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Article

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The effects of acoustic vibration on fibroblast cell migration

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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. Fibroblast cells are known to respond to physical cues generated in the extracellular matrix and it is thought that such cues are important regulators of the wound healing process. Many conditions are associated with poor wound healing, so there is need for treatments/interventions, which can help accelerate the wound healing process. The primary aim of this research was to investigate the effects of mechanical stimulation upon the migratory and morphological properties of two different fibroblast cells namely; human lung fibroblast cells (LL24) and subcutaneous areolar/adipose mouse fibroblast cells (L929). Using a speaker-based system, the effects of mechanical stimulation (0-1600Hz for 5 minutes) on the mean cell migration distance (μm) and actin organisation was investigated. The results show that 100Hz acoustic vibration enhanced cell migration for both cell lines whereas acoustic vibration above 100Hz acoustic vibration was found to decrease cell migration in a frequency dependent manner. Mechanical stimulation was also found to promote changes to the morphology of both 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. Mechanical stimulation at all the frequencies used here was found not to affect cell viability. These results suggest that low-frequency acoustic vibration may be used as a tool to manipulate the mechano-sensitivity of cells to manipulate cell migration and which may be used to aid wound repair.

Key words: Acoustic vibration; Mechanical stimulation; Fibroblast Cell Migration; Actin reorganisation; wound healing

1. Introduction

Cells, by their very nature, have evolved to respond to external physical cues and it is now known that there is a complex interplay between the physical extracellular microenvironment and cellular function [1],[2] and [3]. Cells sense their physical surroundings by converting mechanical forces and distortions into biochemical signals, via the activation of diverse intracellular signalling pathways, through a process known as mechanotransduction [4]. Little is known about mechanotransduction, however work on eukaryotic cells is helping to unravel the complexities of this process. For example, it is known that stretch-sensitive ion channels [5] are key regulators of this process, along with an architectural control of mechanotransduction, through a mechanochemical coupling between the cell surface and nucleus [6]. We also 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 [4] and [7].

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 mechanical stimulation (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 40-50Hz (delivered through a vibrating platform) in combination with exercise, helped to improve patient muscle strength, functional ability and general wellbeing [8]. While, Weinheimer-Haus et al [9], found that low intensity mechanical stimulation (45Hz) significantly increased the wound closure rate in diabetic mice, compared to those mice that were not exposed to mechanical stimulation. Similarly, Wang et al [7] used a dynamic motion platform to apply mechanical stimulations (32-37Hz) 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 [7].

At the cellular level Aryaei and Jayasuriya [10] found that mechanical stimulation can be used to improve osteoblast attachment and proliferation [10]. Kulkarni et al [11], found that mechanical stimulation can inhibit bone reabsorption. Similarly, Wu et al [12] found that mechanical stimulation produced an anabolic effect on bone through the inhibition of osteoclast differentiation and Kim et al [12], found that low-magnitude high-frequency (LMHF) mechanical stimulation enables the osteogenic process of human mesenchymal stromal cells (hMSCs). Such studies highlight the potential of using mechanical stimulation as a novel therapeutic treatment for a range of conditions, particularly those associated with a loss of bone mass. However, knowledge on the effects of mechanical stimulation on other cell types is lacking.

We were interested to demonstrate whether mechanical stimulation delivered via acoustic stimulation, could be used to enhance cell migration. For this work we used fibroblast cells, as these cells play an important role in wound healing. We hypothesised that mechanical stimulation would influence the migratory properties of fibroblast cells, as these cells have been shown to be mechanosensitive [13] and respond to mechanical signals generated within the extracellular matrix [14]. Results show that mechanical stimulation delivered to fibroblast cells in culture, can enhance, or decrease, cell migration in a frequency-dependent manner.

2. Materials and Methods

2.1. Mechanical stimulation via acoustic vibration

To mechanically stimulate cells we used a speaker-based system. The speaker-based system was built using a 0.2W super-thin, waterproof Mylar speaker (45mm) and an Arduino microcontroller board for control (Fig. 1). This system enables mechanical stimulation to be applied (via a sinusoidal waveform) to the underside of the cell culture dish to which the cells adhere, at frequencies ranging between 100-1600Hz.

2.2. Calibration of mechanical stimulation 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 2 for results).

2.3. Cell culture

Human lung fibroblast cells (LL24) and mouse fibroblast cells (L929) were used in this study as fibroblasts are involved in the wound healing process. The two different fibroblast cells were used as they originate from different tissue types with mechanically different properties and functions. The cells were obtained from the European Collection of Animal Cell Cultures (ECACC). The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2mM L-glutamine (Invitrogen), 10% foetal bovine serum (FBS) (sigma, UK) and 1% penicillin- streptomycin in T75 cell culture flasks, at 37˚C in a humidified atmosphere containing 95% air and 5% CO2.

2.4 Cell Imaging

All images obtained during 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 filamentous actin the He-Ne 543nm laser was employed.

2.5. Mechanical stimulation of cells

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.

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 after 4 hours so as to determine mean migration distance (µm).

2.6. Mechanical stimulation and actin organisation

Given that cell morphology and the actin cytoskeleton undergo reorganisation prior to and during cell migration, fluorescence microscopy was used to document the effects of mechanical stimulation upon cell morphology and actin organisation. The LL24 and L929 cells were seeded into 35mm cell culture dishes at a density of 2×10^4cells/cm^2 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 minutes at 0, 100, 200, 400, 800, or 1600Hz and then chemically fixed to preserve their morphology.

For fixation the cells were washed (×1) in phosphate buffered saline solution (PBS) for 5 minutes and then fixed at room temperature in 10% paraformaldehyde for 10 minutes. Following fixation the cells were washed with PBS and permeabilised for 20 minutes at room temperature using 0.5% Triton-X 100. The cells were then washed (x1) with PBS and the actin filaments labelled by staining with Rhodamine-Phalloidin according to the manufacturer's instructions (Cytoskeleton Inc.). After staining, the dishes were thoroughly washed with PBS to eliminate any background staining and the cells were imaged using an excitation wavelength of 543nm. 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 (Fig. 7 & 8).

2.7. MTT cell viability 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 LL24 and L929 cells were seeded into 35mm cell culture dishes at 2×10^4cells/cm^2 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 minutes at 0, 100, 200, 400, 800, or 1600Hz and the MTT assay carried out. Briefly, MTT solution (5mg/ml) was added to each dish and the cells left to incubate at 37°C, 5% CO_2 for 2 hours. 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 540nm using a 96-well plate reader. Each experiment was carried out three times to ensure repeatability.

2.8 Statistical Analysis

Statistical analysis to test for significance between the mean of the control and individual treatments was carried out using an unpaired, two-tailed Student's *t*-test at 95% confidence limit.

3. Results and discussion

3.1. Speaker 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 (Fig. 1 left). This system allows the frequency of mechanical stimulation to be controlled via an Arduino controller. In order to determine that 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 (data not shown). Therefore, frequencies between 100 and 1600Hz were used, so as to accurately 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 (Fig. 1 right).

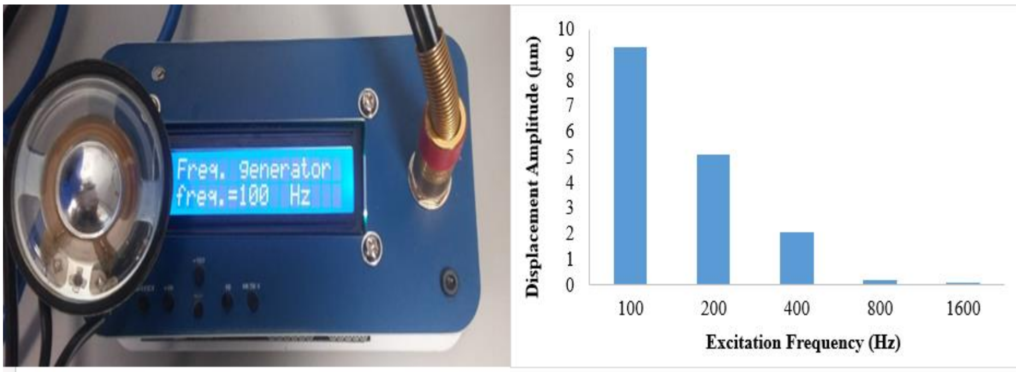

Fig. 1 Speaker-based system (left) and calibration of frequency *versus* amplitude of displacement of the inside bottom surface of a 35mm cell culture dish to which the cells adhere (right).

3.2. The effect of mechanical stimulation on cell migration.

In order to investigate if mechanical stimulation has any effect on the migration of individual fibroblast cells, time-lapse microscopic imaging and subsequent cell tracking using ImageJ software was carried out. Cells were mechanically stimulated using 100, 200, 400, 800, or 1600Hz for 5 minutes and migration was subsequently recorded over a 4-hour period. As can be seen from Figure 2, mechanical stimulation for 5 minutes at 100Hz significantly enhanced (approx. 10% increase) the mean migration distance (µm) of human lung fibroblast (LL24) cells, 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 mechanical stimulation frequency increased (and amplitude decreased) resulting in a decrease in the mean migration distance (µm) of approximately 50% (at 1600Hz), compared to the control population.

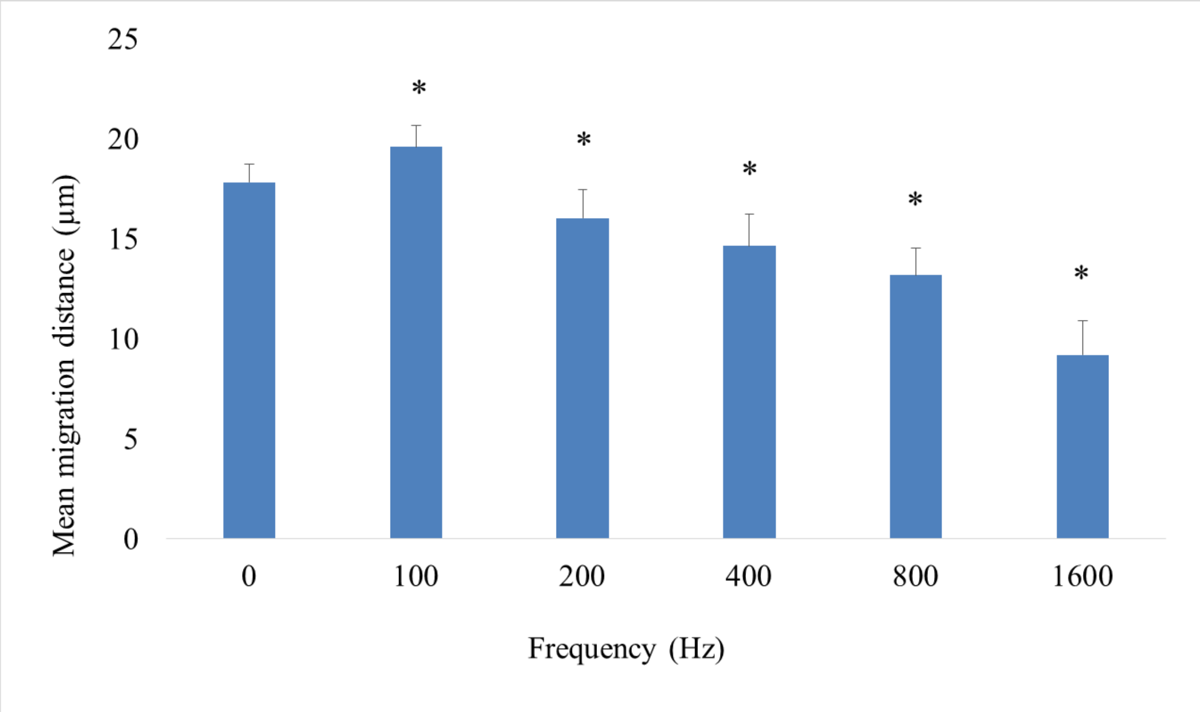

Fig. 2 Total distance travelled (µm) over a 4-hour period for LL24 fibroblast cells (n=30) versus frequency of mechanical stimulation (Hz). Error bars represent standard deviation. * denotes Significance ($P < 0.05$).

The results show a similar trend for the LL29 cells, in that mechanical stimulation at 100Hz resulted in a significant increase in the mean cell migration distance (µm) (approximately 6%) compared to the control population ($p<0.05$) (Fig.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 ($p>0.05$).

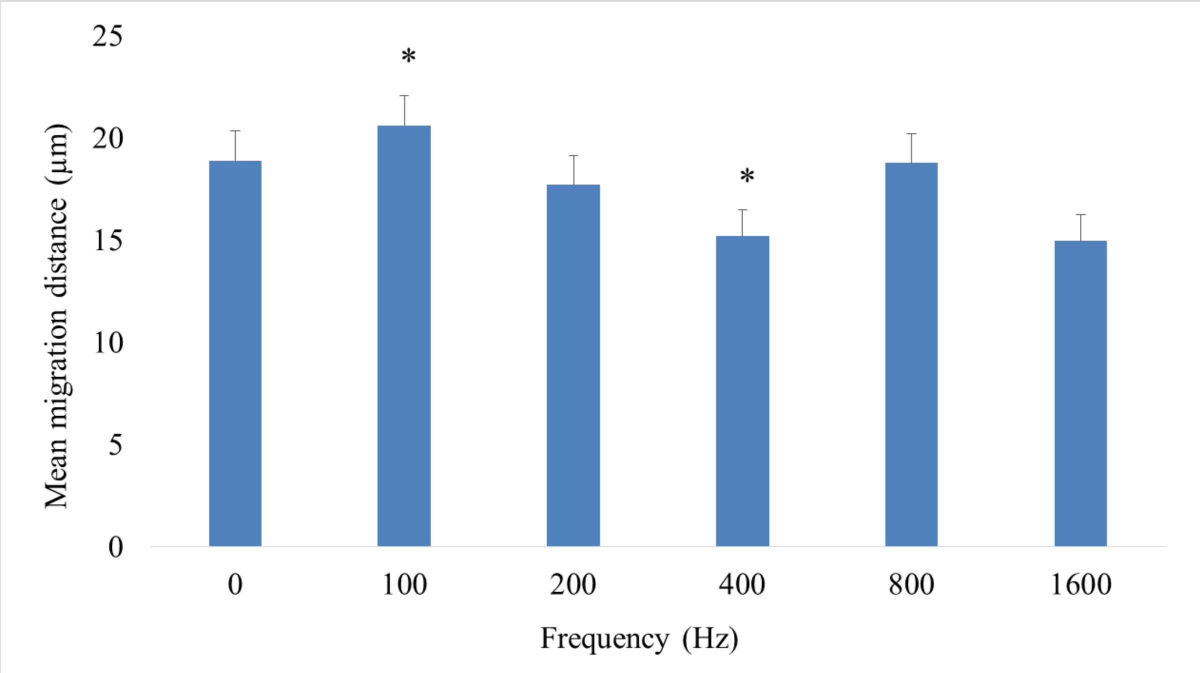

Fig. 3 Total distance travelled (µm) over 4 hours for L929 fibroblast cells (n=30) versus frequency (Hz) following mechanical stimulation. Error bars represent standard deviation. * denotes Significance ($P < 0.05$).

These results 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. To our knowledge this is the first example of investigating the effects of mechanical stimulation delivered via acoustic vibration on cell migration. The results are somewhat consistent with similar work reported in the literature. For example, Aryaei and Jayasuriya [10] found that mechanical stimulation, in the form of shear stress, can be used to enhance osteoblast adhesion and proliferation. Similarly, Ito et al [15] found that mechanical stimulation (100-1000Hz) of L929, HeLa and human umbilical vein endothelial cells (HUVECs) had no effect on cell morphology, or adhesion. In contrast, mechanical stimulation of mouse embryonic fibroblasts at 1 kHz 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.

These results are interesting as they indicate that the use of mechanical/acoustic stimulation may have potential therapeutic benefits for example, enhancing the wound healing process. However, more work is required to investigate the underlying mechanisms as well as the migratory response of other cell types, e.g. human dermal fibroblast and gingival fibroblast cells to mechanical stimulation and the effects of mechanical stimulation on collective cell migration. Our preliminary work in this area using wound healing assays indicates that the enhanced migratory rate following 100Hz migration diminishes 2-3 hours post stimulation. Therefore, we aim to investigate the effects of intermittent stimulation on cell migration e.g. hourly exposure to the stimulus.

3.3 *Effects of mechanical stimulation on cell viability*

In order to determine if the vibration-induced decrease 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 cell viability of LL24 or L929 cells at any of the frequencies used here. Trypan blue cell viability assays were also carried out and the results support those obtained from the MTT assays (data not shown).

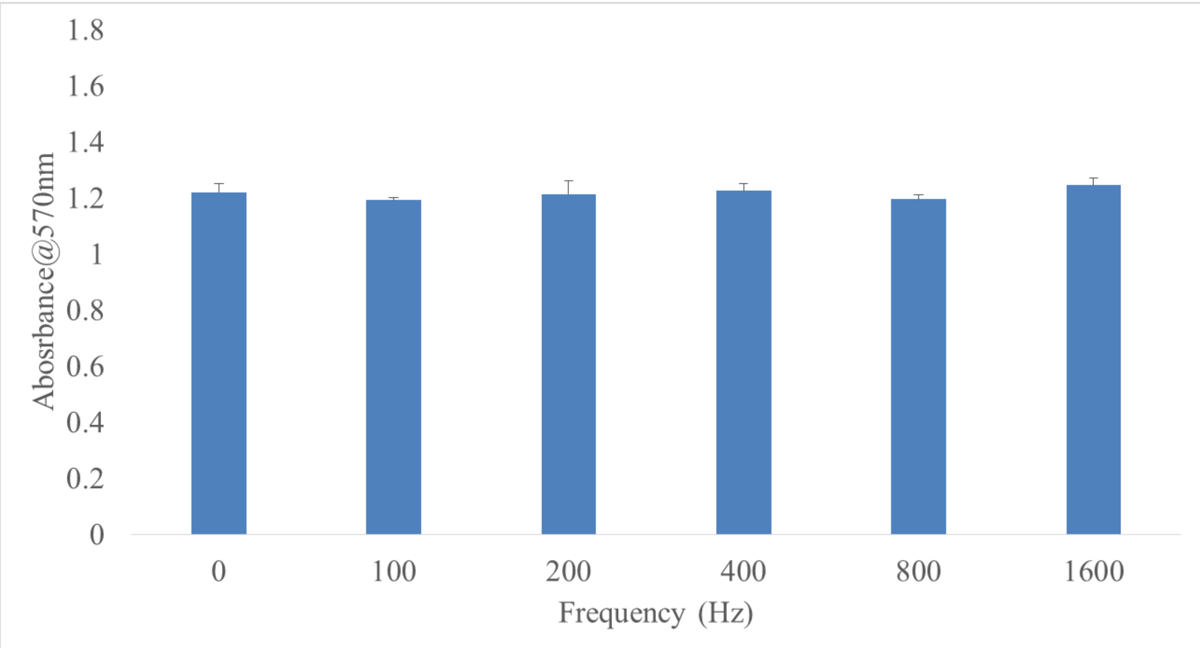

Fig. 4 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of LL24 cells for 5 minutes. Error bars represent standard deviation.

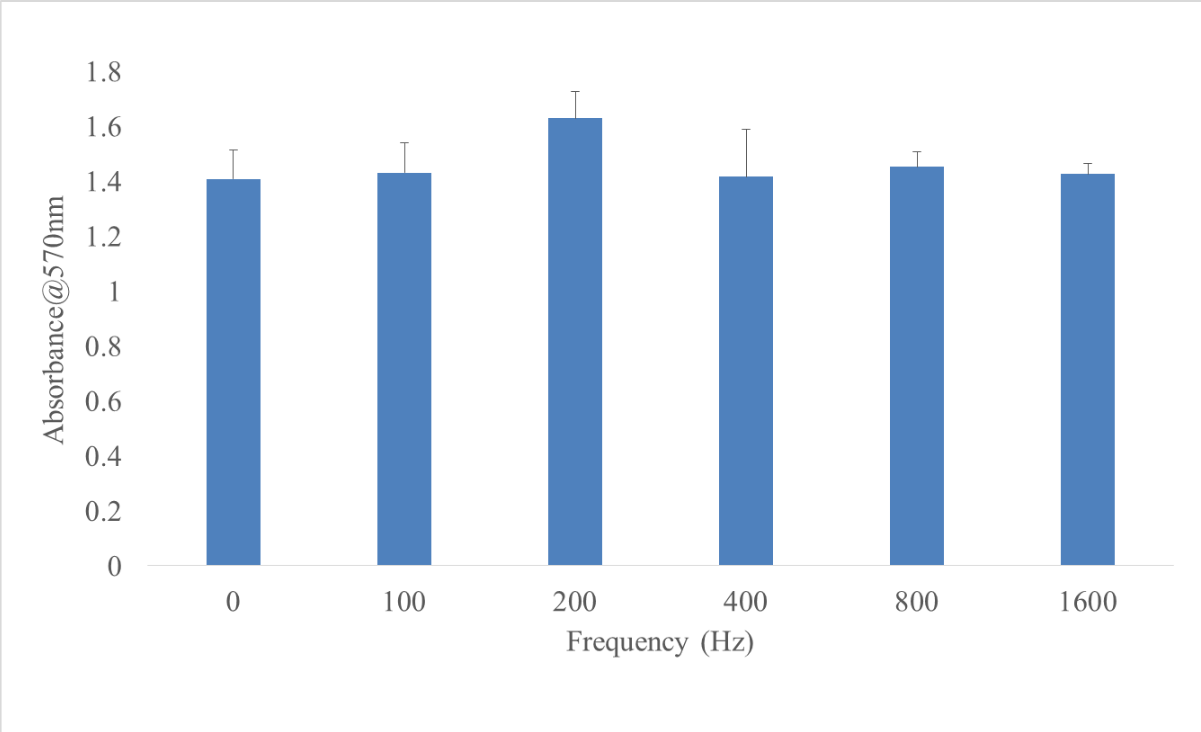

Fig. 5 MTT assay results showing absorbance versus frequency (Hz) following mechanical stimulation of L929 cells for 5 minutes. Error bars represent standard deviation.

3.4. 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 [16], the latter of which has been shown to stimulate cell migration [17]. 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 mechanical stimulation of both 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 Fig. 6) and an increase in stressfiber formation/density, compared to the control. However, there were no notable differences in the F-actin organisation as frequency increased (Fig. 6).

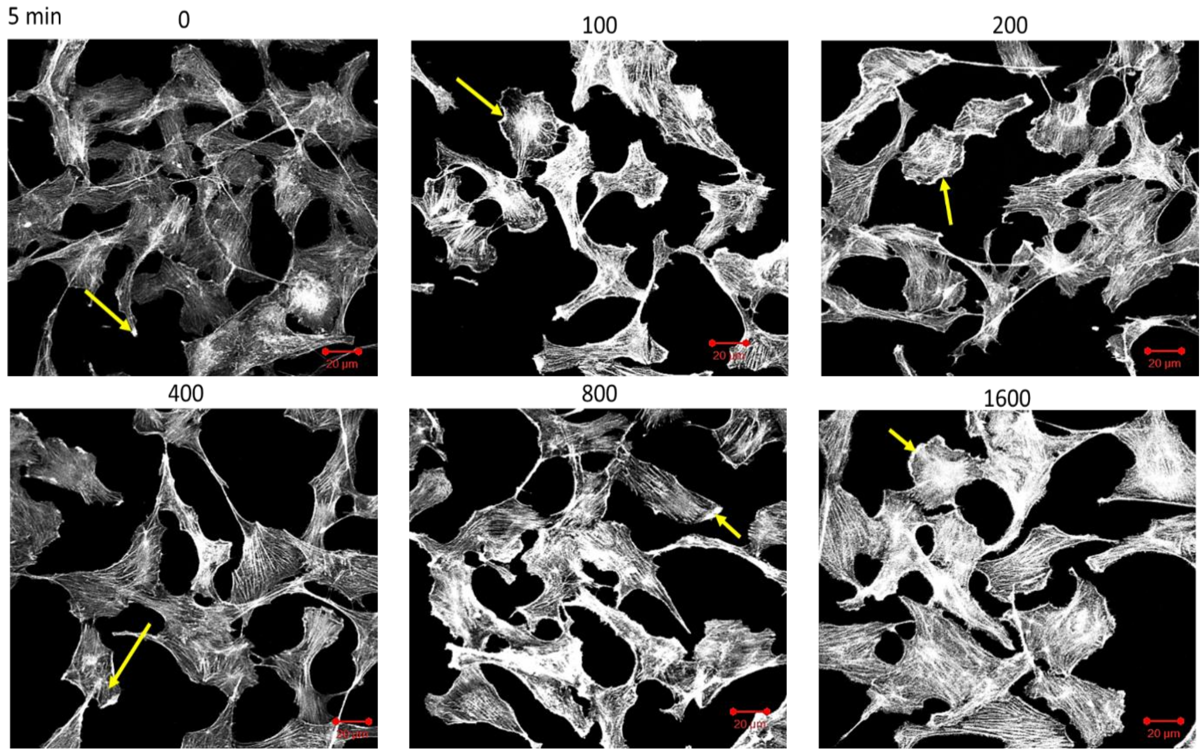

Fig. 6 F-actin organisation in LL24 cells following mechanical stimulation (0Hz to 1600Hz) for 5 minutes. Yellow arrows highlight lamellipodia/ruffling.

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 (Fig. 7).

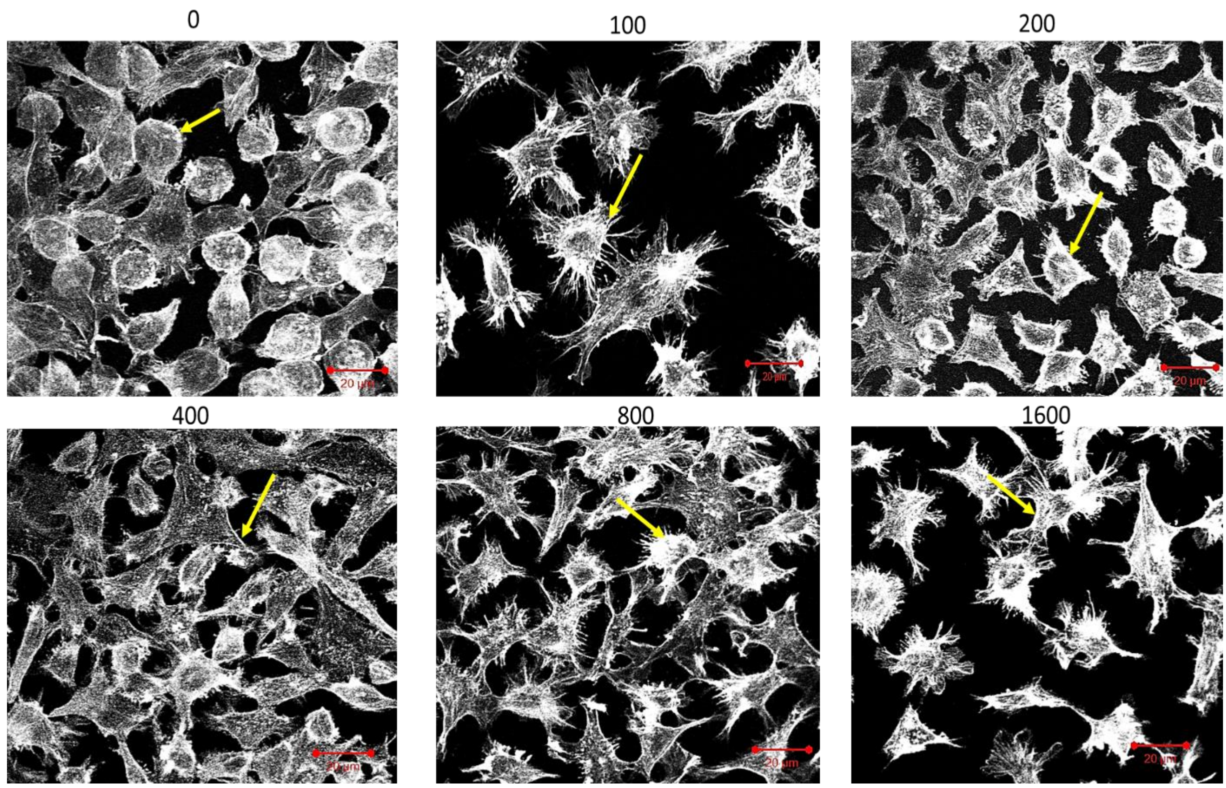

Fig. 7 F-actin organisation in L929 cells following mechanical stimulation (0Hz to 1600Hz) for 5 minutes Yellow arrows highlight lamellipodia/ruffling.

Following actin staining and confocal imaging a more detailed analysis was carried out to try and quantify changes to cell morphology. Morphological changes are highlighted in Fig.8 and are grouped into three categories; cells displaying a rounded morphology, cells displaying prominent filopodia and cells displaying prominent lamellipodia/ruffling.

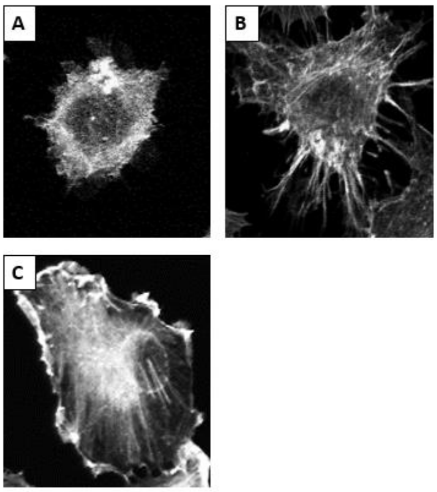

Fig. 8 Confocal microscopy showing (A) rounded shape morphology, (B) cells with filopodia and (C) cells displaying lamellipodia/membrane ruffling.

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 and number of cells with prominent filopodia was recorded. Fig. 9 displays the results for LL24 cells and Fig. 10 displays the results for L929 cells. As can be seen from Fig. 9, the control population contained cells that displayed a predominantly rounded morphology with many cells in this population also displaying some degree of 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 Fig. 10, 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.

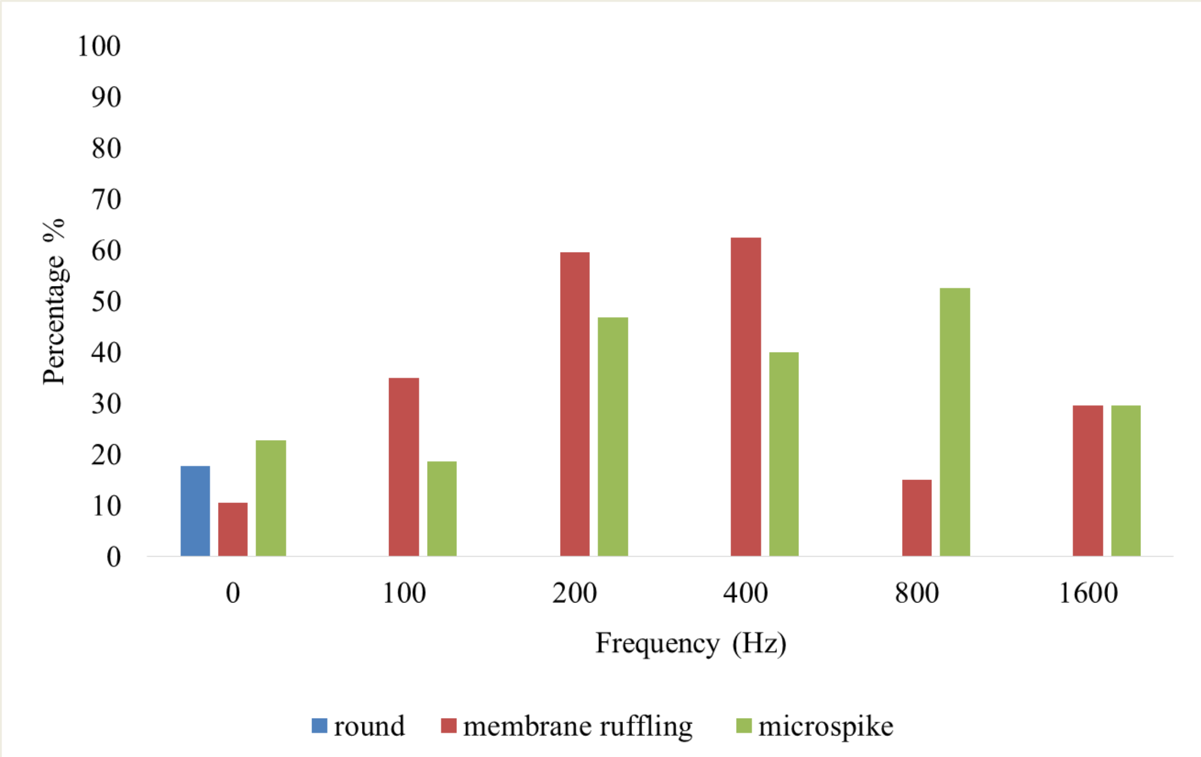

Fig. 9 Image analysis of LL24 cells stimulated for 5 mins showing the percentage of cells being round, membrane ruffling and filopodia.

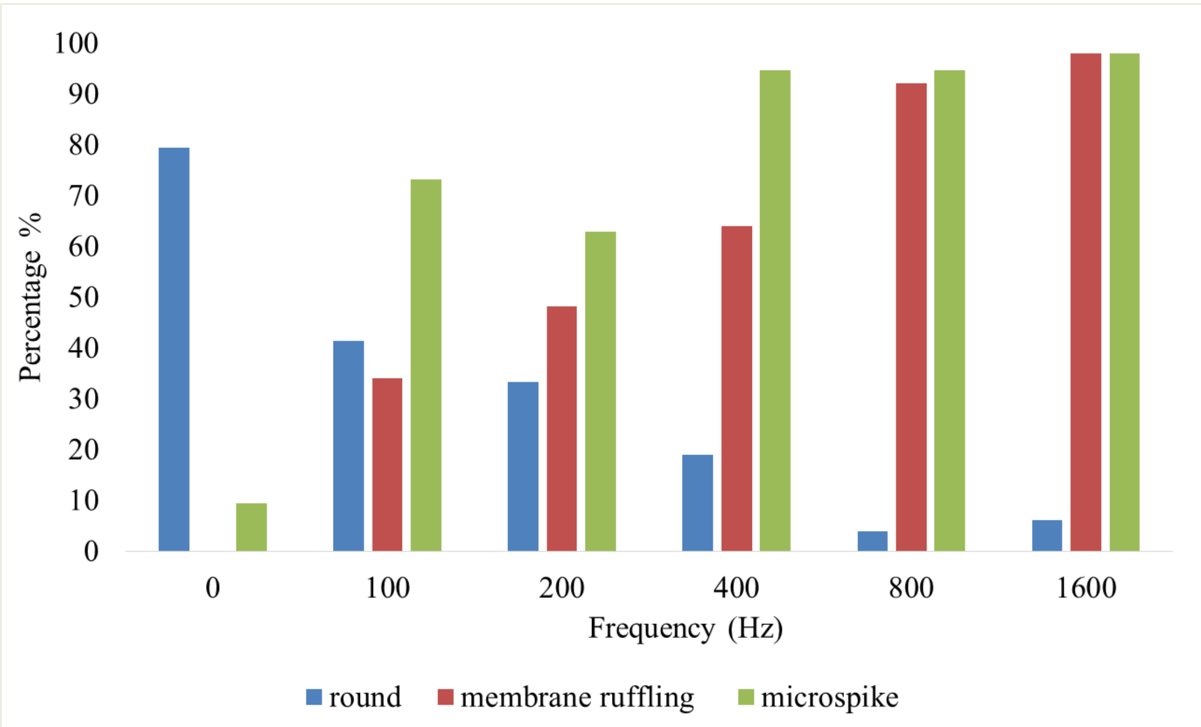

Fig. 10 Image analysis of L929 cells stimulated for 5 mins showing the percentage of cells being round, membrane ruffling and filopodia.

Two alternate 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) [18]. Although most cells in culture express both lamellipodia and filopodia, 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.

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 [19]. 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 [20] and was observed here for both cell lines. 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 clear 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 [21].

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.

This work set out to determine the effects of mechanical stimulation on fibroblast cell migration and to our knowledge this is the first time this has been explored. The results show that acoustic vibration can enhance (100Hz) and decrease (200-1600Hz) cell migration without affecting cell viability. At present, the underlying mechanisms behind these results are unknown and clearly more work needs to be done in this area so 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.

4. Conclusion

We have shown that mechanical stimulation (100-1600Hz) applied via acoustic vibration, can affect cell fibroblast cell migration in a frequency-dependent manner. In particular, mechanical stimulation at 100Hz significantly increased the mean cell migration distance for both cell types studied here. 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 the decrease in migration was less uniform when compared to that of the LL24 lung cells. 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. Acoustic vibration promoted more lamellipodia in the LL24 lung fibroblast cells, while the formation of filopodia was more prominent in the L929 cells.

The results obtained are interesting and may lead to developments in novel wound care technologies that exploit mechanical means to stimulate wound repair/regeneration.

Conflict of interest statement

There are no conflicts of interest.

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