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Control of Cellular Adhesion and Myofibroblastic Character with Sub-Micrometer Magnetoelastic Vibrations

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Abstract

The effect of sub-cellular mechanical loads on the behavior of fibroblasts was investigated using magnetoelastic (ME) materials, a type of material that produces mechanical vibrations when exposed to an external magnetic AC field. The integration of this functionality into implant surfaces could mitigate excessive fibrotic responses to many biomedical devices. By changing the profiles of the AC magnetic field, the amplitude, duration, and period of the applied vibrations was altered to understand the effect of each parameter on cell behavior. Results indicate fibroblast adhesion depends on the magnitude and total number of applied vibrations, and reductions in proliferative activity, cell spreading, and the expression of myofibroblastic markers occur in response to the vibrations induced by the ME materials. These findings suggest that the subcellular amplitude mechanical loads produced by ME materials could potentially remotely modulate myofibroblastic activity and limit undesirable fibrotic development.

Keywords

Mechanotransduction; magnetoelastic; vibrations

Introduction

The ability to repair damaged tissues by replacing lost cells is one of the paramount functions of living organisms. However, after sustaining damage to tissues, such as that caused by biomedical implants, the wound healing response may enter a phase of fibroplasia or fibrosis. This process can become pathogenic if it proceeds without control. Excessive fibrosis results from uncontrolled myofibroblastic activity, particularly the production and significant remodeling of extracellular matrix components (ECM)(Wynn, 2007). Myofibroblasts, derived from local mesenchymal cells (e.g. fibroblasts), are essential

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Conflict of Interest

The authors have no other conflicts of interest to declare.

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to the wound healing process, as they produce ECM components to promote wound contraction. This process allows epithelial or endothelial cells to reach the site of injury and repair damaged tissue (Wynn, 2007, 2008). When a persistent irritant, such as an implant, is present, the growth factors and cytokines that promote myofibroblastic activity are continually expressed, resulting in the overproduction of ECM components (Wynn, 2007). The excessive accumulation of these components at the soft tissue-implant interface can lead to the destruction of healthy tissue architecture and the mechanical instability of an implant, which presents a crucial impediment to the long-term success of many implantable devices (Wynn, 2007, 2008; Ziats et al., 1988).

Current treatments for reducing implant fibrosis include pharmaceutical options and pre-implantation design. Anti-fibrotic drugs attempt to alleviate the effects of fibrosis by controlling inflammation, myofibroblastic activity, and angiogenesis (Wynn, 2007, 2008), whereas biomaterial surface modifications focus on altering surface chemistry with biomaterial coatings that diminish the fibrotic response post-implantation (Anderson et al., 2008; Ziats et al., 1988). While these methods have attenuated the fibrotic response and have remarkably improved stable tissue integration, there is still no treatment process that ultimately prevents fibrous marsupialization of the soft tissue-implant interface (Anderson et al., 2008; Luttkhuizen et al., 2006; Lydon et al., 1985; Pendegrass et al., 2006; Ziats et al., 1988).

In this work, we explore the potential of a non-invasive method to remotely control cellular adhesion and behavior at the soft tissue-implant interface. The underlining principle of this study is based upon the innate properties of magnetoelastic (ME) materials. ME materials are magnetostrictive, which allows them to convert magnetic energy into mechanical energy and *vice versa* (Grimes et al., 2002). As a result, these materials change shape in response to a magnetic field. These changes are in the form of a uniaxial deformation and occur in response to DC and AC magnetic fields, resulting in a static or dynamic (vibration) strains (Grimes et al., 2002, 2011). The deformation seen by the ME material is a function of the initial length of the material and the strength of the applied magnetic field (Grimes et al., 2002, 2011), therefore the magnitude of this deformation can be controlled through pre-fabrication design of the ME material and the parameters of the applied magnetic field. However, the maximum possible deformation of ME materials is still on the order of sub-micrometers (sub-micron)(Grimes et al., 2002, 2011), which makes these materials good candidates for *in situ* use because their functionality does not present a physical danger to surrounding soft tissue. Additionally, the movement of these materials generates a magnetic field that can be remotely detected (Cai et al., 2000, 2001; Cai and Grimes, 2000; Grimes et al., 1999, 2000, 2002, 2011; Grimes and Kouzoudis, 2000; Jain et al., 2000, 2001; Kouzoudis and Grimes, 2000; Loisel and Grimes, 2000; Stoyanov and Grimes, 2000). This secondary magnetic field has been shown to be dependent on the mass applied to the surface of the ME material and the stiffness (Young's modulus) of the surrounding microenvironment (Grimes et al., 2002, 2011). For these reasons, ME materials have already seen use in a variety of biomedical applications by functioning as physical sensors for temperature (Jain et al., 2000, 2001), pressure (Grimes et al., 1999; Jain et al., 2001; Kouzoudis and Grimes, 2000), liquid viscosity and flow velocity (Grimes et al., 2000; Grimes and Kouzoudis, 2000; Loisel and Grimes, 2000; Stoyanov and Grimes, 2000), and

as chemical sensors for carbon dioxide, pH, and ammonia (Cai et al., 2000, p. 2, 2001; Cai and Grimes, 2000).

Previous work has shown that sub-micron vibrations induced by the ME materials (for simplicity, we refer them as the ME vibrations) are capable of preventing cell adhesion *in vitro* without inducing apoptosis or cellular necrosis (Vlaisavljevich et al., 2011), and that an ME material can be functionalized for long-term use *in vivo* without sacrificing its magnetostrictive functionality (Holmes et al., 2012; Vlaisavljevich et al., 2013). This work expands upon these findings by investigating the cellular mechanisms dictating the observed responses to ME vibrational loading. We hypothesize ME vibrations direct cell behavior through matrix adhesion contacts (focal adhesion complexes) that connect cells to the ME substrate (Chiquet et al., 2007, 2009). This process is known as mechanotransduction and occurs when mechanical loads create stresses on sites of focal adhesions. These stresses are conducted through the cell membrane or cytoskeleton and initiate signaling cascades that can induce changes in cell morphology, adhesion, or gene expression (Chiquet et al., 2003, 2007, 2009; Lambert et al., 1998; Wang et al., 1993). This process is essential for maintaining connective tissue homeostasis, as tissues must constantly adapt to a dynamic range of physiologic loads (Kjaer, 2004). Mechanotransduction has been investigated through many methods, such as axial stretching of elastic membranes (Wang et al., 2007) or imposing fluid shear stress on cell cultures (Chen et al., 1999). However, this process is not fully understood, and relatively little is known about the effects of sub-cellular mechanical loads on cell behavior. Therefore, we sought to accomplish three primary objectives in this work: 1) characterize how physical properties (mechanical parameters of applied vibrations) affect cell adhesion, 2) characterize the effect of ME vibrations on cell proliferation and morphology, and 3) determine if ME vibrations can influence myofibroblastic character.

Materials and Methods

The magnetoelastic material, Metglas 2826MB ($\text{Fe}_{40}\text{Ni}_{38}\text{Mo}_4\text{Bi}_8$) a commercial magnetoelastic film purchased from Metglas Inc. was used for all experiments. The material (thickness = 26 μm) was mechanically sheared into strips (5 mm $\times$ 12.7 mm) and annealed for 2 hours at 125°C to reduce the internal stress of the material. Annealed ME strips were coated with Parylene-C using a PDS 2010 LABCOTER™ 2 Parylene deposition system. Parylene-C coated ME materials were functionalized for cell adhesion by etching the Parylene-C surface for 30 seconds with O_2 plasma.

L929 fibroblasts (ATCC) were used for all experiments. Cells were maintained in culture medium composed of Dulbecco's Modified Eagles Medium D5796 supplemented with 10% FBS and 0.5% Penicillin-Streptomycin (ATCC). Cells were washed in PBS (pH 7.4) and Trypsinized (0.05% Trypsin/0.53 mM EDTA in HBSS; Cellgro) to induce cell detachment for experimental seeding. Live cell staining was performed using Calcein-AM (Invitrogen) and all imaging was performed on an Olympus BX51 upright microscope unless otherwise stated. Preliminary experiments established that cells (seeded at 1×10^4 cells/cm²) became adherent after approximately 6 hours and reached 70–80% confluence after approximately 48 hours at 37 °C and 5% CO_2 on plasma etched Parylene-C coated ME substrates.

To stimulate cells with sub-micron vibrational loads, ME materials were activated using the ME vibrational loading system previously developed (Vlaisavljevich et al., 2011). Control vibration negative groups (cells cultured on ME materials that were not magnetically activated) were seeded and imaged concurrently with experimental groups. Cellular adhesion data is presented as the percentage of remaining adherent cells in comparison to non-vibrated controls, unless otherwise indicated. The ME substrates, having a vibration resonant frequency of approximately 165 kHz (Holmes et al., 2012) were activated by an AC magnetic field (sweeping frequencies of 160–170 kHz with a step of 500 Hz).

All experiments were performed in triplicate unless otherwise stated, and all statistical analyses were performed using student's t-test on JMP software; p-values less than 0.05 ($p < 0.05$) were considered significant.

Load Profile

The magnetic field generated by the *in vitro* loading system was tuned to produce the maximum strain of ME materials (12 ppm - a limit predetermined by innate material properties (Grimes et al., 2002)). For the ME materials used in this study, this strain provides an approximate displacement of 0.15 μm , where displacement is defined as the product of strain and initial length (12.7 mm) of the ME materials.

In order to load cells with vibrations of lower magnitude, the frequency range of the applied magnetic field was adjusted to provide a vibrational amplitude two-thirds ($2/3$) of maximum magnitude, as further frequency shifts would substantially diminish the vibration amplitude. The change in vibration amplitude was verified in real time by monitoring the change in the secondary magnetic field response of the ME material. This magnitude of vibration provided an approximate displacement of 0.10 μm . ME vibrations of each magnitude were applied to cells for 1 hour following 48 hours of incubation. Vibrations of 0.15 μm were also applied for 30 minutes (half of a typical loading duration) to determine if cell response was dependent on loading time. The interval between each loading cycle was adjusted through the controlling software for the ME vibrational loading system. Cells were loaded with ME vibrations for 1 hour with 1 second or 10 second delays between each applied vibration. A 1 second delay is the minimum time required for the control system to activate, interrogate, and record data from ME materials (Vlaisavljevich et al., 2011), and a 10 second delay provided an order of magnitude change to understand how this recover time affects the cellular response.

Proliferation

ME vibrations were applied to cells cultured on plasma etched Parylene-C ME substrates after 6 or 48 hours of incubation. Live staining and imaging was performed on all samples immediately following loading application of the 48 hour samples. Changes in proliferation behavior were verified through the expression of Ki-67. Ki-67 was stained using Ki-67 (SP6) primary antibody (Abcam) with Alexafluor-488 conjugated secondary antibody and cell nuclei were stained using 4',6'-diamidino-2-phenyl-indole dihydrochloride (DAPI; Millipore). Ki-67 positivity was calculated by dividing the number of Ki-67 positive cells by the number of total cells (quantified via direct cell counting).

Morphology

Cells were fixed with paraformaldehyde, dehydrated with ethanol, and coated with 5 nm of platinum in preparation for imaging on a JEOL 6400-JSM scanning electron microscope. SEM micrographs were analyzed for shape factor and cell area using BIOQUANT OSTEO™ software. Shape factor is defined as cell 'roundness', where 0 corresponds to a straight line and 1 corresponds to a perfect circle, and cell area is the two dimensional area of coverage for an individual cell.

Cytoskeleton and Focal Adhesion Organization

Cells were fixed in paraformaldehyde, permeabilized with 0.1% Triton-X-100, and blocked with 1% BSA. Actin was stained using TRITC-conjugated phalloidin (Millipore), vinculin was stained with anti-vinculin (SPM227) primary antibody (Abcam) paired with Alexafluor-488 secondary antibody (Abcam), and cell nuclei were stained with DAPI. Shape factor and average cell area were quantified using BIOQUANT OSTEO™ software.

Western Blot Analysis

Cells were immersed in a lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, and 50 mM Tris, pH 8.0) and detached by scraping to produce cell lysates. Cell lysates were collected 24 and 48 hours after seeding for non-vibrated groups and 48 hours and 72 hours after seeding (with vibrations applied at 48 hours) for vibrated groups. These solutions were mixed at 1:1 with Laemmli loading buffer (Bio-Rad) prior to performing electrophoresis; protein content was normalized using BCA protein assay (Thermo Scientific). The electrophoresis gel was transferred to a 0.45 µm nitrocellulose membrane and blocked with 5% BSA. Anti-vinculin (SPM227), anti-FAK (EP695Y), anti-TGF-β1 RII (S250), and anti-GAPDH (GA1R - loading control) primary antibodies (Abcam), were used with horseradish peroxidase conjugated secondary antibodies (Santa Cruz) to identify bands of vinculin (130kDa), FAK (120kDa), TGF-β1 RII (60kDa), and GAPDH (40kDa), respectively. Protein was detected using ECL-plus (Thermo Scientific) with a LAS-4000 Fujifilm image reader.

Results and Discussion

In all experiments, treating adherent cells with ME vibrations for one hour reduced the number of adherent cells by 50 to 70%, which was statistically significant in every experiment. This result indicates that the effects of loading adherent cells with ME vibrations are consistent and reproducible across different cultures. By investigating the effect of loading parameters and characterizing the response of adherent cells, the discoveries in this work will provide a foundation for tuning or optimizing sub-micron mechanical loads to incite a desired cellular response.

Cell Adhesion is Dependent on Loading Parameters

Modulating the amplitude of applied vibrations revealed that cellular adhesion is dependent on the magnitude of applied vibrations. Both vibration amplitudes (0.10 µm and 0.15 µm) showed significantly lower levels of cell adhesion compared to non-vibrated controls as well as significantly different levels of cell adhesion in comparison to each other (Figure 1).

A regression analysis also showed that a relationship exists between cell adhesion and the duration of the ME vibration treatment.

The application of ME vibrations with a 1 second period resulted in significantly less cellular adhesion when compared to non-vibrated controls, but the application of vibrations with a 10 second period showed no significant differences in cell adhesion between either non-vibrated controls or samples loaded with a 1 second period (Figure 2). The lack of statistical significance between the 10 second period group and either the 1 second period or non-vibrated groups, which are significant from each other, suggests that the level of cell adhesion for the 10 second period group is in between the non-vibrated and 1 second period groups. It is possible that an extended interval (10 seconds) between subsequent vibrations fails to initiate the same signaling pathways that induce the changes in cell behavior observed from loading applications with shorter intervals (1 second). Conversely, this result could be attributed to differences in the total number of applied vibration cycles between groups (i.e. an extended delay interval results in fewer applied vibrations). The latter explanation is supported by data presented above, which demonstrates that longer loading times (1 hour) have a greater effect on cell adhesion than shorter (30 minute) loading times. By demonstrating that cell adhesion is dependent on both the magnitude and total number of applied ME vibrations, these results establish that ME vibrations affect cell adhesion in a dose-dependent manner.

ME Vibrations Reduce Cell Proliferation

Loading cells 6 hours (when cells become adherent) and 48 hours (when cells become 70–80% confluent) after seeding both provide significant reductions in cell adhesion (Figure 3). No differences in cell adhesion between samples loaded at 6 hours and samples loaded at 48 hours were observed. This result shows that ME vibrations can control cell adhesion in both early and late stages of cell proliferation, and suggests that ME vibrations can control proliferation.

In order to better understand the effect of ME vibrations on fibroblast proliferation, the expression of Ki-67, a protein expressed only in proliferating cells (Scholzen and Gerdes, 2000), was evaluated. Specific anti-body stains for Ki-67 showed a significant reduction in proliferating cells immediately following and 42 hours after cells were loaded with ME vibrations (Figure 4). This result verifies that ME vibrations can reduce the proliferative activity of fibroblasts. The exact mechanism may involve both the direct response of the fibroblasts to the ME vibrations themselves, in addition to an indirect effect due to the induced detachment and ultimate reattachment of the fibroblasts in response to the vibrations. In either case these data indicate that ME loading induce fibroblasts to express a more quiescent phenotype.

Cell Morphology Changes due to ME Vibrations

SEM micrographs suggest that cells respond to ME vibrations by becoming smaller and rounder in morphology, when compared to cells cultured on non-vibrated substrates (Figure 5A–B). A morphological analysis confirmed this observation, as significant differences in cell area and shape factor exist between cells loaded with ME vibrations and non-vibrated

controls (Figure 5C–D). In this analysis, cell area is defined as the two-dimensional space on the substrate surface occupied by each cell, and shape factor is a scale of “roundness” from 0 to 1, where 0 and 1 correspond to a straight line and a perfect circle, respectively. Thus, the observation that cells become both significantly smaller and rounder in response to ME vibrations provides evidence that sub-micron vibrational loads can reduce cell spreading. The distribution of cell area and shape factor more clearly illustrates this phenomenon, as a shift to a higher percentage of smaller, rounder cells in vibrated groups is apparent (Figure 5E–H). This observation demonstrates that phenotypic changes occur in response to ME vibrations in cells prior to detaching and in cells that retain adherence to the substrate surface. Similar studies of cell response to mechanical vibrations have been performed by applying low frequency (less than 20 Hz) uniaxial (Jungbauer et al., 2008) and biaxial (Xu et al., 2016a) strains to cells cultured on flexible membranes. These studies demonstrated that fibroblasts reorient their alignment in response to these low frequency strains. The loss of adhesion and phenotypic changes observed in this work indicate that the ME vibrations in the 10^5 Hz range elicit a different behavioral response. A similar response would likely be observed for different frequencies in this range, but different behaviors could potentially be triggered with ME vibrations at frequencies on a different order of magnitude (e.g. 10^4 or 10^6 Hz). However, testing these frequencies in these ranges requires the development of new ME material systems.

Modulation of Myofibroblastic Expression with ME Vibrations

The cytoskeletal organization (actin) and mature focal adhesion density (vinculin) of fibroblasts cultured on ME materials prior to vibrational loading resembles that of myofibroblasts (Hinz, 2010), and the application of ME vibrations results in a reorganization of cytoskeletal components and focal adhesion expression that is similar to a quiescent fibroblast (Figure 6Ai–iv). Additionally, cell area and shape factor analyses (Figure 6Av–vi) correspond with the results observed from morphological analyses (Figure 5). Western blots for vinculin and FAK verify that ME vibrations modulate mature focal adhesion formation, as indicated by a decrease in vinculin and FAK expression in cell lysates collected immediately after the application of ME vibrations (Figure 6B). Mechanical tension has been shown to play an integral role in guiding the differentiation of fibroblasts (as well as other mesenchymal cell types) into myofibroblasts (Hinz et al., 2001), which, due to the high elastic modulus of conventional tissue culture dishes, will induce fibroblasts to express a myofibroblastic phenotype *in vitro* (Hinz, 2010). The elastic modulus of the ME material (1.0–1.7GPa (Grimes et al., 2002)) is likely to induce the same phenotypic change in fibroblasts as conventional culture dishes. Thus, it is likely that fibroblasts cultured on ME substrates without magnetic activation are expressing a myofibroblastic phenotype, which is consistent with these observations. The reversal of this phenotype following the application of ME vibrations demonstrates that these loads induce myofibroblastic cells to express a more quiescent phenotype. Further, substrate stiffness has also been linked to cell adhesion. Thus, vibrations may promote a pseudo-soft matrix condition at this submicron level. Cells are believed to extract information from their environmental conditions on the submicron scale, and cell sensing of matrix stiffness is a process that can be reset when matrix modulation occurs (Ricca et al., 2013; Liu, 2016). In soft matrix environments, others have suggested that two mechanisms contribute to a cells’ time-dependent behavior: 1) activation

of integrin, and 2) dissociation of integrin-ECM complexes, a result of a lower ability to resist forces (Xu et al., 2014, 2016b). These mechanisms may also be at play in this work tying vibration character (i.e. increasing amplitudes emulate softer matrix environments) to changes in cell adhesion and additionally a more rounded phenotype. This discovery has the potential to fulfill a significant clinical need, as myofibroblasts are often responsible for many fibrotic pathologies (including implant fibrosis) once they begin uncontrollably replacing healthy parenchymal tissue with collagenous scar tissue (Hinz, 2010; Wynn, 2007, 2008).

The observed increase in vinculin and FAK expression in cell lysates collected 24 hours after loading with ME vibrations indicates that mature focal adhesions begin reforming over time. In addition to the formation of contractile actin stress fibers and mature focal adhesions, myofibroblasts can also be characterized by the expression of alpha-smooth muscle actin (α -SMA), and TGF- β 1 RII (Hinz et al., 2001; Hinz, 2010; Schmid et al., 1998). However, the expression of α -SMA is common in a variety of other healthy cell types (such as vascular smooth muscle cells)(Schmid et al., 1998). Therefore, the expression of TGF- β 1 RII in response to ME vibrations was evaluated. TGF- β 1 RII expression in cells cultured on ME materials increased over time prior to vibrational loading and decreased in response to ME vibrations, which is consistent with previous results (Figure 6B). Interestingly though, following the application of ME vibrations, the expression of TGF- β 1 RII did not increase over time (Figure 6B). This result suggests ME vibrations have an enduring effect on myofibroblastic expression even though focal adhesions begin reforming over time. Furthermore, excessive expression of TGF- β 1 RII has been directly correlated to hypertrophic scarring (a form of fibrosis)(Schmid et al., 1998). TGF- β 1 RII expression is also required to bind with TGF- β 1, the primary cytokine regulator of myofibroblastic activity (Hinz et al., 2001). Thus, these results indicate that ME vibrations can reduce the myofibroblastic expression of cells in all stages of myofibroblastic differentiation, and may reduce the susceptibility of quiescent fibroblasts to becoming myofibroblastic or even reverse the differentiation of myofibroblastic cells. The exact mechanisms responsible for this phenomenon have not been completely elucidated, but behavioral similarities between cells cultured on soft substrates and cells loaded with ME vibrations suggest these loading parameters trigger the same signaling pathways as a soft ECM microenvironment, promoting a pseudo-soft matrix condition at the submicron level. Investigating this connection will be the focus of future work.

Conclusion

Fibrosis resulting from the uncontrolled myofibroblastic activity is a major obstacle preventing the stable integration of many implantable devices. Sub-micrometer vibrations in the kHz range, have been presented as a novel approach to modulate the excessive production of ECM components and significant remodeling of healthy tissue architecture. This work has established that fibroblasts exhibit a dose-dependent response to the sub-micron vibrations, and has provided evidence that these vibrations induce fibroblasts to express a quiescent phenotype as demonstrated by a reduction in proliferation behavior and characteristic changes in cell morphology. Further, the results of this work suggest that these sub-micron vibrations can remotely control the expression of myofibroblastic activity as

evidenced by their ability to modulate the organization of the actin cytoskeleton, formation of mature focal adhesions, and the expression of the myofibroblastic marker TGF- β 1 RII. Overall, this work expands upon the clinical potential of incorporating ME materials as a part of the implant to treat fibrosis based on their unique ability to remotely control myofibroblastic activity.

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Figure 1: Cell adhesion is dependent on vibration amplitude and loading duration.

Qualitative images of cell adhesion on ME materials that are non-vibrated (A), loaded with 0.10 μm (B), and 0.15 μm (C) vibrations. Inset shows cell adhesion after 30 minute loading with 0.15 μm vibrations. Quantitative assessment (D) shows significantly ($p < 0.05$) different levels of cell adhesion on each loading group, demonstrating that the amplitude of applied vibrations can selectively control cell adhesion to a substrate. Regression analysis (E) shows significant ($p < 0.05$) probability that cell adhesion is also dependent on duration of vibrational loading. *,† indicate statistically lower ($p < 0.05$) levels of cell adhesion than non-vibrated controls and all other groups, respectively.

Figure 2: Cell adhesion is dependent on the vibration period (time between applied vibrations). Qualitative images of cell adhesion on ME material surfaces that are non-vibrated (A), vibrated with a delay interval of 10 seconds (B), or vibrated with a delay interval of 1 second (C). Quantitative assessment of cell adhesion (D) shows significantly less cell adhesion on samples with a 1 second delay interval in comparison to non-vibrated controls, but no significant differences in cell adhesion were observed between any other groups. This result suggests that the period of sub-micron vibrations effects the response of adherent cells. Visual representation of differences in delay interval between loading groups (E). All vibrations were 0.15 μm in magnitude. * statistically significant ($p < 0.05$) differences in cell adhesion compared to non-vibrated control.

Figure 3: Submicron ME vibrations can modulate cell adhesion during early or late stages of cell proliferation.

Fibroblasts cultured on non-vibrated ME materials (A) and ME materials loaded with submicron vibrations (0.15 μm; 160–170 kHz) 6 hours (B) or 48 hours (C) after seeding; all groups were stained and imaged after 48 hour vibration. Significant ($p < 0.05$) differences in cell adhesion, quantified via direct cell counting, were observed between both loading groups and non-vibrated controls (D) but no significant differences in cell adhesion were observed between loading groups. This observation suggests that sub-micron vibrations may also effect proliferation behavior in addition to inducing immediately detachment. * indicate statistically significant differences ($p < 0.05$) in cell adhesion compared to non-vibrated control.

Figure 4: ME vibrations reduce proliferative character of fibroblasts.

Cells, fixed with 4% paraformaldehyde immediately (A-B) or 42 hours (C-D) after loading application (0.15 μ m from 150–170 kHz applied 6 hours after seeding), were stained using primary Ki-67 anti-body (Abcam) with Alexfluor-488 conjugated secondary anti-body (Abcam) and DAPI (Millipore) to visualize Ki-67 presence and cell nuclei, respectively. The Ki-67 positivity index (E-F), defined as the ratio of Ki-67 positive cells to total cells as determined by direct cell counting, was significantly different between non-vibrated and vibrated groups for both time points. * indicate statistically significant differences ($p < 0.05$) in Ki-67 positivity compared to non-vibrated controls.

Figure 5: ME vibrational loading induces a reduction in cell spreading.

Sub-micron vibrations (0.15 μm , 160–170 kHz) were applied 48 hours after seeding. Cells imaged on a JEOL JSM-6400 SEM (A-B) and measured for average area (C) and shape factor (D) using ImageJ software. Quantitative analysis showed significant differences in cell area and shape factor between vibrated and non-vibrated groups. The distribution of average cell area (E) and cell shape factor (F) is presented as a 20-bin histogram. * indicate statistically significant ($p < 0.05$) differences in cell area and shape factor compared to non-vibrated control.

Figure 6: Changes in cytoskeletal organization, focal adhesion formation, and expression of TGF- β 1 receptors occur in response to ME vibrations.

Following vibrational loading (applied 48 hours after seeding), cells were fixed and stained using phalloidin (Invitrogen) (Ai-Aii) and anti-vinculin antibody (Abcam) with FITC conjugated secondary (Abcam) (Aiii-Aiv) to mark the actin cytoskeleton and sites of focal adhesions (vinculin), respectively. Inset shows magnified view of actin fibers. Fluorescent images, quantified using ImageJ software, showed a significant reduction in average cell area (Av) and increase in cell shape factor (Avi). Western blot analyses was performed for vinculin (Avii), focal adhesion kinase (FAK; Avii), and TGF- β 1 RII (Bi)

using anti-vinculin (Abcam), anti-FAK (Abcam), and anti-TGF- β 1 RII primary antibodies with HRP-conjugated secondary anti-bodies (Santa Cruz). Endogenous TGF β -1 (Bii) as determined by ELISA assay demonstrates that sub-micron vibrations do not significantly affect the expression of TGF β -1. Lysates were collected 24 hours (non-vibrated; Biii), 48 hours (non-vibrated and vibrated; Biv-v) and 72 hours (vibrated; Bvi) after seeding. Results show vinculin and FAK expression increase from 24 to 48 hours, decrease after vibrational loading, and increase again after loading (72 hours). TGF- β 1 RII expression also increases from 24 to 48 hours and decreases after vibration, but remains low after loading. * indicate statistically significant differences ($p < 0.05$) in cell area and shape factor compared to non-vibrated control.