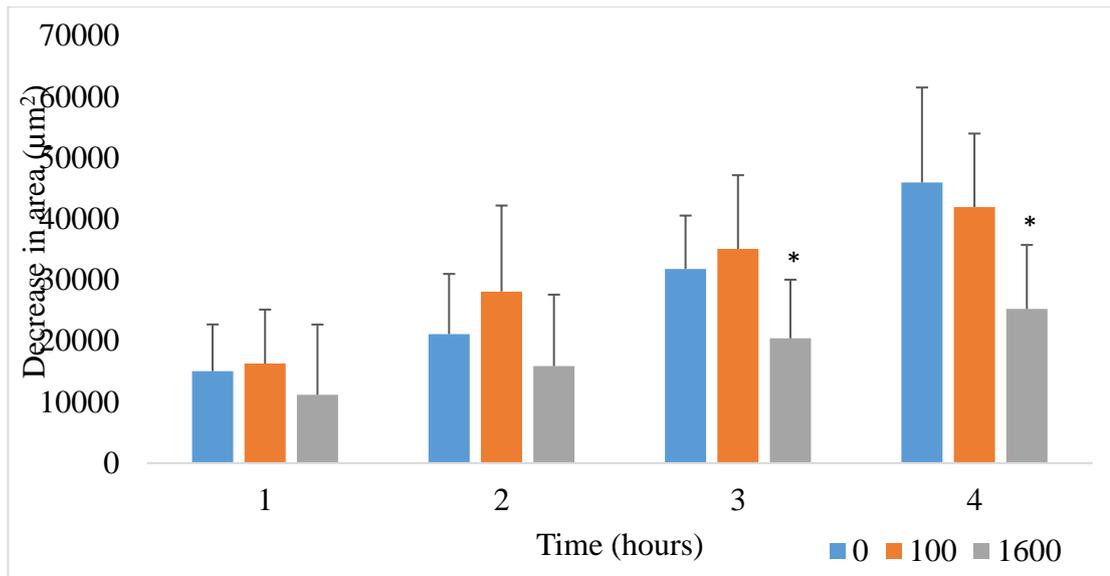
Figure 4.13 Decrease in area (μm^2) over 4-hour period, following mechanical stimulation of HDF cells for 20 minutes. Error bars represent standard deviation. *denotes Significance ($p < 0.05$).

From Figures 4.8-4.13 it can be observed that there was a similar trend seen when all three fibroblast cells were stimulated for 20 minutes. The trend shows that cells stimulated at 100Hz had an increase in wound closure from 1 to 3 hours. However, on the 4th hr the control population seem to have caught up, resulting in an increased wound closure compared to the cells that were stimulated. Overall, with 5 minute stimulation, the LL24 cells and HDF cells have a similar pattern showing that the control population recovered the wound quickly compared to the wounds that were stimulated.

4.4 The effects of intermittent mechanical stimulation on collective cell migration

Previous results shown within this thesis have shown that stimulating LL24 cells at 100Hz for 20 minutes resulted in an increase in collective cell migration up to 3 hours and on the 4th hour the control population had 'caught up' this indicating that the effects of stimulation may wear off. Therefore, it was interesting to see if intermittent mechanical stimulation at 100Hz enhanced the collective cell migration for LL24 (i.e. would the stimulated cells have greater wound closure after 4 hours compared to the control). As can be seen from Figure 4.14, there is a decrease in cells migration of

approximately 51271µm^2 (36%) with 5 minutes single stimulation of 100Hz and approximately 9304µm^2 (7%) with intermittent stimulation every hour for 4 hours when compared to control. Additionally, mechanical stimulation at 100Hz for 5 minutes applying single and four stimulations was also found to result in significantly greater cell migration into the wound area, compared to the control (p<0.05). Figure 4.15 shows the decrease in area (µm^2) when LL24 cells wounds were stimulated at 100Hz for 20 minutes. There was increase in cell migration approximately 13943µm^2 (11%) with single stimulation and approximately 26555µm^2 (22%) with stimulation every hour for 4hours period when compared to control. Figure 4.15, statistical analysis found this difference not to be statistically significant (p>0.05).
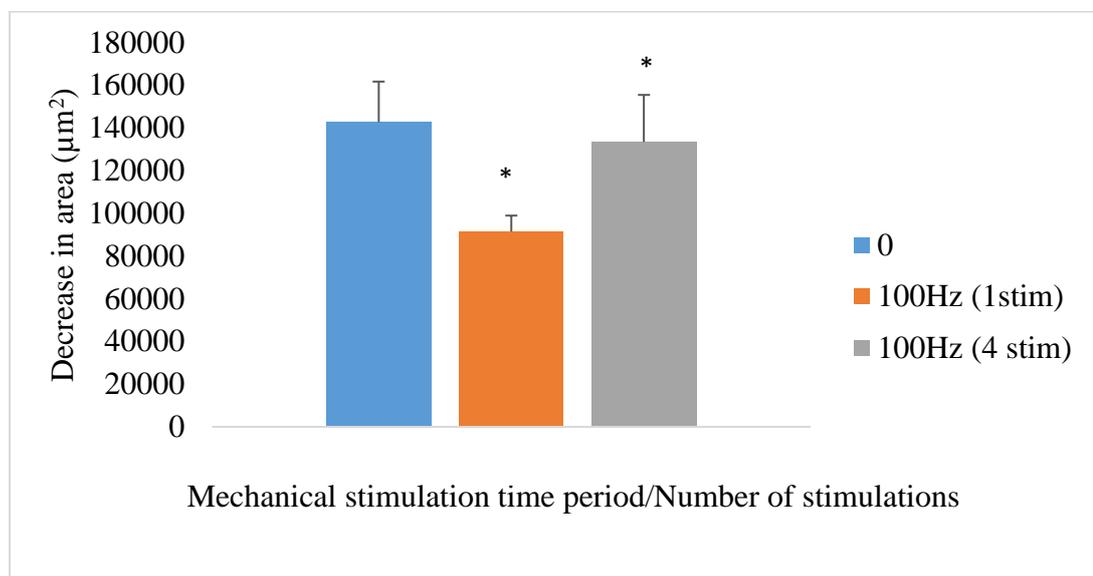
Figure 4.14 Effects of single stimulation versus hourly mechanical stimulation (Hz) for 5 minutes of on LL24 cells. Error bars represent standard deviation. * denotes Significance (p<0.05).
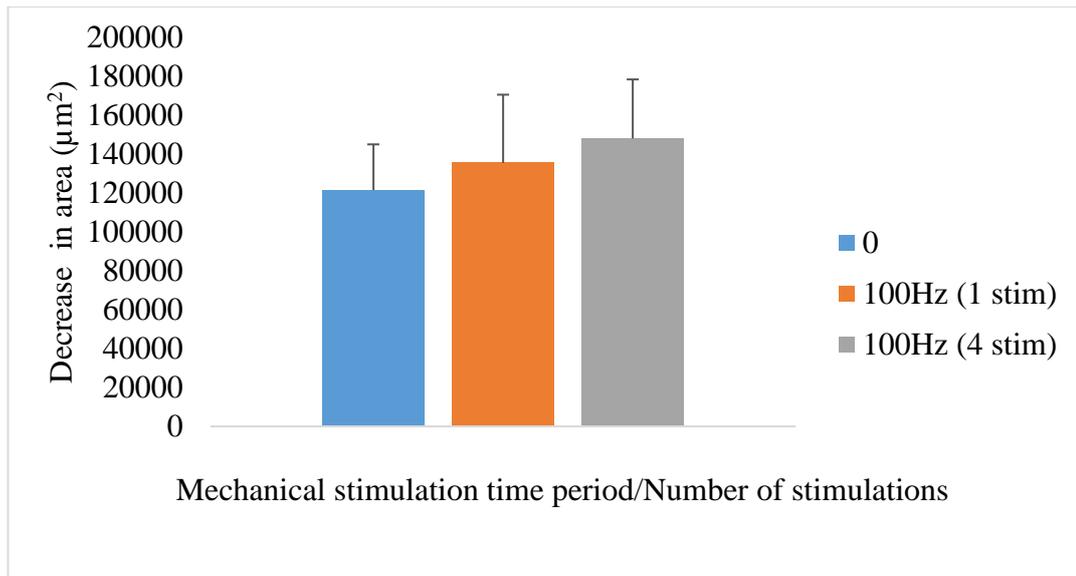
Figure 4.15 Effects of single stimulation versus hourly mechanical stimulation (Hz) for 20 minutes of on LL24 cells. Error bars represent standard deviation.

Figure 4.16 shows the decrease in area (μm^2) when L929 cells wounds were stimulated at 100Hz for 5 minutes. From the figure it shows approximately 40727μm^2 (20%) decrease in wound closure with single stimulation when compared to control and approximately 34467μm^2 (17%) decrease in wound closure when stimulated every hour for 4 hours. Figure 4.42 shows that the statistical analysis found shows the difference is not to be statistically significant (p>0.05). Figure 4.17 shows the decrease in area (μm^2) when L929 cells wounds were stimulated at 100Hz for 20 minutes. This figure shows that the wounds stimulated with single stimulation have a greater wound closure of approximately 34575μm^2 (27%) and with intermittent stimulations of 100Hz have a greater wound closure of approximately 56302μm^2 (44%). Again, Figure 4.17 shows that the difference is not to be statistically significant (p>0.05).
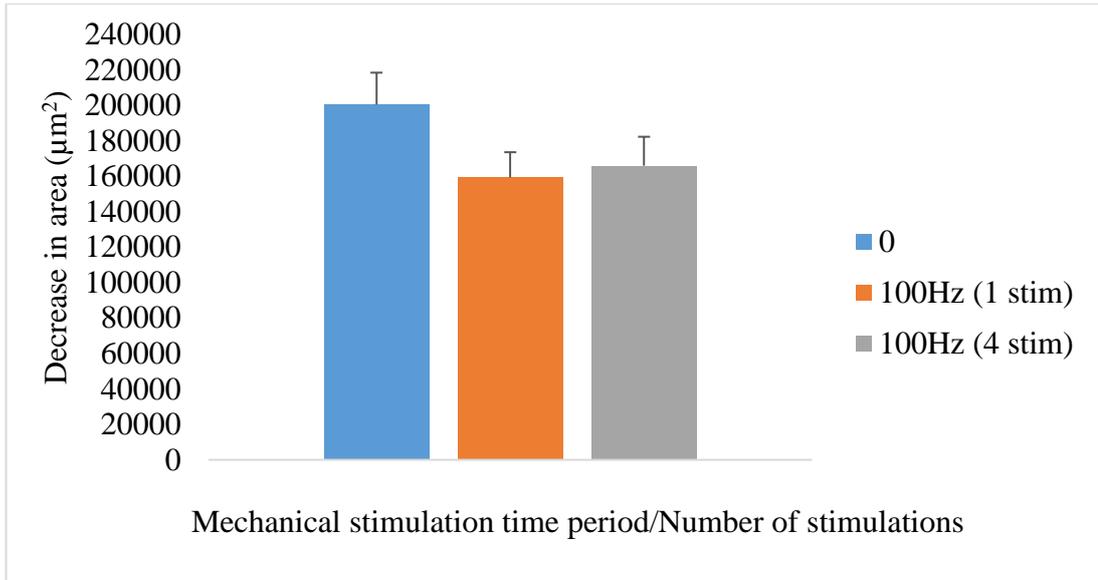
Figure 4.16 Effects of single stimulation versus hourly mechanical stimulation (Hz) for 5 minutes of on L929 cells. Error bars represent standard deviation.
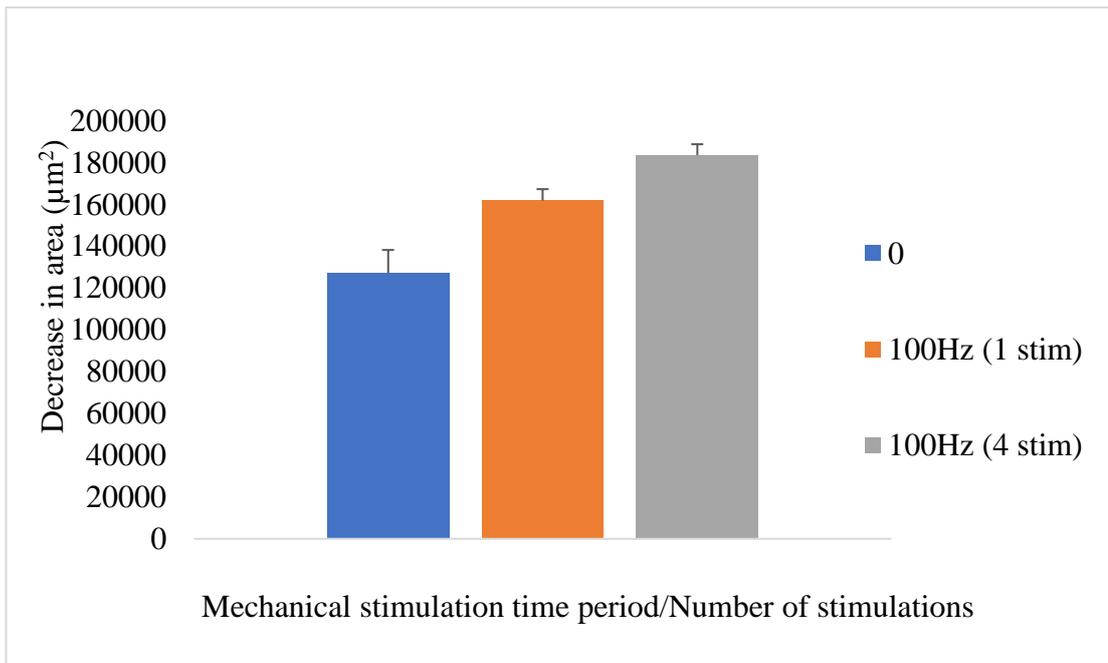
Figure 4.17 Effects of single stimulation versus hourly mechanical stimulation (Hz) for 20 minutes of on L929 cells. Error bars represent standard deviation.

Overall, figures 4.14-4.17, suggest that wounds from both cell lines, at 5 minutes of either single or four stimulations, seem to close the wound slowly in comparison to the control. In contrast to 5 minutes, 20 minute stimulation shows a different trend. Compared to the control, both cell lines show that the cell population with 0 stimulation tend to move slowly compared to single or every hour stimulation.

Stimulation every hour for 20 minutes seems to be more effective in wound closure than single stimulation.

4.5 The effects of intermittent mechanical stimulation on single cell migration

Mechanical stimulation has been shown to increase the mean migration distance in single cell migration when cells are stimulated at 100Hz. Therefore, it was interesting to see if a similar trend occurs with intermittent stimulation on an hourly basis. Figure 4.18 show the mean migration distance (µm) over a 4-hour period, for LL24 fibroblast cells, with a single stimulation and four stimulations at 100Hz for 5 minutes and 20 minutes. As can be seen by Figure 4.18, when comparing the 5 minutes single stimulation at 100Hz with control, there is an increase in mean migration distance of approximately 11.3µm (136%). In contrast, when stimulating the cells on an hourly basis for 5 minutes over a 4-hour period, it can be seen that, there is an increase in cell migration distance of approximately 4.7µm (57%). When comparing the effects of 20 minutes stimulation it was found that there was an increase in mean migration distance of approximately 12.7µm (153%) for a single stimulation and for intermittent stimulation there is an increase in mean migration by approximately 7.5µm (90%) (Figure 4.18). Additionally, mechanical stimulation at 100Hz for 5 minutes with single stimulation and 20 minutes of single and four stimulations was also found to be significant ($p < 0.05$).
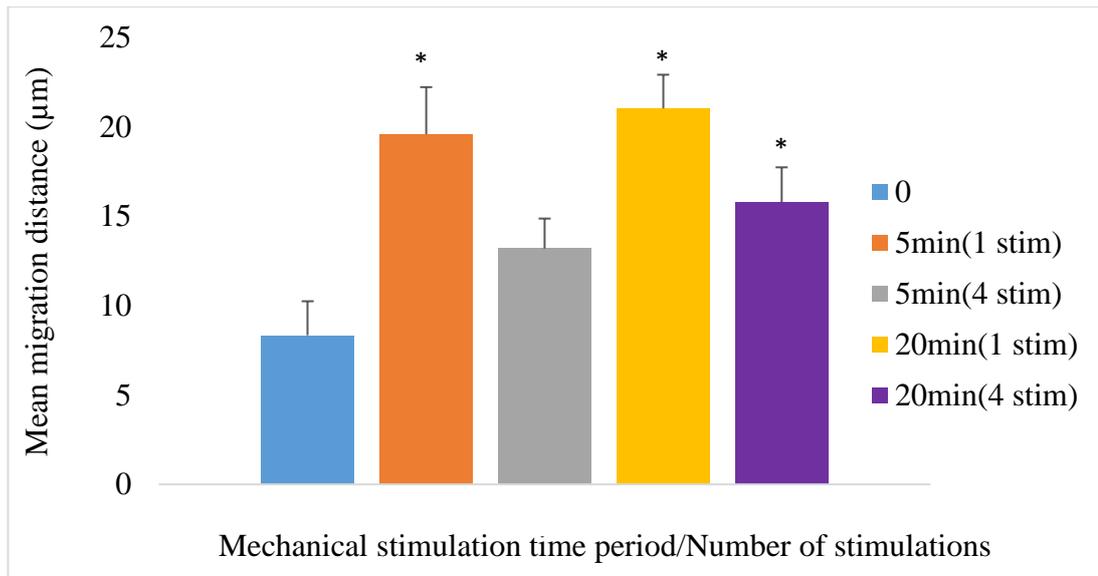
Figure 4.18 Mean migration distance (µm) over a 4-hour period for LL24 fibroblast cells (n=30) versus frequency of mechanical stimulation (Hz) of 5 and 20 minutes of 100Hz. Error bars represent standard deviation. * denotes Significance (p<0.05).

Figure 4.19 represents the data for L929 cells, showing intermittent stimulation at a 100Hz frequency, for 5 minutes and 20 minutes, compared to the control. As can be seen from Figure 4.19, after 5 minutes single stimulation of 100Hz, there is a greater mean migration distance of approximately 7.6µm (58%) compared to the control and for 5 minutes with four stimulations there is a decrease in mean migration distance by approximately 1.8µm (14%). However, compared to the single 20 minutes stimulation to the control, there is an increase in mean migration distance (µm) of approximately 5.9µm (45%) and for intermittent four stimulations there is increase in mean migration (µm) of approximately 1.8 µm (14%). Additionally, mechanical stimulation at 100Hz for 5 minutes with single stimulation and 20 minutes of single stimulations was also found to be significant (p<0.05) in greater single cell migration, compared to the control.
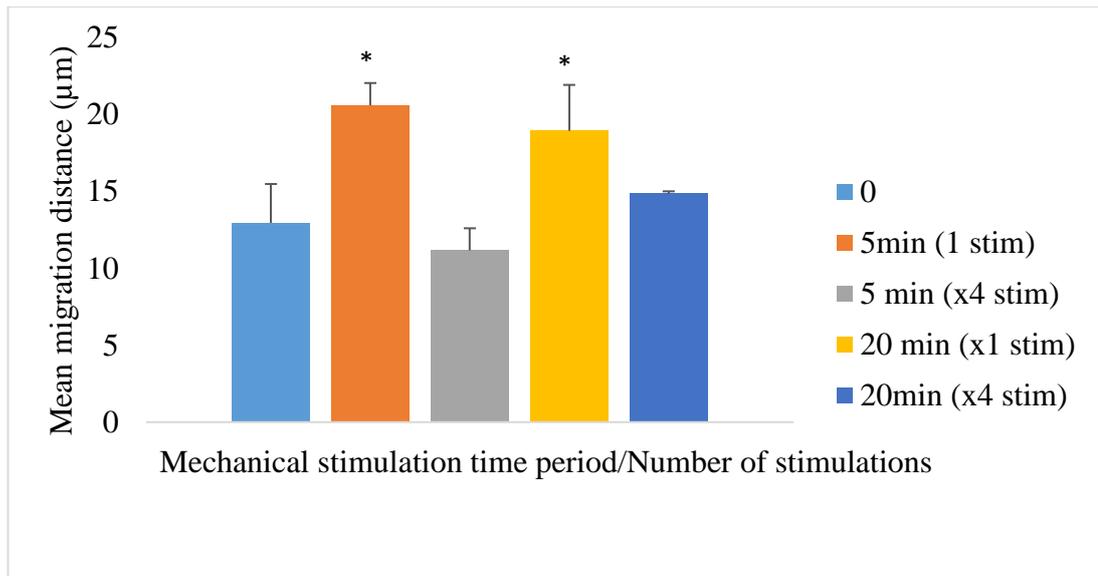
Figure 4.19 Mean migration distance (µm) over a 4-hour period for L929 fibroblast cells (n=30) versus frequency of mechanical stimulation (Hz) of 5 and 20 minutes. Error bars represent standard deviation. * denotes Significance ($p<0.05$).

From the Figures 4.18 and 4.19, there is an opposite trend to collective cell migration. In both cell lines, LL24 and L929 cells show that the mean migration distance was greatest with single 5 minute stimulation and single 20 minute stimulation compared to the control and intermittent stimulation. However, in LL24 cells it shows the cells migrated the most with a single 20 minutes stimulation of 100Hz whereas in L929 cells the cells with a single 5 minutes stimulation migrated the most.

4.6 The effects of mechanical stimulation on the viability of fibroblast cells

In order to determine if the vibration-induced decrease or increase in cell migration was due to changes in cell viability, an MTT assay was carried out. The MTT is a colorimetric assay that relates absorbance to the number of viable cells. A reduction/increase in absorbance (relative to the control) would indicate that the mechanical stimulation has had some effect on the viability (or mitochondrial activity) of the cells. It was observed on all occasions that mechanical stimulation had no effect on the viability of LL24 or L929 cells at any of the frequencies used here. This is evident from Figures 4.20 - 4.21 which show the results of the MTT assay for both LL24 and L929 cells when stimulated for 5 and 20 minutes. As can be seen from the

figures, the level of cell viability (as indicated by the absorbance values) is similar across all frequencies and the control.
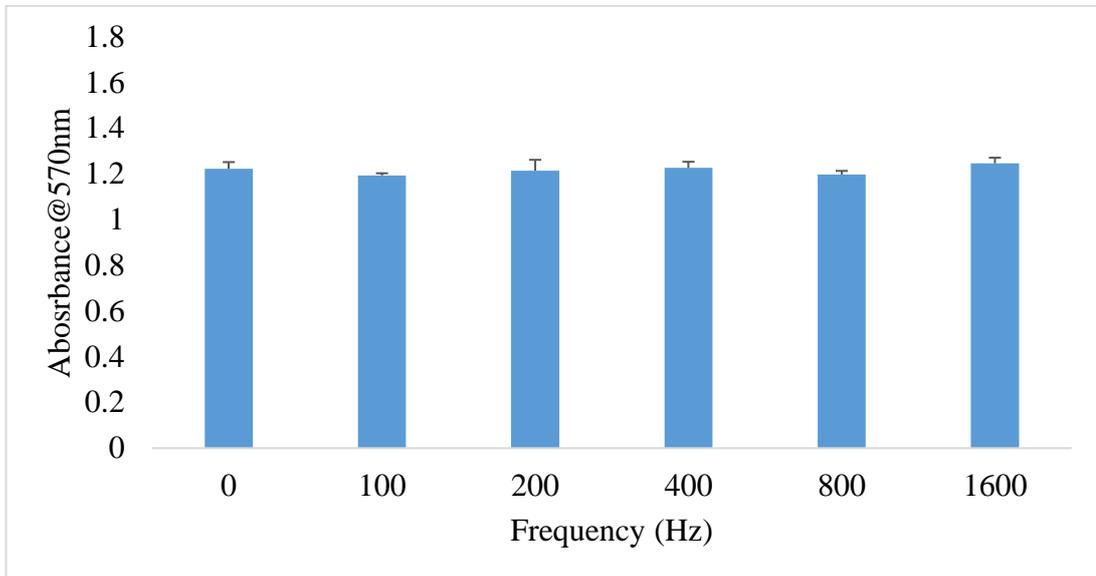
Figure 4.20 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of LL24 cells for 5 minutes. Error bars represent standard deviation.
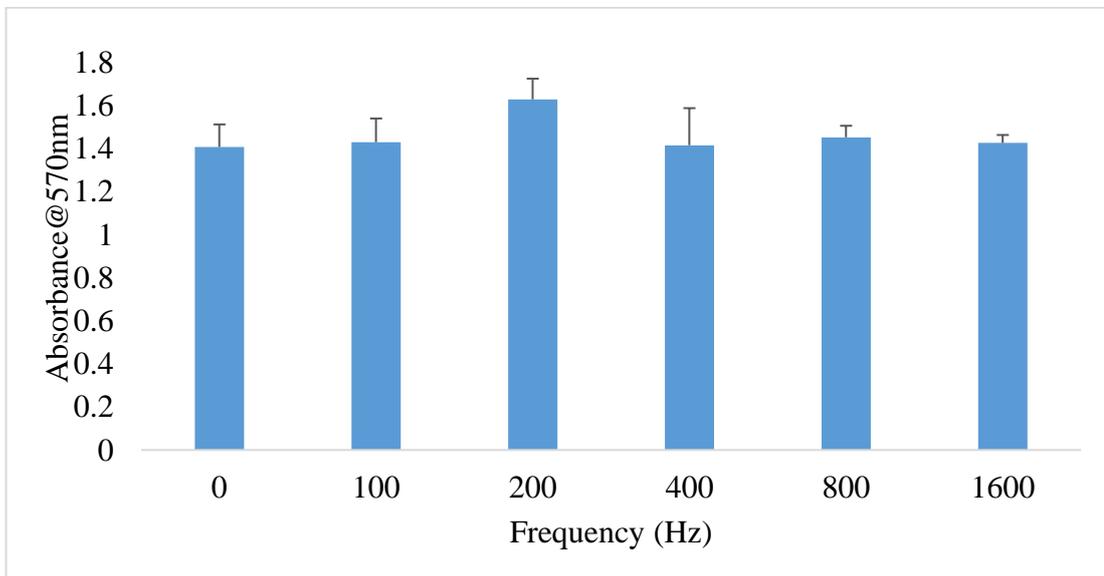
Figure 4.21 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of L929 cells for 5 minutes. Error bars represent standard deviation. * denotes Significance ($p<0.05$).
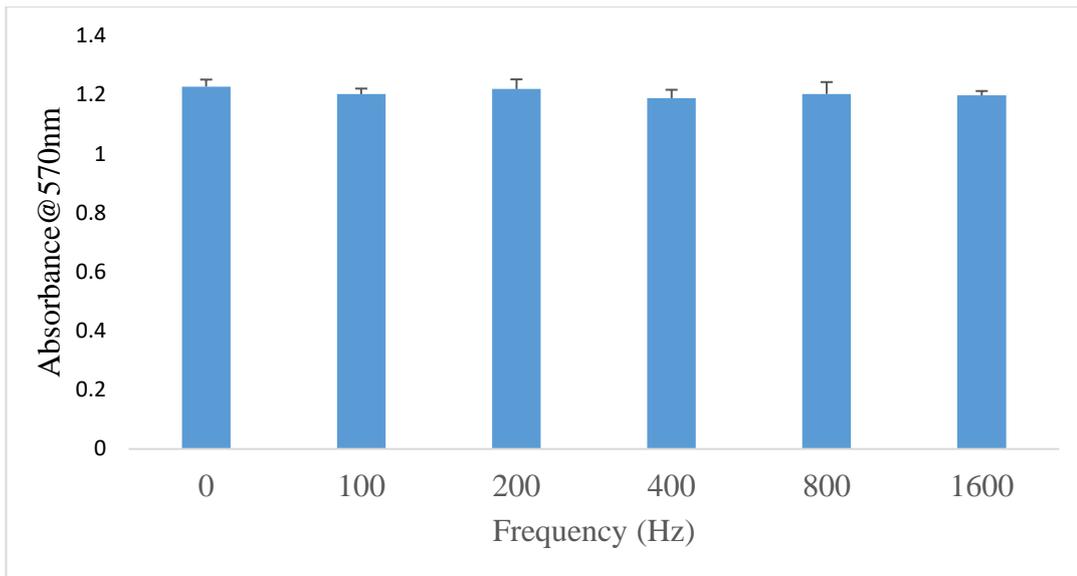
Figure 4.22 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of LL24 cells for 20 minutes. Error bars represent standard deviation.
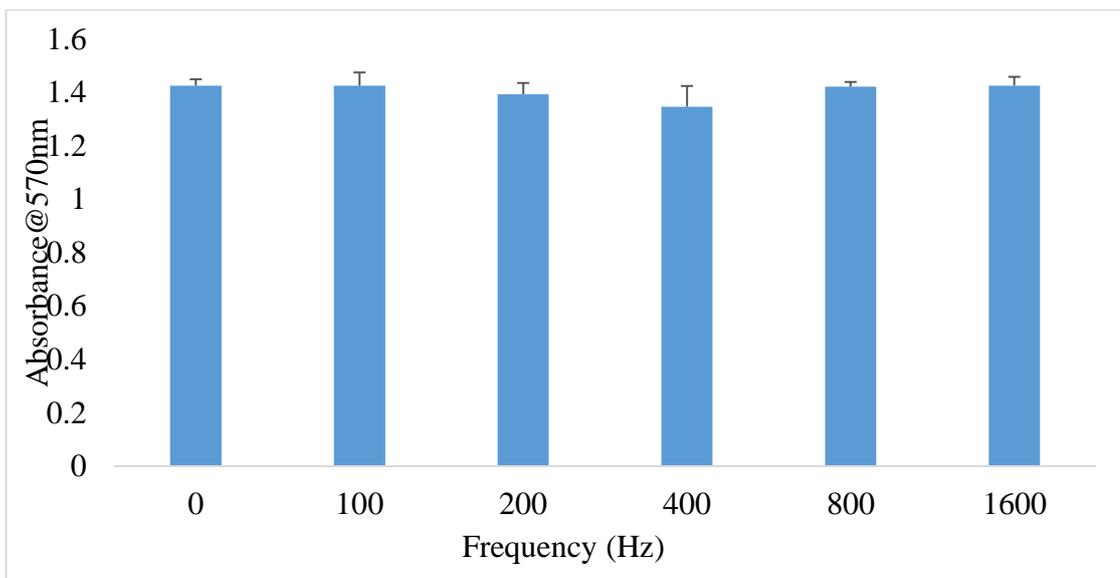
Figure 4.23 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of L929 cells for 20 minutes. Error bars represent standard deviation.

In order to identify if mechanical stimulation was having an effect on the viability of primary cells (HDF), an MTT assay was carried out. As can be seen from Figures 4.24 and 4.25, there was very little difference in the mean absorbance values for all frequencies compared to the control.
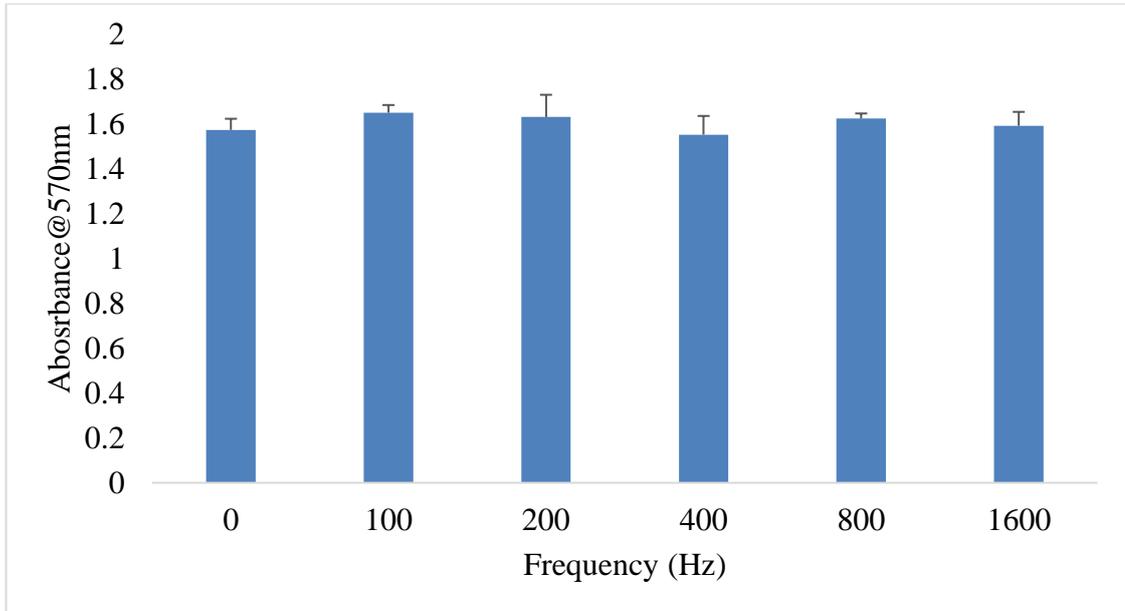
Figure 4.24 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of HDF cells for 5 minutes. Error bars represent standard deviation.
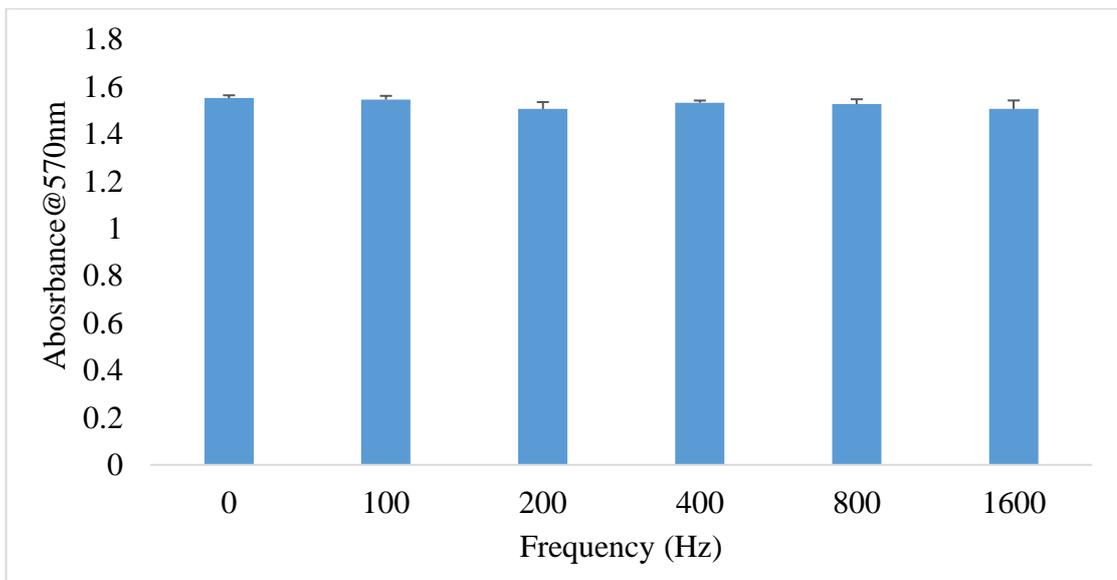
Figure 4.25 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of HDF cells for 5 minutes. Error bars represent standard deviation.

Trypan blue cell viability assays were also carried out and the results support those obtained from the MTT assays. Trypan blue is used to assess viability based on membrane integrity. Viable cells will contain an intact cell membrane which will exclude the trypan blue dye from entering the cell. If, however, the cell becomes non-viable then one of the first indications is loss of membrane integrity. In some

circumstances the trypan blue dye is able to pass freely across the cell membrane staining the cytoplasm of the cell blue. As can be seen from Figures 4.26 and 4.27 there was no obvious differences in the number of viable LL24 and L929 cells following 5 and 20 minutes vibration at all frequencies when compared to the control. These results support the MTT assay and show that the vibration frequencies (and exposure times) used here are not affecting cell viability. This indicates that the observed changes in cell migration are not due to changes in cell viability.
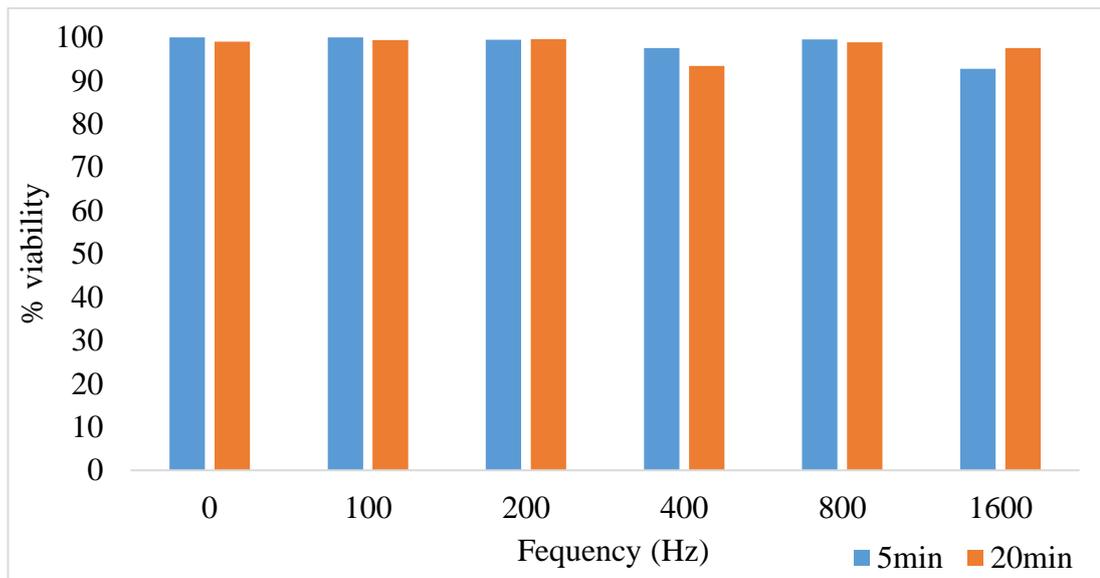
Figure 4.26 Trypan blue assay results showing % viability versus frequency (Hz) following mechanical stimulation of LL24 cells for 5 and 20 minutes.
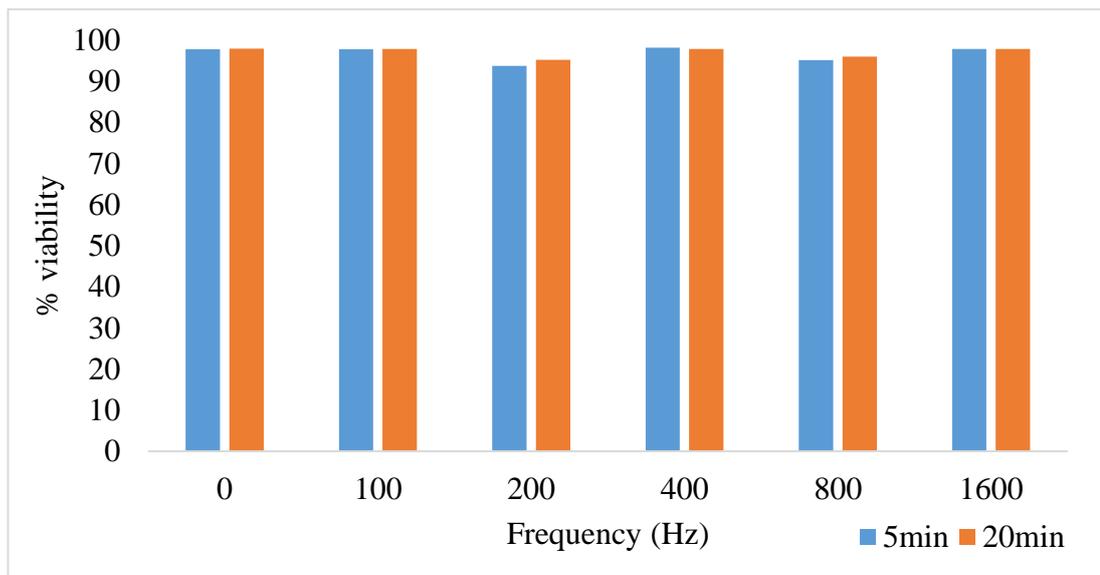
Figure 4.27 Trypan blue assay results showing % viability versus frequency (Hz) following mechanical stimulation of L929 cells for 5 and 20 minutes.

To further test the effects of mechanical stimulation on HDF cell viability a trypan blue assay was carried out. Figure 4.28 shows that for all frequencies mechanical stimulation did not have any effects on cell viability of the HDF cells compared to the control when stimulated for 5 or 20 minutes.
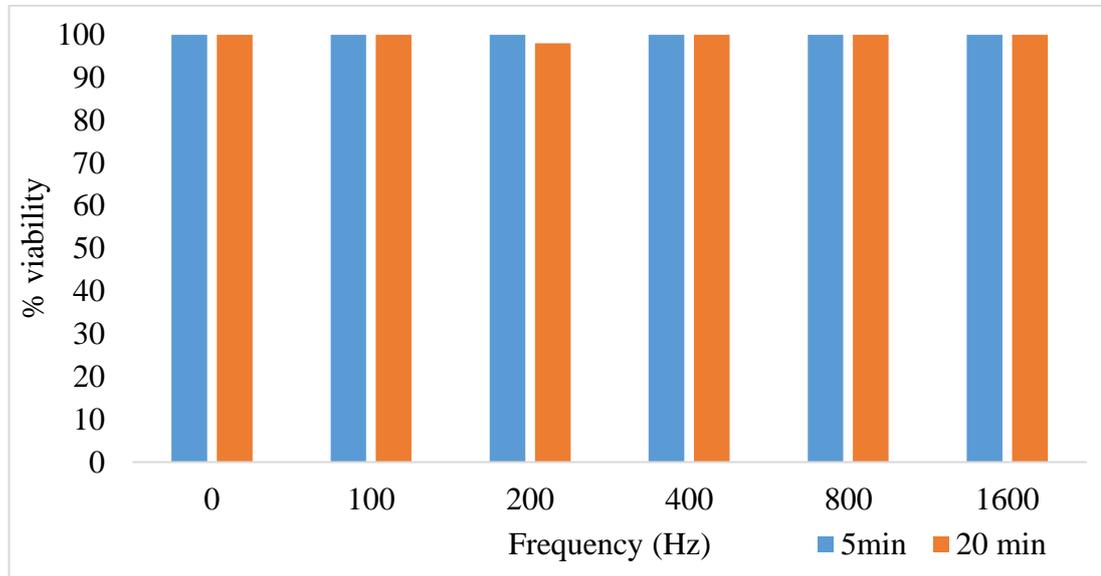
Figure 4.28 Trypan blue results showing % viability versus frequency (Hz) following mechanical stimulation of HDF cells for 5 minutes and 20 minutes. This experiment was repeated three times.

4.7 The effects of mechanical stimulation on cell proliferation

In order on determine if mechanical stimulation had any effect on cell proliferation a cell proliferation assay was carried out on LL24 cells, L929 cells and HDF cells over 72 hours following mechanical stimulation for 5 or 20 minutes at frequencies between 0-1600Hz. Figure 4.29 shows cell number versus frequency (0-1600Hz) following 5 minutes stimulation on LL24 cells. As can be seen from Figure 4.29, there is a steady increase in cell number for all frequencies (and control) over the 72 hr period. Compared to the control, it can be seen that cell proliferation is similar between the control and 100Hz at 24, 48 and 72 hr. After 24 and 48 hours, the cell number is lower for all frequencies in relation to the control. The greatest cell number appears at 72 hours following 100Hz stimulation resulting in 1,248,000 cells/ml. Above this frequency, it seems that there is a steady decrease in cell proliferation as the frequency is increased. For example, after 72 hours at 1600Hz there were only 824,600 cells/ml, this shows that as the frequency increased, less cells proliferated at 72 hours.
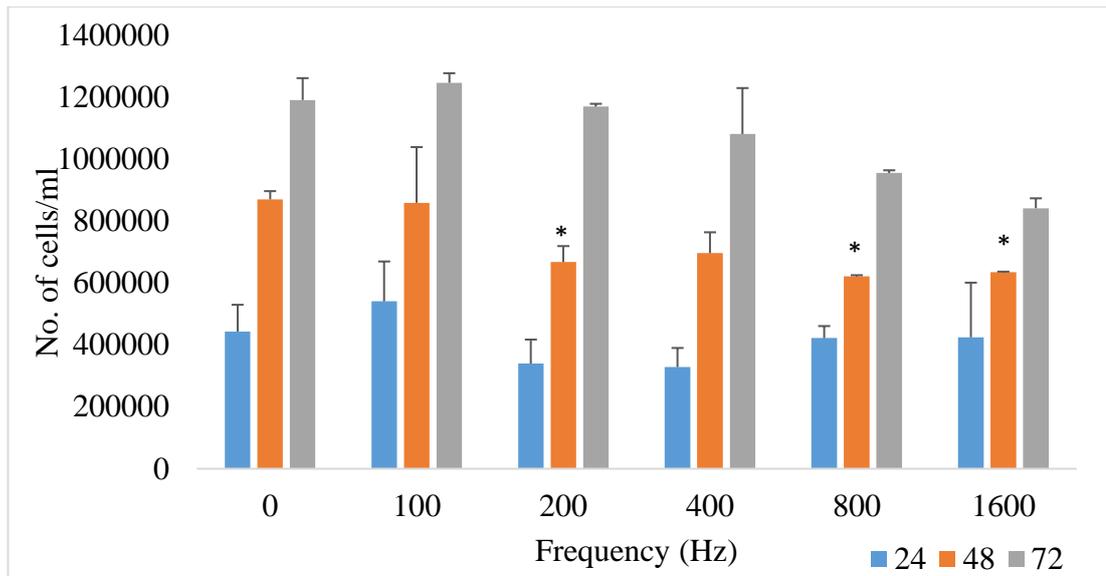
Figure 4.29 Cell number versus frequency (Hz) following mechanical stimulation of LL24 cells for 5 minutes. Error bars represent standard deviation. * denotes Significance (p<0.05).

Figure 4.30 shows cell number versus frequency (0-1600Hz) following 20 minutes stimulation. As can be seen from Figure 4.30 there is a steady increase in cell number for all frequencies over the 72hours. Compared to the control it can be seen that cell proliferation is similar between the control, 100 and 400Hz at 24, 48 and 72hr. While those cells stimulated at 200, 800 and 1600Hz all displayed similar levels of cell proliferation, and which was less in comparison to the control, 100 and 400Hz samples. The maximum cell number of 1,506,250 cells/ml appears at 72 hours following 100Hz stimulation. Above this frequency it seems that there is a slight decrease in cell proliferation as the frequency increases, except at 400Hz. For example, after 72 hours following 100Hz stimulation there were 1,506,250 cells/ml while at 1600Hz there were 994,250 cells/ml.
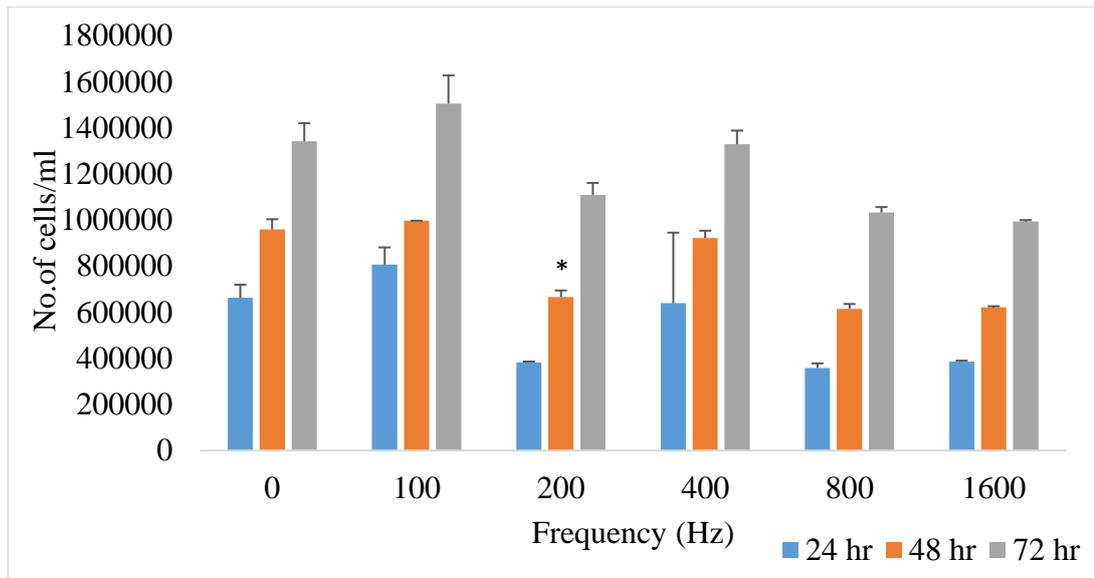
Figure 4.30 Cell number versus frequency (Hz) following mechanical stimulation of LL24 cells for 20 minutes, repeated 3 times. Error bars represent standard deviation. * denotes Significance (p<0.05).

Figure 4.31 shows L929 cell number versus frequency (0-1600Hz) following 5 minutes stimulation. As can be seen from Figure 4.31, compared to control, the trend across 24-72 hours is very similar for all frequencies. However, compared to the control it can be seen that cell proliferation is similar at 100Hz over 24, 48 and 72 hr. After 24 and 48 hours, the cell number is lower for all frequencies. The greatest cell number appears at 72 hours, when stimulated at 100Hz excluding 800Hz and above 100Hz, it seems that there is a decrease in cell proliferation. The greatest cell number, 3,405,000 cells/ml was observed at 72 hours when stimulated at 800Hz followed by control with 3,350,000 cells/ml and then 100Hz 3,245,000 cells/ml. Figure 4.32 shows cell proliferation for L929 cells when mechanically stimulated for 20 minutes. Compared to the control, it can be seen that cell proliferation is similar between the control and 100Hz at 72 hr. At no stimulation the cell number is 24,050,000 cells/ml and at 100Hz there are 2,435,000 cells/ml which are very similar to each other. At 72 hours the highest number of cells is seen at 200Hz with 2,690,000 cells/ml. and the least cell number is seen at 1600Hz with 1,755,000 cells/ml.
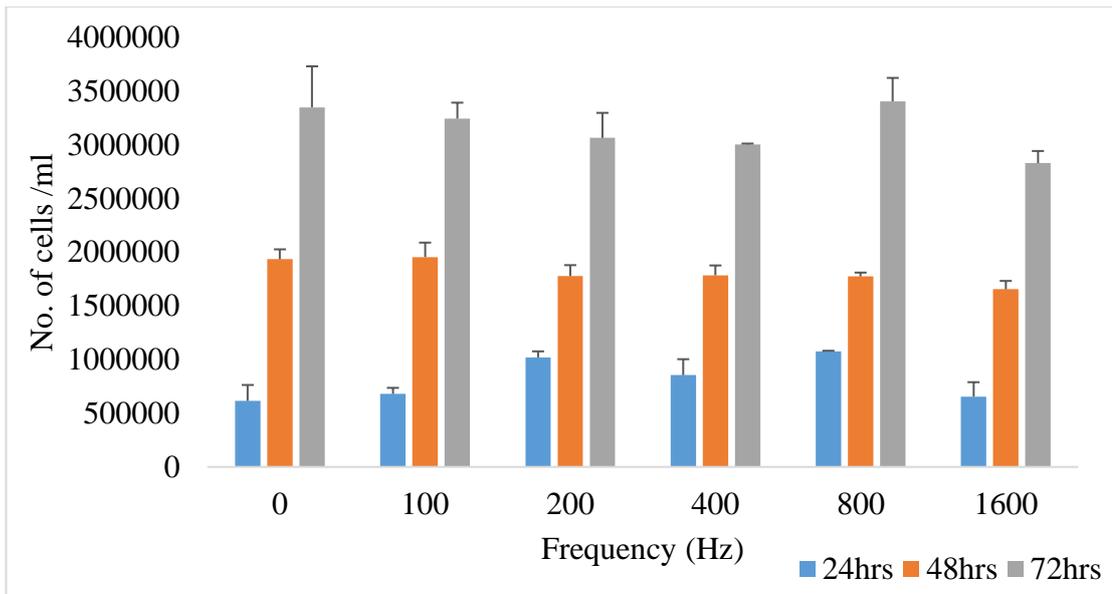
Figure 4.31 Cell number versus frequency (Hz) following mechanical stimulation of L929 cells for 5 minutes. Error bars represent standard deviation.
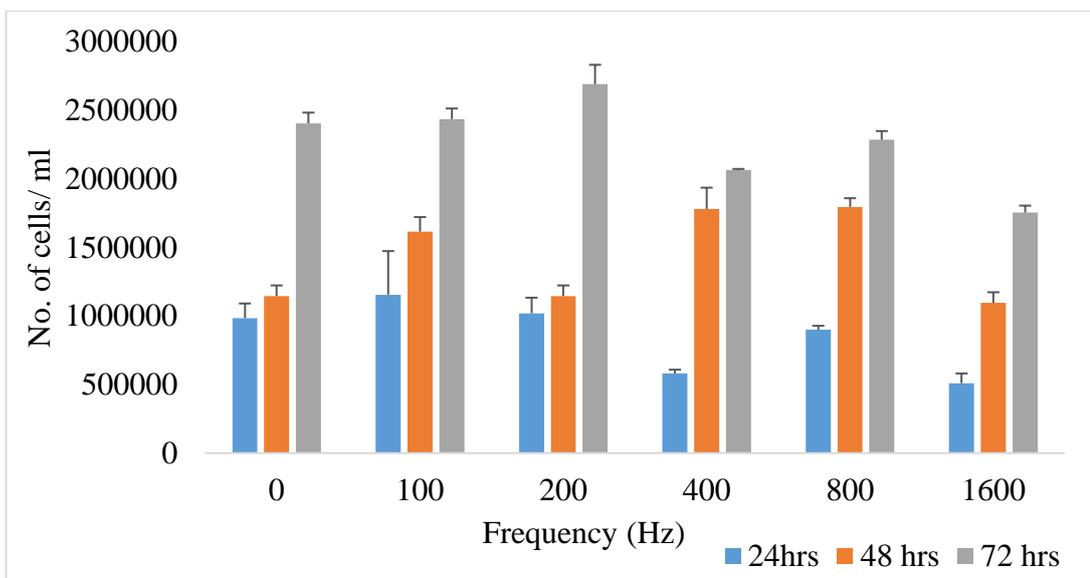
Figure 4.32 Cell number versus frequency (Hz) following mechanical stimulation of L929 cells for 20 minutes. Error bars represent standard deviation.

In order to assess the effects of mechanical stimulation on the proliferation of HDF cells, a cell proliferation assay was carried out over 72 hours. Figure 4.33 shows the results of this assay following 5 minutes stimulation. As can be seen from Figure 4.33 there was a steady increase in cell number over the 72 hr period at all frequencies. The greatest proliferation can be seen at 100Hz followed by the control population. Between 200-800Hz there seems to be a steady decline in HDF proliferation. However, at 1600Hz the level of proliferation was found to be similar to the control. Figure 4.34 shows the cell proliferation of L929 cells when mechanically stimulated

at range of frequencies of 0-1600Hz. As can be seen from Figure 4.34 above 100Hz there is a steady decrease in cell number at 72 hours excluding 400Hz. The greatest proliferation can be seen at 100Hz of approximately 633333 cells/ml followed by the control population 626666 cells/ml. Between 200-800Hz there seems to be a steady decline in HDF proliferation except at 400Hz where the cell number is 580000cells/ml.
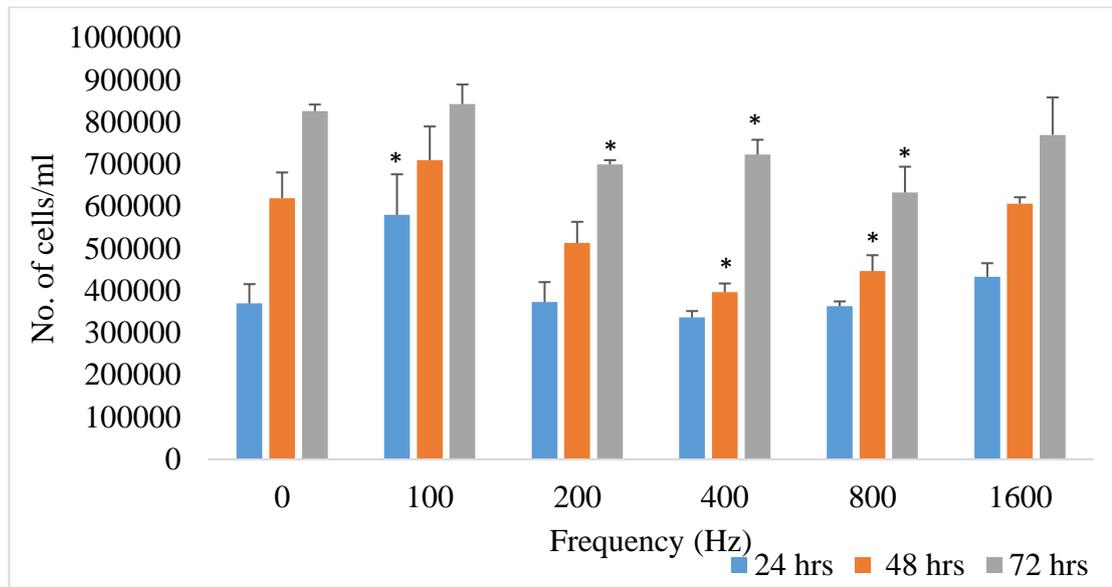
Figure 4.33 Cell number versus frequency (Hz) of mechanical stimulation, on HDF cells for 5 minutes. Error bars represent standard deviation. * denotes Significance (p<0.05).
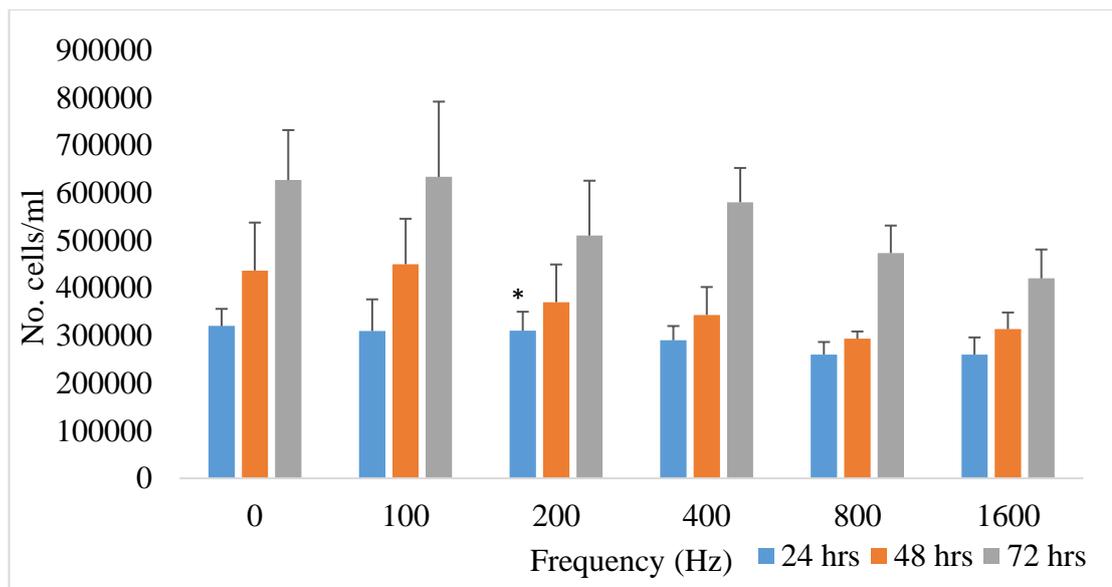
Figure 4.34 Cell number versus frequency (Hz) of mechanical stimulation on HDF cells for 20 minutes. Error bars represent standard deviation. * denotes Significance (p<0.05).

To summarise the results, LL24 and the HDF cells indicate that after 72 hours of cell proliferation, the cells that were mechanically stimulated at 100Hz for 5 minutes and 20 minutes, overall showed the most number of cells per ml. However, after 100Hz, as the frequency increased there was an overall steady decline in the number of cells. The trend was different with the L929 cells, at 5 minute stimulation the greatest number of cells were seen at 800Hz and for 20 minutes of stimulation the greatest cell number was seen at 200Hz.

4.8 The effects of mechanical stimulation on actin organisation

Cell migration is associated with well characterised changes to the cell. Many of these changes are macro-scale structural and morphological changes and include; the formation of lamellipodia/membrane ruffling, membrane blebs and actin filopodia (David J. Barry, 2015), the latter of which has been shown to stimulate cell migration (Antti Arjonen, 2011). Given that fibroblast cells have been shown to be mechanosensitive and that actin remodelling is associated with cell migration, the effect of mechanical stimulation on actin organisation was investigated. Following 5 and 20 minutes mechanical stimulation, both cell lines LL24 cells and L929 cells at 0-1600Hz, the cells were fixed and stained using Phalloidin, which specifically labels actin filaments (F-actin). Confocal microscopy revealed that mechanical stimulation encouraged actin remodelling in both cell types. In particular, for LL24 cells it was observed that there was an increase in lamellipodia/membrane ruffling (identified by the arrows in Figure 4.35) and an increase in stress-fibre formation/density, compared to the control. However, there were no notable differences in the F-actin organisation as frequency increased (Figure 4.35).
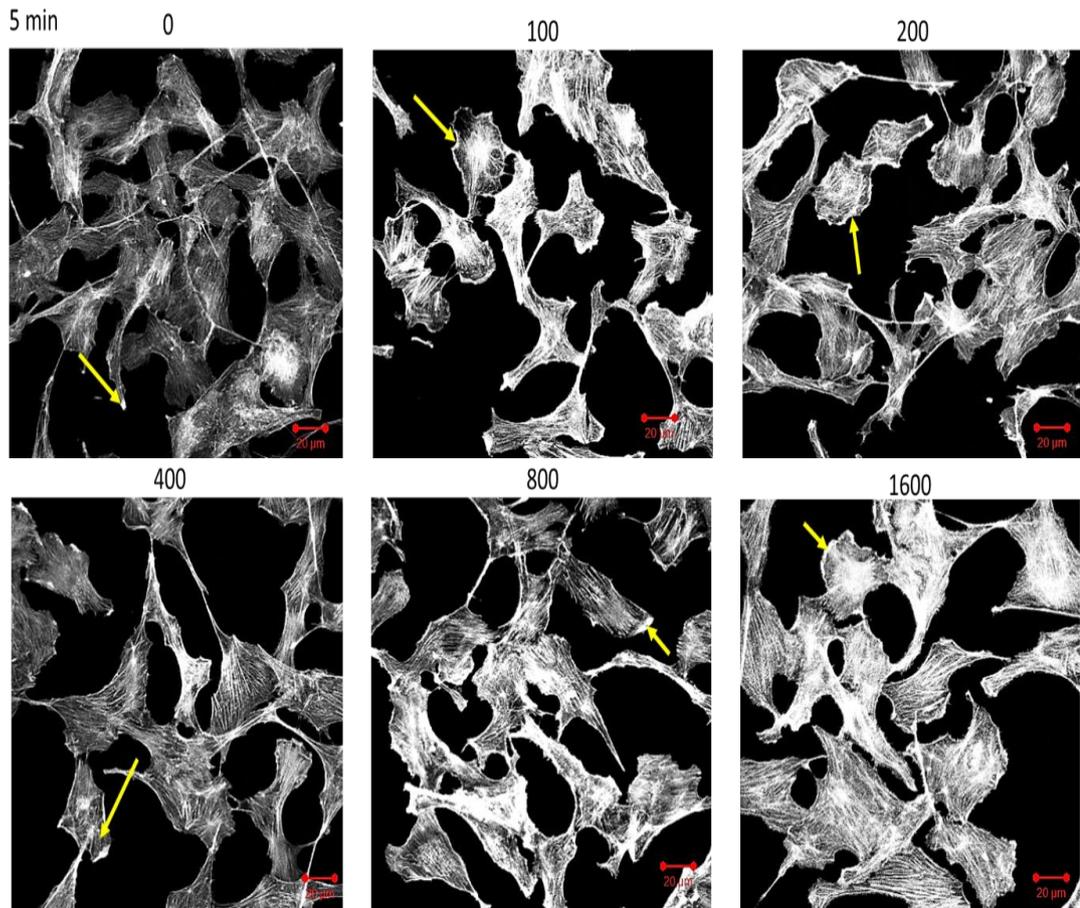
Figure 4.35 F-actin organisation in LL24 cells following mechanical stimulation (0Hz-1600Hz) for 5 minutes. Yellow arrows highlight lamellipodia/ruffling.

Similarly, mechanical stimulation of L929 cells also resulted in a reorganisation of F-actin, however the response was markedly different when compared to that of the LL24 cells. The control L929 cells generally appeared to be less spread compared to the LL24 cells. However, when mechanically stimulated, at all frequencies, there were distinct morphological changes to the L929 cells that were characterised by an increased level of cell spreading and actin filopodia formation (Figure 4.36).
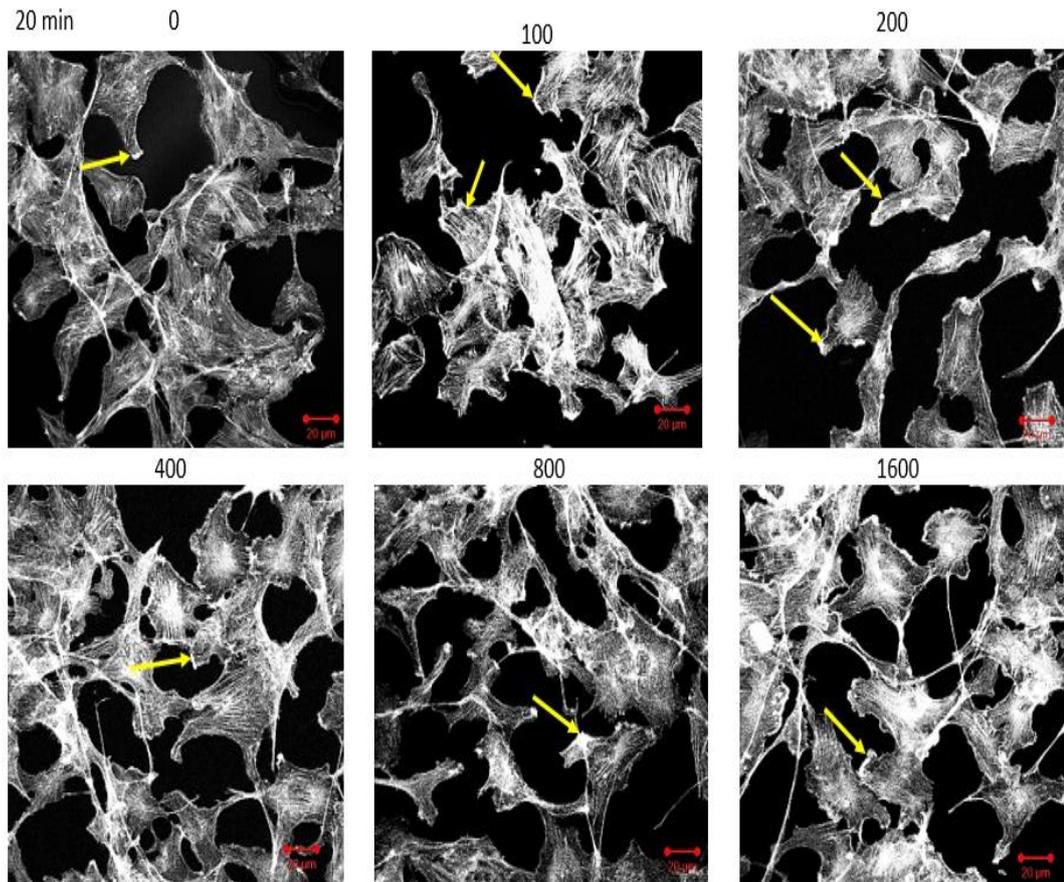
Figure 4.36 F-actin organisation in LL24 cells following mechanical stimulation (0Hz-1600Hz) for 20 minutes. Yellow arrows highlight lamellipodia/ruffling.

As can be seen from LL24 cells there is increased actin at the leading edge of cells. Figure 4.37 and 4.38 both show the actin organisation of L929 cells when mechanically stimulated for 5 and 20 minutes between 0-1600Hz. Overall from the figures, it was observed that there was an increase in lamellipodia/membrane ruffling and filopodia/microspikes compared to the control. More rounded cells were seen in control in relation to other frequencies such as 100Hz-1600Hz. However, when mechanically stimulated, at all frequencies, there were distinct morphological changes to the L929 cells that were characterised by an increased level of cell spreading and actin filopodia formation (Figure 4.38).
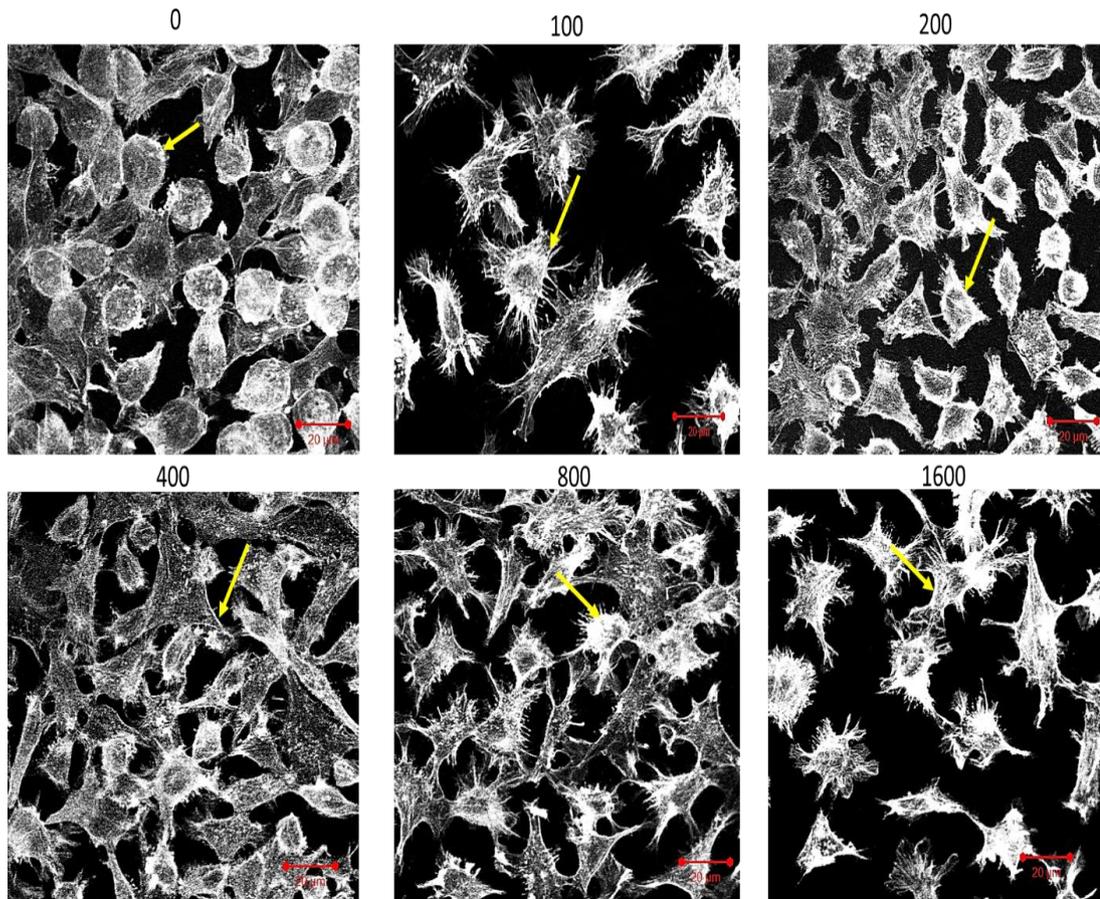
Figure 4.37 F-actin organisation in L929 cells following mechanical stimulation (0Hz-1600Hz) for 5 minutes. Yellow arrows highlight microspikes/filopodia. Red bar represents the scale bar of 20µm.
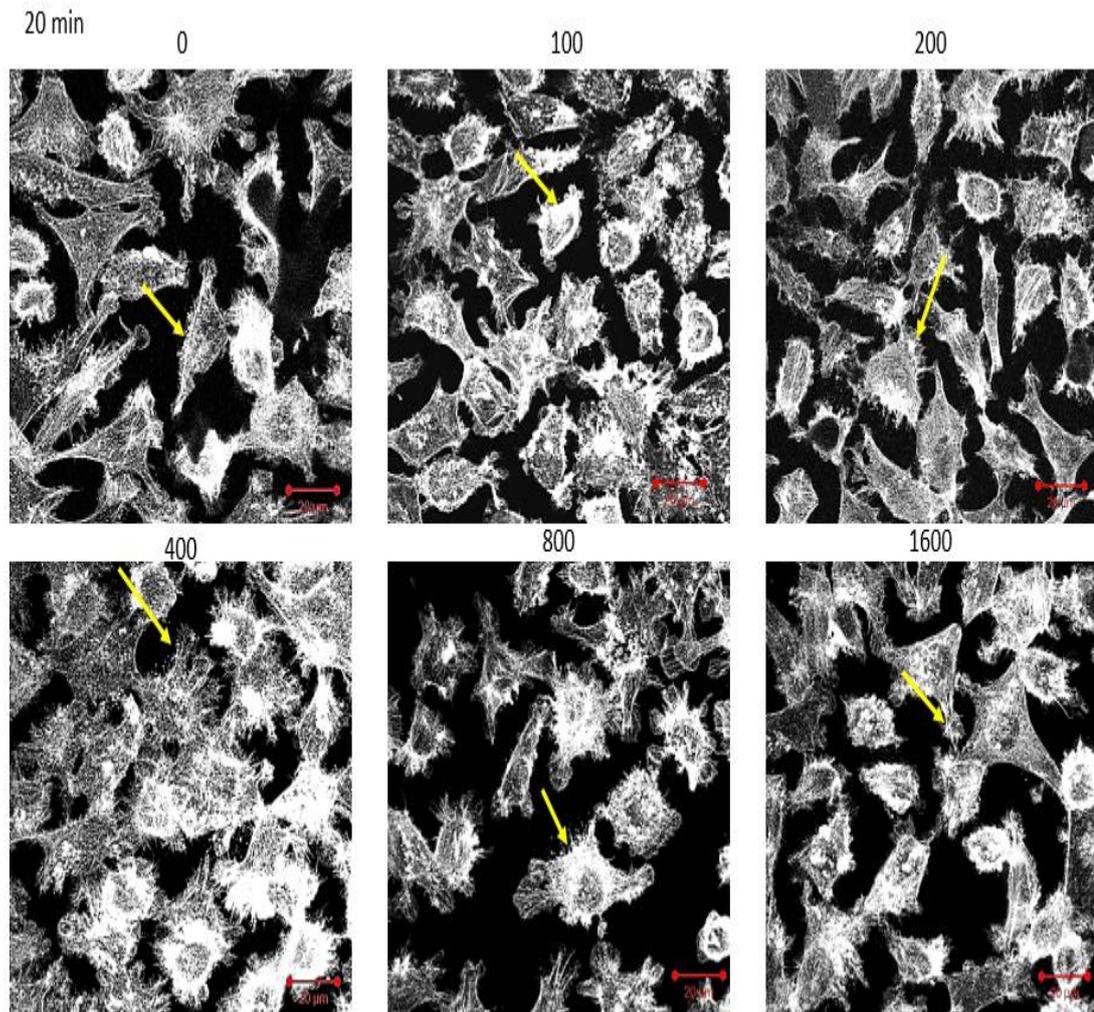
Figure 4.38 F-actin organisation in L929 cells following mechanical stimulation (0Hz-1600Hz) for 20 minutes Yellow arrows highlight microspikes/filopodia.

Following actin staining and confocal imaging a more detailed analysis was carried out to try and quantify changes to cell morphology/actin organisation. Morphological changes are highlighted in Figure 4.39 and are grouped into three categories; cells displaying a rounded morphology, cells displaying prominent filopodia and cells displaying prominent lamellipodia/ruffling.
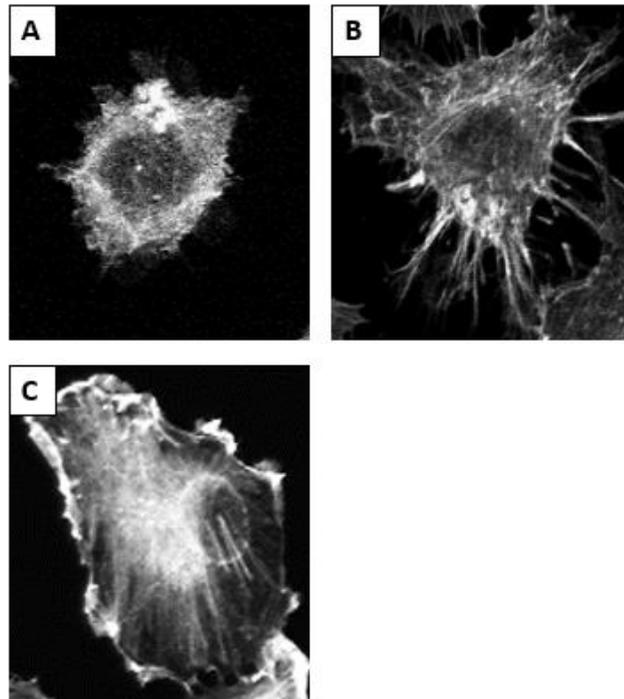
Figure 4.39 Confocal microscopy showing (A) rounded shape morphology, (B) cells with filopodia and (C) cells displaying lamellipodia/membrane ruffling.

For the analysis a total of 200 cells were counted for each frequency (and control) and the percentage of rounded cells, number of cells with prominent lamellipodia/ruffling, filopodia or a rounded morphology was recorded. Figure 4.40 displays the results for LL24 cells and Figure 4.41 displays the results for L929 cells. As can be seen from Figure 4.41, the control population contained cells that displayed a predominantly rounded morphology with many cells in this population also displaying some degree of both lamellipodia and filopodia. Mechanical stimulation at all frequencies resulted in changes to LL24 cell morphology. In particular, mechanical stimulation increased the level of cell spreading, with none of the mechanically stimulated cell populations observed to have cells with a rounded morphology. Mechanical stimulation of LL24 cells also resulted in an increase in the percentage of cells displaying prominent lamellipodia/ruffling and filopodia, with lamellipodia/ruffling being the most prominent morphological feature for the LL24 cell. This increase in lamellipodia/ruffling and filopodia formation can be seen to be somewhat dependent upon mechanical stimulation frequency. For example, the percentage of cells displaying membrane ruffling/lamellipodia increased steadily as mechanical stimulation frequency increased up to 400Hz and then was observed to decrease at 800

and 1600Hz, respectively. While the percentage of cells displaying actin filopodia was found to increase markedly at 200, 400 and 800Hz.

Similarly, mechanical stimulation of L929 cells was found to enhance cell spreading and increase the percentage of cells displaying lamellipodia/ruffling and filopodia. As can be seen from Figure 4.41, the control population of L929 cells contained a high percentage of cells displaying a rounded morphology. However, mechanical stimulation was seen to reduce the percentage of rounded cells in a frequency-dependent manner. Similarly, the percentage of cells displaying lamellipodia/ruffling was observed to increase in a frequency-dependent manner, while the percentage of cells displaying actin filopodia was found to increase markedly above the control population.
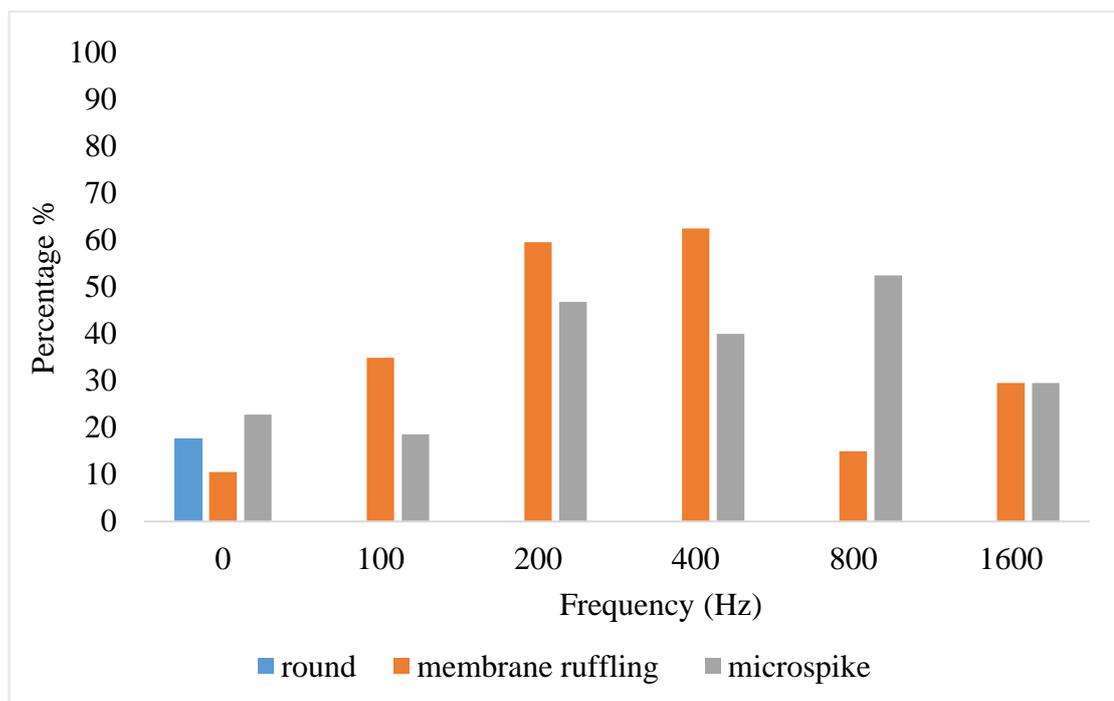
Figure 4.40 Image analysis of LL24 cells stimulated for 5 min showing the percentage of cells displaying a rounded morphology, membrane ruffling and filopodia.
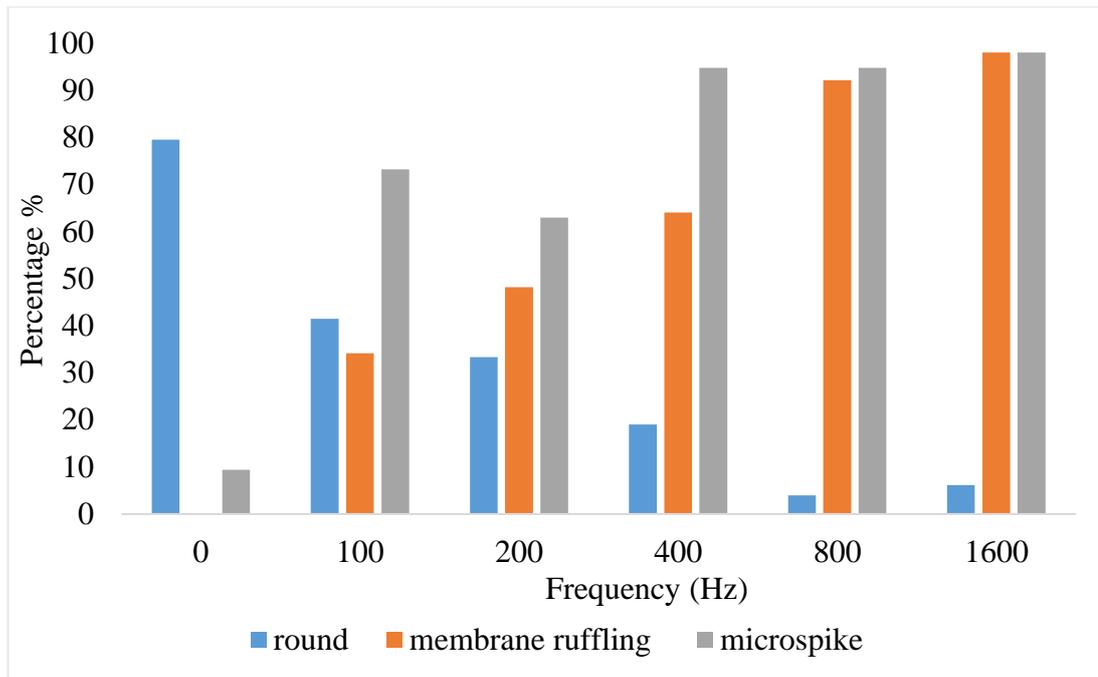
Figure 4.41 Image analysis of L929 cells stimulated for 5 minutes showing the percentage of cells being rounded morphology, membrane ruffing and filopodia.

Figure 4.42 displays the results following mechanical stimulation of LL24 cells for 20 minutes while Figure 4.43 displays the results for L929 cells. As can be seen from Figure 4.42 there were no rounded cells observed between 0-400Hz. Cells displaying clear lamellipodia/membrane ruffling were predominant across all frequencies. Actin filopodia/microspikes were also present across all frequencies. Similar to 5 minutes stimulation, L929 cells having a rounded morphology were observed across all frequencies. However, unlike 5 minutes stimulation there were no obvious differences in the percentage of rounded cells across all frequencies (see Figure 4.43). The level of cells displaying filopodia and microspikes were similar across all frequencies however, the level of cells displaying these actin arrangements was found to increase with frequency up until 800Hz. At 1600Hz the percentage of cells displaying these features had reduced. These results mirror somewhat those from the 5 minutes stimulation highlighting the consistency in response of the cells to mechanical stimulation.
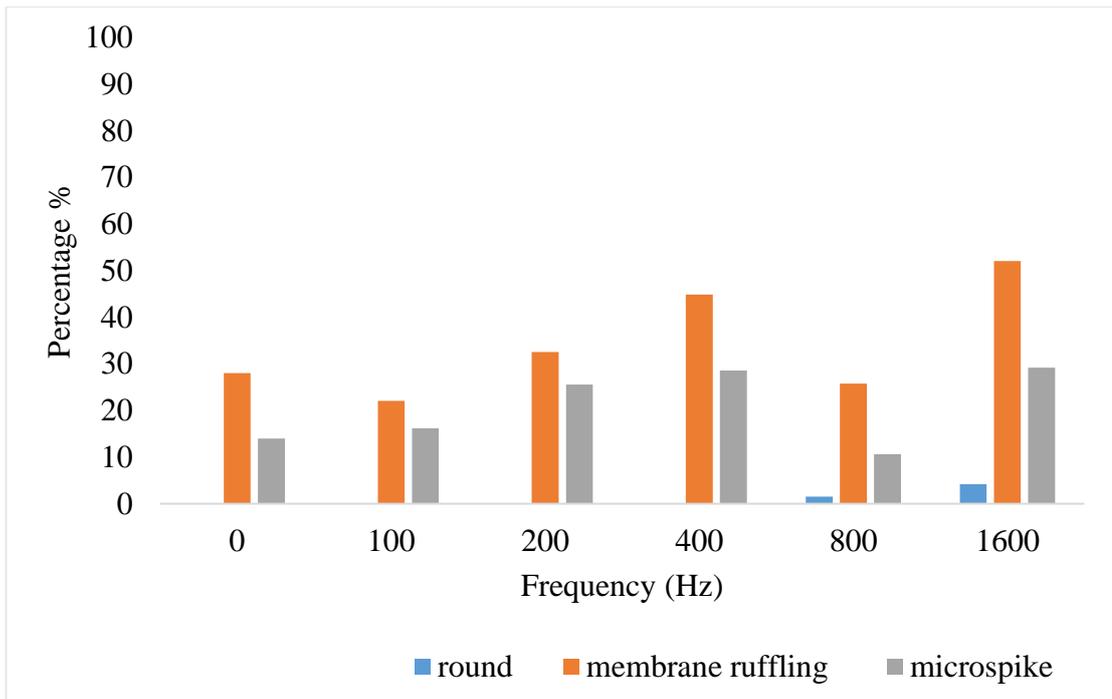
Figure 4.42 Image analysis of LL24 cells stimulated for 20 minutes showing the percentage of cells having a rounded morphology, membrane ruffing and filopodia.
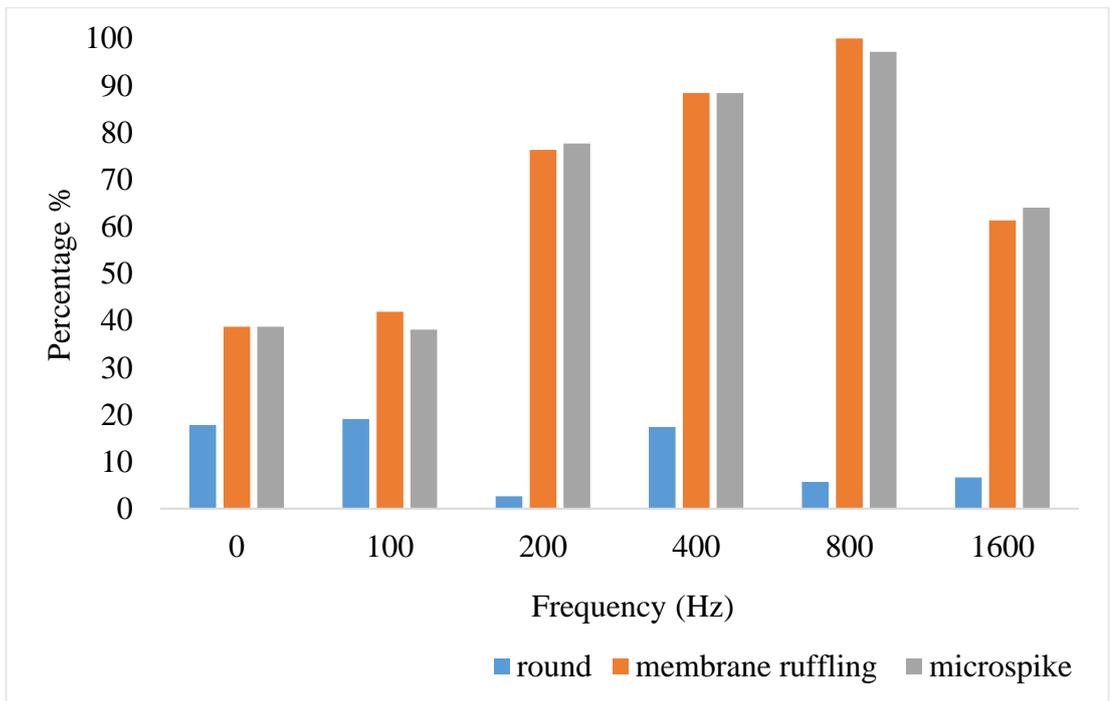
Figure 4.43 Image analysis of L929 cells stimulated for 20 minutes showing the percentage of cells having a rounded morphology, membrane ruffing and filopodia.

As previously seen with LL24 and L929 cells, there were changes to the actin organisation following mechanical stimulation. Therefore, experiments were repeated using HDF cells to see if and/or to what extent mechanical stimulation would have on

the organisation of the actin cytoskeleton. Following 5 and 20 minutes of mechanical stimulation, the cells were fixed and stained using Phalloidin, which specifically labels actin filaments (F-actin). Confocal microscopy was used to capture the images.
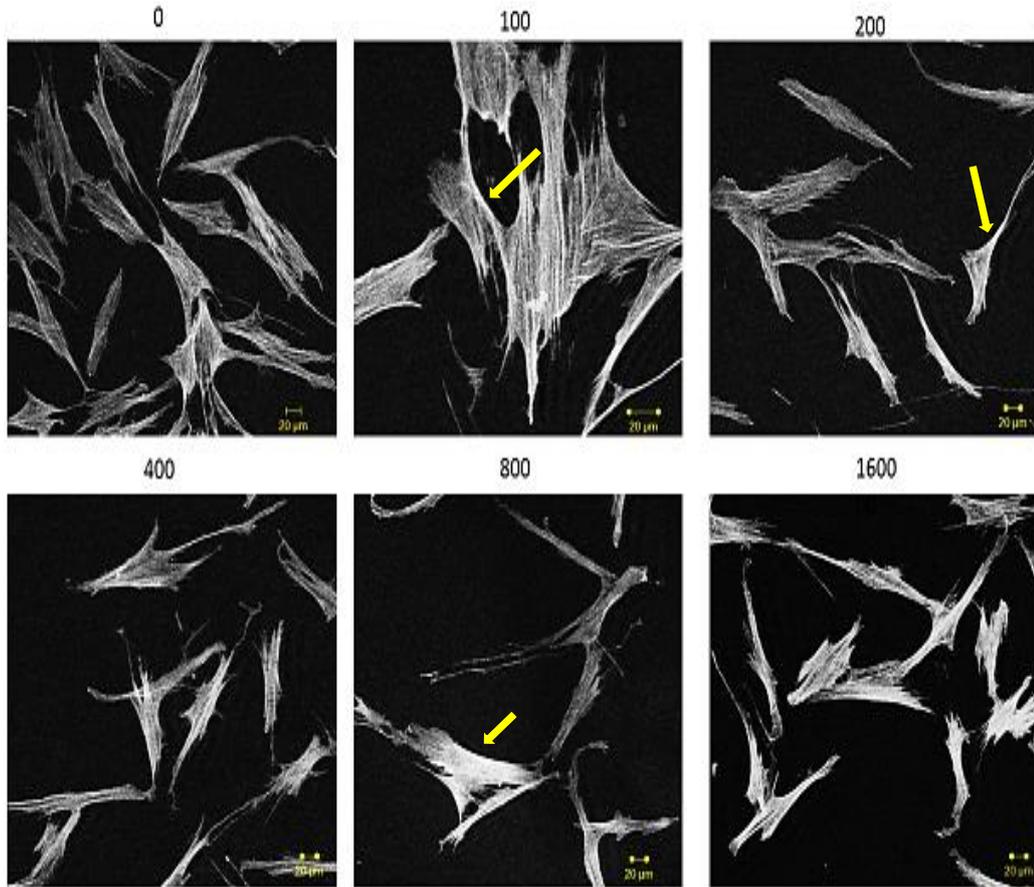
Figure 4.44 Actin on the leading edges of HDF cells when mechanically stimulated for 5 minutes at a range of frequency (0Hz-1600Hz). Images all have a scale bar of 20µm (yellow scale bar shown on images).
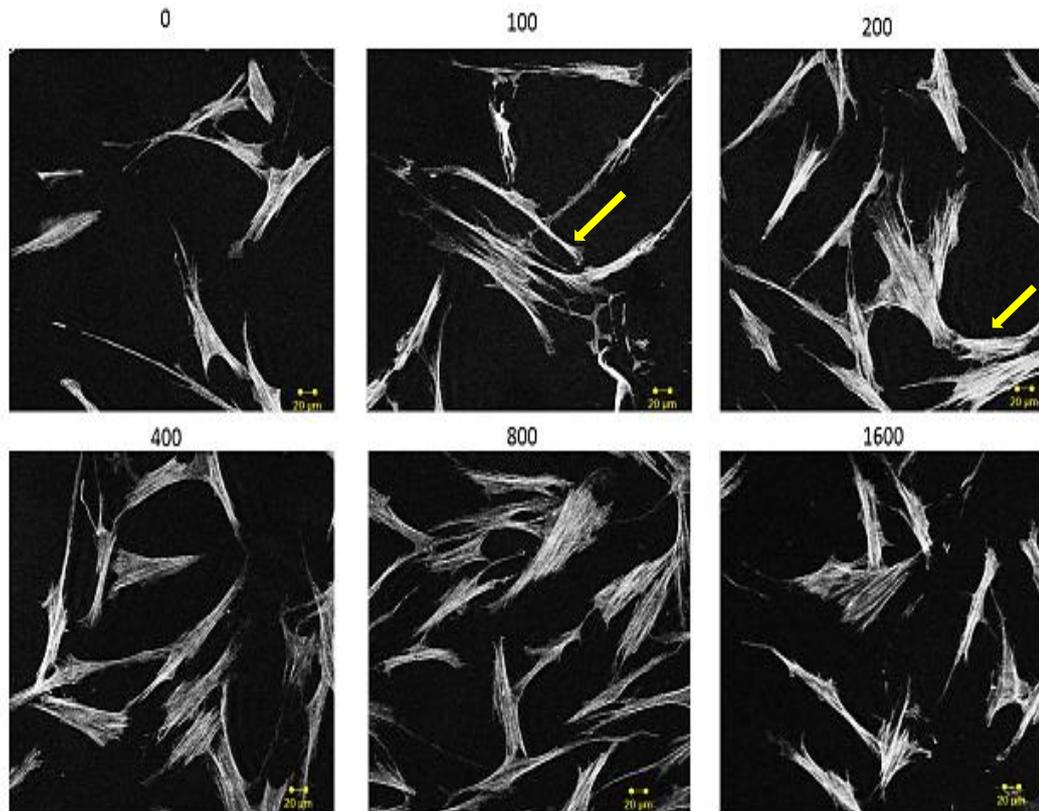
Figure 4.45 Actin on the leading edges of HDF cells when mechanically stimulated for 20 minutes at a range of frequency (0Hz-1600Hz). Images all have a scale bar of 20µm (yellow scale bar shown on images)

Viewing the images, the HDF cells look elongated and have spindle like features, however, the actin stress fibres are visible as they are long elongated fibres, and the increased white thickness seen in the images shows an increase in the amount of actin on the leading edge of cells which suggests that cells are or ready to migrate. Figure 4.44 shows an increased amount of elongated actin fibres visible in the cells that have been stimulated for 5 minutes at a range of frequency (0-1600Hz) compared to the control. Figure 4.45 shows a similar amount of actin in all cells when stimulated or not for 20 minutes at a range of frequency (0-1600Hz).

4.9 The effects of mechanical stimulation on vinculin organisation

Vinculin is important in cell migration as it helps form focal adhesions which link with the actin cytoskeleton. Previous results have shown that there is a change in actin organisation when mechanical stimulation was applied at different frequencies. Therefore, to see if there is any effect with mechanical stimulation on vinculin organisation, cells were stimulated for 5 minutes using a range of frequencies of 0-

1600Hz and then stained for vinculin using a rabbit monoclonal antibody directed against vinculin. Figure 4.46 shows vinculin organisation in LL24 cells versus stimulation frequency. From Figure 4.46 it can be observed that starting from 100Hz, as the frequency increases; the cells tend to appear more spread with an increase in lamellipodia on the leading edge of cells. However, overall there does not seem to be any notable differences in the expression levels and distribution of vinculin within the cells.
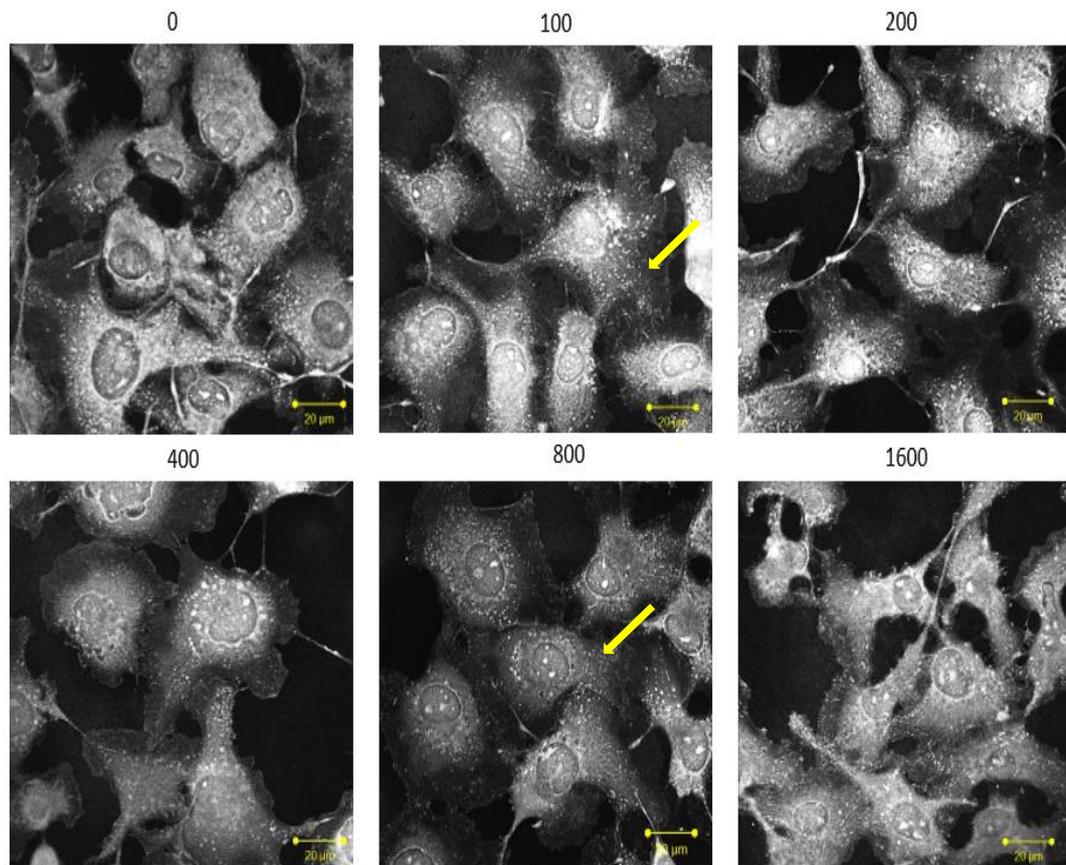
Figure 4.46 Vinculin organisation of LL24 cells following mechanical stimulation (0Hz-1600Hz) for 5 minutes.

As can be seen by Figure 4.47, when mechanical stimulation of 5 minutes was applied to L929 cells at a range of frequencies of 0-1600Hz. The results show that as the frequency increased, the cells appeared more spread with lamellipodium. Vinculin appeared homogeneously distributed and punctate from the nucleus to cell periphery when the cells were stimulated at 0, 100Hz and 200Hz. After 200Hz, vinculin appeared to be punctate around the lamellipodium at the leading edge but was diffuse and concentrated at the nucleus.
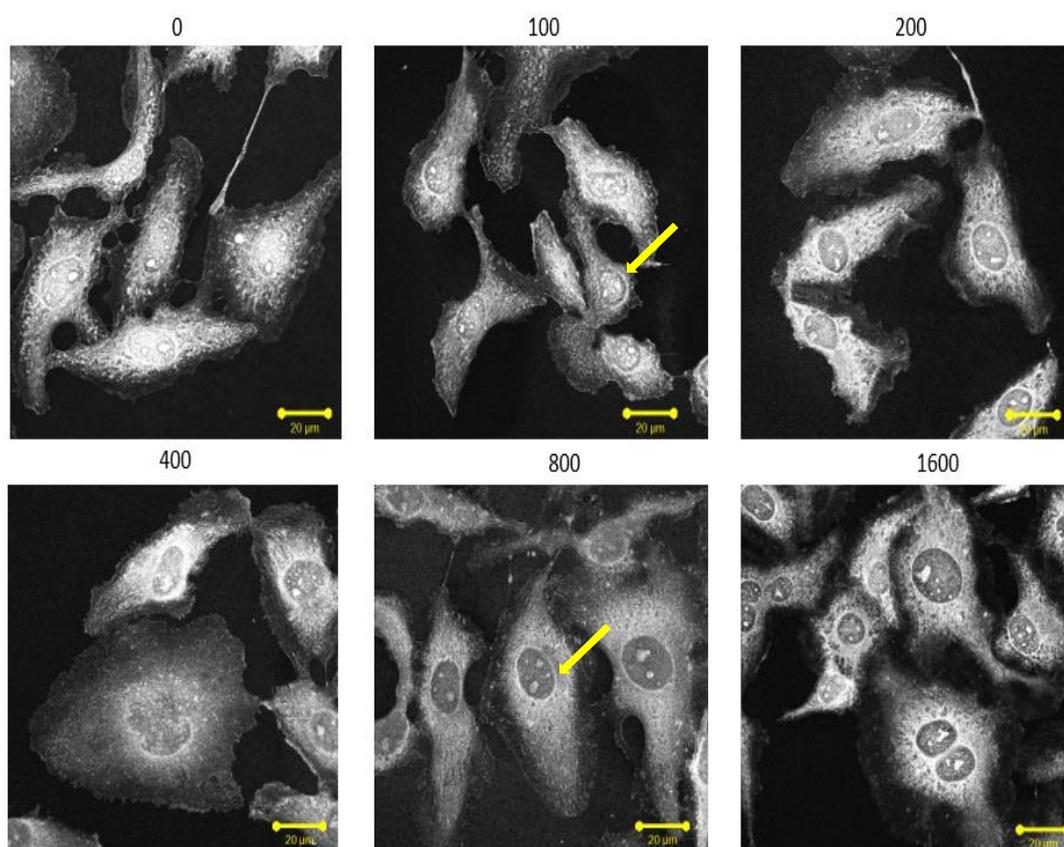
Figure 4.47 Vinculin organisation of L929 cells following mechanical stimulation (0Hz-1600Hz) for 5 minutes.

4.10 The effects of mechanical stimulation on temperature

In order to investigate if temperature is having any effect on cell migration it was important to investigate if there was an increase in temperature when mechanically stimulating cells. Therefore, a thermocouple was attached to bottom of the dish and mechanical stimulation was applied between 0-1600Hz for 20 minutes and the temperature recorded at 1 minute intervals. This was repeated three times to get consistent results. As can be seen from Figure 4.48, there was a steady increase in temperature as the frequency increased. Compared to control at 37.3°C, the temperature at 100Hz is 37.7°C which increased slightly up to 38°C with 1600Hz stimulation. Overall as the frequency increased the temperature also increased which may be causing an effect on how the cells respond to stimulation.
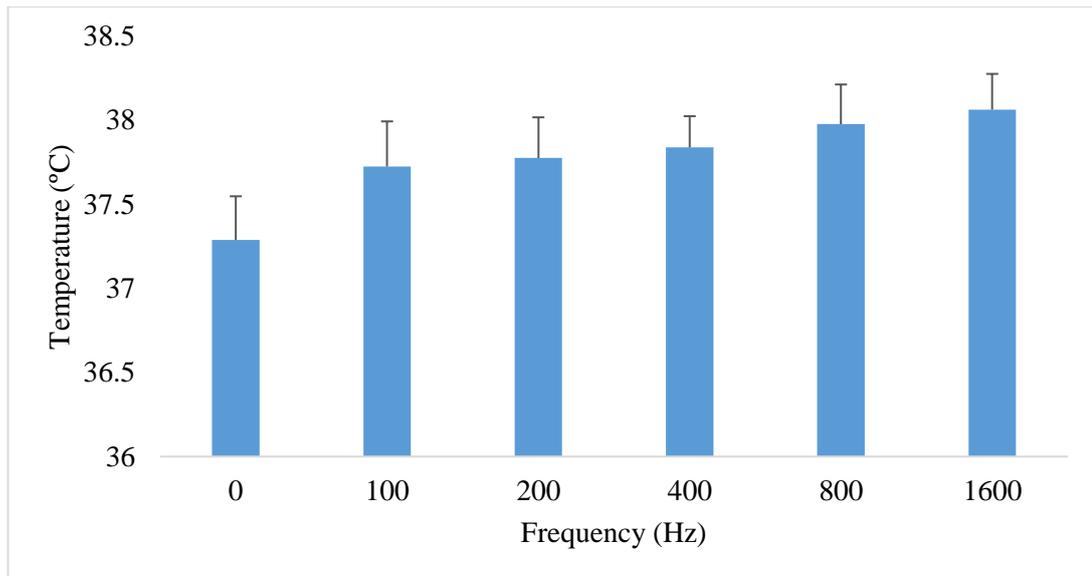
Figure 4.48 Temperature (˚C) versus Frequency (Hz) over 20 minutes. Error bars represent standard deviation. * denotes Significance (p<0.05).

4.11 The effects of vibration amplitude on single cell migration

As can be seen from previous results, that there was an increase in mean migration distance (µm) when LL24 cells were stimulated at 100Hz. At 100Hz the amplitude was recorded to be approximately 9µm. In order to assess the effects of amplitude on cell migration the system was altered so as to control relative amplitude. For these experiments cells were mechanically stimulated for 5 minutes at 100Hz using either 100% amplitude (i.e. original amplitude approximately 9µm) and for 5 minutes with 50% of the original amplitude.
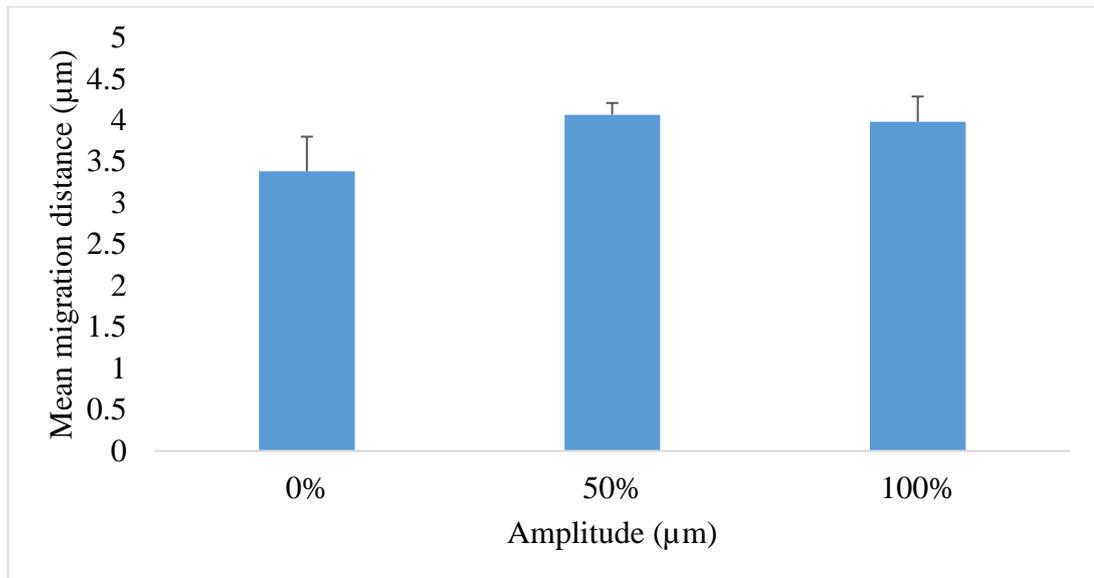
Figure 4.49 Mean migration distance (µm) versus % of amplitude (µm). Error bars represent standard deviation. [*] denotes Significance ($p < 0.05$).

Figure 4.49 shows the mean migration distance (µm) of single cell LL24 cells when stimulated at 100Hz using a range of amplitudes of 0 (no amplitude), 50% (approximately 4.5µm) and 100% (approximately 9.0µm). As can be seen by Figure 4.49, at 50% amplitude there was an increase in mean migration distance (µm) by approximately 0.7µm (21%) compared to the control and at 100% there is an increase of mean migration distance (µm) of approximately 0.6µm 18%. Overall, the mean cell migration at amplitudes of 100 and 50% are very similar. This indicates that amplitude is not having an effect on cell migration.

4.12 The effect of mechanical stimulation on Muscle fibre diameter

In order to investigate if mechanical stimulation had any effect on muscle fibre formation a muscle cell model which readily forms muscle fibres in culture was studied. Briefly, the muscle cells were grown to confluence, and serum levels reduced to 2% as this promotes formation of muscle fibres. Immediately after adding the serum the cells were mechanically stimulated for 5 minutes or 20 minutes at 0, 100 or 1600Hz. Cells were put back in the incubator and stimulated every 24 hours for 4 days and on day 5 images of the fibres were captured and the mean fibre diameter (n=30) measured. These experiments were repeated at least three times to ensure accurate and consistent results.

As can be seen from Figure 4.50 those cells/fibres that were mechanically stimulated had a smaller mean fibre diameter compared to the control. For example, the mean diameter of the control population was 20.2µm whereas the mean diameter for those cells/fibres stimulated at 100Hz was significantly smaller than the control at 16.5µm (p<0.05). While those cells stimulated at 1600Hz had a mean diameter of 17.4µm (p>0.05). In contrast, Figure 4.51 shows the fibres with no stimulation had smaller diameters compared to the diameters that were stimulated at 20 minutes for 100Hz and 1600Hz. There was an increase in mean fibre diameter of approximately 2.3µm (7%) when stimulated at 100Hz and approximately 0.7µm (2%) when stimulated at 1600Hz compared to the control.
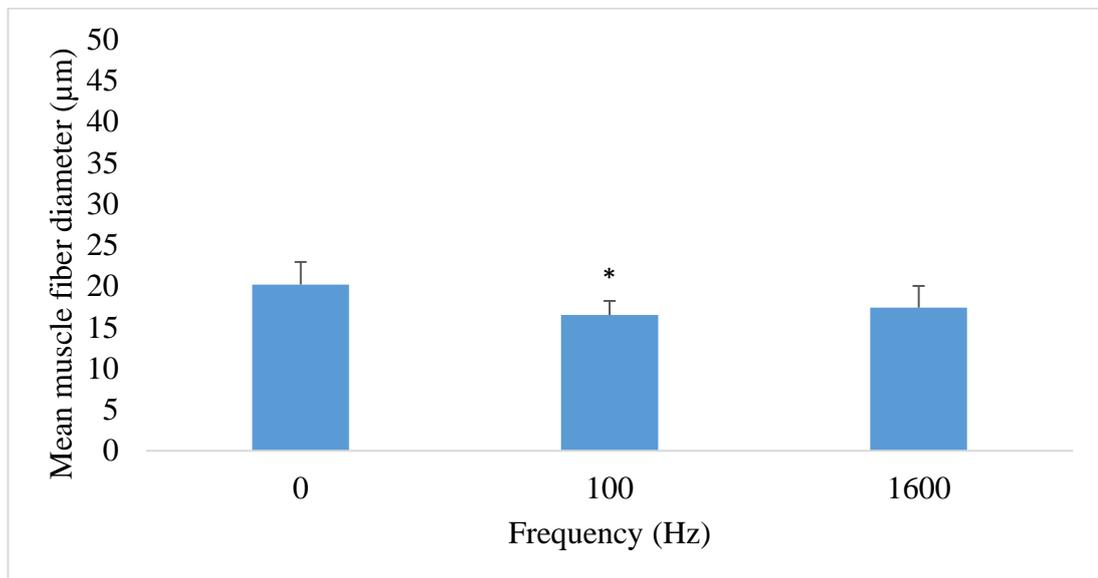
Figure 4.50 Mean muscle fibre diameter (µm) versus frequency (Hz) following mechanical stimulation for 5 minutes every 24hours for 4 days. Error bars represent standard deviation. * denotes Significance (p<0.05).
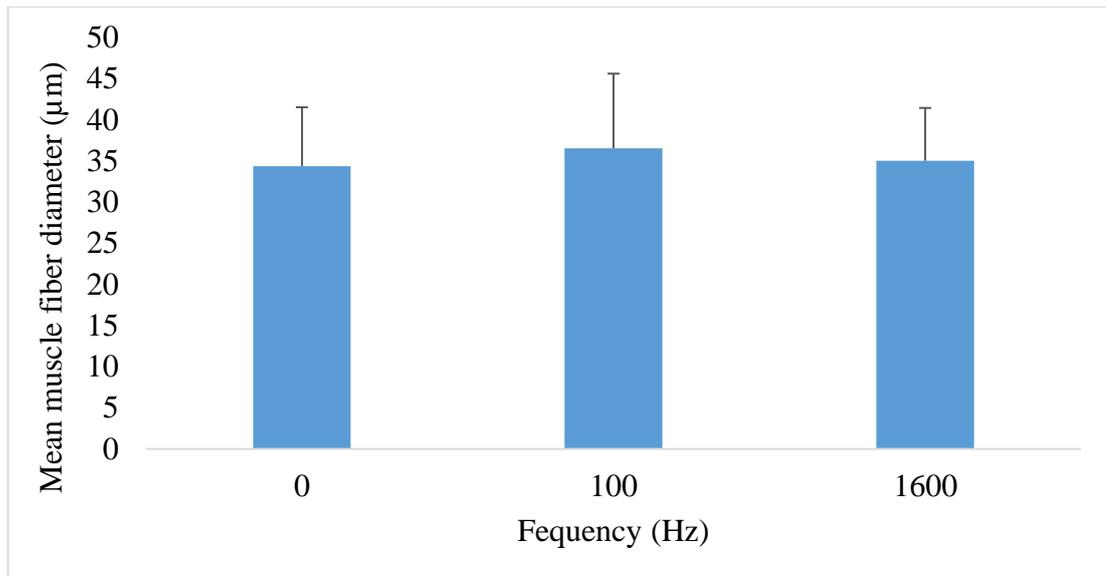
Figure 4.51 Mean muscle fibre diameter (µm) versus frequency (Hz) for 20 minutes every 24 hours for 4 days. Error bars represent standard deviation. * denotes Significance (p<0.05).

The results overall show that when the muscle fibres were stimulated for 5 minutes there was a decrease in the muscle fibre diameters compared to the control but when the muscle fibres were stimulated for 20 minutes at 100Hz there was an increase in muscle fibre diameter.

Chapter 5

5.0 Discussion

The work that has been described in this thesis involved an in-vitro investigation to determine the effects of mechanical stimulation on fibroblast cell migration and muscle fibre formation but to the author's knowledge this is the first time this has been explored. An inbuilt speaker system was developed and was used to apply mechanical stimulations to cells in incubator settings of $37\,^{\circ}C$ and 5% C02. A confocal microscope was used for time lapse imaging for single migration, to take images of scratch assays and to examine actin organisation in the three cell lines; LL24 cells, L929 cells and HDF cells. Vinculin organisation was also identified using confocal microscope but only for LL24 cells. Muscle cells were also stimulated and the images were also taken by using a confocal microscope. The results are compared and outlined in the following chapter showing that acoustic vibration can enhance (100Hz) and decrease (200-1600Hz) cell migration without affecting cell viability. However, at present, the underlying mechanisms behind these results are unknown and clearly more work needs to be done in this area so as to decipher such mechanisms and help us understand more about the mechanobiology of cells. However, the work is promising and may open up new avenues of research that focus on exploiting mechanical means for controlling cell behaviour. For example, the development of novel wound care technologies that use mechanical means to accelerate the wound healing process in patients with conditions associated with poor wound healing.

5.1. Speaker system and calibration

Once the speaker system was built, the system was calibrated by a company called Polytec. Using a laser vibrometer, the displacement values were calculated at the frequency below 100Hz and above 1600Hz the system was unstable (in terms of frequency), as laser vibrometry recorded multiple harmonics outside of this frequency range (See Appendix 1).

5.2 The effect of mechanical stimulation on single cell migration

The effects of mechanical stimulation on single cell migration for 5 minutes show that fibroblast cell migration can be controlled using acoustic vibration and that migration distance is dependent upon vibration frequency. The trend observed was repeatable for both cell lines, particularly the increase in mean cell migration distance (µm) seen at 100Hz. On all occasions a steady decrease in mean cell migration was observed for the lung cells (LL24), with 1600Hz always resulting in lowest mean cell migration distance (µm). The results for the L929 cells somewhat mirrored those of the LL24 cells, however the trend did not always produce a steady decrease in mean cell migration. These results are somewhat consistent with similar work reported in the literature. For example, Aryaei and Jayasuriya found that mechanical stimulation, in the form of shear stress, can be used to enhance osteoblast adhesion and proliferation (Aryaei and Jayasuriya 2015). Similarly, Ito *et al*., found that mechanical stimulation (100-1000Hz) of L929, HeLa and human umbilical vein endothelial cells (HUVECs) had no effect on cell morphology, or adhesion (Ito *et al.,* 2011). In contrast, mechanical stimulation of mouse embryonic fibroblasts at 1kHz was found to increase cell adhesion and alter cell morphology. Such work supports the work presented here, in that cells respond to external physical cues and how they respond seems to depend on the cell type and nature and properties of the mechanical stimulation.

Additionally, from the fibroblast cells stimulated at 20 minutes, the trend observed was similar to 5 minutes for both cell lines. However, for LL24 cells the maximum cell movement was observed at 400Hz stimulation and for L929 400Hz showed the least cell migration. In contrast to LL24 and L929 cells, single cell migration of HDF cells was also investigated for 5 and 20 minutes. Cells that were stimulated for 5 minutes at 100Hz and 1600Hz had greater mean migration distance compared to the control. Although the difference in migratory response to mechanical stimulation was slightly less this may be due to the primary cells derived from human can behave differently in culture conditions depending on the genetics and age of individuals from whom the tissue was derived they are also not well characterized, have limited life span, and are known to be slow in proliferation. Moreover, with every passage, the characteristics of primary cells change. The results showed that the cells stimulated at 1600Hz migrated the least and the cells stimulated at 100Hz had a greater mean

migration distance. These findings were very similar to LL24 cells. These results are hard to explain as there has been no previous work that has looked at these aspects. However, the possible reasons for increase of migratory distance at 100Hz and decrease above 100Hz could be due to the fact that fibroblast cells are one of the major cell types responsible for transferring mechanical signals into biological events, especially expression of ECM genes. Above 100Hz, the cells may have abnormal mechanical loads which can affect diverse cellular functions including cell proliferation and alteration of the composition of the ECM leading to a decrease in migratory distance (Manuyakorn *et al.,* 2016). It is likely that fibroblasts from different parts of the body respond differently to the same stimuli as fibroblasts from individual anatomical sites show variable levels of expression from large groups of expressed genes (Gaston *et al.,* 2012). Overall, fibroblast cells may experience extracellular and intercellular changes which can result in altered gene and/or protein expression perhaps due to the vibration exposure, as it is widely accepted that cells are inherently sensitive to their surroundings. It has previously been proposed in vivo that fibroblasts contain the necessary intracellular machinery to remodel the ECM in response to mechanical stimulation. In other parts of the body, fibroblast expression levels of extracellular glycoproteins have a direct connection to applied mechanical stress. Dermal fibroblasts such as HDF cells have also been shown to increase collagen type I and fibronectin synthesis in response to mechanically induced strain. Similar effect is detected in ligament fibroblasts, which secrete collagen I and III when subjected to axial strain. Also cardiac fibroblasts are known to remodel their ECM in response to mechanical stimuli and laryngeal fibroblasts have been shown to increase mRNA levels of several ECM genes, both proteins and proteases, following mechanical vibration. A recent study has also showed that laryngeal fibroblasts not only upregulate ECM related mRNA, but also secrete matrix proteins, including collagen I and fibronectin, when exposed to mechanical vibration (Garston *et al.,* 2012). However, in this thesis, a short stimulation was applied in-vitro, thus it is unlikely that ECM is involved as a cellular response was seen immediately rather than over 48 hours. Therefore, an alternative reason for the results shown could possibly be due to the tensegrity model.

The possible explanation could be when a mechanical stress is applied to cells, in this case a mechanical stimulation, the stimulation then alters cell shape and structure. This

may lead to changes in the internal cytoskeleton, that is composed of a network of microfilaments and microtubules and are linked by adhesion receptors to the cell nucleus. These rearrangements in proteins of the cell cytoskeleton can possibly lead to adaptive responses. Overall, cell spreading on the extracellular matrix has been associated with increased tyrosine phosphorylation of focal adhesion kinases (Bockholt and Burridge, 1993). Changes in cell sensing of its neighbours secondary to distortional changes from physical loading, might invoke mechanisms where changes in cell architecture activate classic signalling pathways.

The increase of cell migration at 100Hz could be due to the fact that when vibration is applied the cell is connected through its cytoskeleton to cell surface receptors that link to the extracellular matrix (Ingber, 1997). The cytoskeleton then generates a tension via an actinomyosin filament by creating an isometric tension that generates an internal "pre-stress". Then the external mechanical loads (vibration) enforces on a pre-stressed structure. Then the membrane attaches to the substrate, through focal adhesions, which pulls through the C-Src Tyrosine Kinase (CSK) complex. As the microtubules are assembled, they exert forces, which causes the cells to become mobile if the assembly is at the leading edge of the cell. The force is generated by protein polymerization and the interaction of the growing microtubule end with a specific attachment might modify the force (Dogterom and Yurke, 1997). However, if the force starts from a focal adhesion point in this case the stimulation of 100Hz, then the force generated will be felt throughout the cell body, activating specific signalling systems which alter stress in one part of the cell leading to affect another part causing the cells to migrate faster.

5.3 The effect of mechanical stimulation on collective cell migration

With respect to collective cell migration (wound healing), results found that with 5 minutes of mechanical stimulation, at both 100Hz and 1600Hz, the rate of wound healing was slower when compared to the control. However, when 20 minutes of mechanical stimulation was applied for 100Hz, wound closure was seen to accelerate compared to the control. However, after a few hours the apparent effect of the stimulation seems to decrease, with those cells that were not stimulated (0Hz), seemingly having a greater rate of cell migration in the final hour (hour 4), suggesting that the stimulation may 'wear off'. The mechanical environment of the wound site is

also of fundamental importance for the rate and quality of wound healing. It is known that mechanical stress can influence wound healing by affecting the behaviour of cells, but it remains unclear how mechanical forces affect the healing of cells. It has been reported that tensile forces are known to affect the behaviour of cells within epithelia, and the material properties of extracellular matrices, such as substrate stiffness. They also have been shown to affect the morphology, proliferation, differentiation and migration of many different cell types (Evans *et al.,* 2013).

Overall in all three cell lines; LL24 cells, L929 cells and HDF cells all showed that stimulation at 100Hz for 20 minutes enhanced the wound closure up to the third hour and then the control caught up on the 4th hour. This could be due to the mechanotransduction mechanisms when the initial vibration/ mechanical stress was applied to the cells. A similar study applied stress on normal human dermal fibroblasts cells via cyclic strain (24 h of cyclic axial stretching (10 cycles min (-1)). The results showed that the cells reoriented themselves perpendicular to the direction of stretching associated with reduced cellular apoptosis and unchanged proliferation. Stretching did not increase collagen synthesis but did elevate collagen degradation. However, the biological effects appeared to be mediated by the integrin and Wnt mechanotransduction pathways, which transmitted the mechanical stimulus via cell–substrate interactions, cell–cell junctions and indirect cell–cell communications (Huang *et al.,* 2013).

Collective cell migration was mainly focussed for wound healing and wound healing occurs as a cellular response to injury. The cells mainly involved in wound healing are the activation of fibroblasts, endothelial cells, and macrophages (Clark 2001). In this study, fibroblast cells were mainly used because fibroblast proliferation is involved in the restoration of structure and function in the wound site (Mensah *et al.,* 2001). To study wound healing in vitro many studies have used the scratch assay which has several advantages including; its simplicity, ability to mimic the migration of cells in vivo, and capacity to isolate variables by testing the interaction between distinct cell types. These are powerful reasons to use this classic method to study wound-closure mechanisms during the healing process (Rodrigues-menocal *et al.,* 2011).

However, HDF cells showed that at 20 minutes of stimulation at 100Hz the cell migration was enhanced up to 3 hours, but then in the 4th hour the control caught up. In general, human dermal fibroblasts play essential roles in cutaneous wound repair and remodelling. Fibroblasts play a pivotal role in wound healing. After a wound is made, cells are stimulated by the availability of empty space, the cells at the edges of the newly created gap proliferate and move toward the centre of the denuded area until the wound is closed (Ascione *et al.,* 2016). However, previous studies have also suggested that primary cells and cell lines of epithelial or endothelial origin perform sheet migration if a scratch is made on a cellular monolayer. This involves a specific response to cell injury at the scratch site as well as response to deconstraining cells observing the appearance of unpopulated substrate space, which is sufficient to cause sheet movement. In collective cell migration, cell proliferation may also occur under these conditions, but it is not required for most of the gap-filling response and can be separated (Nikolic *et al.,* 2006, Poujade *et al.,* 2007, Vitorino & Meyer 2008).

The statistical analysis from all scratches showed no statistically significant differences in scratches with or without stimulation Even though experiments were repeated and the trend was seen again, it is thought that this was due to the fact that the scratches were made by hand using a pipette tip which led to the scratches being varied, as some scratches were wider than others. It is possible to suggest that narrow scratches tend to migrate more, as the cells are closer and can send signals or information to other cells quickly, whilst in the wider ones there is obviously a larger distance involved so migration may be reduced as signalling is slower. Another possibility is that there could be an increase in errors when analysing the scratches. As the scratches were drawn around using tools from ImageJ, there is a fairly high possibility of human error. At present we do not understand the mechanisms that seemingly drive/reduce cell migration following mechanical stimulation. Clearly more work needs to be done in this area, however the results are exciting and show that this approach may have potential when applied to disorders that have a slow wound healing response, such as chronic illnesses like diabetes where wounds heal very slowly.

5.4 The effects of intermittent mechanical stimulation on collective cell and single cell migration

The results in Figures 4.40-43 show that the wounds stimulated with one or four stimulations for 5 minutes, are both slower in terms of cell migration compared to the control. However, at 20 minutes the cells stimulated with four stimulations seem to close the wound gap move quickly compared to the control. Compared to the single cell migration, the LL24 cells migrated the most, with a single 20 minutes stimulation of 100Hz, whereas in L929 cells the cells with a single 5 minute stimulation migrated the most. This research is fairly new and at present we do not understand the mechanisms that seemingly drive/reduce cell migration with single or multiple stimulations. The possible explanations for the enhanced cell migration could be due to the cells converting the mechanical cues from repetitive stimulation into biochemical signals (mechanotransduction) and the structures of proteins and protein networks being altered upon mechanical load. The cellular microenvironment consists of protein networks of varying biochemical and physical properties, including matrix composition, dimensionality and stiffness, all of which have been shown to co-regulate cell function, migration, differentiation, tissue homeostasis and organ development. Cell migration causes cell adhesion (and de-adhesion), spreading, and contraction. First, cells form protrusions at their leading edge, driven by actin polymerization. Second, these protrusions are attached to the substrate thorough the formation of focal adhesions. Third, these focal adhesions are connected to actin stress fibres that are tensed thorough the contractile activity of myosin motors. Finally, the focal adhesions at the rear end of the cells de-adhere under the influence of time and contractile force (Paluch *et al.,* 2015). Previous studies have stated the case for collective cell migration, i.e. that a migrating cell group exerted much larger forces than a single cell, providing evidence for the collective nature of group movement (du Roure *et al.,* 2005). It should be added that confluent fibroblastic cells can also respond to a scratch wound, but appear to do so primarily as independent cells, not as cohorts (Matsubayashi *et al.,* 2004).

5.5 The effects of mechanical stimulation on cell viability

Detecting cell viability is important in all studies to see if any external parameters have any effect on the cells. Figures 4.8-4.16 do suggest that the cells are viable when mechanical stimulation was applied from 100Hz to 1600Hz, however they are sensitive to stimulation hence there is a response with cell migration. Viability assays were carried out to see if the decrease in migration above 100Hz was due to changes in cell viability, i.e mitochondrial activity. For the LL24 cells and L929 cells, HDF cell viability was assessed using a trypan blue assay and MTT assay. In the MTT assay the viable cells convert MTT into a purple coloured formazan product with an absorbance maximum near 570 nm. If the cells die, they lose the ability to convert MTT into formazan, which results in less colour formation. The exact cellular mechanism of MTT reduction into formazan is not well understood, but it is possible that NADH or similar reducing molecules that transfer electrons to MTT are involving specific mitochondrial enzymes, thus leading to MTT's use as a measuring mitochondrial activity assay. MTT has been considered to be reduced by the activity of mitochondrial dehydrogenases in living cells (Riss 2013). As MTT assays rely on a mitochondrial reductase to convert the tetrazole to formazan, the conversion is dependent on the number of viable cells. Overall, there is no difference in the mitochondrial activity seen when compared to the control, however, there is a possibility that by applying mechanical stimulation to the cells, the cells may have increased enzymatic activity without actually having an effect on cell number or cell viability. Therefore, a simple Trypan blue exclusion assay was carried out to test for cell vaibility. From the results, it can be seen that the percentage of viability is approximately 90-100% which shows that when stimulation is applied to the cells the cell membrane is not compromised thus leading no uptake of trypan blue dye. This could be due to the fact that stimulation changes the cellular structures, such as ion channels, integrin complexes, and cell-cell adhesions. These changes enable the influx and efflux of ions or the activation of signalling cascades, resulting in altered cell shape and altered production of proteins (Hoffman *et al.*, 2011). The cytoskeleton, which is made up of actin, microtubules, intermediate filaments and their cross-linkers is the support framework determining the cellular shape and stiffness (Sugawara *et al.*, 2008). Integrins anchor to the extracellular matrix (ECM) and mechanically bind to

the cytoskeleton, forming a trans-membrane structure. The stiffness could be preventing the cells membrane being affected leading the cells to be viable.

To support the strength of fibroblast cells a previous study by Kashiwazaki *et al.,* investigated the effect of mechanical vibration of cervical trachea, chest wall and femoral muscle on cough reflex sensitivity, perceptions of urge-to-cough as well as dyspnoea. Mechanical vibration was applied by placing a vibrating tuning fork on the skin surface of cervical trachea, chest wall and femoral muscle. Chest wall vibration is frequently used as physiotherapy in patients complaining of sputum production and ~100 Hz is commonly used in clinical practice. They found that not only chest wall but also cervical trachea vibration inhibited both cough reflex sensitivity and perception of urge-to-cough (Kashiwazaki *et al.,* 2013). As LL24 cells are from the human lung, this shows that cells can withstand vibrational frequencies of 100Hz and is already being used in clinical practice to inhibit cough reflex and the to urge to cough.

Cells such as HDF cells showed that cell viability was not affected by mechanical stimulation this may be due to fact that the HDF cells are more robust and resistant to mechanical deformation. This is because the cells are from the dermis of adult skin, responsible for producing the extracellular matrix forming the connective tissue of the skin, and play a crucial role during wound healing and protect skin from UVA-induced photo-aging. From this it can be evident that the HDF cells are programmed to protect the under cells, tissues which may be the reason why the cells are viable (Fleissner *et al.,* 2016).

5.6 The effects of mechanical stimulation on cell proliferation

From the results it was found that when mechanical stimulation was applied there was an increase in the number of cells. For example, after 72 hours, stimulation at 100Hz showed an overall increase in the number of cells in LL24 cells and HDF at 5 and 20 minutes. In all of the cell proliferation assays there was a similar trend observed showing the cells stimulated at 100Hz were shown to have the greatest number of cells/ml and then as the frequency increases the number of cells present decreases. Previous studies have looked at mechanical vibration and cell proliferations, such as

Zhang *et al.,* who demonstrated that low-magnitude, high-frequency (LMHF) mechanical vibration caused a reduction in human periodontal ligament stem cell (hPDLSC) proliferation in the range of 20–180 Hz and stated that other studies have thought that this was due to the mechanics of PDLCs when related to stretch or compressive stresses. Mechanical vibration at a frequency of 40 Hz to 90 Hz was more encouraging for the osteodifferentiation of PDLSCs. The study showed that mechanical vibration at 50 Hz frequency was more favorable for PDLSC osteogenic differentiation. The process for increase in cell proliferation when stress is applied is still unclear. However, the possible explanation for the increase in cell numbers at 100Hz could be due to mechanical force, i.e vibration, being recognized by the cells and transduced into a cellular signal that controls transcriptional activity leading to enhanced proliferation. However, other studies have also demonstrated either increased or decreased proliferation after cyclic tension loading of Periodontal ligament cells (PDLCs) (Li et al., 2013). Another study reported that proliferation of BMSCs was increased following exposure to continuous perfusion, or to a combination of cyclic compression and perfusion (Jagodzink *et al.,* 2008). Luu *et al.,* found that BMSC proliferation was positively influenced by LMHF mechanical vibration (Luu *et al.,* 2009). In contrast, Zhou *et al.,* found that BMSC proliferation was decreased after vibration treatment (Zhou *et al.,* 2011). Rubin *et al.,* found out that vibration was capable of inhibiting 3T3-L1 cell proliferation after exposure to vibration for 2 or 3 days, whereas other work by Patel *et al.,* has found no effect of LMHF mechanical vibration on 2T3 cell proliferation (Rubin *et al.,* 2001; Patel *et al.,* 2009). From these studies it can be noted that effects of LMHF mechanical vibration on cell proliferation may be a reflection of the different cell types being studied, culture conditions and the vibration applied.

Previous studies have looked at the mechanical force in regulating cell growth and proliferation, and also mechanical stretch treatment to the proliferative capacity of BMSCs. A study by Song *et al.,* found that the proliferation of bone marrow mesenchymal stem cells significantly increased after exposure to a 1-Hz stretch stimulation within 15–60 min at an 8% strain (Song *et al.,* 2007). However, a study by Zuo *et al.,* found that 8% CMS had no significant influence on cell viability during a 24 to 72 hour period (Zuo *et al.,* 2014). Another study by Luu *et al.* found that 6 weeks of low-magnitude mechanical signals of 90-Hz applied for 15 min/day for 5 days a

week overall showed an increase in the marrow-based stem cell population by 37% and the number of MSCs by 46%. There are many studies that have shown mechanical stimulation to have no effect on cell proliferation and the mixed findings can be explained by the different conditions in the experiments, including frequency and how the mechanical stimulation was applied, the type of cells, culture media used, as well as the wide range of loading parameters used.

5.7 Effects of mechanical stimulation on actin organisation

Two alternative forms of actin machinery coexist at the leading edge of most motile cells; lamellipodia (persistent protrusion over a surface) and filopodia (sensory and exploratory functions to steer cells depending on cues from the environment) (Meijillano *et al.,* 2004). Although most cells in culture express both lamellipodia and filopodia, where the levels of each structure is thought to be cell-specific, the response of the LL24 and L929 cells to mechanical stimulation was found to differ. Lamellipodia were the more prominent actin structure formed by the LL24 cells, while filopodia were the most prominent actin structure formed by the L929 cells (Figures 4.23-4.26).

When adhesions to the substrate are weak, contraction of the actin filaments occurs, causing the lamellipodium to bend upwards, resulting in ruffling and transient retraction (Giannone *et al.,* 2007). Such events are characterized by an extension of the cellular leading edge before retraction, or ruffling, occurs. This type of pattern of migration has been noted in fibroblast cells and was observed here for both cell lines (Bear *et al.,* 2002). Given that the formation of lamellipodia is associated with cell migration and that mechanical stimulation of LL24 cells at 400Hz, resulted in the highest percentage of cells displaying lamellipodia, one may expect the mean cell migration distance (µm) to be greatest at 400Hz; this however was not the case as migration was greatest at 100Hz. Similarly, for L929 cells both membrane ruffling and filopodia were found to be greatest following mechanical stimulation at a frequency of 1600Hz, however the least cell migration occurred at 1600Hz and the greatest at 100Hz (which consequently also produced cells having the lowest percentage of lamellepodia). Thus it is possible from these results that actin remodelling occurs due to mechanical stimulation and the extent to this remodelling

seems to be dependent, somewhat, on frequency of vibration. This observation agrees with previous work, whereby it was shown that cortical actin remodelling is dependent on the rate of applied stress (Pravincumar *et al.,* 2012).

It is unknown why such structural differences are observed between the different cells, but it should be noted that although both are fibroblast cells, one cell is of human origin and one sourced from a mouse. Also, they originate from distinctly different tissues (i.e. LL24 cells are derived from the lung, while L929 cells are derived from subcutaneous areolar/adipose tissue) and it is known that fibroblast cells, although structurally similar, can differ genetically depending on where they originate within the body. However, although both cells appeared to respond differently to mechanical stimulation, from a structural point of view, their migratory response was similar, particularly at 100Hz. It is still unknown about how the physical mechanisms operate in modifying the vibration-induced response in transmitting mechanical signals to the cell. However, the cytoskeleton is intimately involved in transmitting and amplifying the oscillatory mechanical signal by the up-regulation of genes important for cytoskeletal remodelling. When mechanical force was transmitted thourough the cytoskeleton to the nucleus this resulted in a biochemical cascade and it has been shown that unidirectional steady flow-induced actin thick stress fibre formation in cells is due to exposure to a mechanical stimulus.

Overall there was no obvious difference seen in actin organisation of the HDF cells. This may be due to the fact that they are primary cells; they may respond to force differently to LL24 cells and L929 cells. Phalloidin is a toxin from the "Death Cap" toadstool (*Amanita phalloides*) that binds actin and is used to stain actin in cells. Binding is specific for F-actin and is a great tool to study this distribution of F-actin in permeabilised cells, since fluorescent analogues can be synthesized that retain actin binding. Phalloidin binds to actin at the junction between sub-units and because this is not a site at which many actin-binding proteins actually bind, most of the F-actin in cells is available for phalloidin labelling (Allen and Jamney 1994). When stimulation is applied to HDF cells it is possible to suggest that the actin organisation is not being affected; this is because the force generated externally of the cell it is not passed along the cytoskeleton in the same response as LL24 cells or L929 cells, which is perhaps the reason why no changes of actin organisation were seen. A study by

Rodríguez-Viciana *et al*. reported that the actin cytoskeleton changed from a bulky number of thin, microfilament bundles, extending across the entire cytoplasm in undifferentiated MSCs, to a few thick actin filament bundles located at the periphery of differentiated cells (Rodríguez-Viciana *et al*., 1997). Another study by Titushkin discovered that the actin cytoskeleton changed from thick stress fibres in hMSCs into the thinner filamentous network in osteoblasts cells. Even so, changes in the cytoskeleton of human periodontal ligament stem cells (hPDLSCs) after vibration may be one of the mechanisms of the biological effects (Titushkin and Cho 2009). Therefore, no obvious changes could be seen in actin organisation except that they are differently shaped to LL24 cells and L929 cells; they are long, not rounded so it was less obvious to see any lamellipodia, or filopodia, forming. However, other than staining, the cells behaved similarly to other fibroblast cells.

5.8 The effects of mechanical stimulation on vinculin organisation

Looking at Figures there is no notable difference in the LL24 cells except as the frequency increases the cells tend to have more lamellipodia and filopodia. The vinculin appeared to be more punctate in the L929 cells. Vinculin is important in the regulation of cell adhesion but the mechanisms underlying its stabilizing function on focal adhesion in cells is unclear. However, during cell migration, vinculin activation occurs at the protruding cell front where focal complexes mature into tension dependent FAs. In areas where tension is insufficient, FAs become labile and disassemble. This explains the formation of adhesions in migrating cells, whereby adhesion sites grow in a restricted area at the cell front with highest Rho activity (this is a protein used for cell migration) and tension. FAs would then disassemble in the lamella in front of the nucleus where Rho activity and subsequently tensile forces, are reduced (Pertz *et al.,* 2006).

5.9 The effects of mechanical stimulation on temperature

The overall results showed an increase in temperature as the frequency increased. This is due to the fact that, when the speaker stimulates sound via vibration, heat is also being transmitted. This then warms up the base of the dish and possibly could be the reason for the increase in cell migration. At 100Hz the temperature had increased by

0.4 degrees C compared to the control and at 1600Hz the temperature increased by 0.7 degrees C; this may be the reason why there is an increase in cell migration as the 35mm dish is placed into the incubator with conditions of 37ºC and 5 % CO_2 and onto the speaker and then stimulation is applied. A study by Iijima 1998 looked at the effects of temperature on cell migration and proliferation during wound repair in a gastric epithelial cell line. The cells located on the wound edge formed lamellipodia and showed active ruffling movement until complete repair was achieved. The results showed cell migration was much more important in the 39°C group, while cell proliferation was dominant in the 33°C group in the GSM06 cell model of wound repair (Iijima, 1998). However, temperature affects the speed of sound by changing the density of the medium in which a sound waves travels. When the temperature of a medium increases so does the speed of sound through that medium. Sound waves require a medium in order to travel. A medium can be a solid, liquid, or a gas such as air. The speed of sound depends on both the elasticity and density of the medium. When the elasticity increases and density decreases of a medium, sound is allowed to travel faster. Higher temperatures lower the density of a medium, which puts less resistance on the sound waves which possibly could be reason why cells stimulated at frequency above 100Hz move slower. Perhaps, different cells react differently with increases in temperature. As, in this thesis, fibroblast cells and muscle cells were stimulated, it is possible suggest that cells respond differently with temperature conditions at 100Hz compared to 1600Hz.

5.10 The effects of amplitude on single cell migration

Vibration of an object is always caused by an excitation force. This force may be externally applied to the object, or it may originate inside the object. The rate (frequency) and magnitude of the vibration of a given object is determined by the excitation force, direction, and frequency. To see if displacement was having any effect, experiments were carried out using 100Hz, as previous results showed that there was an increase in mean migration distance when cells were stimulated at 100Hz. However, from the displacement graph it can be seen that, the amplitude at 100Hz was the highest and then rapidly decreases as the frequency increased due to the frequency response of the excitation apparatus. Taking this into consideration the difference in

amplitude was investigated and it was shown that when the cells were stimulated at 100% (9.0µm) amplitude and 50% (4.5µm) the migratory distance of cells was very similar. This shows that the difference in amplitude has similar effects and that there is no obvious difference in cell behaviour with changing amplitude. A difference was only seen when compared to the control. This is because frequency is what determines the pitch of a sound; it is the rate per second of a vibration constituting a wave and therefore the higher the frequency, the higher the pitch. As the frequency was the same for both with 100% amplitude and 50% amplitude the cells behaved very similarly. The amplitude is the only objective measurement of the degree of change (positive or negative) in atmospheric pressure (the compression and rarefaction of air molecules) caused by sound waves. It is known that sounds with greater amplitude will produce greater changes in atmospheric pressure from high pressure to low pressure. As amplitude is having no effect, but frequency is, this could be due to the fact that cells respond to force and change the cell shape and structure, which leads to changes in the internal cytoskeleton, especially microfilaments and microtubules, which are linked by adhesion receptors to the cell nucleus resulting in changes in gene expression.

5.11 The effect of mechanical stimulation on muscle fibre diameter.

From the results, the result show that when the muscle cells/fibres were stimulated at 100Hz and 1600Hz there was an increase in diameter when they were stimulated for 20 minutes. The reasons for the increase in diameter is yet unknown, although applying vibration to muscles is used widely across the globe but not much has been done to understand the effects of mechanical stimulation on muscle cells. However, to speculate about the possible reason for the enhancement of cell/fibre diameters, this could be due to the increase in mechanical signals as stimulation was applied every day for four days. These signals are vital for the development and maintenance of skeletal muscle, but the mechanisms that convert these to biochemical signals are still unknown. It has been previously stated if there is a deformation imposed on a muscle, then there are changes in cellular and molecular conformations that link the mechanical forces with biochemical signals, and the close integration of mechanical signals with electrical, metabolic, and hormonal signaling may disguise the aspect of the response that is specific to the mechanical forces. The conformational change

mechanically induced via vibration may directly activate downstream signaling and may trigger messenger systems to activate signaling indirectly. The effectors of mechanotransduction include the ubiquitous mitogen activated protein kinase (MAP) and phosphatidylinositol-3' kinase (PI-3K), which have well described receptor dependent cascades, but the chain of events leading from mechanical stimulation to biochemical cascade is still not clear (Burkholder, 2007).

Muscle fibre types are generally defined by the particular myosin heavy chain isoforms that they express, but many other components contribute to a fibre's physiological characteristics. Skeletal muscle fibre type can have a profound impact on muscle diseases, the aging-induced loss of muscle mass and strength. As C2C12 was used in this thesis, a previous study is of interest by Wang *et al.,* who looked at the effects of vertical vibration (VV) treatment with frequencies of 5, 8, or 10 Hz on the expression of extracellular matrix proteins and myogenic regulatory factors (MRFs), as well as myotube formation, in C2C12 myoblasts. They showed that VV stimulation is safe and effective at stimulating myogenesis in C2C12 myoblasts and that VV treatment at frequencies of 8 to 10 Hz can stimulate the expression of ECM proteins and MRFs in myoblasts and, in turn, increase myotube formation (Wang *et al.,* 2010). Therefore, stimulation of 20 minutes every day may be causing an increase in ECM proteins and MRFs in myoblasts, which overall increases the myotube formation, leading them to have greater diameter. It is also possible that the expansion could be due to the fact that muscles are made up of tiny fibres and processed by synthesizing proteins. The stimulation perhaps causes an increase in synthetisation for more protein and more mass.

However, the difference in the average muscle fiber formation between 100Hz and 1600Hz is small and this may be due to the relatively small number of measurements that were made. As 7 measurements were taken on each muscle fibre it was hard to draw lines from one side of the diameter to the other. This may have caused some differences. As only 7 measurements were taken perhaps more readings should have been taken to get an accurate measurement. Some fibres were merging into the other so it was hard to distinguish separate muscle fibres.

Chapter 6

6.0 Conclusion

The work in this thesis has shown that mechanical stimulation (ranging from 100-1600Hz) applied via acoustic vibration, can affect fibroblast cell migration in a frequency-dependent manner, thus highlighting the potential to use mechanical stimulation to enhance and reduce cell migration.

6.1 Cell Migration Studies

With respect to collective cell migration (wound healing), results show that with 5-minutes of mechanical stimulation, at both 100Hz and 1600Hz, the rate of wound closure was slower in comparison to the control for LL24 cells and HDF cells. However, the L929 cells showed that vibration at 1600Hz had the greatest collective cell migration occurred followed by control then 100Hz. When 20 minutes of mechanical stimulation was applied at 100Hz to LL24 cells, wound closure was seen to accelerate in comparison to the control, up to the third hour; thereafter the wounds with 0Hz seemingly had a greater rate of cell migration in the final hour (hour 4) and this trend was also similar for HDF and L929 cells. At present we do not understand the mechanisms that seemingly drive/reduce cell migration following mechanical stimulation. Clearly more work needs to be done in this area, however the results are exciting and show that this approach may have potential when applied to disorders that have a slow wound healing response, such as chouronic illnesses like diabetes where wounds heal very slowly. However, more work would need to be done in this area to further assess this hypothesis using an in vivo model.

To support the wound healing findings, the effects of mechanical stimulation on actin organisation was investigated. Mechanical stimulation was also found to affect cell morphology and actin organisation, with acoustic vibration increasing the formation of lamellipodia and filopodia in both cell lines. Overall there were no obvious differences in the level of actin stress fibres between both lines, but there was a clear

difference in the amount of actin at the leading edges of LL24 cells. When a cell initiates migration, a thin veil of cytoplasm builds up at the leading edge of the cell, named lamellipodia. Lamellipodia are formed via a network of actin and are found at the front of the migrating cell. Acoustic vibration promoted more lamellipodia in the LL24 lung fibroblast cells, while the formation of filopodia was more prominent in the L929 cells. These results support, in part, the hypothesis that changes to the migratory properties of cells via mechanical stimulation are linked to changes in the organisation of the actin cytoskeleton. For actin organisation on HDF cells there were no obvious visible differences seen except perhaps an increase of white image intensity at the edges of the cells suggesting an increase of actin.

Following actin staining, vinculin staining was carried out. The results showed that for LL24 cells as the frequency increases, the cells tend to appear more spread, with an increase in lamellipodia on the leading edge of cells. However, overall there does not seem to be any notable differences in the expression levels and distribution of vinculin within the cells. For LL29 cells, as the frequency increased the cells appeared to be more spread with lamellipodium. Vinculin appeared homogeneously distributed and punctate throughout the cytoplasm when the cells were stimulated at 0, 100Hz and 200Hz. After 200Hz, the vinculin appeared to be punctate around the lamellipodium at the leading edge.

Mechanical stimulation was also found to have an effect on single cell migration. In particular, mechanical stimulation at 100Hz increased the mean cell migration distance for some cell types studied here. However, unlike collective cell migration, mechanical stimulation for 5 minutes at 100Hz seemed to have a positive effect on cell migration. For the LL24 lung fibroblast cells, mechanical stimulation at frequencies above 100Hz resulted in a steady decrease in the mean cell migration distance without affecting cell viability. A similar response was seen with the L929 cells, however, for L929 the decrease in migration was less uniform when compared to that of the LL24 lung cells. However, when 20 minutes stimulation was applied to both cell lines, the trend was similar to the cells' response to 5 minutes stimulation. However, for LL24 cells, stimulation at 400Hz had a greater mean migratory distance and for L929 cells the greater mean migratory distance was seen at800Hz; without these two points the trend would be similar to 5 minutes. Following the work on LL24 cells and L929 cells, single cell migration for HDF cells was investigated. The results

show at 5 minutes of stimulation at 100Hz and 1600Hz there was an increase in mean cell migration distance and at 20 minutes of mechanical stimulation there was also an increase in mean migration at 100Hz, without affecting cell viability.

Intermittent stimulation was investigated and found that with single cell migration, single stimulation tends to increase cell migration compared to hourly stimulations. However, for collective cell migration an opposite trend was seen. By stimulating every hour, at 100Hz, the cells migrated a greater distance. This is possibly due to the cells communicating more, sending signals as a group rather than individually, in a collective cell migration, as compared to single migration.

In conclusion, these results show that mechanical stimulation can be used to enhance or decrease fibroblast cell migration in a frequency dependent manner and that different cells from different tissue origins may respond differently to force/mechanical stimulation. It would appear that mechanical stimulation results in changes to the cytoskeleton, which may act as a conjugate to help convert external physical stimuli into a biological response, in this instance cell migration.

Temperature was investigated to see if temperature has any effect on cell migration. The results show that as the stimulation increased the temperature increased too, although this was only very slightly. This may have affected the results; however, more work still needs to be done here. Moreover, to see if amplitude was having an effect on cell migration, the amplitude was reduced and compared to a control and 100%. The results show that there is hardly any difference between the different amplitudes, as the cell migratory distance appeared to be similar, therefore it can be deduced that amplitude is not affecting the cell migration.

Overall it has been shown that 100Hz seems to be the best frequency to accelerate single cell migration, also for wound healing for 20 minutes and for an increase in muscle fibre diameters.

6.2 Muscle cells studies

Another approach to this thesis was to look at how muscle cells respond to stimulation. Many people currently use vibrating platforms in health and clinical settings as a means for improving muscle function, however it is not yet fully understood in terms

of what is happening at the cellular level. Therefore, it was interesting to see if mechanical stimulation had any effect upon muscle cells, such as an increase, or decrease, in the muscle fibre diameters. The results obtained show that after 5 minutes of mechanical stimulation at 100 and 1600Hz for four days, the muscle fibres with no stimulation had greater diameters compared to the ones that were stimulated. However, after 20 minutes of stimulation there was a different trend, where those muscle cells that were stimulated at 100Hz had greater diameters compared to the control and those stimulated at 1600Hz. This indicates that applying stimulation for 20 minutes increases the muscle fibre diameters.

Chapter 7

7.0 Suggestion for Future studies

The work that has been outlined in this thesis was focused on investigating the effects of mechanical stimulation upon fibroblast cell migration and muscle fibre formation. The work is novel and has shown that mechanical stimulation, delivered via acoustic vibration, can be used to control the level of fibroblast cell migration and muscle fibre formation. The work however has its limitations. Therefore, the following are suggestions to further this research.

7.1 Cell Migration Studies

Firstly, the work has focused on using acoustic vibration using a speaker-based system. Is it the acoustic vibration that is important, or could the same effects be achieved using mechanical stimulation delivered via non-acoustic methods, e.g. thourough a piezo actuator. To address this issue, a new system could be developed which uses piezo actuators to deliver vibration. Also, another approach would be to use a device that would be able to control displacement amplitude more effectively. The device used in this study did not have a resistor to control the amplitude. Therefore, all the experiments could be repeated with controlled amplitudes. This would enable us to understand the effects of amplitude of displacement on cell migration.

During this study it was found that stimulation at 100Hz was consistent in enhancing the migration of fibroblast cells. Previous research has focused on lower frequencies, so it would therefore be interesting to look at the effects of frequencies below 100Hz on cell migration. Similarly, the vibration exposure times used here were essentially carefully chosen following a review of the current literature. Therefore, exposure time should be investigated more closely and scientifically to look at the effects of exposure time on cell migration. I would also suggest that it would be interesting to investigate the effects of intermittent mechanical stimulation using a range of frequencies outside

of 100Hz and 1600Hz and to compare the effects of single cell migration and collective cell migration.

I would also suggest that possibly repeating the experiments using different cell lines, e.g. stem cells, might be interesting to see if they respond in a similar/different way to the LL24 cells, L929 cells and HDF cells, as previous research has suggested that stem cells do differentiate when vibration stimulation is applied. Another option could be to apply mechanical stimulation to cancer/tumour cells and see if stimulation accelerates, or decelerates, the mean migration distance, or increases, or decreases, the wound closure process in cancer patients.

As actin and vinculin was stained in this study, other adhesion tests could be carried out, such as ECM adhesion assays to look at the variety of extracellular matrix proteins, or endothelium adhesion assays, which could be carried out to measure the interactions of either leukocytes or tumour cells with vascular endothelium. However, other assays such as the western blot assay could be carried out to quantify changes to actin and vinculin.

Other approaches would be to try and elucidate the underlying mechanisms responsible for the cells to respond to mechanical stimulation in this manner. Also to use in vivo studies results from this thesis, which then can be incorporated into novel wound care technology and then stimulation can then be applied directly to wounds to accelerate wound healing in patients. Finally, is to incorporate vibration to cells on 3D surfaces, such as scaffolds; this would be interesting as certain surfaces accelerate wound healing and applying vibration could rapidly close wounds compared to a normal wound closure.

This study has provided interesting results; however, a different approach could be applied to image the cells using Atomic force microscopy. As it is not fully understood how cells respond to force, in particular mechanical stimuli may act to activate membrane channels which then allow the passage of ions, such as calcium, into the cell. Such studies could be carried out using a combination of both atomic force and fluorescence microscopy to apply forces to cells and measure any changes in

intracellular ions and changes in the structure of the cytoskeleton. Similar studies using both AFM and fluorescence could involve investigating if forces applied to the cellular surface cause changes in focal adhesion formation. In this thesis it has been shown that with an increase of stimulation this also increases the temperature, which could be a factor that could be considered for further investigation. AFM could be used to study how temperature affects the mechanical properties of cells, by carrying out force indentation measurements on cells over a range of temperatures.

7.2 Muscle Cell Studies

Due to time constraints the work presented on the effects of mechanical stimulation on muscle fibre formation presented in this thesis is somewhat limited. Moving forward with this work it would be interesting to vary the parameters, such as frequency/amplitude of vibration, exposure time and intermittent stimulation. Similarly, it would be interesting to look at using a cell model which does not readily form muscle fibres, so as to see if fibre formation could be enhanced with these cells.

Previous research has been carried out at Liverpool John Moores University that looked at controlling cell behaviours thorough the development of novel patterned surfaces for use as cell substrates. Therefore, another suggestion would be to investigate the combined effects of mechanical stimulation and patterned substrates on muscle fibre formation and fibre alignment. Alterative suggestions for the work presented in this thesis would be to investigate the effects of mechanical stimulation on muscle fibre cell viability and function using more established bio-assays.

References

ALBERTS, B. 2013. Impact Factor Distortions. Science, 340, 787-787.

ALLEN, P. G. & JANMEY, P. A. 1994. Gelsolin displaces phalloidin from actin filaments. A new fluorescence method shows that both Ca2+ and Mg2+ affect the rate at which gelsolin severs F-actin. The Journal of biological chemistry, 269, 32916-32923.

ANDREOLI, A., MONTELEONE, M., VAN LOAN, M., PROMENZIO, L., TARANTINO, U. & DE LORENZO, A. 2001. Effects of different sports on bone density and muscle mass in highly trained athletes. Medicine & Science in Sports & Exercise, 33, 507-511.

ARYAEI, A. & JAYASURIYA, A. C. 2015. The effect of oscillatory mechanical stimulation on osteoblast attachment and proliferation. Materials Science and Engineering: C, 52, 129-134.

ASCIONE, F., VASATURO, A., CASERTA, S., D'ESPOSITO, V., FORMISANO, P. & GUIDO, S. 2016. Comparison between fibroblast wound healing and cell random migration assays in vitro. Experimental Cell Research, 347, 123-132.

BACABAC, R. G., SMIT, T. H., VAN LOON, J. J. W. A., DOULABI, B. Z., HELDER, M. & KLEIN-NULEND, J. 2006. Bone cell responses to high-frequency vibration stress: does the nucleus oscillate within the cytoplasm? The FASEB Journal, 20, 858-864.

BEAR, J. E., SVITKINA, T. M., KRAUSE, M., SCHAFER, D. A., LOUREIRO, J. J., STRASSER, G. A., MALY, I. V., CHAGA, O. Y., COOPER, J. A., BORISY, G. G. & GERTLER, F. B. 2002. Antagonism between Ena/VASP Proteins and Actin Filament Capping Regulates Fibroblast Motility. Cell, 109, 509-521.

BOCKHOLT, S. M. & BURRIDGE, K. 1993. Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. Journal of Biological Chemistry, 268, 14565-14567.

BRUCE ALBERTS, A. J., JULIAN LEWIS, MARTIN RAFF, KEITH ROBERTS, AND PETER WALTER. 2002. Molecular Biology of the Cell, New York, Garland Science.

BRUCE ALBERTS, D. B., KAREN HOPKIN, ALEXANDER D JOHNSON, JULIAN LEWIS, MARTIN RAFF, KEITH ROBERTS, PETER WALTER 2013. Essential Cell Biology.

BURGER, E. H. & KLEIN-NULEND, J. 1999. Mechanotransduction in bone—role of the lacuno-canalicular network. The FASEB Journal, 13, 101-112.

BURKHOLDER, T. J. 2007. Mechanotransduction in skeletal muscle. Frontiers in bioscience: a journal and virtual library, 12, 174-191.

BUTEZLOFF, M. M., ZAMARIOLI, A., LEONI, G. B., SOUSA-NETO, M. D. & VOLPON, J. B. 2015. Whole-body vibration improves fracture healing and bone quality in rats with ovariectomy-induced osteoporosis. Acta cirurgica brasileira, 30, 727-735.

CHAFFEY, N. 2003. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. Molecular biology of the cell. 4th edn. Annals of Botany, 91, 401-401.

CHOW, S. K. H., LEUNG, K. S., QIN, J., GUO, A., SUN, M., QIN, L. & CHEUNG, W. H. 2016. Mechanical stimulation enhanced estrogen receptor expression and callus formation in diaphyseal long bone fracture healing in ovariectomy-induced osteoporotic rats. Osteoporosis International, 27, 2989-3000.

CONDE, C., ARIAS, C., ROBIN, M., LI, A., SAITO, M., CHUANG, J.-Z., NAIRN, A. C., SUNG, C.-H. & CÁCERES, A. 2010. Evidence for the Involvement of Lfc and Tctex-1 in Axon Formation. The Journal of neuroscience : the official journal of the Society for Neuroscience, 30, 10.1523/JNEUROSCI.5420-09.2010.

CRUZ-CHU, EDUARDO R., MALAFEEV, A., PAJARSKAS, T., PIVKIN, IGOR V. & KOUMOUTSAKOS, P. 2014. Structure and Response to Flow of the Glycocalyx Layer. Biophysical Journal, 106, 232-243.

CURTIS, A. S. G., REID, S., MARTIN, I., VAIDYANATHAN, R., SMITH, C. A., NIKUKAR, H. & DALBY, M. J. 2013. Cell Interactions at the Nanoscale: Piezoelectric Stimulation. IEEE Transactions on NanoBioscience, 12, 247-254.

DOGTEROM, M. & YURKE, B. 1997. Measurement of the Force-Velocity Relation for Growing Microtubules. Science, 278, 856-860.

DU ROURE, O., SAEZ, A., BUGUIN, A., AUSTIN, R. H., CHAVRIER, P., SIBERZAN, P. & LADOUX, B. 2005. Force mapping in epithelial cell migration. Proceedings of the National Academy of Sciences of the United States of America, 102, 2390-2395.

EDIONWE, J., HESS, C., FERNANDEZ-RIO, J., HERNDON, D. N., ANDERSEN, C. R., KLEIN, G. L., SUMAN, O. E. & AMONETTE, W. E. 2016. Effects of whole-body vibration exercise on bone mineral content and density in thermally injured children. Burns, 42, 605-613.

EVANS, N. D., OREFFO, R. O. C., HEALY, E., THURNER, P. J. & MAN, Y. H. 2013. Epithelial mechanobiology, skin wound healing, and the stem cell niche. Journal of the Mechanical Behavior of Biomedical Materials, 28, 397-409.

FLEISSNER, F., BONN, M. & PAREKH, S. H. 2016. Microscale spatial heterogeneity of protein structural transitions in fibrin matrices. Science Advances, 2.

GASTON, J., QUINCHIA RIOS, B., BARTLETT, R., BERCHTOLD, C. & THIBEAULT, S. L. 2012. The Response of Vocal Fold Fibroblasts and Mesenchymal Stromal Cells to Vibration. PLOS ONE, 7, e30965.

GIANNONE, G., DUBIN-THALER, B. J., ROSSIER, O., CAI, Y., CHAGA, O., JIANG, G., BEAVER, W., DÖBEREINER, H.-G., FREUND, Y., BORISY, G. & SHEETZ, M. P. 2007. Lamellipodial Actin Mechanically Links Myosin Activity with Adhesion-Site Formation. Cell, 128, 561-575.

GILCHRIST, C. L., WITVOET-BRAAM, S. W., GUILAK, F. & SETTON, L. A. 2007. Measurement of intracellular strain on deformable substrates with texture correlation. Journal of Biomechanics, 40, 786-794.

HARVEY LODISH, A. B., S LAWRENCE ZIPURSKY, PAUL MATSUDAIRA, DAVID BALTIMORE, AND JAMES DARNELL. 2000. Molecular Cell Biology, New York, W. H. Freeman.

HOFFMAN, N. J. & ELMENDORF, J. S. 2011. Signaling, cytoskeletal and membrane mechanisms regulating GLUT4 exocytosis. Trends in Endocrinology & Metabolism, 22, 110-116.

HOLMES, R. D. A. K. C. 2011. Actin Structure and Function. Annual Review of Biophysics, 40, 169-186.

HORIO, T. & MURATA, T. 2014. The role of dynamic instability in microtubule organization. Frontiers in Plant Science, 5.

HUANG, C., MIYAZAKI, K., AKAISHI, S., WATANABE, A., HYAKUSOKU, H. & OGAWA, R. 2013. Biological effects of cellular stretch on human dermal fibroblasts. Journal of Plastic, Reconstructive & Aesthetic Surgery, 66, e351-e361.

HUMPHRIES, J. D., WANG, P., STREULI, C., GEIGER, B., HUMPHRIES, M. J. & BALLESTREM, C. 2007. Vinculin controls focal adhesion formation by direct interactions with talin and actin. The Journal of Cell Biology, 179, 1043-1057.

IIJIMA, K. 1997. Effect of temperature on cell migration and proliferation during wound repair in cultured temperature-sensitive gastric epithelial cells. Juntendo Medical Journal, 43, 419-425.

INGBER, D. E. 2006. Cellular mechanotransduction: putting all the pieces together again. The FASEB Journal, 20, 811-827.

INGBER, D. E. 2008. Tensegrity and mechanotransduction. Journal of Bodywork and Movement Therapies, 12, 198-200.

ITO Y, K. T., AGO Y, NAM K, HIRAKU K, MIYAZAKI K, MASUZAWA T, KISHIDA A. 2011. Nano-vibration effect on cell adhesion and its shape. Biomed Mater Eng, 21, 149-58.

IVASKA, J., PALLARI, H.-M., NEVO, J. & ERIKSSON, J. E. 2007. Novel functions of vimentin in cell adhesion, migration, and signaling. Experimental Cell Research, 313, 2050-2062.

JAGODZINSKI, M., BREITBART, A., WEHMEIER, M., HESSE, E., HAASPER, C., KRETTEK, C., ZEICHEN, J. & HANKEMEIER, S. 2008. Influence of perfusion

and cyclic compression on proliferation and differentiation of bone marrow stromal cells in 3-dimensional culture. Journal of Biomechanics, 41, 1885-1891.

KÄS, J., STREY, H., TANG, J. X., FINGER, D., EZZELL, R., SACKMANN, E. & JANMEY, P. A. 1996. F-actin, a model polymer for semiflexible chains in dilute, semidilute, and liquid crystalline solutions. Biophysical Journal, 70, 609-625.

KIM, D.-H. & WIRTZ, D. 2013. Focal adhesion size uniquely predicts cell migration. The FASEB Journal, 27, 1351-1361.

KIM, I. S., SONG, Y. M., LEE, B. & HWANG, S. J. 2012. Human Mesenchymal Stromal Cells are Mechanosensitive to Vibration Stimuli. Journal of Dental Research, 91, 1135-1140.

KOMRAKOVA, M., HOFFMANN, D. B., NUEHNEN, V., STUEBER, H., WASSMANN, M., WICKE, M., TEZVAL, M., STUERMER, K. M. & SEHMISCH, S. 2016. The Effect of Vibration Treatments Combined with Teriparatide or Strontium Ranelate on Bone Healing and Muscle in Ovariectomized Rats. Calcified Tissue International, 99, 408-422.

KULKARNI, R. N., VOGLEWEDE, P. A. & LIU, D. 2013. Mechanical vibration inhibits osteoclast formation by reducing DC-STAMP receptor expression in osteoclast precursor cells. Bone, 57, 493-498.

LAMBIE, E. J. 1996. Cell–cell communication: Receptor function at the junction. Current Biology, 6, 1089-1091.

LAURENS, N., KOOLWIJK, P. & DE MAAT, M. P. M. 2006. Fibrin structure and wound healing. Journal of Thrombosis and Haemostasis, 4, 932-939.

LI, L., HAN, M., LI, S., WANG, L. & XU, Y. 2013. Cyclic tensile stress during physiological occlusal force enhances osteogenic differentiation of human periodontal ligament cells via ERK1/2-Elk1 MAPK pathway. DNA and cell biology, 32, 488-497.

LUU, Y. K., CAPILLA, E., ROSEN, C. J., GILSANZ, V., PESSIN, J. E., JUDEX, S. & RUBIN, C. T. 2009. Mechanical Stimulation of Mesenchymal Stem Cell Proliferation and Differentiation Promotes Osteogenesis While Preventing Dietary-Induced Obesity. Journal of Bone and Mineral Research, 24, 50-61.

MANUYAKORN, W., SMART, D. E., NOTO, A., BUCCHIERI, F., HAITCHI, H. M., HOLGATE, S. T., HOWARTH, P. H. & DAVIES, D. E. 2016. Mechanical Strain Causes Adaptive Change in Bronchial Fibroblasts Enhancing Profibrotic and Inflammatory Responses. PLoS ONE, 11, e0153926.

MATSUBAYASHI, Y., EBISUYA, M., HONJOH, S. & NISHIDA, E. 2004. ERK Activation Propagates in Epithelial Cell Sheets and Regulates Their Migration during Wound Healing. Current Biology, 14, 731-735.

MATSUMOTO, T., SATO, D. & HASHIMOTO, Y. 2016. Individual and combined effects of noise-like whole-body vibration and parathyroid hormone treatment on

bone defect repair in ovariectomized mice. Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine, 230, 30-38.

MATTILA, P. K. & LAPPALAINEN, P. 2008. Filopodia: molecular architecture and cellular functions. Nat Rev Mol Cell Biol, 9, 446-454.

MAZZARELLO, P. 1999. A unifying concept: the history of cell theory. Nat Cell Biol, 1, E13-E15.

MEJILLANO, M. R., KOJIMA, S.-I., APPLEWHITE, D. A., GERTLER, F. B., SVITKINA, T. M. & BORISY, G. G. 2004. Lamellipodial Versus Filopodial Mode of the Actin Nanomachinery: Pivotal Role of the Filament Barbed End. Cell, 118, 363-373.

MENSAH, A. Y., SAMPSON, J., HOUGHTON, P. J., HYLANDS, P. J., WESTBROOK, J., DUNN, M., HUGHES, M. A. & CHERRY, G. W. 2001. Effects of Buddleja globosa leaf and its constituents relevant to wound healing. Journal of Ethnopharmacology, 77, 219-226.

PALUCH, E. K., NELSON, C. M., BIAIS, N., FABRY, B., MOELLER, J., PRUITT, B. L., WOLLNIK, C., KUDRYASHEVA, G., REHFELDT, F. & FEDERLE, W. 2015. Mechanotransduction: use the force(s). BMC Biology, 13, 47.

PATEL, M. J., CHANG, K. H., SYKES, M. C., TALISH, R., RUBIN, C. & JO, H. 2009. Low magnitude and high frequency mechanical loading prevents decreased bone formation responses of 2T3 preosteoblasts. Journal of Cellular Biochemistry, 106, 306-316.

PÉREZ-CASTEJÓN C, P.-B. R., LLORENTE M, PES N, LACASA C, FIGOLS T, LAHOZ M, MAESTÚ C, VERA-GIL A, DEL MORAL A, AZANZA MJ. 2009. Exposure to ELF-pulse modulated X band microwaves increases in vitro human astrocytoma cell proliferation. Histol Histopathol, 24, 1551-61.

PERTZ, O., HODGSON, L., KLEMKE, R. L. & HAHN, K. M. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature, 440, 1069-1072.

POLLARD, T. D. & COOPER, J. A. 2009. Actin, a central player in cell shape and movement. Science (New York, N.Y.), 326, 1208-1212.

POUJADE, M., GRASLAND-MONGRAIN, E., HERTZOG, A., JOUANNEAU, J., CHAVRIER, P., LADOUX, B., BUGUIN, A. & SILBERZAN, P. 2007. Collective migration of an epithelial monolayer in response to a model wound. Proceedings of the National Academy of Sciences of the United States of America, 104, 15988-15993.

PR, #XE8, , D., CECCARELLI, G., VISAI, L., BENEDETTI, L., IMBRIANI, M., CUSELLA DE ANGELIS, M. G. & MAGENES, G. 2013. High-Frequency Vibration Treatment of Human Bone Marrow Stromal Cells Increases Differentiation toward Bone Tissue. Bone Marrow Research, 2013, 13.

PRAVINCUMAR, P., BADER, D. L. & KNIGHT, M. M. 2012. Viscoelastic Cell Mechanics and Actin Remodelling Are Dependent on the Rate of Applied Pressure. PLOS ONE, 7, e43938.

RAMAEKERS, F. C. S. & BOSMAN, F. T. 2004. The cytoskeleton and disease. The Journal of Pathology, 204, 351-354.

RICHTER, C.-P., BAYON, R., IZZO, A. D., OTTING, M., SUH, E., GOYAL, S., HOTALING, J. & WALSH JR, J. T. 2008. Optical stimulation of auditory neurons: Effects of acute and chronic deafening. Hearing Research, 242, 42-51.

RODRIGUEZ-MENOCAL, L., SALGADO, M., FORD, D. & VAN BADIAVAS, E. 2012. Stimulation of Skin and Wound Fibroblast Migration by Mesenchymal Stem Cells Derived from Normal Donors and Chronic Wound Patients. Stem Cells Translational Medicine, 1, 221-229.

RUBIN, C., TURNER, A. S., MÜLLER, R., MITTRA, E., MCLEOD, K., LIN, W. & QIN, Y.-X. 2002. Quantity and Quality of Trabecular Bone in the Femur Are Enhanced by a Strongly Anabolic, Noninvasive Mechanical Intervention. Journal of Bone and Mineral Research, 17, 349-357.

SCHUHFRIED, O., MITTERMAIER, C., JOVANOVIC, T., PIEBER, K. & PATERNOSTRO-SLUGA, T. 2005. Effects of whole-body vibration in patients with multiple sclerosis: a pilot study. Clinical Rehabilitation, 19, 834-842.

SCHWARTZ, M. A. 2010. Integrins and extracellular matrix in mechanotransduction. Cold Spring Harbor perspectives in biology, 2, a005066.

SHI, H.-F., CHEUNG, W.-H., QIN, L., LEUNG, A. H.-C. & LEUNG, K.-S. 2010. Low-magnitude high-frequency vibration treatment augments fracture healing in ovariectomy-induced osteoporotic bone. Bone, 46, 1299-1305.

SMITH, M. L., GOURDON, D., LITTLE, W. C., KUBOW, K. E., EGUILUZ, R. A., LUNA-MORRIS, S. & VOGEL, V. 2007. Force-Induced Unfolding of Fibronectin in the Extracellular Matrix of Living Cells. PLOS Biology, 5, e268.

SONG, G., JU, Y., SHEN, X., LUO, Q., SHI, Y. & QIN, J. 2007. Mechanical stretch promotes proliferation of rat bone marrow mesenchymal stem cells. Colloids and Surfaces B: Biointerfaces, 58, 271-277.

SUGAWARA, Y., ANDO, R., KAMIOKA, H., ISHIHARA, Y., MURSHID, S. A., HASHIMOTO, K., KATAOKA, N., TSUJIOKA, K., KAJIYA, F., YAMASHIRO, T. & TAKANO-YAMAMOTO, T. 2008. The alteration of a mechanical property of bone cells during the process of changing from osteoblasts to osteocytes. Bone, 43, 19-24.

THIEVESSEN, I., FAKHRI, N., STEINWACHS, J., KRAUS, V., MCISAAC, R. S., GAO, L., CHEN, B.-C., BAIRD, M. A., DAVIDSON, M. W., BETZIG, E., OLDENBOURG, R., WATERMAN, C. M. & FABRY, B. 2015. Vinculin is required for cell polarization, migration, and extracellular matrix remodeling in 3D collagen. The FASEB Journal, 29, 4555-4567.

TITUSHKIN, I. A. & CHO, M. R. Controlling cellular biomechanics of human mesenchymal stem cells. 2009 Annual International Conference of the IEEE Engineering in Medicine and Biology Society, 3-6 Sept. 2009 2009. 2090-2093.

UZER, G., MANSKE, S. L., CHAN, M. E., CHIANG, F.-P., RUBIN, C. T., FRAME, M. D. & JUDEX, S. 2012. Separating Fluid Shear Stress from Acceleration during Vibrations in Vitro: Identification of Mechanical Signals Modulating the Cellular Response. Cellular and molecular bioengineering, 5, 266-276.

VITORINO, P. & MEYER, T. 2008. Modular control of endothelial sheet migration. Genes & development, 22, 3268-3281.

WANG, C.-Z., WANG, G.-J., HO, M.-L., WANG, Y.-H., YEH, M.-L. & CHEN, C.-H. 2010. Low-magnitude vertical vibration enhances myotube formation in C2C12 myoblasts. Journal of Applied Physiology, 109, 840-848.

WANG, H., BRENNAN, T. A., RUSSELL, E., KIM, J.-H., EGAN, K. P., CHEN, Q., ISRAELITE, C., SCHULTZ, D. C., JOHNSON, F. B. & PIGNOLO, R. J. 2013. R-Spondin 1 promotes vibration-induced bone formation in mouse models of osteoporosis. Journal of molecular medicine (Berlin, Germany), 91, 1421-1429.

WEHRLE, E., LIEDERT, A., HEILMANN, A., WEHNER, T., BINDL, R., FISCHER, L., HAFFNER-LUNTZER, M., JAKOB, F., SCHINKE, T., AMLING, M. & IGNATIUS, A. 2015. The impact of low-magnitude high-frequency vibration on fracture healing is profoundly influenced by the oestrogen status in mice. Disease Models & Mechanisms, 8, 93-104.

WEINHEIMER-HAUS, E. M., JUDEX, S., ENNIS, W. J. & KOH, T. J. 2014. Low-Intensity Vibration Improves Angiogenesis and Wound Healing in Diabetic Mice. PLOS ONE, 9, e91355.

WU, S.-H., ZHONG, Z.-M. & CHEN, J.-T. 2012. Low-Magnitude High-Frequency Vibration Inhibits RANKL-Induced Osteoclast Differentiation of RAW264.7 Cells. International Journal of Medical Sciences, 9, 801-807.

ZHANG, C., LI, J., ZHANG, L., ZHOU, Y., HOU, W., QUAN, H., LI, X., CHEN, Y. & YU, H. 2012. Effects of mechanical vibration on proliferation and osteogenic differentiation of human periodontal ligament stem cells. Archives of Oral Biology, 57, 1395-1407.

ZHOU, S., BUENO, E. M., KIM, S. W., AMATO, I., SHEN, L., HAHNE, J., BLEIBERG, I., MORLEY, P. & GLOWACKI, J. 2011. Effects of age on parathyroid hormone signaling in human marrow stromal cells. Aging Cell, 10, 780-788.

ZUO, B., ZHU, J., LI, J., WANG, C., ZHAO, X., CAI, G., LI, Z., PENG, J., WANG, P., SHEN, C., HUANG, Y., XU, J., ZHANG, X. & CHEN, X. 2015. microRNA-103a Functions as a Mechanosensitive microRNA to Inhibit Bone Formation Through Targeting Runx2. Journal of Bone and Mineral Research, 30, 330-345.

2006. MECHANOTRANSDUCTION INVOLVING MULTIMODULAR PROTEINS: Converting Force into Biochemical Signals. *Annual Review of Biophysics and Biomolecular Structure,* 35**,** 459-488.

2011. Actin Structure and Function. *Annual Review of Biophysics,* 40**,** 169-186.

Appendix 1: Publication and awards arising from work in this thesis

The work carried out as part of this thesis has resulted in a publication.

1. Mohammed T, Murphy MF, Lilley F, Burton DR, Bezombes F. (2016) The effects of acoustic vibration on fibroblast cell migration. *Materials Science and Engineering: C.* Volume 69, Pages 1256–1262. https://doi.org/10.1016/j.msec.2016.07.037

Awards:

June 2014:   Best Poster presentation at GERI Annual Research Symposium (GARS).

Jan 2015:   IET scholarship (£1000)

Jan 2015:   Runner up of British Federation of Women Graduates (BFWG) travel Bursary.

Mar 2015:   Awarded £3000 from the Hilda Martindale trust for the representation of women in Engineering.

June 2015:   Best Poster Presentation at Faculty of Engineering and Technology LJMU.

Oct 2015:   Best presentation award at The Annual Presentation day for BFWG.

May 2016:   Best oral presentation at Faculty of Engineering and Technology LJMU.

May 2016:   Certification for being a reviewer for Elsevier.

Appendix 2: Displacement measurements.
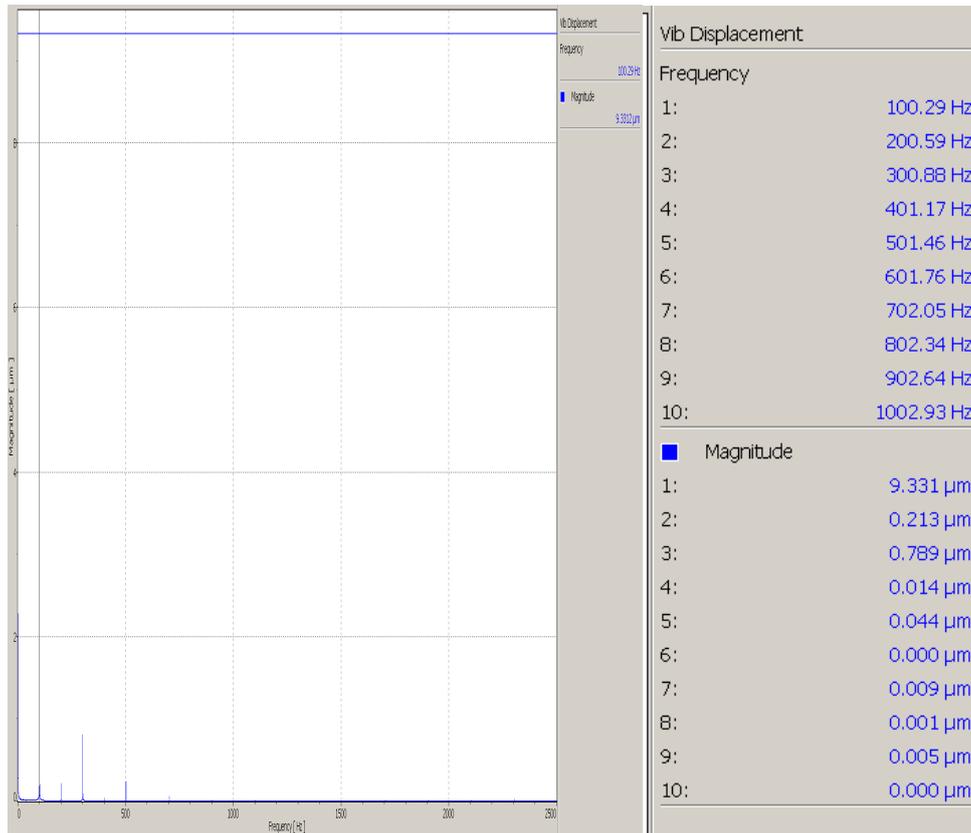
Figure 1 Displacement measurements by Polytec. Results show that a single excitation is not possible as multiple harmonics are observed at a frequency of 100Hz.
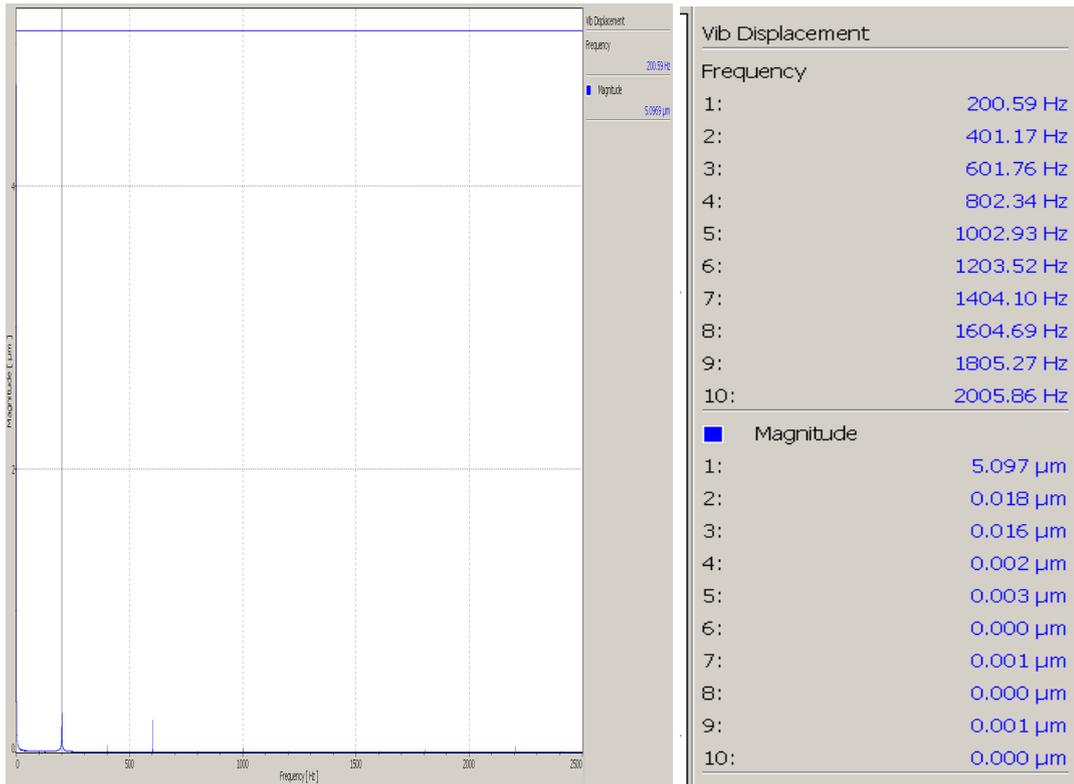
Figure 2 Displacement measurements by Polytec. Multiple harmonics, but at a very low displacement amplitude at frequency of 200Hz.
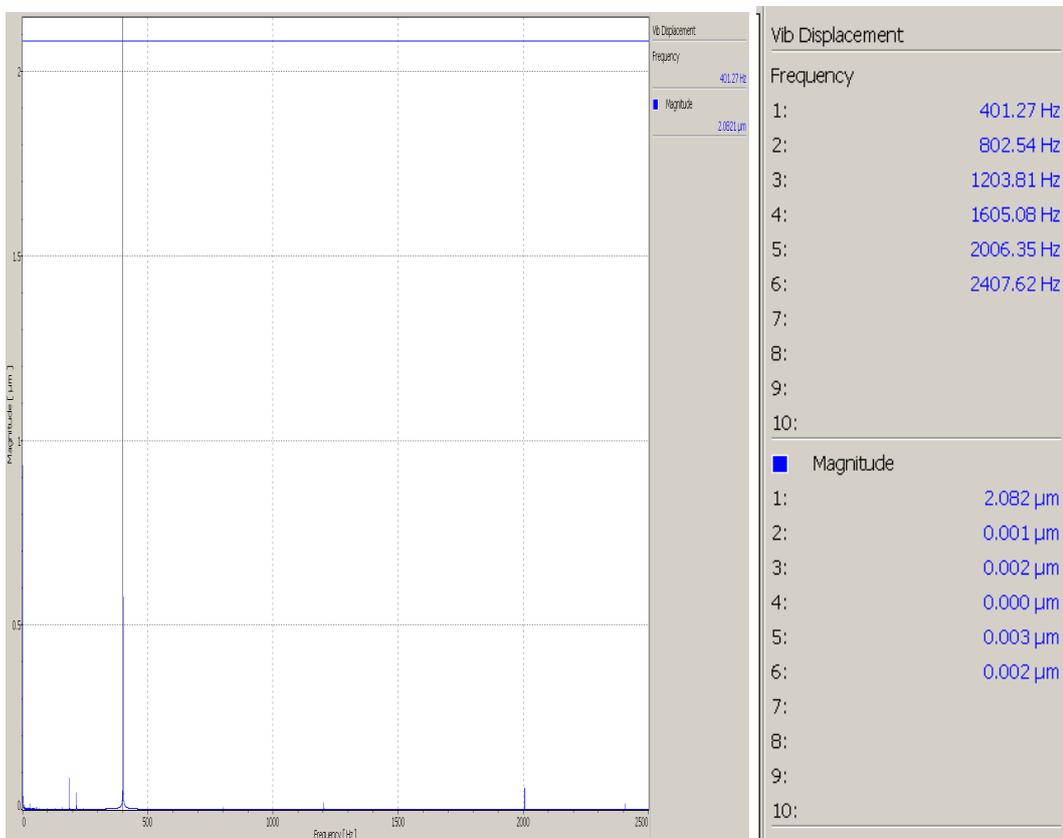

Figure 3 Displacement measurements by Polytec. Multiple harmonics, but at a very low displacement amplitude at frequency of 400Hz.
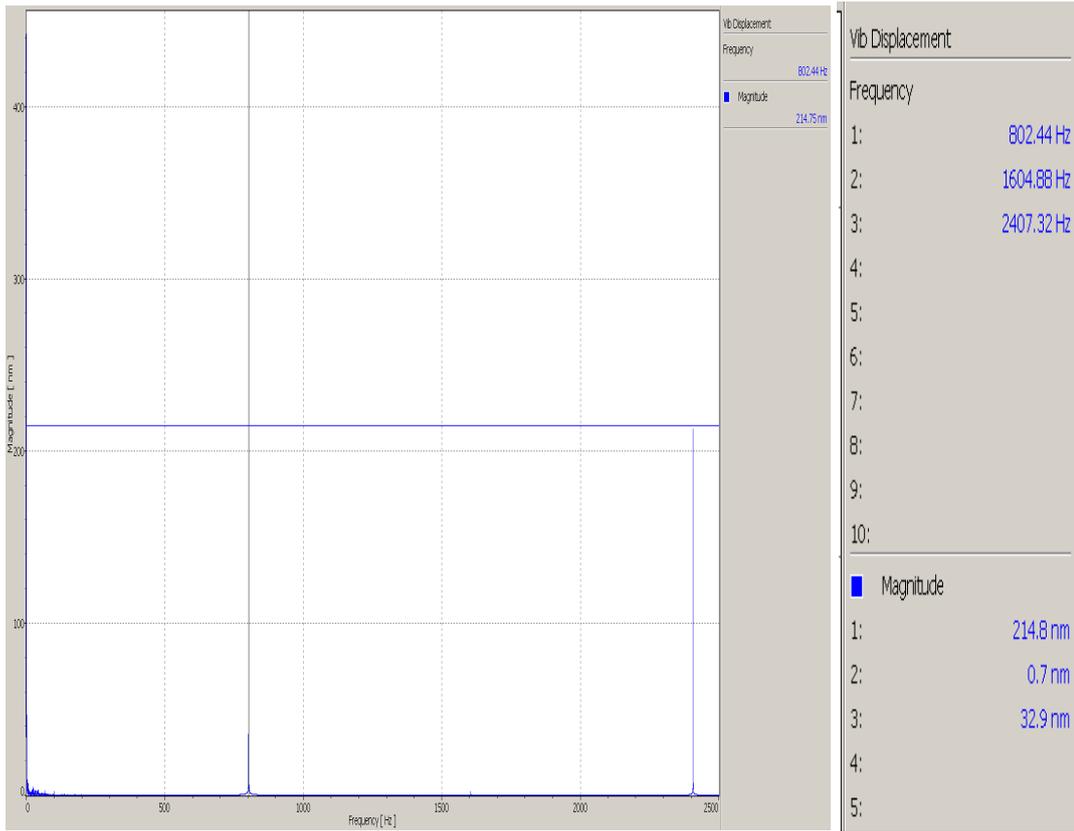
Figure 4 Displacement measurements by Polytec. Greater contribution from the 3rd Harmonic