INVESTIGATION OF MECHANICAL VIBRATION EFFECTS ON BREAST CANCER CELLS

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ABSTRACT

INVESTIGATION OF MECHANICAL VIBRATION EFFECTS ON BREAST CANCER CELLS

In this doctoral dissertation, low magnitude mechanical signals (LMMS, <1g in magnitude) were used to test the stress shielding model hypothesized on breast cancer cells. The hypothesis was that the breast cancer cells will be sensitive to mechanical vibrations and will respond to these vibrations. It was similarly used to test the adipogenic differentiation of Lamin A/C knockdown (by siRNA) bone marrow-derived mesenchymal stem cells. It is known that Lamin A/C plays a role in the nucleus and intracellular organization in these cells and affects gene expression by chromatin regulation. The hypothesis was that if these cells are deprived of the organization for the nucleus, they will be sensitive to mechanical vibrations, but that the mechanical vibrations cannot restore the effect of lamin A/C on gene regulation.

We investigated the effects of high-frequency low-density mechanical signals (LMMS) on cell proliferation, apoptosis, cell cycle, protein expression, differentiation, cytoskeleton and phenotypic change processes.

According to findings, LMMS caused cell cycle arrest in the aggressive type of breast cancer cells and slowed proliferation. Non-aggressive breast cancer has not responded to LMMS. In mammary epithelial cells, LMMS has not shown an effect that triggers proliferation. In the mesenchymal stem cell model, Lamin A/C knockdown accelerated adipogenic differentiation. Although LMMS in these cells decreased the rate of adipogenic differentiation, it was not sufficient to restore the baseline.

ÖZET

MEKANİK TİTREŞİMLERİN MEME KANSERİ HÜCRELERİ ÜZERİNDEKİ ETKİLERİNİN ARAŞTIRILMASI

Bu doktora tezinde, yüksek frekanslı düşük yoğunluklu mekanik sinyaller (LMMS) meme kanseri hücreleri üzerinde hipotezlenen “stress shielding” modelini test etmek için kullanılmıştır. Meme kanseri hücrelerinin mekanik titreşimlere duyarlı olacağı ve bu titreşimlere yanıt oluşturacağı hipotezlenmiştir. Benzer şekilde kemik iliğinden alınmış ve siRNA uygulaması ile Lamin A/C knock down edilmiş mezenkimal kök hücrelerin adipojenik farklılaşmalarını test etmekte kullanılmıştır. Bu hücrelerde

Lamin A/C nin çekirdek ve hücre içi organizasyonda rol oynadığı, kromatin regülasyonu ile gen ekspresyonunu etkilediği bilinmektedir. Bu hücrelerin çekirdek için organizasyondan mahrum kalsalar da mekanik titreşimlere duyarlı olacağı ancak mekanik titreşimlerin gen regülasyonu üzerinde lamin A/C nin kaybolan etkisini geri getiremeyeceği hipotize edilmiştir.

Yüksek frekanslı düşük yoğunluklu mekanik sinyallerin (LMMS) bahsi geçen hücre modellerinde proliferasyon, apoptoz, hücre döngüsü, protein ekspresyonu, farklılaşma, hücre iskeleti ve fenotipik değişim süreçlerindeki etkileri incelenmiştir.

Bulgulara göre LMMS; agresif tip meme kanseri hücrelerinde hücre siklusunda arreste yol açarak çoğalmayı yavaşlatmıştır. Agresif olmayan tip meme kanserinde ise LMMS’e karşı yanıt oluşmamıştır. Meme epitel hücrelerinde LMMS, çoğalmayı tetikleyen bir etki göstermemiştir. Mezenkimal kök hücre modelinde ise, Lamin A/C knock down adipojenik farklılaşmayı hızlandırmıştır. Bu hücrelerde LMMS, adipojenik farklılaşma hızını azaltsa da kontrol hücreleri ile aynı seviyeyi yakalamakta yetersiz kalmıştır.

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CHAPTER 1

INTRODUCTION

1.1. Mechanical Signals and Mechanobiology

1.1.1. Mechanobiology

Development of organs, maintenance, and functions of tissues, differentiation and growth of cells depend on mechanical forces as well as chemical environment and genetic base [1-3]. Human body consists complex hierarchical structures that are all regularly exposed to mechanical forces and rearrangements [4]. The most obvious example of an organ system where mechanical forces are apperant is the musculoskeletal system. Not only musculoskeletal system functions in its own domain to provide basis and support for locomotion, and contraction, it also supports other organ systems such as in the movement of food in gastointestinal system, and pumping of the blood for circulatory system [5]. Function of all other organ systems also involve mechanical loads, such as reception of sound or pressure by the nervous system, or volumetric capacity of respiratory system [6-8]. The functional health of these systems may depend on not only reception of external mechanical loads, but also creation of internal mechanical loads as well.

Biomechanics is the study of structure and function in living systems by using principals of mechanics. In 1973 when the International Society of Biomechanics was first founded, biomechanics field was only focused on mechanics of movement of human beings [9, 10]. But then, this field has expanded to different species and different organ systems, tissues and cells. Biomechanics focuses on how organs and systems behave during exercise or in basic movements such as walking [11, 12], swimming [13], running [14], and what are the specific conditions that apply in sports like basketball [15], cycling [16], tennis [17, 18], golf [19] and martial arts [20]. Musculoskeletal system is in the focal point of most biomechanics studies. While mechanobiology focuses on mechanical force transduction (as in form of activation or deactivation of signalling pathways) and response

generation (as a functional or phenotypical change) in cellular and molecular level. Wolff's law, stating that bone remodels according to the forces it faces, provides a base for mechanobiology phenomenon [21-23]. This "form follows function" concept can be best seen in tennis players arm condition [24]. Their bones in their dominant racket using arms are significantly thicker compared to their contralateral arm (Figure 1.1).

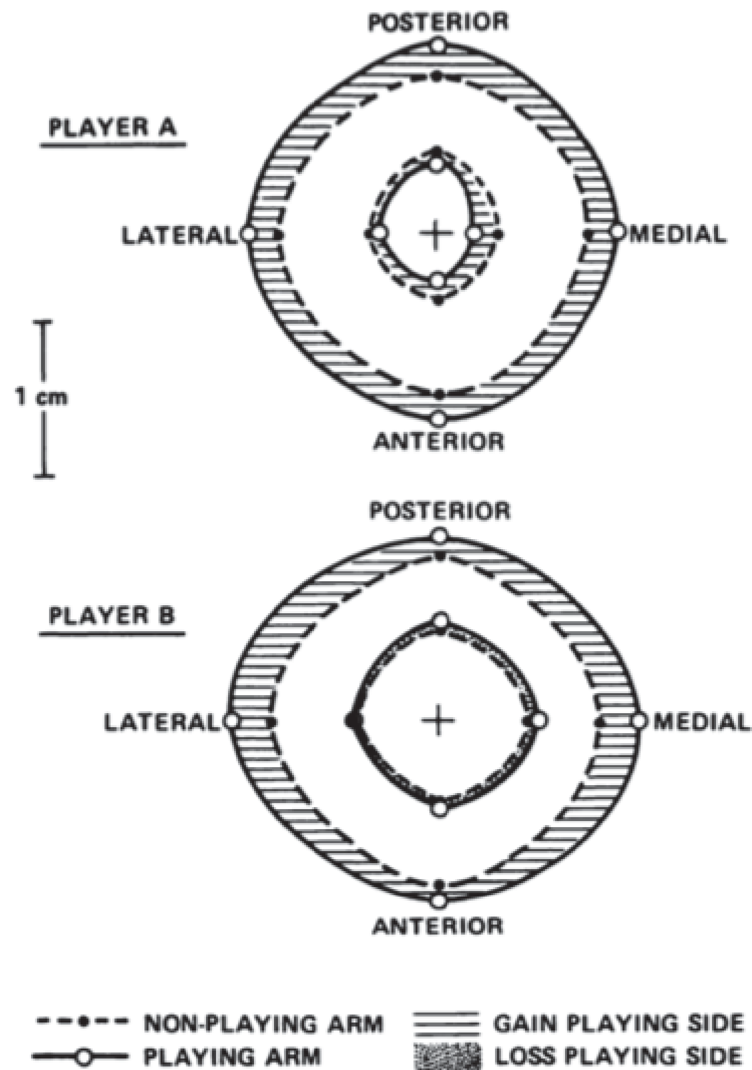

Figure 1.1. Tennis arm [25]. Dominant racket using arms are thicker.

Functional adaptation to mechanical signals is not specific for bone tissue [26]. Blood flow in vasculature, stretching in a muscle and peristaltic movement in the esophagus are examples of mechanical cues for functional regulation [27]. In the cells, chromosome movement, cell contractility and motility are the basic examples of

processes that have mechanical regulators [28].

Mechanobiology focuses on both mechanosensation and mechanotransduction. Mechanosensation is the term that refers to perception of the mechanical stimuli to generate a response *via* intracellular signal pathways [29]. Mechanosensation is a broadly conserved aspect in organisms, starting from early metazoans [30]. Translation of the mechanical stimuli to a response is enabled by mechanotransduction [31]. Mechanical stimuli are perceived by mechanosensors, but, transmission of mechanical stimuli into a response is not specific for mechanoreceptor cells, mechanotransduction is an ability that all cells have [32].

Cellular sensing of mechanical signals is dependent on the signal properties and cellular characteristics. Mechanotransduction starts at the cellular membrane. Signals are received by integrins and receptors to be delivered through the cellular membrane. Mechanical signals are then transmitted through the cytoplasm *via* cytoskeleton and reach the nucleus. After it is processed in the nucleus, the cell generates an appropriate response, such as a specific gene expression related to growth, migration or differentiation.

Extracellular matrix (ECM) is one of the most important actors in the transduction of mechanical cues. As an example, it mediates force transfer in muscle to sensory cells *via* strech receptors [33]. Properties of extracellular matrix are important in force distribution and transduction to cells. Flexible ECM makes it easier to transmit even the slightest deformation while harder ECM requires bigger deformations created for the force to reach the cells [34].

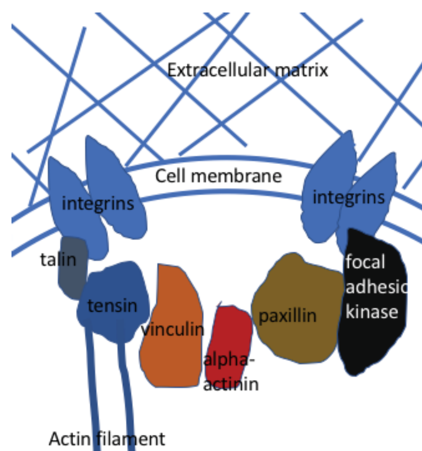

Figure 1.2. Focal adhesion complex.

Integrin receptors on the cellular membrane are the most deliberately characterized force sensors of the cells [35]. Integrin proteins are formed by heterodimers of one alpha and beta subunits that go through the cellular membrane [36]. Integrins, which have more than 20 types characterized, are not spreaded throughout the cellular membrane evenly [36]. They have regions where they are clustered, which are called "focal adhesions" [36, 37].

These focal adhesions are complexes formed at stress fiber ends, and several other cytoskeletal associated proteins such as vinculin or talin [38-40]. These proteins connect integrin mediated focal adhesions to the cytoskeleton (Figure 1.2). Cytoskeleton is another key element in the mechanotransduction. It is a network of microfilaments, microtubules and intermediate elements and acts on the shape, stiffness, inner molecular transport of the cell [1, 41]. Cytoskeleton enables the cell to transmit the force both from inside to outside and from outside to inner cell [42]. These mechanical cues play important roles on important biological functions such as stem cell differentiation, migration or formation of a tumor [43-45]. When the mechanical cues are transmitted into the cell, the physical state changes may trigger specific molecules to expose certain binding sites or enable phosphorylation to trigger signalling pathways [46-48]. For example in Drosophila embryos, environmental mechanical cues are of essential importance for differentiation and developmental stages [49]. Changes in these environmental forces lead to changes in differentiation.

Cytoskeleton also generates stability in the cell *via* balancing the forces *via* actomyosin filaments creating a tension in the cell. By this tension, cell stays in a balanced state, by adhesions to extracellular matrix and microtubules inside. This phenomenon is named as "tensegrity" and used a powerful tool in computational models to predict mechanical behaviours of a cell (Figure 1.3) [1, 3].

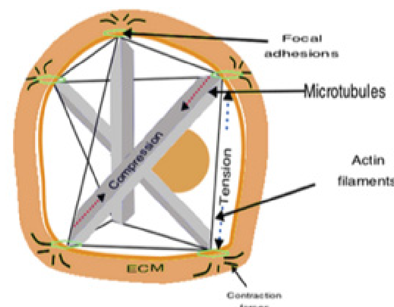

Figure 1.3. Tensegrity in schematic form in an animal cell [50].

Mechanical forces, actin of cytoskeleton, may lead to a response in change in cell stiffness, morphology or cell migration [42, 51-53]. But recent studies also showed that cells do not need an attachment point for migration. Lammermann et al. showed that dendritic cells can move on collagen matrices without any attachments involving integrins [54, 55]. This non-attached state provides a low adhesion to the extracellular environment and makes the migration process easier.

The signal transmitted through cytoskeletal actin fibers, then reaches to nucleus. The nuclear envelope is connected to actin cytoskeleton *via* the protein complex LINC (Linker of Nucleoskeleton and Cytoskeleton; Figure 1.4) [56]. There are several proteins characterized to be involved in this complex such as, SUN proteins and NESPRINs [57]. Through this complex; the mechanical signal reaches nucleoskeleton, which is made up of lamins [58]. Lamin nucleoskeleton serves as an anchor for the chromatin, defining euchromatin and heterochromatin sites [59]. Transmitted force, *via* its effect on nucleoskeleton, may lead to changes in gene expression [59-61].

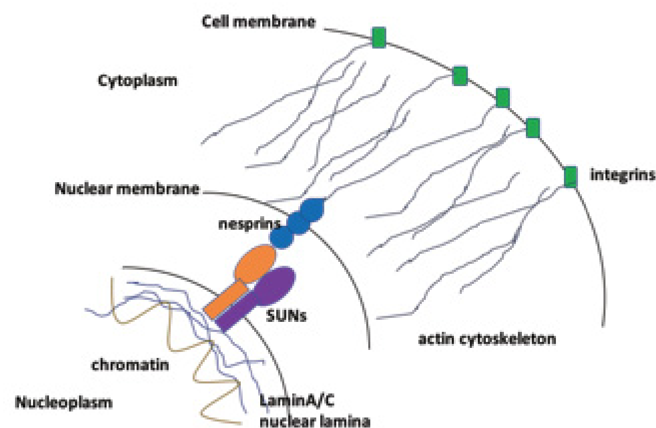

Figure 1.4. LINC connecting cytoskeleton and nucleoskeleton.

All the cells in the human body live on adapting to the mechanical forces that affect them. These forces organize the physiology of some cell types during development and homeostasis, as well as for some cells (e.g. muscle and bone cells). In situations such as diminution or complete loss of mechanical forces (due to long-term bed rest or space travel), functional and organizational disturbances occur in cells [62, 63]. Likewise, cancer cells have a distorted organization in cellular tissues, both nuclear and cellular infrastructure [64-66].

1.1.2. Mechanical Signals

There are four basic forces, known as fundamental forces: gravitational, electromagnetic, strong nuclear forces and weak nuclear forces [67, 68]. These forces are active in all living beings. Gravitational force of the earth, is modeled as a continuous classical field [68] translated to different forms in living systems, organs, tissues and cells, such as fluid shear, compression, tension etc. (Figure 1.5) [69-71]. A body reacts to a force, by accelerating or changing its velocity, to an extent inversely as its mass. Everything else is a consequence of the gravitational force on bodies [68].

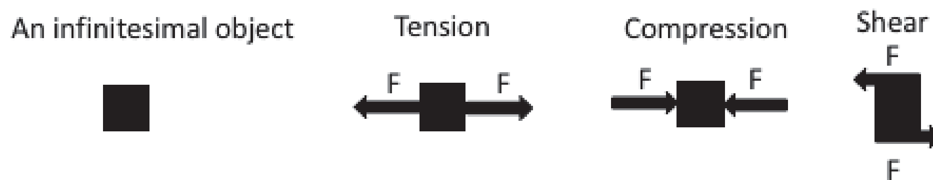

Figure 1.5. Representation of physical forces on an infinitesimal object.

These forces are active regulators of biological functions in both health and disease conditions [72-74]. But they are also used to study biological functions and their medical benefits. Motion of a cell or a blood cell in a vein, creates shear stresses and pressure by its motion relative to the surrounding fluid [75]. For a stationary cell, blood flow in the vein, for epithelial cell as an example, create shear stress and pressure [76]. Muscle contraction and extracellular matrix adhesion also creates forces on surrounding cells [77, 78]. External application of mechanical forces helps in regulating cell and tissue behaviour in disease conditions.

1.2. Cellular Responses to Mechanical Signals

Tension changes cell shape and cytoplasmic pressure. The difference between extracellular and intracellular pressure is balanced *via* cell surface tension. In the cell, the actin network by the plasma membrane forms a cell cortex to balance cell surface tension.

So the cell surface tension is directly transduced to the cytoskeleton [79]. Tension was reported to increase proliferation in aortic endothelial cells *in vitro* [80]. Tensional stress on an *in vivo* model of bone distraction resulted in increase in bone morphogenetic factor expression, specifically BMP2 and BMP4 [81]. In mouse melanoma cell line, B16F1, tension resulted in increased microtubule outgrowth [82].

Compressive forces are natural for chodrocytes and dental cells. Compressive stress in embryonic stem cells resulted in chondrogenic differentiation on PDMS (polydimethylsiloxane) scaffolds [83]. In periodontal ligament cells, compression forces induce osteoclastogenesis *via* NF-κB (nuclear factor kappa B) activator upregulation [84]. In human dental pulp cells, compressive stresses was reported to promote cell cycle and increased expression of BMP2 in short term [85]

Compressive forces are also reported on mouse bone marrow mesenchymal cells on collagen matrices to promote upregulation of osteogenic genes and mineralisation [86].

Shear forces are most apperant in vasculature as fluid shear resulted from blood flow. Endothelial cells are constantly exposed to flow shear and act as a barrier for the underlying tissue. Shear stress applied *in vitro*, was reported to regulate cell orientation and shape in endothelial cells [87]. Cells located in regions of shear stress aligns according to blood flow, ressisting the drag [88, 89]. Cellular membrane itself is suggested to act as a mechanosensor in sensing fluid shear [90]. In the cell, the shear force triggers G protein activation and NO (nitric oxide) production [91-93].

As the cells form monolayers, external forces are transduced to neighboring cells. Here, Newton's second law of Dynamics is the model for this force transduction. Every force applied, creates an acceleration. [94]. And that creates a drag force on neighboring cells. F=m.a; where F is force, m is mass and a is acceleration.

Figure 1.6. Force creates acceleration on constant mass.

In motion of cell monolayers, such as migration in epithelial cells [95], traction forces are exerted by the leader cell to the following cells. Cell to cell connections are the key regulators in this process [96]. And the forces are thereby propagated and decayed over a distance (Figure 1.7) [97]. This force propagation enable cells to move as cell sheets [98].

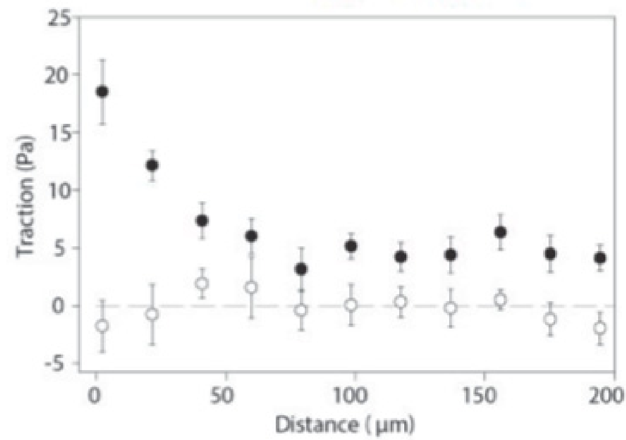

Figure 1.7. Traction forces in cell monolayers [97].The average normal traction decays slowly with distance from the edge (filled symbols), whereas the average parallel traction is negligible and independent of the distance from the edge (open symbols). Error bars indicate standard errors.

1.3. Methods for Mechanical Stimulation in Mechanobiology

Current methods for mechanically stimulating cells are various [99-101]. Choosing a method depends on cell type and its natural environment. It may be fluid shear for renal epithelial cells [102], while it needs to be cyclic stretch for cardiac muscle cells [103] and compression for cartilage [104].

1.3.1. Compression Systems

Compressive forces are delivered to cell *in vitro* in various ways. Use of hydrostatic pressure on cells and tissues is a frequently used technique for cell compression (Figure 1.8) [105, 106]. With this technique, positive or negative pressurization can be obtained [107] The technique is simple and provides homogenity in delivering the force and enables many application regimens in static or transient. In this technique, the force is transmitted through the liquid therefore there is no direct contact with the platen. Another advantage of the technique is that it is applicable to both adherent or non-adherent cell types but the gas pressure between the platen and the liquid that is compressed requires additional consideration and calculations [108]. Higher frequencies with lower magnitudes create insignificant pressurization in between [109].

In another variation to hydrostatic pressurization, compressive loading can be obtained *via* direct application of the platen. This variation is preferred in tissue explant studies, especially in cartilage, in order to mimick *in vivo* conditions better [110]. It also enables to study cell-extracellular matrix composites. This technique has disadvantages in delivering the force homogeniously, since the frictional forces will differ on the surfaces where platen and the specimen are in touch, as well as the delivery of nutrients from medium may be affected under this sort of loading.

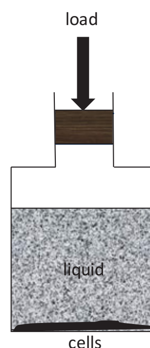

Figure 1.8. Hydrostatic pressurization for cell compression.

Compressive systems are used in different types of cells [111, 112]. Compression bioreactors with a cyclic compression regimen of 1 Hz for 4 weeks combined with strain of 0.22% for 4 hours per day are osteoinductive on MSCs grown on PCL-TCP scaffolds, osteogenesis related gene expression and ALP activity were increased. Also, the

experimental group showed increase in calcium deposition compared to control. This also shows the importance of exercise in fate selection and differentiation [113].

Compressive conditions were used in effort to mimick orthodontic force with an *in vitro* model of human peirodontal ligament like tissue. The tissue was grown on poly L lactide scaffolds [114]. Hydrostatic pressure was used to model bladder outler obstruction (BOO) *in vitro* with MYP3 (rat urothelial) cells. This model enabled high levels of ATP release as in disease condition. Increasing pressure yielded in respectively increasing levels of ATP release. And high pressure was reported as a trigger of acute caspase 1 activation [115]. HC11 cells (mammary epithelial) were exposed to hydrostatic pressure in order to stimulate milk accumulation, resulting in reduction of short circuit current and change in tight junction proteins' expression. This shows hydrostatic pressure during milk accumulation enables lactation *via* these molecular changes [116]. Cyclic hydrostatic pressure was found to enhance development and functional stabilization of chondrocytes using bone marrow derived stem cells and fat pad derived multipotent stromal cells seeded on agarose gels in chondrogenic medium [117]. Dynamic hydrostatic pressure also had a stabilizing effect on chondrogenesis in cells derived from embryonic limb bud grown in high density micromass culture. Pressure also reduced expression of hypertrophic genes [118]. A novel hydrostatic pressure bioreactor with a flexible membrane to transmit the pressure, was efficient in 3D cartilage regeneration *in vitro* [119].

1.3.2. Cell Strecthing Systems

Tensile forces are delivered to cells *via* cell stretching systems. In these systems, a flexible membrane is used a base where cells are seeded onto. Various modalities are possible when stretching the cells (Figure 1.9).

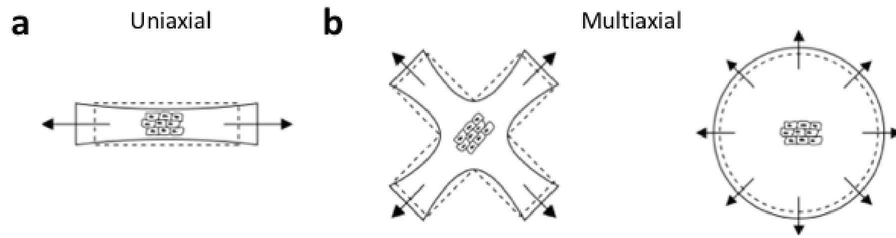

Figure 1.9. Uniaxial (a) and multiaxial (b) cell stretching [120].

1.3.2.1. Uniaxial Stretching

In uniaxial strecthing, the cells are stretched in one direction (Figure 1.9a). In the first study that used uniaxial stretching, a spring was used to create tension to rabbit cranial sutures [121]. Since then, use of a membrane to deliver tensile forces *in vitro* became popular. Different designs with the membrane were used: parallel membranes [122, 123] [124], single-cell membranes [125], microchips with membranes [126], 3D gels [127, 128].

Uniaxial systems are easy to construct and physiologically relevant for vascular cells [129, 130]. These properties make them popular in the stretch studies. On the other hand, delivery of the force is not homogenous therefore, it is difficult to control the strain [106]. But, this property are used as preferred in specific studies [131, 132]. System can be modified to deliver controlled non-homogenous strain intentionally.

Uniaxial cyclic streching reduced tenogenic markers in human MSCs under effect of strech activated calcium channel blocker (SACC), gadolinium and mediated non-apoptotic cell death; revealing the importance of SACC in mechanosensation and tenogenic differentiation [133]. Human bone marrow cells were used in a non-uniform stretching study, cell orientations and threshold for migration in nonuniform strain fields were determined [134]. Uniaxial stretching was used to determine natural mechanical properties of the skin, on breast skin specimens taken during mastectomy. A universal model for stretch response in breast skin was developed [135]. Uniaxial cyclic stretch was reported to induce myogenesis in C2C12 myoblasts and resued inhibitory effects of TZD, a substance used to control blood sugar in diabetes patients, in myotube formation [136].

1.3.2.2. Multiaxial Stretching

Multiaxial stretching indicates bi- or circular stretching systems to deliver strain in more than one direction (Figure 1.9b). Biaxial stretching systems have a rectangular or a cruciform shaped membrane [137-139]. Circular systems deliver strain in all directions [140-142]. These systems are indented either by a platen or vacuum [143, 144]. A commercial system named as Flexcell, uses vacuum for indentation [145, 146].

The homogenity problem is on the table for these systems too. In systems other than intentional heterogeniousity of the strain is designed [142, 143], the outer regions of the membrane may have different strain fields than the central region. Although the situation is better in multiaxial systems than uniaxial systems, it still decreases the usable cell culture area [147, 148].

The multiaxial systems are harder to build and are not neccessarily more relevant for vascular system studies. However, multiaxial systems are preferred for connective tissue studies, which experience stretching in more than one direction naturally [149]. Biaxial loading was reported to trigger forming of mature elastic fibers and alignment on collagen fibers toward a native phenotype in tissue engineered blood vessels [150]. Osteogenic activity in terms of expression of osteopontin and CBFA1 was increased under anisotropic axial strain in primary rabbit periosteal cells [151].

1.3.3. Fluid Shear Systems

1.3.3.1. Parallel Plate Flow Chambers

Parallel plate flow systems have been used since 1970s (Figure 1.10). First study by Krueger et al., was successful in culturing cells in a parallel plate system and achieving shear stress values from 10^{3} to 10 dynes/cm^2. In parallel plate systems viscosity of the fluid is important in generating the shear stress of interest [87, 152]. Temperature control and chamber design are also important parameters to obtain a steady laminar flow [153-156]. To obtain a disturbed flow, different designs may be applied to flow chambers. A

step-flow design enables to study cell behaviour under different flow regimes in one setup [157, 158]. Pulsatile or oscilatory flow regimes can also be produced in step flow chamber [157]. Chamber design modifications are not limited to these, flow chambers are modeled to mimick natural environment for the tisuue of interest. Branched designs for vessels [159], chambers for cocultures [160, 161] or 3D conditions [161, 162], and different coatings for the chambers [156, 163-165] are used for different needs.

Figure 1.10 Parallel plate flow [120].

1.3.3.2. Cone and Plate Systems

Cone and plate system studies are similar to parallel plate systems (Figure 1.11). Studies with bovine pulmonary artery and rabbit aortic epithelial cells reported change in cell shape and orientation [166-168]. Cell density, growth rate and cell adhesion were also reported to change under fluid shear applied in a cone-plate system [169, 170].

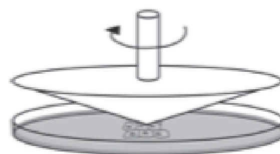

Figure 1.11. Cone and plate system [120].

1.3.3.3. Orbital Shaker Systems

This technique is applied *via* a standard orbital shaker (Figure 1.12). Therefore it is relatively easy. Studies include measurements about cell morphology [171, 172],

alignment and proliferation. This technique is preferred beacuse it is easy to apply, provides flexibility on cell culture plates [171] and experimental times. On the other hand, shear stress delivered to cells is difficult to measure [173] and for data collection, system must be stopped so real-time evaluation and media sampling are not possible.

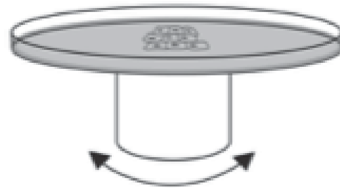

Figure 1.12. Orbital shaker flow system [120].

1.3.3.4. Microfluidic Techniques

Microfluidic devices are micro-scale set ups to deliver fluid shear to cells of interest. Capillary tubes or PDMS chips are used often. Fluid shear is acquired *via* a pumping system [174, 175]. Capillary tubes are produced form polypropylene or polyethylene [176-178]. Chip production needs a master design for micropatterning by photolithography [179, 180]. Physical responses such as phenotypical changesi proliferation and adhesion of epithelial cells can be easily determined in these systems [175, 181].

1.3.4. Single-cell Technologies

As the technology advances, it became possible to work with single cell systems. Measuring forces that are created in or on a single cell or applying a mechanical stimuli on a cell is possible. This area of Lab-in-a-Cell (LIC) systems enable specific biochemical of physical changes that occur on a single ion channel, or a cellular response to a drug [182].

For single cell studies, tubing equipments[183] microneedle techniques [184]and micropipette aspiration [185], optical traps [186], optical stretching [187] and magnetic

trapping methods [188] are used to exert physical forces on a single cell. Micropatterned surfaces or La- on-a-chip (LOC) techniques are also used for determination of single cell responses for environmental cues [189]. Cellular mechanical responses such as cellular stiffness can be measured by atomic force microscopy at the single cell level [190].

1.3.5. Gravitational Unloading

Absence of mechanical signals also regulates cell behaviour. One can easily see the effect of absence of mechanical signals on long term bedrest patients or in astraunots. The absence of mechanical signals are studied as gravitational unloading *in vitro*.

Gravitational unloading was applied on human umbilical vascular endothelial cells for 24 hours. Gene expression in inflammation and adhesion factors were increased, and the effect was reversible with re-loading of the cells. Similar effects were also seen with shear stress which indicate mechanical loading may be using some common pathways [191].

In muscle tissues, unloading creates an atrophic response. This response is seen gradually in slow extensors first, then fast extensors and lastly in fast flexors. Myonuclear number is decreased per mm of fiber length in unloaded fibers. Slow muscles are more responsive to unloading than fast muscles [192]. Gravitational unloading in the space also leads to cardiovascular problems. Human endothelial cells were used to model the situation under simulated microgravity for ten days and caspase3, bax and bcl2 proteins were increased. Accumulation of collagen type I and III and cytoskeletal changes were present. Release of soluble factors was decreased for VEGF, BDNF and endothelin 1 [193]. Deterioration of muscles in long bed rest also affects motoneurons and mitochondrial bioenergetics possibly due to heat loss [194].

1.3.6. Mechanical Vibrations as a Stimulator in Mechanobiology

Mechanical vibrations are defined as a reciprocating motion of a body or a medium forced from an equilibrium state. There are two extreme points about this

equilibrium state that the vibrating body oscillates between. Vibration creates a deformation on the substance that it is acting on, like any other mechanical force.

Vibrations can be classified into three categories as: free, forced and self excited. When a system is set in motion with an initial input only, system vibrates freely. Systems comes back to equilibrium and stops eventually. When a vibrating systems energy dissipated, by friction or an opposite force, it is said to be damped. And the third type is forced vibration in which the system is stimulated by an external input.

When a signal acts on a particle, it moves the particle and the surrounding ones. Each system behaves uniquely in this way, mainly depending on the frequency of the input and the composition of the system. The vibration is distributed throughout the system and this external force generates internal stress and a reaction [195]. In our system, periodic forced vibrations are used in sinusoidal wave form.

Low magnitude high frequency signals (LMMS; less than 1 g in magnitude and between 30-100 Hz frequency) are frequently used for bone and muscular tissues. These signals trigger adaptive, anabolic responses in skeletal tissues [196]. Increasing bone mass and attenuating bone loss in post menopausal [197] and adolescent [198] women, children with cerebral palsy [199, 200], adolescents with down syndrome [201] and individuals with adolescent idiopathic sclerosis related osteopenia [202]. Unlike drug treatments and physical rehabilitation which are sometimes impossible in severe disease conditions, low magnitude mechanical signals are safe, natural and easy to apply. Finding the right window of effectiveness of LMMS for different conditions still is an expanding research area [203-205]. Bone cells are responsive to low magnitude mechanical signals [21, 206, 207]. Mesenchymal stem cells are also responsive to LMMS and it plays role in their fate selection towards osteogenic lineage [208].

Low magnitude mechanical signals (LMMS) with characteristics of small magnitude and high frequencies (also called high frequency oscillations, low intensity vibrations, etc. in the literature) are pressure waves that can be generated with a biomedical device in repetitive fashion. A surface or a platform then transmits these mechanical signals to biological systems, including the whole body, a local tissue, or an *in vitro* cell culture system [209, 210]. In the literature of mechanical vibrations for biomedical usage, the magnitude of the mechanical waves is often provided in terms of their proportion to the earth's gravitational pull (g), (where 1 g is equal to 9.81 m/ s^2) frequency of mechanical loads is generally depicted with number of repetitions in a given

second (Hz).

Mechanical vibrations that are the subject of this study are considered low magnitude and high frequency as they have lower magnitude and higher frequency compared to physiological loads that arise from daily activities. The general assumption is that when the magnitude of the mechanical signal is lower than 1 g, it is considered to carry low intensity [209]. It is possible to use mechanical vibrations of higher magnitude for the augmentation of sports training, but the applications for regenerative medicine are comparatively limited. This difference perhaps arises from the subject of safety, because high intensity vibrations have safe exposure threshold on the order of seconds, as defined by the International Standards Organization's "Human exposure to mechanical vibration and shock" (ISO-2631). Even though a healthy adult is recommended to be exposed to high intensity vibrations for very brief amounts of time, low intensity range vibratory signals can be received safely for hours [211]. From a logical perspective, compliance to safety recommendations should be even more important for an elderly or injured person who is seeking augmentation of rehabilitation from mechanical vibrations. Perhaps that is the reason for LMMS signals' having broader range of applications in regenerative medicine [209, 212].

Mechanical vibrations are frequently used in rehabilitation medicine, since they are the simplest and purest form of vibratory energy application [213-216]. Vibration therapies are often recommended to patients who are not able to do physical exercise either because of a disease or aging [209]. Whole body vibration therapies have vibratory signals around 20-50 Hz. While focal vibratory therapies have much higher frequencies such as 300-400 Hz [217]. Human body is exposed to low frequency vibrations 1-3 Hz with high magnitudes around 2000-3000 strain, frequently and high frequency (10-50 Hz) low magnitude (postural muscle contractions) in daily life [218]. Vibration therapies are mostly used to benefit in conditions such as muscle training, muscle soreness and strenghten bone metabolism [219]. Whole body vibration (WBV) training for 6 months were reported to increase muscle strength in post-menapausal women and increased bone mineral density of the hip [220]. In another study, WBV training in men resulted in increase in growth hormone levels and decrease in cortisol levels [221]. WBV was also tested on blood circulation and found to increase the circulation in calf and thigh after training [222].

Vibration is anabolic to bone, muscle and tendons. It mimicks motion and exercise and is beneficial for muscle function and coordination. In molecular level, bone

metabolism is positively affected. Vibration induces osteogenic differentiation of mesenchymal stem cells. Mechanisms of this anabolic effect and its potential uses in medicine are still strongly explored in the literature [223-225].

In elderly or diseased, mechanical disuse, unloading or underloading of tissues is a big problem that leads to bone, muscle losses. Animal models are used to model these situations to provide treatment options. A very frequently used mechanical disuse model is hindlimb loading *via* tail suspension of rats [226]. Hindlimb loading for one month significantly reduces bone formation rates. LMMS signals are effective in restoring this bone loss at 0.25g, 90Hz and 10min/day regimen [227]. In a similar study with mice, an LMMS regimen of 0.2g, 90Hz 15min/day for three weeks prevented disuse and restored osteoblast numbers [24, 212].

LMMS applications were also effective in restoring bone loss occuring from estrogen deficiency due to aging in females. Ovariectomy in rats is a useful model for estrogen deficiency. An LMMS regimen of 3 g, 45 Hz 30 min/day for 90 days, significantly enhanced bone formation [228]. In a similar model, vertebra operation was performed and 3 months post-operative application of another regimen of 3.9g 90Hz 15min/day for 35 days increased trabecular and cortical bone quantity and whole bone biomechanical properties [196, 229]. Effect of signal frequency was determined in another study by application of two different vibration regimens. LMMS at 0.15g 10min/day for 28 days at 90Hz was effective in increasing bone formation rate while 45 Hz with the same regimen was not [196].

Bone fractures under estrogen deficiency is another important medical condition in post menopausal women [230]. Daily application of LMMS 0.3g, 35 Hz 20min/day for 8 weeks significantly increases fracture healing and mineralization in ovarectomized rats [231].

LMMS also eases the integration process of bone tissue with an implant. The tissue-implant integration in bone implants is a crucial process, that may lead to loosening of implant if failed [232]. LMMS signals were shown to be beneficial in bone recovery around implant site [233].

Other areas where LMMS was used in attempt of treatment; were obesity [234], cancer [235], osteogenesis imperfecta [236] and lipopolysaccharide induced inflammation [237].

1.4. Cancer Mechanobiology

Cancer is characterized by uncontrolled growth. In recent years, research has provided more to this definition. Hanahan et al. proposes hallmarks of cancer that a normal cell acquires during carcinogenesis [238]. These hallmarks include sustaining chronic proliferation, resisting cell death, inducing angiogenesis, activation of invasion and metastasis. The underlying mechanisms of these hallmarks are genomic instabilities that lead to mutations to activate signalling circuits that normally growth factors activate.

Cancer is one of most frequent reasons of death worldwide. There is no complete cure for cancer and therapautic approaches often lowers the life quality of the patients. Therefore, prevention strategies are important [238-242]. Prevention strategies usually includes a healthy diet and lifestyle, physical activity, less sun exposure and substance use [243]. A negative correlation between physical activity and cancer incidence was first considered in the literature in 1945 [244-248]. Since then, physical activity has been shown to lower incidence of colon, prostate, endometrium, breast and lung cancers [245, 249-251]. Mechanisms of this effect are suggested to be as effects of exercise on body fat, hormonal and immune system [250]. But, the direct mechanisms are still unknown [252-254].

Tumors are known to be facing mechanical forces, such as fluid pressure [255]. This pressure is shown to be drawing drug substances therefore, reducing the drug delivery and cancer cells to surrounding tissues leading to tumor invasion and metastasis [256]. Uncontrolled cell growth in the tumor increases inner solid stress [257]. Most anticancer therapies target this inner-tumor solid stress *via* decreasing compressive forces [258-260]. As an example, paclitaxel and docetaxel drugs act *via* this mechanism [261]. Tumor shrinkage leads to blood flow and lowers the interstitial fluid pressure in mouse and breast cancer patients. But this is not a permanent end for cancer, because cancer cells develop drug resistance and regrow to increase solid stress in the tumor [262-264].

It is shown that metastatic cells sense compressive stress *in vitro* and compressive stress leads to invasion and phenotypic changes [252, 263], *in vivo* application of compressive stress was also shown to be inducing oncogenes in mouse colon [265]. Additionally, melanoma cells by changing the cell skeletons through mechanical forces, the shape and orientation and attachment properties to the surface [266]. Low mechanical stimulation with severe ultrasound causes programmed cell death in human leukemia

cells and cell gene it also changes the expression profiles [267].

The absence of biomechanical forces as well as their presence affects cancer cells. For example, it can lead to significant metabolic changes in cancer cells in a gravitational environment, cell infrastructure and reproduction, affect the cycle and lead to metastatic susceptibility to resistance to programmed cell death in cancer cells. In addition, when melanoma cells released in gravity-free medium are transferred to the subcutaneous tissues of mice, create tumors much larger than controls left in normal gravity and have more spreading potential. The response of cancer cells to presence of biomechanical forces (or their absence) have not been investigated sufficiently as compared to the response of normal cells. Despite this lack, existing data suggest that biomechanical forces may also have certain regulatory effects in cancer cells.

Tumor extracellular matrix is shown to be stiffer than a normal extracellular matrix. High molecular weight polysaccharides are found in tumor extracellular matrices such as hyaluronan. In a normal extracellular matrix, this increase in pressure is balanced by collagen fibers and infiltrating myofibroblasts. In tumor extracellular matrix, cancer cells do not cooperate and increase force by activating cancer associated fibroblasts to produce more extracellular matrix. Therefore force distribution is not uniform in tumor tissue. This leads to nonuniform disruption in surrounding tissues and limiting of nutrient delivery and oxygenation which in turn increases invasion and metastasis and resistance to chemotherapy, radiotherapy and immunotherapy.

Besides this, most cancerous cells (eg, breast, bladder, pancreas and lung, leukemia and lymphoma, cancerous fibroblasts) are softer than normal cells. Increased in breast cancer cells, softness correlates positively with the metastatic potential, i.e. softer the cell, faster in the spreading. In addition, focal adhesion kinase (FAK) expression and activation are observed above normal levels, indicating that these tumor cells are an indication of their readiness to move at any moment. MCF7 cells, were shown to be compliant than normal cells (Figure 1.13) [268]. They were also shown to have a smaller Young's modulus, *via* AFM indentation method, than MCF10A cells [269]. MCF7 cells were also shown to be vulnerable to low intensity and frequency pulsed electromagnetic fields of 20 Hz [270]. A study, showed that, breast epithelial and cancer cell lines MDAMB231, MCF7 and MCF10A can be seperated according to their deformability *via* a microfluidic device [271]. And this study also indicates that, higher flexibility is related with a less differentiated state of the cell, and tumor initiating cells fall into this category.

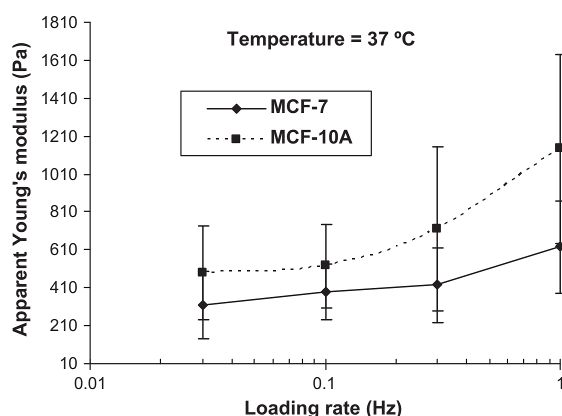

Figure 1.13. MCF7 cells are more compliant than MCF10A cells, shown by AFM indentation method [269].

Cell to cell contacts are also necessarily weak, and the cancer cells on invasion site, move independently of each other. The peripheral mechanical forces (ECM and other cells) of cancer cells are perceived from microenvironment less than in normal cells. If the microenvironment is thought of as a bioengineering structure, the forces generated by the system, acts on the rigid elements of the system rather than on soft elements. This is a phenomenon called "stress shielding". As a biological example of "stress shielding", bone-hip implant interaction can be considered [272, 273]. The hip implant, being more rigid than the bone, is loaded more heavily comepared to bone. This leads to disuse osteoporosis in the bone.

Cancer extracellular matrix, being more rigid than cancer cells, may bear mechanical loads and cancer cells are spared from the mechanical loads. Re-application of such mechanical loads on cancer cells may change the character or behaviour of cells. The "stress shielding" model of load bearing of breast cancer cells was not tested in the literature before this study.

In the treatment of breast cancer, the life and quality of life of the patient are also being extended by developing medical and technological applications [274]. However, due to breast cancer, patient losses are high and about 90% of these deaths are due to metastasis [274, 275].

In order for metastasis to take place, cancer cell must leave the tissue and transit into circulation [276]. At this stage, epithelial -derived cancer cells use epithelial to

mesenchymal transition (EMT) [277]. The EMT is a natural process in developmental stages [99]. In this process, epithelial cells lose their connection with their neighboring cells and can settle in different places within the organism [278]. In cancer development on the other hand, the EMT process leads to increased motility and tissue invasion, providing an environment for the formation and spread of malignant tumors [279]. For example, in epithelial and ovarian cancers, tumor cells use EMT signalling pathways to gain a more aggressive character [280-282]. It has also been reported that aggressive and metastatic breast cancer cells have mesenchymal characteristics, and expression of EMT genes is increased in these cells [283, 284].

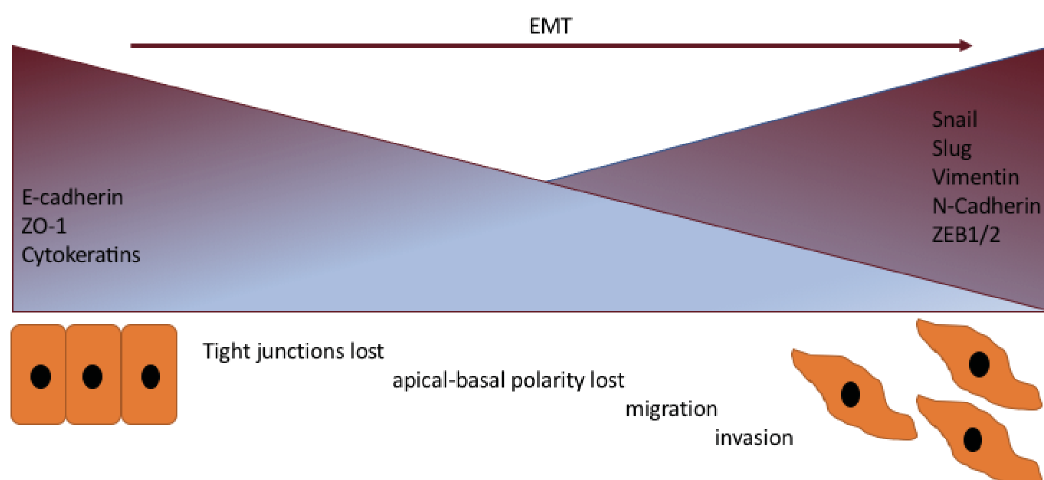

Figure 1.14. Changes in marker gene expressions and cell behavior in EMT.

The most important and first molecular marker seen in breast cancer cells in the EMT process is the decrease in E-cadherin expression in cells (Figure 1.14) [285]. It is known that transcription factors such as Snail / Slug family factors, Twist, Zeb1 and Zeb2 play an active role in this decrease [275]. Snail and Twist act together to trigger ZEB1 expression [286], thus reducing the expression of E-cadherin and the cells weaken and lose their binding to neighboring cells [276]. Subsequently, with the morphological changes in the cell skeleton, the cell becomes ready for migration [276]. This change begins with G-actin polymerization, which leads to a tip that will lead to migration in the cell [99].

In the literature, the efficacy of the EMT process in breast cancer, the process of

cell invasion and metastasis is known [287]. In this process, the direction and magnitude of intracellular forces are determinants. Cell-cell connections and connexins in the EMT process have been shown to be regulated by efficient transcription factors in the EMT process [288-292]. Decreased cell connections cause loss of apical-basal polarity and reduced structural support in cells [293]. The increase in the expression of vimentin, a mesenchymal marker, changes the organization of the cortical actin skeleton and forms membrane protrusions. The reorganization of the actin cytoskeleton and the formation of stress fibers also enhance cell contractility [294]. Vimentin plays a role in network deterioration and altered cell stiffness, and in the direction of cell invasion [295]. Mechanical forces outside the cell as well as within the cell have been shown to be effective in this process. The increase in extracellular medium stiffness activates EMT pathways with TWIST1 activation and enhances cell invasion [296].

The effects of mechanical signals on EMT have been demonstrated in developmental processes. In the formation of tissues and organs, intracellular mechanical forces also play important roles. The formation of mesodermin and the closing of the notochord in the embryos of Xenopus laevis occur only when the stiffness of these tissues is at a level that is torsion-proof [297, 298]. Actomyosin contractility allows the dorsal opening to close in the Drosophila embryo [299, 300]. In this closure, the forces created by apoptosis are also determinants [301]. The EMT process can be triggered by increasing the Twist expression by externally applied forces on the Drosophila embryos [299, 300].

Mechanical effects are the effects of lung, kidney and mammary tissue involution and development of mammals [302-305]. Anomalins in extracellular mechanical forces are known to play a role in breast cancer metastasis. Increased cell stiffness has been shown as a trigger for metastasis [306-308]. For the interaction of externally applied forces with EMT, the studies carried out are limited. It has been demonstrated on the pulmonary epithelial cells and on the heart valve produced by tissue engineering methods that the EMT process is activated by activating different signal pathways depending on the magnitude and direction of the cyclic stretching force [309, 310]. It has been shown here that different pathways are used for EMT activation induced by normal and pathological tissue force changes. It is not known if the LMMS affects EMT process, one of the effective pathways of metastasis in breast cancer.

1.5. Mechanical Forces in Stem Cell Differentiation

Mesenchymal stem cells, have the capacity to differentiate into both osteoblastic and adipogenic lineages [311]. MSC fate determination is affected by environmental clues, such as chemical and physical signals, since bone formation is in-part regulated by mechanically induced differentiation of these progenitor cells [312]. Mechanical-responsiveness of MSCs play critical role in bone regeneration [313]. When MSCs are subjected to unloaded conditions, bone formation is reduced and adipogenic differentiation is favored [314]. Mechanical vibrations has been shown to prevent muscle degeneration, promote osteogenesis and to stimulate osteogenic differentiation for mesenchymal stem cells in mouse bone marrow [315-318]. At cellular level, these low magnitude mechanical signals operate through similar signalling pathyways as high magnitude mechanical signals increasing focal adhesion strength and activating RhoA [37]. However, in contrast to high magnitude strain low magnitude mechanical signals requires LINC (Linker of Nucleoskeleton and Cytoskeleton) mediated actin-nucleus connectivity [319]. Absence of mechanical signals are on the other hand, is related with increased adipogenesis in bone marrow mesenchymal stem cells [314].

Critically, in aging where MSC cell mechanoresponsiveness is reduced, physical exercise can not prevent adipogenesis in aging bone [320]. Although MSC fate determination has many key players.

LINC complex functions as a bridge to transmit mechanical signals to inner nucleus [321]. In the nucleus, LINC is anchored by the Lamin A/C meshwork, which is involved in maintenance of nuclear structure and support [322]. Lamin A/C is an intra-nuclear intermediate filament meshwork placed between inner membrane of the nucleus and chromatin, and in addition to providing mechanical support, is also involved in cellular signalling and gene control [322]. Lamin A/C adapts to incoming mechanical challanges by changing its structure and binding partners and thus tought to regulate cell response and fate [321, 323]. In this way, mutations and catabolic changes in LINC complexes and LaminA/C result in abnormal cellular mechanical response.

Lamin A/C also plays role in cellular mechanical repsonse [324]. For example, when MSCs differentiate towards bone lineage, cell stiffness get elevated in parallel to the increase in Lamin A/C [325]. Loss or defective lamin nucleoskeleton also leads to adipogenesis and reduces osteogenesis [326]. Loss of Lamin A/C has a role in premature

muscle differentiation and muscle dystrophies. In situations that the Lamin A/C nucleoskeleton is disrupted or defective, cell displays improper mechanical response, cell's nucleo-connectivity is reduced. Lamin A/C loss and/or mutations are linked to certain diseases muscular dystrophies and lipodystrophies such as, familial partial lipodystrophy of Dunningan type (FPLD) and Emery Dreifuss muscular dystrophy (EDMD) [327]; Hutchinson-Gilford progeria syndrome [328] and Charcot Marie Tooth disorder [329].

Critically, previous studies showed that LINC mediated cytoskeleton-nucleus connectivity is crucial in regulating signalling pathways and differentiation in response to mechanical challanges [330, 331]. But the exact mechanism of how changes in Lamin A/C regulates MSCs ability to respond to mechanical challanges remains unclear. In normal aging, LINC complex is weakened as LaminA/C nucleoskeleton is disrupted [332, 333]. In respective mouse models, the cells are less responsive to mechanical signals, adipogenesis is increased in bone marrow and early aging is observed [334, 335]. These findings together, suggest that there is a strong connection between lamin nucleoskeleton, nuclear mechanosentivity, aging and differentiation.

1.6. Aim of the Study

Vibration signals are anabolic to normal tissues. In disease conditions on the other hand, such as cancer and aging, mechanosensing and mechanotransduction pathways in cells are not as effective as normal cells. Therefore cells cannot sense and/or create an appropriate response for external mechanical signals. In this study, direct application of low magnitude mechanical signals on the selected cells was hypothesized to restore the mechanoresponsiveness in cells.

According to this hypothesis, the study consists of two questions:

- Breast cancer cell mechanoresponsiveness determination.
    - Does LMMS change the behaviour of breast cancer cells?
- Mesenchymal stem cells' responsiveness determination.
    - Does LMMS affect the increased rate of adipogenesis resulted from LaminA/C knockdown in mesenchymal stem cells?

For the first part, LMMS has been used to restore partially unloaded state of breast cancer cells; MCF7, MDAMB231 and normal breast epithelial cell line MCF10A. In the

second part, LMMS has been delivered to bone marrow derived mesenchymal stem cells which are in the increased adipogenesis state.

CHAPTER 2

METHODS

2.1. Application of Low Magnitude Mechanical Signals (LMMS)

2.1.1. LMMS Generation Device

LMMS was generated and delivered to cells by a custom-made platform in vertical direction [37], and the signal quality was continuously observed with real-time accelerometer (K-Beam, Kistler, Amhers, NY, USA), measurements were obtained by Labview 2010 Signal Express (National Instruments, Austin, TX, USA) software (Figure 2.1 and 2.2).

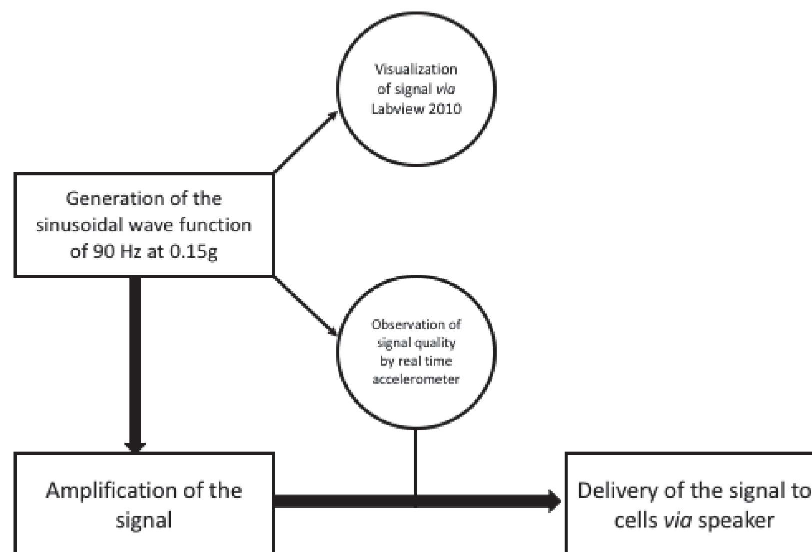

Figure 2.1. Schematic representation of LMMS application method.

Figure 2.2. LMMS generating device. (a) View of the signal. (b) signal generator (top) and amplifier (bottom) (c) delivery of the signal to the culture plate via speaker.

2.1.2. Breast Cancer Cell Lines

Experimental cells were exposed to LMMS daily at 90 Hz and 0.15 g (1 g=Earth's gravitational pull), for 15 min/day, 5 days/week in ambient conditions, during which control plates were subjected to sham loading (Table 2.1).

Table 2.1. Experimental timeline.

D-1	D0	D1	D2	D3	D4	D5
-	-	++	+	++	+	++
D6	D7	D8	D9	D10	D11	D12
-	-	+	++	+	+	++
D13	D14	D15	D16	D17	D18	D19
-	-	+	+	+	+	++

"D" designates days. On day -2 cells are seeded. On D1 LMMS starts daily.
- : LMMS is not applied.
+ : LMMS is applied.
++: LMMS is applied and analyses are done.

2.1.3. Mesenchymal Stem Cells

Acute response to LMMS, FAK phosphorylation was measured immediately after mechanical loading. Cells were subjected to LMMS twice (20 min, 0.7g, 90 Hz.). Phosphorylation levels were measured by western blot analysis.

For the effect of LMMS in adipogenic differentiation, cells were treated with adipogenic growth medium that includes dexamethasone and insulin for 7 days. Samples for protein and expression analysis were collected on days 0, 3, 5 and 7. Adipogenic marker, adiponectin were analyzed by western blot and qPCR. Experimental data and imaging were analyzed by Image J and Prism softwares.

2.2. Cell Culture Conditions

2.2.1. Maintenance of Breast Cancer Cell Lines

MDA-MB-231, MCF7 (American Type Culture Collection, VA, USA) breast cancer cells were used in this part of the study. Cells were cultured in high glucose DMEM (Thermo Scientific HyClone, UT, USA) with 1% Pen/Strep (Biological Industries, Israel) and 10% FBS (Biological Industries, Israel) addition. MCF10A (American Type Culture Collection, VA, USA) human breast epithelial cells were used as non-cancerous epithelial controls. MCF10A cells were cultured in DMEM:F12 medium (Sigma, MO, USA) swith 20 ng/ml EGF (Sigma, MO, USA), 0.5 ug/ml hydrocortisone (Sigma, MO, USA), 100ng/ml choleratoxin (Sigma, MO, USA), 10 ug/ml insulin (Sigma, MO, USA), 1% Pen/Strep (Bio- logical Industries, Israel), 5% donor equine serum and 2 mM L-glutamine addition. Cells were kept in 37°C and 5% CO_2 except vibratory loading protocol, during which they were exposed to the ambient conditions. For all experiments cells were cultured in 24 well plates (Corning, NY, USA) with a 640 cells/mm^2 density to prevent overpopulation during 3 weeks and culture medium was changed every two days. For scratch wound assay only, 6 well plates were used with the same seeding density. For all experiments, day of plating was considered

as day (-2) and cultures were maintained for a range of days, including D1, D3, D5, D9, D12 and D19 (Table 2.1). At the designated time points, experiments were terminated for further analysis.

2.2.2. Maintenance and Adipogenic Induction of Primary Mesenchymal Stem Cells

Studies with mesenchymal stem cells were performed in Dr. Rubin's Lab, University of North Carolina at Chapel Hill, NC, USA. Primary bone marrow derived mesenchymal stem cells were provided by Dr. Rubin. Cells were grown in regular IMDM medium (Gibco) supplied with 10% fetal bovaine serum and 1% penicillin/streptomycin cocktail. For adipogenic differentiation experiments, induction media was MEM-alpha medium (Gibco) supplied with 5 μg/ml insulin and 0.1 μM dexamethasone.

2.2.3. SiRNA Treatment of Mesenchymal Stem Cells

SiRNA was used to regulate LaminA/C expression *via* RNA interference mechanism. RNA interference is useful for targeting specific single genes [336]. A non-viral delivery method was used. Lamin A/C (SiL) and control (SiC) siRNAs (Invitrogen, order number: 81507044) were suspended in RNase free water to make 20uM solutions and kept in -80ºC after aliquoted. Cells were seeded 10500 cells/cm^2. After 24 hours of seeding, cells were treated with 0.75 μl/ml siRNA, 2.25 μl/ml Lipofectamine (Invitrogen) and 100 μl/ml OPTI-MEM media (Gibco).

For an acute response to LMMS, the medium was changed with regular IMDM medium on the next day after siRNA addition. Then, cells were starved with IMDM medium that do not contain serum and antibiotics, overnight before mechanical signal application.

For long term response in differentiation, medium change and starvation steps were excluded and adipogenic differentiation medium was added onto cells on the next day of siRNA treatment.

For a successful Lamin A/C knockdown, siRNA dose was determined by western blot analysis and knockdown was assessed by western blot and qPCR.

2.3. Cell Viability

Trypan blue exclusion method was used to determine number of cells for experimental and control groups, for breast cancer cell lines. Live cells exclude trypan blue dye whereas dead cell do not. After cell suspension is mixed with the dye, a viable cell will appear clear under the microscope whereas a dead cell will appear blue [337].

Detachment of cells after LMMS application was also assessed. Cells were trypsinized and diluted with 0.4% trypan blue dye (Gibco, Invitrogen, NY, USA) dye in 1:1 ratio and counted with a Neubauer hemocytometer.

Cell viability was also determined by MTT assay. MTT tetrazolium reduction method is a useful technique in high throughput screening of cell viability. MTT salt is positively charged and taken up by viable eukaryotic cells. The formazan product appears when MTT is metabolized, in presumably proportional amount to number of viable cells [338]. The color change is measured by spectrophotometer. Cells were incubated with 0,5 mg/ml MTT (Amresco LLC, OH, USA) for 4 hours. After the incubation tetrazolium salts were dissolved in DMSO and colorimetric measurements was done at 570 nm with a background subtraction at 650 nm.

2.4. Calcein Staining

Cell viability was also assessed with Calcein staining. In this assay, acetoxymethyl ester of calcein (calcein-AM), is used. It gets inside viable cells and is converted to green fluorescent calcein *via* esterase activity and stays in the cell. The loss of calcein is determined by flow cytometry [339] Calcein-AM cell permeant dye (Life Technologies, Oregon, USA) was used to stain MDA-MB-231 cell at days D5, D12 and D19, with 30 min incubation in the dark, MCF7 cells were stained for D5 only.

2.5. Cell Cycle and Apoptosis Analysis

Experimental and control groups were subjected to cell cycle analysis based on the DNA content of the cells *via* propidium iodide (PI) staining. Propidium iodide is an intercalating agent, which has increased fluorescence if bound to DNA or RNA [340]. Cells were collected in 1x cold PBS solution and then fixed with EtOH. After overnight incubation at -20°C, cells were permeabilized with 0.1% Triton x-100 in PBS and treated with RNase A. Finally, cells were incubated with PI and analyzed with FACS Canto (BD Biosciences, CA, USA) with low flow rate. Based on binomial distribution of PI signal gating and doublet distinction was done according to the area of the signal peaks (Figure 2.3) with a cell cycle analysis software (Modfit LT, Verity Software, USA).

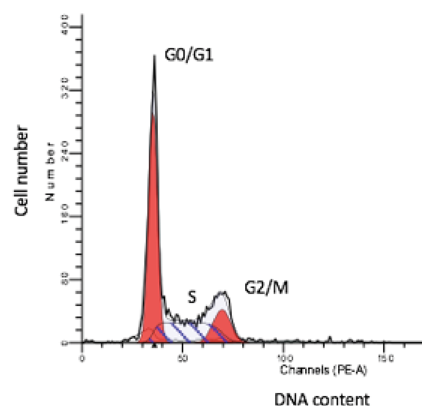

Figure 2.3. Representative image for cell cycle analysis with PI staining.

Fractions of apoptotic, live and dead cells were quantified using Annexin V – PI (BD Pharmingen, NJ, USA) staining based on the specifications instructed by the provider. Annexin V has a natural affinity for phosphatidyserine, which is exposed on the cell surface after apoptosis is initiated. Annexin V is labeled by FITC (fluorescein isothiocyanate) for detection [341]. Cells were collected and washed twice with PBS then suspended in binding buffer and stained with Annexin V (FITC) and PI dyes. Cells were analyzed with FACS Canto where single stain and unstained cells were used to set event Gates (Figure 2.4).

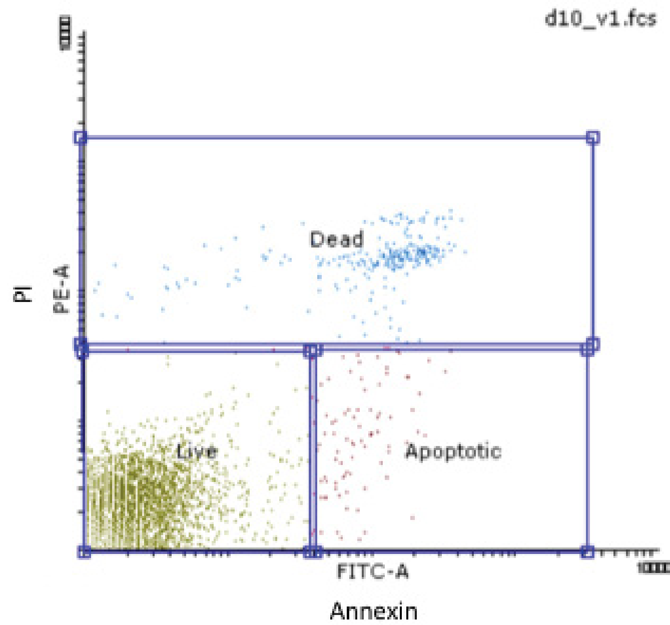

Figure 2.4. Representative image for apoptosis analysis and gating cell populations in flow cytometry.

2.6. Scratch Closure

In vitro scratch wound closure assay is an inexpensive and easy method to measure cellular migration [342]. Scratch closure rates for both groups were quantified using an artificial scratch mark made with the tip of a 200 µl pipette. Scratches were visualized immediately and after 24 hrs of incubation for both groups using a microscope (CKX41, Olympus, Japan) with image processing software (DP2- BSW, Olympus, Tokyo, Japan). The gap between cells was measured from 3 different regions on a single scratch, repeated 10 times within sample. The percent change of average gap length between 0 and 24 hrs was reported as an indicator of gap closure. Mechanical signal was not applied to cells during this 24 hrs period.

2.7. Protein Expression by Western Blot

Western blot technique is used to seperate proteins based on their molecular weight, in gel electrophoresis. By their sizes, proteins can be easily identified. Specific antibodies for proteins form bands specific for each protein and gives a fluorescent color, that enables the detection on transfer membrane. By the band thickness, amount of protein can be measured [343]. Samples were collected immediately after LMMS application, with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.24% sodium deoxycholate, 1% Igepal, pH 7.5) containing phosphatase and protease inhibitors (25mM NaF, 2mM Na_3PO_4, aprotinin, leupeptin, pepstatin and phenylmethylsulfonylfluoride (PMSF)), after washing with cold PBS on ice. Cells were scaped off from the surface and collected into tubes as cell lysates.

Lysates were sonicated for 4x3 seconds on ice, then centrifuged at 15000 rpm for 10 minutes at 4ºC. Supernatant was taken to a new tube and BCA assay (Thermo) was performed. Samples were then prepared according to the protein concentrations with loading buffer and double distilled water. After 5 minutes of boiling, samples were centrifugated 10000 rpm for 3 minutes and loaded into 14% SDS-PAGE gels as 20ug protein. Then, they were transferred to polyvinylidene difluoride (PVDF) membranes and membranes were blocked with 5% milk diluted in Tris buffered saline with Tween 20.

Blots were then incubated overnight with the antibody of interest. For the following secondary antibody incubation at room temperature for an hour, horseradish peroxidase conjugated secondary antibodies diluted 1:5000 were used (Cell Signalling). ECL solutions (Thermo Scientific) were used to detect chemiluminescence.

2.8. Gene Expression by RT-PCR

RT-PCR method was used to measure mRNA levels of our genes of interest. This method in particular, enables the observation of amplification in real time *via* measuring the fluorescent signals in each cycle. For data analysis $\Delta\Delta$CT method is used to determine relative changes in gene expression to a reference gene [344]. Total RNA isolation was performed by using PureLink RNA Mini Kit following the manufacturer's protocol

(Invitrogen, Cat# 2 830 8). Purity and concentration of isolated RNA was determined by nanodrop spectrophotometer (ND-1000 Spectrophotometer). Two step RT-PCR was performed. cDNA synthesis was done by Fermentas First Strand cDNA Synthesis Kit (Thermo, Cat#K1622) with 1000 ng/µl template RNA. Results were analysed with Delta-Delta Ct Method. For control TBP primers were used.

Total RNA extraction from collected samples was done using Qiagen RNeasy Mini Kit. After concentration determination, cDNA was obtained as 1ug in 20ul per reaction by RT-PCR. Expression levels of Suns, Nesprins, LaminA/C and Adiponectin were analyzed by qPCR using B2M and RPLP0 as reference genes *via* real-time PCR performed on Bio-Rad iCycler. Every reaction had 0.5 µM of forward and reverse primers, and SYBR green mix from BioRad was used.

2.9. Fluorescence Imaging

In breast cancer cell lines, cellular morphology and actin ultrastructure was visualized using phalloidin (actin filaments: red color, Alexa Fluor 488, Invitrogen, USA) staining after fixation with 4% paraformaldehyde and membrane permeabilization with 0.1% TritonX in PBS, in breast cancer cell lines. Images were obtained by fluorescence microscopy (CKX71, Olympus, Japan) and processed with an image processing software (Image J, USA).

In Lamin A/C knockdown cells, cells were fixed with 4% paraformaldehyde and incubated with phalloidin (Alexa Fluor 488, Invitrogen), DAPI (nucleus: blue color, Molecular Probes) and anti-LaminA/C primary antibody (red color, Cell Signalling) and analyzed by fluorescence microscopy, in mesenchymal stem cells. Nuclear circularity and area was determined manually in Image J.

CHAPTER 3

RESULTS

3.1. Studies with Breast Cancer Cell Lines

3.1.1. Cell Viability

During 19 days of culture a steady increase was observed in sham control MDA-MB-231 cells as measured with Trypan Blue cell counts. At the end of 19 days, on average number of cells increased 108-fold compared to baseline controls (Figure 3.1). Number of low magnitude mechanical stimulation (LMMS) treated cells also showed a steady increase during experimental period with an average increase of 92-fold compared to baseline controls. LMMS group had 41%, 32% and 18% (all $p<0.05$) less number of MDA-MB-231 cells at D9, D12 and D19 compared to controls.

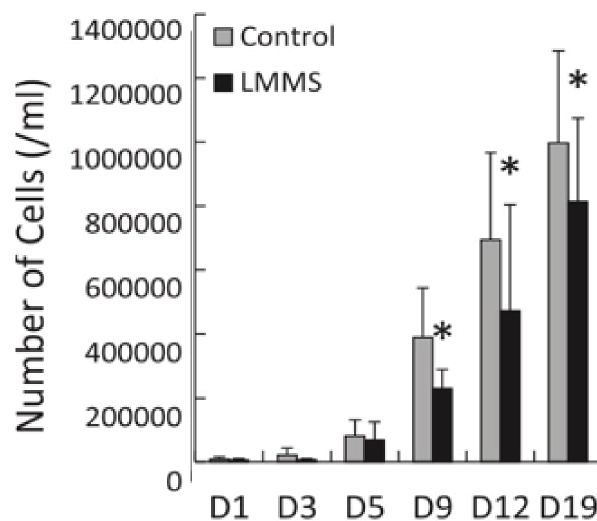

Figure 3.1. Trypan blue dye exclusion in MDAMB231 cells LMMS group had 41%, 32% and 18% (all $p<0.05$) less number of MDA-MB-231 cells at D9, D12 and D19.

Similar to Trypan blue readings, cell viability signals of MDA-MB-231 as documented with MTT assays for sham control cells showed a steady increase during the experimental protocol (Figure 3.2). At D19 MTT signal showed 25-fold increase compared to baseline controls. Compared to sham controls, LMMS treated cells showed 62%, 18% and 50% (all $p<0.05$) less MTT signal during D5, D9 and D19, respectively.

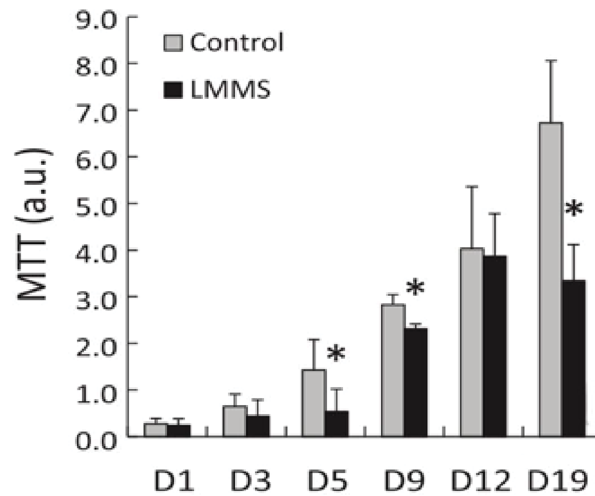

Figure 3.2. MTT in MDAMB231 cells. LMMS treated cells showed 62%, 18% and 50% (all $p<0.05$) less MTT signal during D5, D9 and D19.

To test if the reduced number of cells in LMMS groups observed because of cellular detachment from culture plate, cells in collected media was counted for both groups using Trypan blue stain (Figure 3.3). No significant differences (all $p>0.1$) were detected for number of detached cells in experimental days (Figure 3.3).

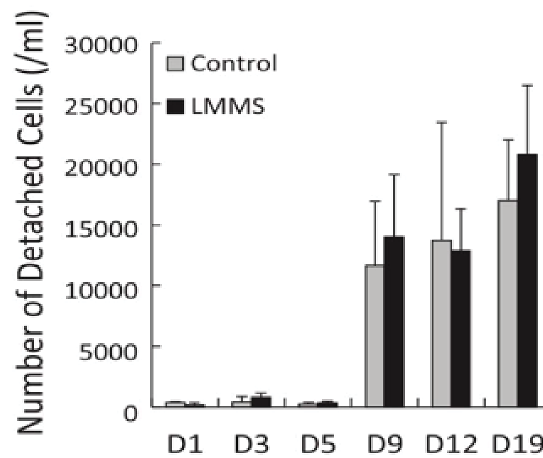

Figure 3.3. Detached cell number determination in MDAMB231 cells. No significant differences (all p>0.1) were detected for number of detached cells.

In spite of observed reduction in cell numbers for breast epithelial cancer cells (MDA-MB-231), similar loading with LMMS affected non-cancerous breast epithelial cells (MCF10A) differently (Figure 3.4). At the D5, LMMS group had 8% more MTT activity (p<0.01) compared to controls. At D12 and D19, MTT activity of MCF10A cells in LMMS group had a non-significant 6% and 1% (p=0.20 and 0.55, respectively) difference compared to sham controls.

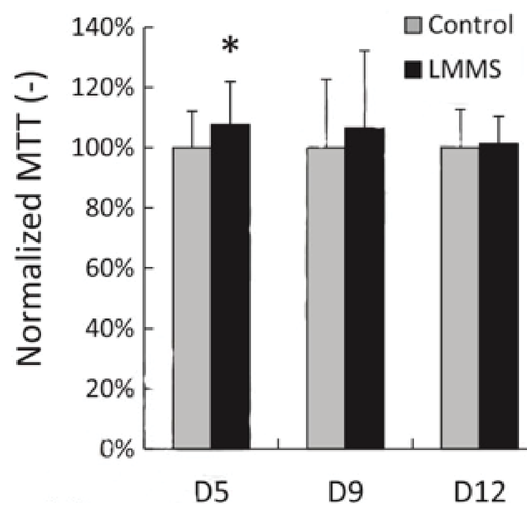

Figure 3.4. MTT in MCF10A cells. MCF10A cells in LMMS group had a non-significant 6% and 1% (p=0.20 and 0.55, respectively) difference compared to sham controls. At the D5, LMMS group had 8% more MTT activity (p<0.01).

According to MTT analysis, cell proliferation is significantly decreased 8% on day 12 (p=0.03) and increased 15% on day 19 (p=0.002) compared to controls for MCF7 cells (Figure 3.5). Experiment was done as three repeats each had eight replicates.

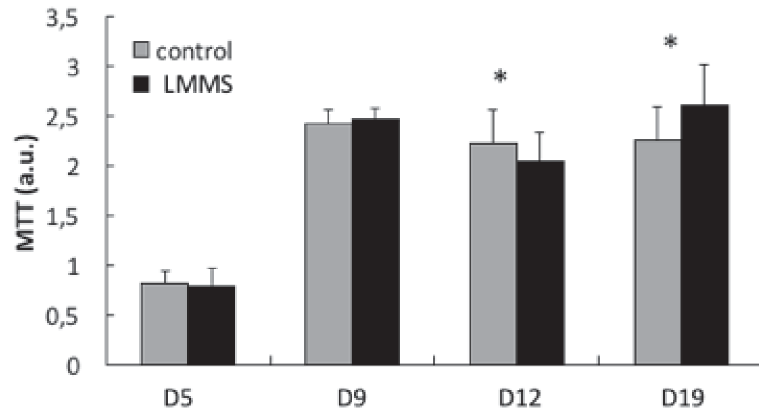

Figure 3.5. MTT in MCF7 cells. Cell proliferation is significantly decreased 8% on day 12 (p=0.03) and increased 15% on day 19 (p=0.002) compared to controls for MCF7 cells.

But these results are not complementary with tryphan blue staining and counting results. According to trypan blue staining, no significant difference was observed between groups throughout the experimental period. And also, the trend was quite different compared to MTT results (Figure 3.6). Experiment was done as three repeats each had four replicates.

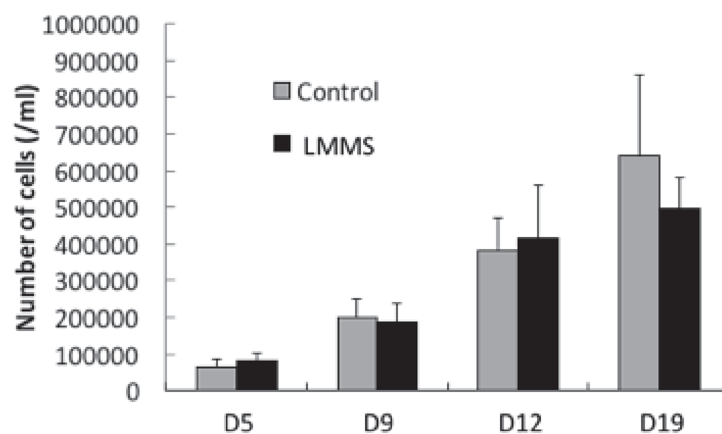

Figure 3.6. Trypan blue dye exclusion in MCF7 cells. No significant difference was observed between groups throughout the experimental period.

The effect of LMMS on the morphology and ultrastructure of MDA-MB-231 cells were determined by fluorescence microscopy (Figure 3.7). At D1 no significant difference was observed in individual cellular area, cellular circularity and actin content (Figure 3.7 c-e). However at the end of first week cancer cells that received daily LMMS had 32% (p=0.02) stronger fluoresce signal with 12% (p=0.04) smaller circularity compared to control cells.

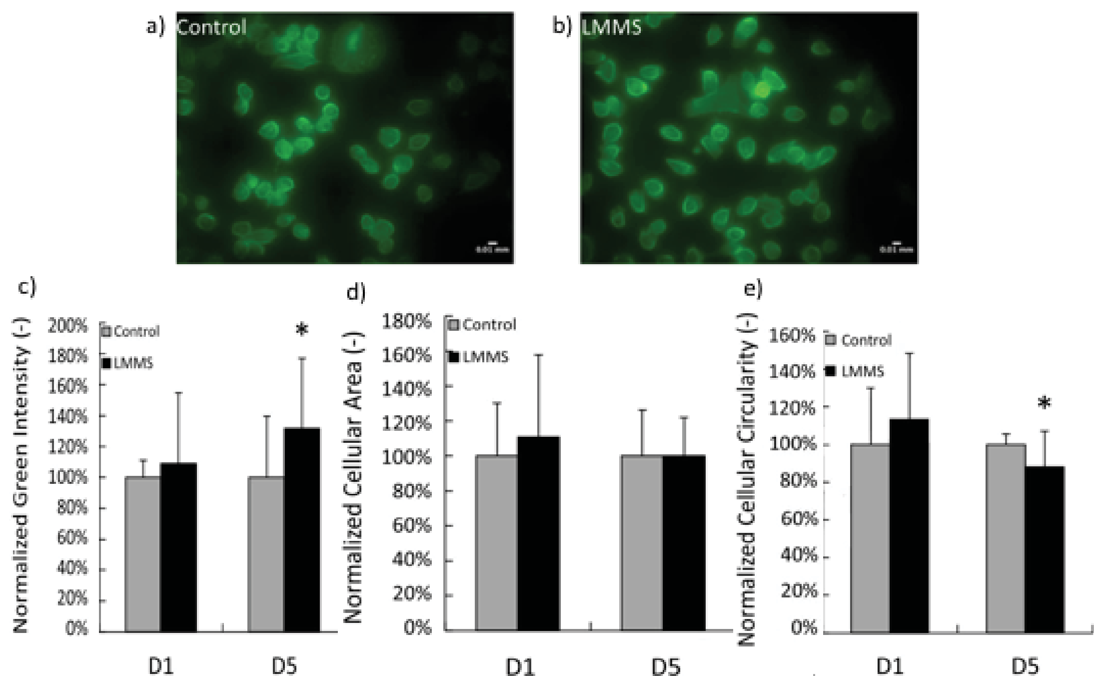

Figure 3.7. Morphological changes in MDAMB231 cells. Top:Representative fluorescence microscopy imaging of cells. Bottom: (a) Phalloidin staining intensity as a marker for cytoskeletal actin. At the end of first week cancer cells that received daily LMMS had 32% (p=0.02) stronger fluoresce signal. (b) and (c) Area and circularity respectively, at the end of first week cancer cells that received daily LMMS had 12% (p=0.04) smaller circularity compared to control cells. measured in Image J.

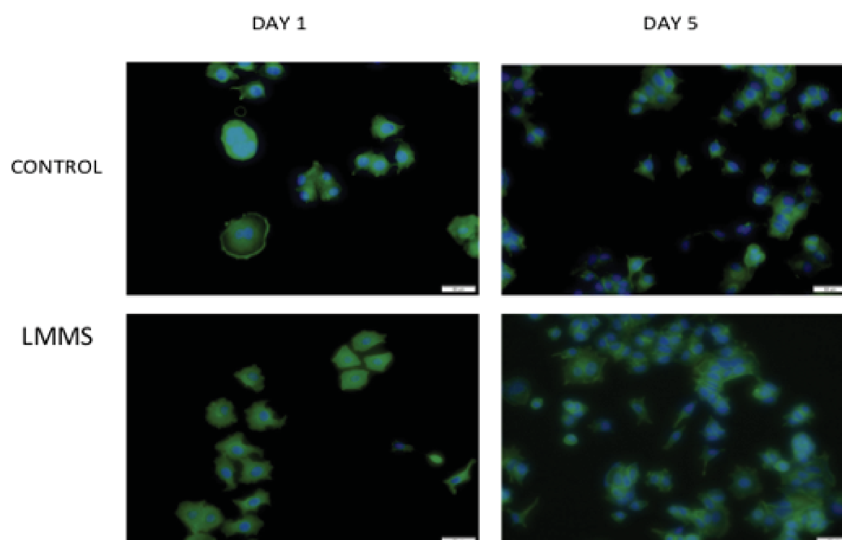

Figure 3.8. Morphology of MCF7 cells on day 1 and 5 by fluorescence microscopy.

3.1.2. Calcein Staining

Fraction of live cells at D12 showed a similar reduction (3%, p=0.02) as determined by Calcein-AM staining (Table 3.1).

For MCF7 cells, Calcein-AM staining revealed significant differences for 5 days of LMMS between control and LMMS groups. But, in the second repeat of experiment, a different histogram was observed, so the two repeats were not pooled (Table 3.2).

Table 3.1. Calcein staining of MDAMB231 cells.

	D5	D12	D19
Control	93.3±1.4	92.1±1.0	92.8±1.2
LMMS	94.7±1.4	89.5±1.2*	93.5±0.6

(*p<0.05).

Table 3.2. Calcein staining for MCF7 cells, in two seperate experiments.

	D5 (1)	D5 (2)
Control	84.23±1.1	99.5±1.14
LMMS	82.27±0.93	99.78±0.10
	(p=0.08)	(p=0.01).

3.1.3. Cell Cycle and Apoptosis

In an effort to explain the reduction of cell numbers and viability in LMMS treated cultures, cell cycle analysis was performed using PI staining. Unfortunately, cultures from both sham control and LMMS groups at D1, D3 and D5 lacked enough number of cells for analysis therefore cultures from D9, D12 and D19 were reported here. At D9, LMMS group had 3% (0.03) more fraction of cells in G1 phase while 24% (p=0.03) less fraction of cells in G2 phase compared to sham controls (Figure 3.9). At D12, LMMS group had 18% (p=0.02) less fraction of cells in G2 phase. In contrast to previous time points, LMMS treated cells at D19 had 3% (p=0.05) less fraction of cells at G1, while 27% (p=0.05) more fraction cells in G2 compared to controls. No significant differences were observed in the fraction of cells that are in S phase between LMMS and controls for the duration of experiment (Figure 3.9).

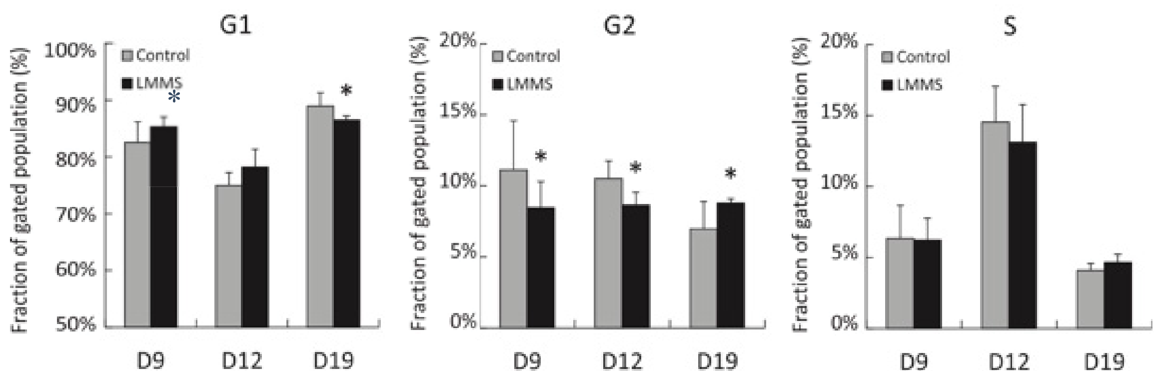

Figure 3.9. Cell cycle changes in MDAMB231 cells. At D9, LMMS group had 3% (0.03) more fraction of cells in G1 phase while 24% (p=0.03) less fraction of cells in G2 phase. At D12, LMMS group had 18% (p=0.02) less fraction of cells in G2 phase. In contrast to previous time points, LMMS treated cells at D19 had 3% (p=0.05) less fraction of cells at G1, while 27% (p=0.05) more fraction cells in G2 compared to controls.

Control and LMMS cells were stained with Annexin V – PI documented for the apoptotic status of experimental cells (Figure 3.10). No difference (all p>0.15) in the fraction of apoptotic cells were observed between control and LMMS groups (Figure 3.10). Fraction of dead cells in LMMS group was 51% (p<0.01) and 28% (p=0.05) larger

at D12 and D19 compared to controls (Figure 3b). At D12, fraction of live cells had small but a significant reduction (1.1%, p<0.01) compared to control cells (Figure 3.10).

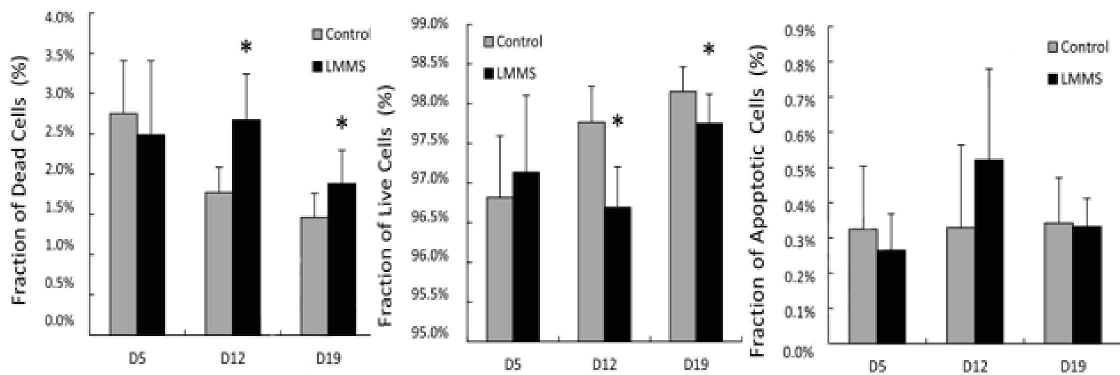

Figure 3.10. Apoptotic behaviour in MDAMB231 cells. No difference (all p>0.15) in the fraction of apoptotic cells were observed between control and LMMS groups. Fraction of dead cells in LMMS group was 51% (p<0.01) and 28% (p=0.05) larger at D12 and D19 compared to controls. At D12, fraction of live cells had small but a significant reduction (1.1%, p<0.01) compared to control cells.

MCF7 cells showed a different trend than MDAMB231 cells. They had an increase of 12% (p=0.04) in S phase on day 5 and a decrease of %14 (p=0.01) in G2 phase on day 19 (Figure 3.11). This experimental set had 24 replicates.

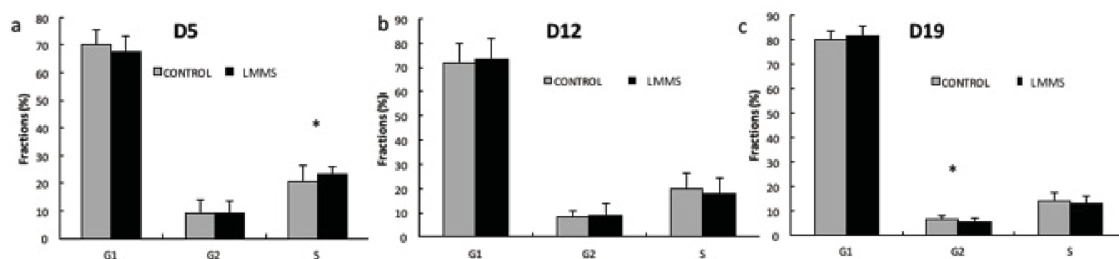

Figure 3.11. Cell cycle changes in MCF7 cells. MCF7 cells had an increase of 12% (p=0.04) in S phase on day 5 and a decrease of %14 (p=0.01) in G2 phase on day 19.

The apoptotic behaviour of MCF7 cells showed significant difference for early apoptotic population. On day 5, this population was increased 2 fold (p=0.04) (Figure 3.12). This experimental set had 6 replicates.

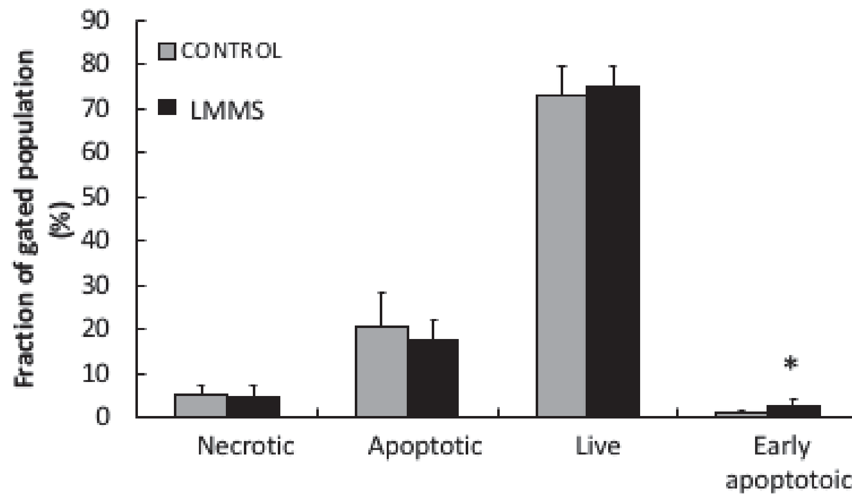

Figure 3.12. Apoptotic behaviour of MC7 cells. On day 5, fraction of early apoptotic population was increased 2 fold (p=0.04)

3.1.4. Scratch Wound Closure Assay

MDAMB231 cells showed no difference between experimental and control group on D5, D9, D12 and D19 (Figure 3.13). All samples showed full scratch closure at the end of D19. This result suggests that cell migration was not affected by LMMS in MDAMB231 cells.

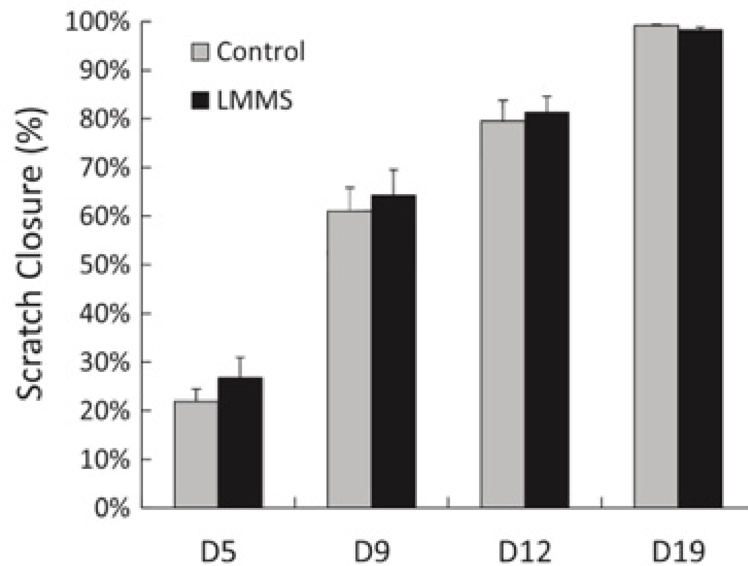

Figure 3.13. Scratch closure in MDAMB231 cells. MDAMB231 cells showed no difference between experimental and control group on D5, D9, D12 and D19. All samples showed full scratch closure at the end of D19.

3.1.5. LINC and EMT Markers Expression in Breast Cancer Cell Lines

LaminA/C was shown to be relevant in several cancer types. For breast cancer it was shown to be diagnostically important. LINC proteins, as being in touch with the nuclear lamina, were also shown to be down regulated in breast cancer. These proteins were also important to predict survival and cancer stages. In our previous study, we showed that breast cancer cell line MDAMB231, goes into cell cycle arrest when encountered with LMMS. According to the literature this may suggest autopaghy or cellular senescence which is a natural mechanism that inhibits cancer growth in benign tumors. Lamin depletion in tumorigenic stress also results in nuclear envelope proteins degredation. So these changes and any possible therapautic effects of LMMSs may be subject to investigation.

Preliminary data for characterization of breast cancer cell lines based on LaminA/C and LINC protein expression levels, showed that MDAMB231 and MCF7 cells are quite different, while MCF10A and MDAMB231 have similar characteristic according to their LaminA/C and LINC protein expressions (Figure 3.14).

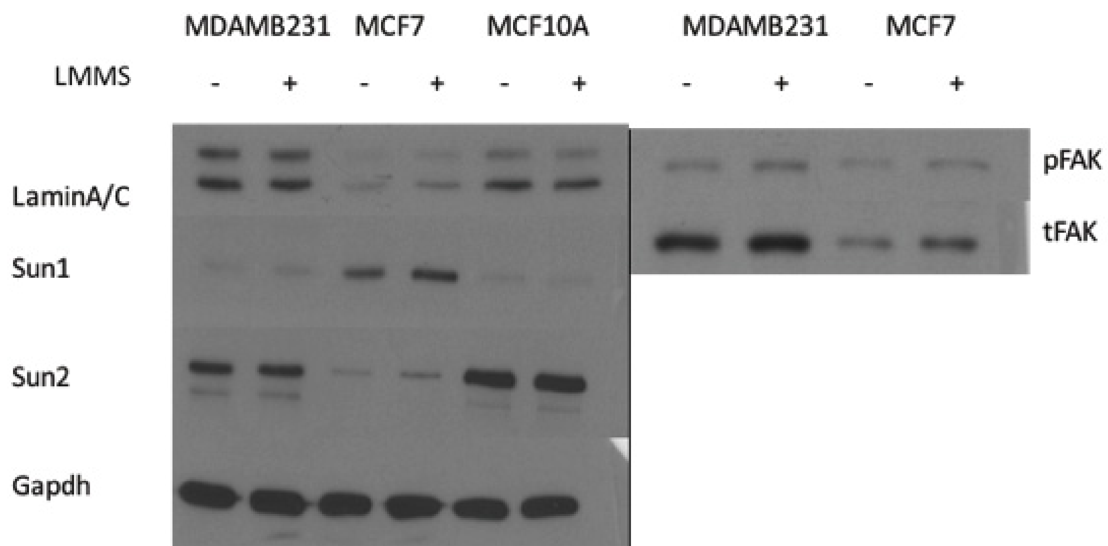

Figure 3.14. Expression of LINC members and FAK phosphorylation response to LMMS in breast cancer cells. MDAMB231 and MCF7 cells are quite different, while MCF10A and MDAMB231 have similar characteristic according to their LaminA/C and LINC protein expressions.

For the first study of marker expression levels, ZO1 and Snail2 showed an increase in LMMS group compared to control, on day 9 (Figure 3.15).

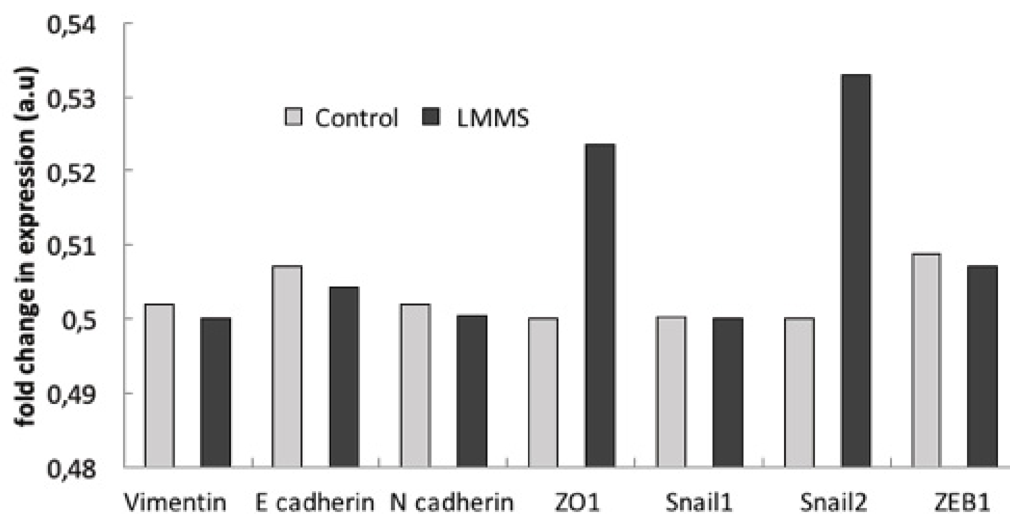

Figure 3.15. Expression of EMT markers in MDAMB231 cells. ZO1 and Snail2 showed an increase in LMMS group compared to control, on day 9.

3.2. Studies with Mesenchymal Stem Cells

3.2.1. SiRNA Knock-down

SiRNA amount to be added onto cells was chosen according to western blot result. Among doses, 0.75 µl/ml was observed as the most effective dose for knockdown and the study continued with this chosen dose (Figure 3.16).

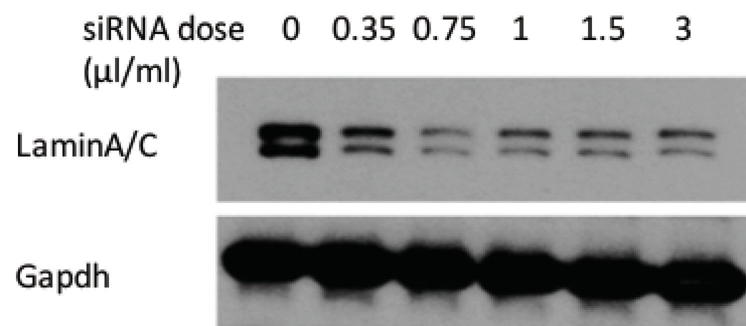

Figure 3.16. Determining the dose of siRNA for MSCs. 0.75 µl/ml was observed as the most effective dose for knockdown.

3.2.2. Nuclear Shape and Area

SiRNA for LaminA/C knock down treated cells (SiL cells) showed reduced nuclear circularity and abnormal nuclear shape (Figure 3.17) compared to control cells (SiC). This finding is in concordance with the literature [29]. Loss of LaminA/C, disrupts the nucleoskeleton leaving the nuclear in an abnormal shape (Figure 3.17).

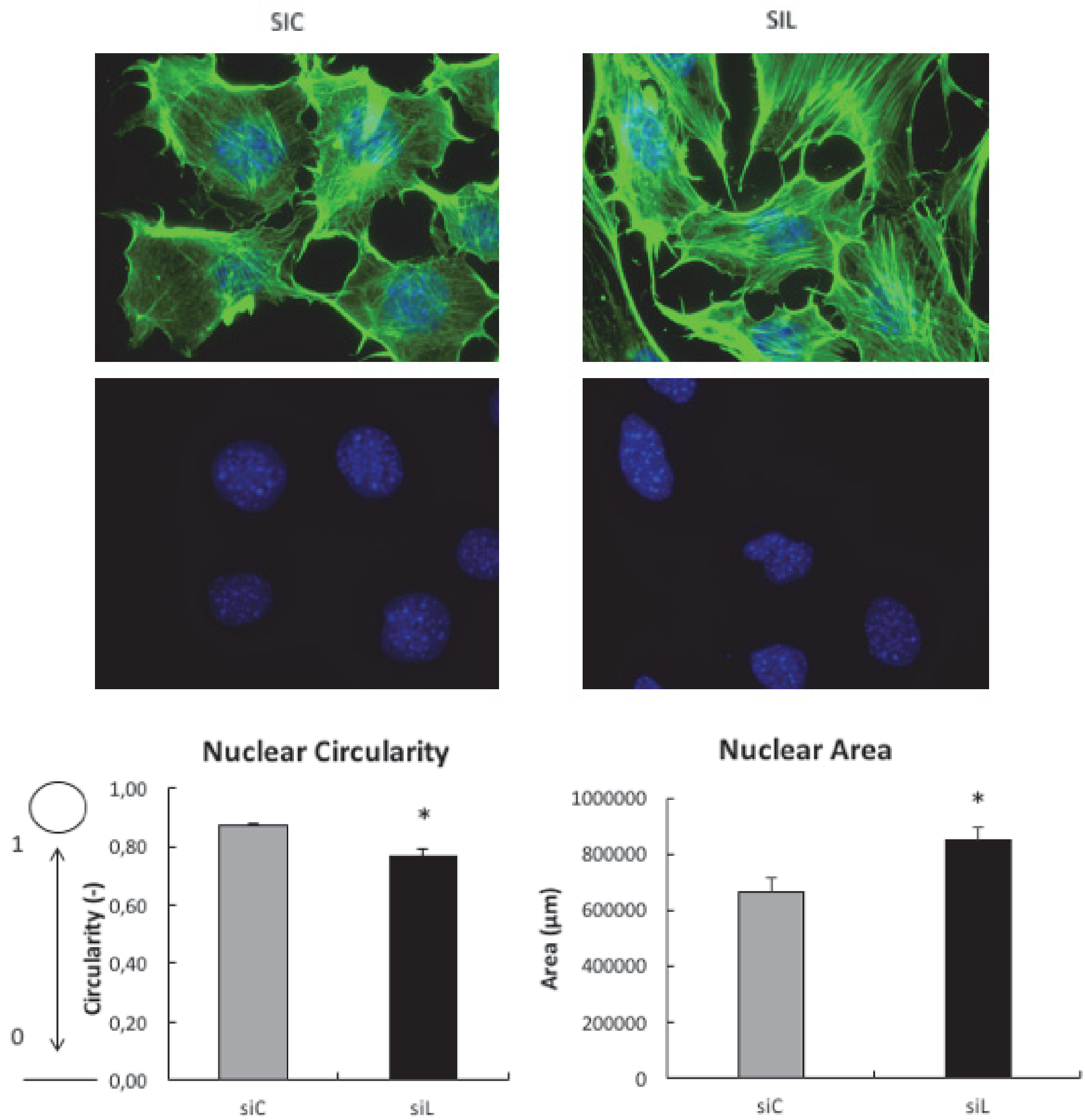

Figure 3.17. Change in cell shape and area. SiL cells showed 11% reduced nuclear circularity ($p<0.01$), 28% increased nuclear area ($p<0.01$) and abnormal nuclear shape. (Blue: Dapi, nucleus; Green: Phalloidin, actin).

3.2.3. Protein Expression Changes

3.2.3.1. Acute Response (FAK Phosphorylation) for LMMS

In acute response to LMMS, cells responded with an increase in FAK phosphorylation (42%, $p<0.05$, Figure 3.18) suggesting that these cells are still responsive to LMMS even if the nuclear lamin A/C network is knocked down. Interestingly, SiL-Ctrl cells also showed increased FAK phosphorylation compared to SiC-Ctrl cells. This baseline increase may suggest for a compensatory mechanism in generating a response. This needs further investigation.

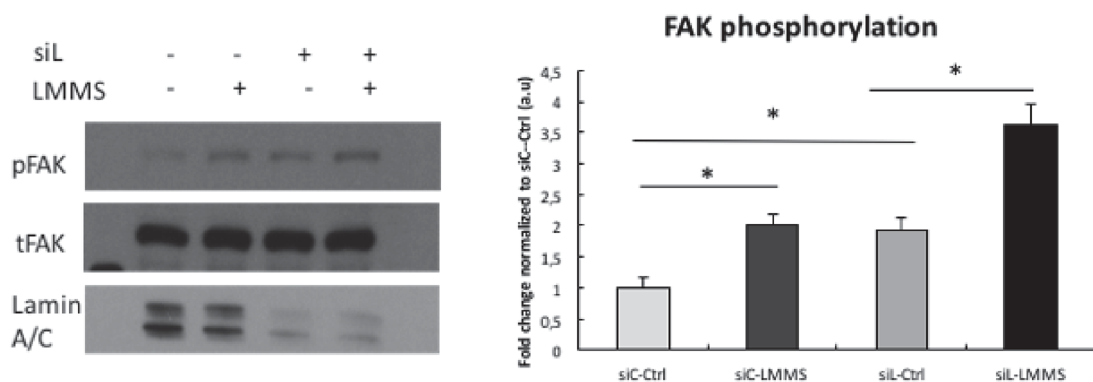

Figure 3.18. Acute response as FAK phosphorylation in MSCs. In acute response to LMMS, cells responded with an increase in FAK phosphorylation (42%, $p<0.05$).

3.2.3.2. Adipogenesis in LaminA/C Knockdown MSCs

In long term, SiL MSCs showed increased (186%, $p<0.01$) adiponectin (APN) protein levels averaged over 7 days, which shows that LaminA/C deficiencies led to adipogenesis (Figure 3.19). As shown in Figure 3.21 introduction of LMMS twice daily for 20 minutes for 7 days, lowered APN 16%. According to our results, LMMS is slowing down the favored state of adipogenesis caused by siRNA knockdown on days 5 and 7.

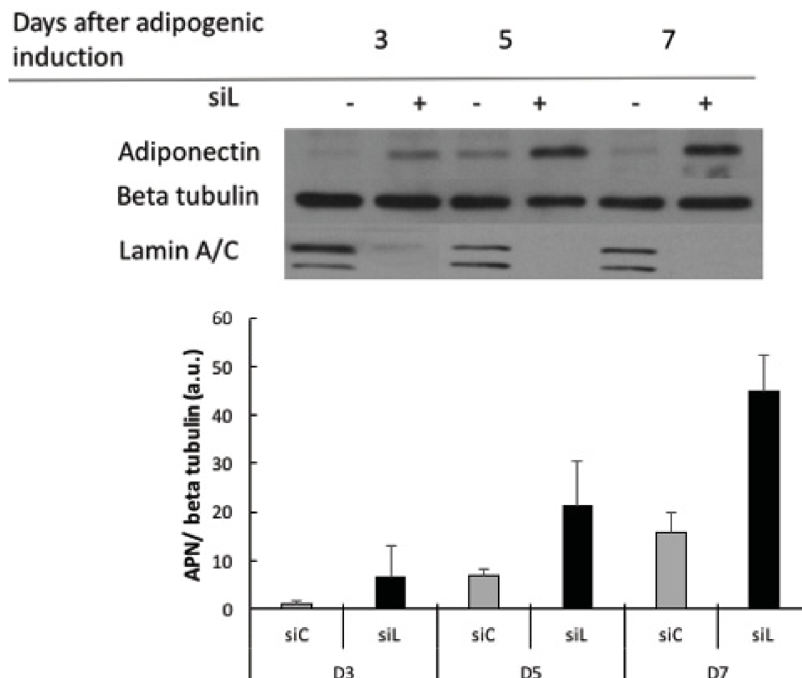

Figure 3.19. Adipogenetic marker APN expression in LaminA/A knockdown MSCs. The rate of adipogenesis is higher in knockdown cells. SiL MSCs showed increased (186%, $p<0.01$) adiponectin (APN) protein levels averaged over 7 days.

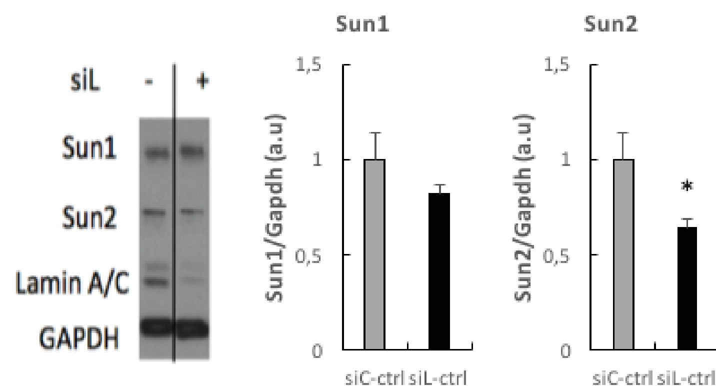

Figure 3.20. Expression of LINC proteins Sun1 and Sun2 in LaminA/C knockdown MSCs. Sun2 protein expression was decreased 33% ($p<0.05$).

LINC proteins Sun1 and Sun2 levels were determined by western blot (Figure 3.20). Sun2 levels were decreased significantly while Sun1 levels remained unchanged as also shown in qPCR results (Figure 3.20). These results may be interoreted as the LINC complex is partially functional that the cell is able to generate a response for LMMS.

3.2.3.3. Differentiation Under Effect of LMMS

LMMS signal lowered the rate of adipogenesis in siL cells as seen in a trend throughout 7 days. Though it was not sufficient enough to return it to the SiC baseline, there was a consistent trend of reduction. SiL group showed increased rate of adipogenesis compared to SiC group, as well. LaminA/C knockdown was also still effective on D5 and D7 as shown in the blot (Figure 3.21).

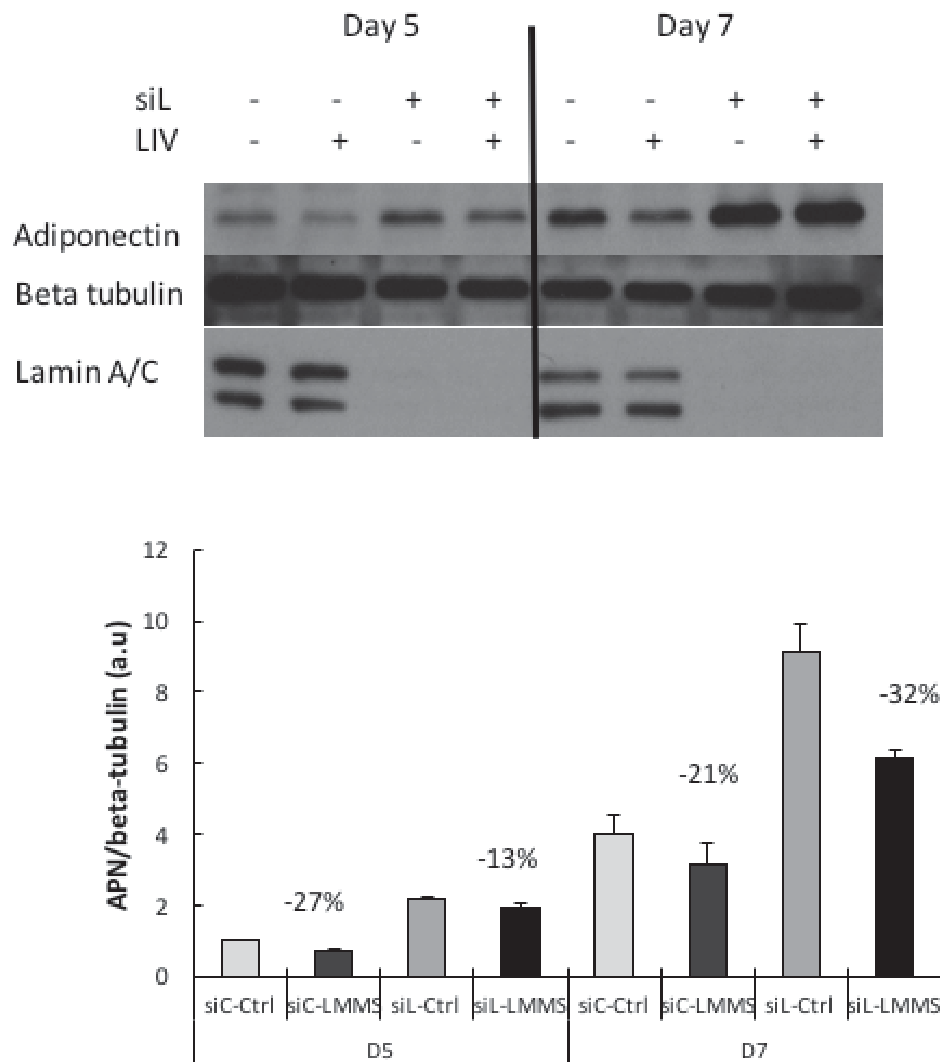

Figure 3.21. The change in differentiation rate in LaminA/C knockdown MSCs under LMMS. LMMS signal lowered the rate of adipogenesis in siL cells as seen in a trend throughout 7 days ($p>0.05$) SiL group showed increased rate of adipogenesis compared to SiC group ($p>0.05$). LaminA/C knockdown was still effective on D5 and D7 as shown in the blot.

3.2.4. Gene Expression Changes

Adiponectin gene expression levels were not significant, though qPCR method revealed a similar trend in adiponectin expression levels as the protein expression levels (Figure 3.22). SiL controls had higher adiponectin levels than SiC samples. And LMMS decreased adiponectin on D7 in both SiC and SiL group and on D5 in SiL group.

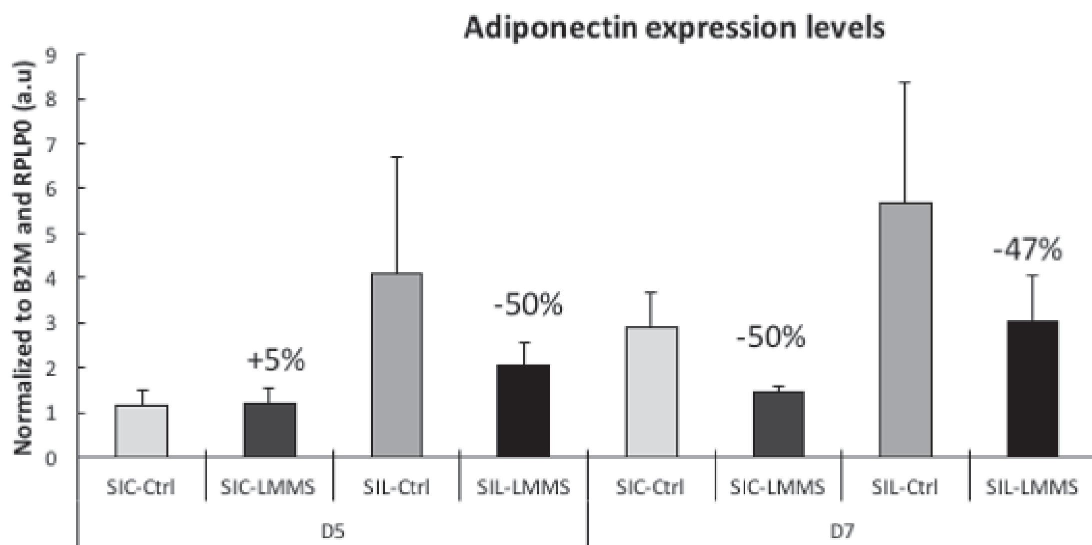

Figure 3.22. APN gene expression levels in LaminA/C knockdown cells under LMMS. SiL controls had higher adiponectin levels than SiC samples. And LMMS decreased adiponectin on D7 in both SiC and SiL group and on D5 in SiL group ($p>0.05$).

CHAPTER 4

DISCUSSION

Mechanical loads are important regulators of cellular functions such as cellular division, cytoskeletal arrangement, gene expression and homeostasis. All cells and tissues are subject to mechanical loads [24, 345, 346]. When these mechanical loads are absent, many cellular functions are impaired or out of control, such as growth, proliferation, fate determination, tissue integrity [347].

Mechanical signals are anabolic for normal tissues and cells. Bone tissue is one of the well-characterized tissues under mechanical loading. Bone cells are responsive to many different forms of mechanical signals such as cyclic stretch [345], shear stress [348, 349] and static pressure [350] as well as low magnitude mechanical signals. In absence of mechanical signal such as in space travel, bone turnover shifts towards catabolism [24, 317, 346]. Absence of mechanical loads such as in sedantary lifestyle, directs bone marrow progenitors to adipogenesis. Presence of physical exercise shifts this fate selection of the progenitor cells towards osteogenesis [351, 352]. Low magnitude mechanical signals (LMMS), enhances osteogenesis even after mechanical unloading [212]. LMMS triggers anabolic responses in osteoprogenitor cells [353, 354], for adipogenesis these responses are not well characterized [355].

Tenocytes are another example of studied cells under mechanical loading. Tenocytes showed an increased collagen production under cyclic stretch [356]. Mechanical loading was also reported to increase chondrogenesis of mesenchymal stem cells [357]. In other tissues and cell types, effects of low magnitude mechanical signals are not well investigated.

Under disease conditions, low magnitude mechanical signals are advantageous in a therapeutic way. Since mechanosensing and mechanotransduction pathways are impaired LMMSs are useful in triggering and recovery of the pathways.

In our study, LMMS signals were used to show their recovering and protective effects in breast cancer and LaminA/C knock-down bone marrow derived mesenchymal stem cells (BM-MSC). These cell types have reduced mechanosensitivity due to changes in their mechanotransduction machinery. Breast cancer cell lines, have altered

cytoskeleton to adapt their stiff tumor environment and facilitate migration and invasion. BM-MSCs with LaminA/C knockdown, have their nucleoskeleton impaired. This loss, alters the force transduced into the nucleus.

In the study, we hypothesized the breast cancer cells would respond to LMMS and their behavior would change. According to our "stress shielding" model, LMMS was used to restore partially unloaded state of breast cancer cells. Throughout the study, breast cancer cell lines showed a steady proliferation. LMMS slowed the rate of proliferation in experimental group of MDAMB231 cells. Non-cancerous control cell line MCF10A cell proliferation was not affected. MCF7 cells on the other hand, did not show a clear pattern under LMMS application. Cell proliferation studies showed LMMS can be beneficial in suppression of growth in MDAMB231 cells.

To explain the reduction in proliferation, apoptosis and cell cycle studies were done. MDAMB231 cells did not show any significant difference in apoptotic phenotype after LMMS treatment. Instead, LMMS led MDAMB231 cell to arrest in G1 phase [358]. Migratory potential was not altered as wound healing assay showed.

LMMS was reported as anabolic in normal musculoskeletal tissue *in vivo* and in recovery from mechanical unloading [212], as a suppressor in adipogenesis in fat pads [351], liver [359], and bone marrow [234]. In these studies, no harming effects were reported in normal controls. Under *in vivo* conditions, daily loading regimen was reported as beneficial for skeletal health and was harmless for animal longevity [235]. Taken together, literature shows that LMMS is harmless if not anabolic for normal tissues and cells whereas mostly advantageous for diseased cells and tissues. In another *in vivo* study, survival rate of mice with ovarian cancer was not increased by LMMS but, tumor incidence with fewer organs was reported [360]. Our results also showed that cancer cell growth was not stopped, but delayed as proliferation and cell cycle analyses showed. Therefore, LMMS might be regulating the cancer initiation but not progression. For cancer research, optimization studies for duration, frequency and amplitude are needed.

In our study, LMMS directly affected proliferation of MDAMB231 cells. Cancer cells are less stiff than normal cells, previous studies shows this as a characteristic for cancer cells in breast [360], ovarian [361], lung [362], bladder [363], leukemia [364], and gastrointestinal cancers [365]. Cancer cell invasion is directly proportional to cellular compliance [366]. As cancer cells lose their ability to adhere to the extracellular matrix, their metastatic capacity increases [367]. Total number of glycopeptides in the cells from malignant tumors were reported as reduced to about two-thirds of the level in the

nonmalignant cells [368]. The loss of integrins, laminins and other proteins that form or interact with the basement membrane pairs with their lacking mechanical signals in their tumor extracellular environment. Aggressively metastatic cells were reported to have less force interactions with their environment [369]. Mechanical signals regulate many functions in cells and as cells try to adapt their mechanical environment, their survival, proliferation and invasion capabilities are affected accordingly [370]. Other than integrin mediated adhesions, cells also use side-to-side junctions that connect epithelial cells together. These connection proteins have anchoring sites for extracellular portions and transmembrane portions to connect cytoskeleton. Adaptor complexes mediates adherens junctions connections to cytoskeleton and desmosomes to intermediate filaments. These junctions also provide mechanical support and intracellular signalling recruitment based on position of nucleus [371]. The cell to cell connections are more prominent in MCF7 cells, while MDAMB231 cells grow rather individually. Mechanical vibrations, therefore, may be acting on individual characteristics of MDAMB231 cells.

Our study was designed in 2D environment, to reach more comprehensive results, behavior of cancer cells may be investigated in 3D environment. Therefore, their tumor forming capabilities may be better understood. Using cancer cell lines is another limitation since their behavior do not always represent the disease scenario. For better translational results, primary cancer cells may be tested in the system, or animal studies should be considered.

The scratch wound assay is a good way to obtain preliminary information about cells' migratory behavior but it is not an absolute decider. Scratch wound results may be affected from environmental and experimental conditions. It does not show a clear distinction between whether the cells are proliferating to cover the wound or they are migrating to cover the wound. Mitomycin agent to inhibit growth, may be used in this assay to get better migration results.

Our cell cycle arrest findings are in concordance with the literature. Avvisato et al.. have previously reported that shear stress results in a similar G1 arrest in colon cancer cells [372]. Cell cycle arrest as response to mechanical loading may be a common behavior among cancer cells. In absence of mechanical loading, such as spaceflight [346], MCF7 cells were reported to cycle more and have increased proliferation [373]. In another study on mechanical unloading, constant weightlessness triggered metastatic spread and inhibited apoptosis in thyroid carcinoma cells [374]. G1 arrest on the other hand, is coupled with increased invasion in several studies [375]. These data suggest that

mechanical signals should be studied further to uncover its regulatory effects on cell cycle in various cancer cells.

Cancer cells have the common feature as being softer than their normal counterparts. Given the previous studies on different cancer types, under LMMS, cancer cells are not affected like normal cells. The decrease in proliferation we reported in our study, may be a common behavior among cancer types. The mechanical signal used in studies is of critical importance. Mechanical vibrations have negative effects on growth and division while compression forces increases invasiveness for breast cancer cells [252]. Cyclic stretch in another study was reported to promote growth on lung cancer cells [376]. Tumor environment or circulating cancer cells would have different responses to mechanical signals. Therefore, studies should be designed accordingly.

Exercise as a mechanical load in our everyday life, is said as protective and even therapeutic for cancer. Studies show that circulating and muscle specific miRNAs have an adaptive role between exercise and cancer prevention and suppression [377-379], possibly *via* their DNA repair and checkpoint functions [380]. Exercise also induces myokines which have a role in prevention of colorectal cancer [381]. Physical exercise, has a decreasing effect on adipose mass therefore reducing the amount of carcinogens [382-385], this is another factor that reduces the risk of cancer. Exercise also helps reducing the level of circulating insulin that act as a cell proliferating agent [386, 387]. Protecting effect exercise on cancer is usually explained *via* its enhancing effect on immune system [388, 389], therefore enabling for the body to fight cancerous cells. But the exact mechanisms how exercise regulates protective effects for cancer is still unclear.

Here in this study, we hypothesized that reduction in cell proliferation is a component of this protection MDAMB231 cells showed a clear reduction in cell proliferation and arrest in cell cycle, MCF7 cells did not seemed to be affected by LMMS signals. In this difference, signal optimization and application regimens surely plays a significant role, these two different cell lines have different mechanosensitivities, cellular stiffness and cytoskeletal stiffness [390, 391]. And there are other differences between these breast cancer cell lines. Furthermore, tumorigenic characters of MCF7 and MDAMB231 cells are different [392]. MCF7 cells are estrogen dependent to form a tumor, while MDAMB231 cells are aggressively tumorigenic and metastatic. MCF7 cells are primary tumor cells, with estrogen and progesterone receptors. MDAMB231 cells do not have any estrogen receptors. MDAMB231 cells are invasive by their extracellular matrix (ECM) degrading matrix metalloproteinase (MMP) enzymes [393]. Considering

MDAMB231 cells were more responsive to mechanical signals, and MCF7 cells did not respond with a reduction in proliferation. Thus, we may conclude that LMMS interferes with the progression of the cancer, not the initiation.

In breast tissue, mesenchymal stem cells play a role in cancer progression and metastasis. They form an environment for cancer cells to create tumors [394, 395]. These cells have potential to differentiate into adipose cells in breast and with aging, adipogenesis increases [396]. Breast cancer cells from patients were reported to have altered Lamin A/C status [323, 397]. In our study, we also showed breast cancer cell lines have different profiles for Lamin A/C and LINC proteins. Thirty-eight percent of breast cancer patient samples do not have Lamin A/C [398]. A recent study showed Lamin A/C upregulation in breast cancer cells increased reattachment [399]. Therefore, the difference in Lamin A/C and LINC profiles between breast cancer cells need more investigation.
In breast cancer metastasis, epithelial to mesenchymal transition is an important path that cells undergo. There are other ways to acquire metastatic capabilities such as MET (mesenchymal to epithelial transition) [400] or collective migration [401]. EMT is not a required pathway [402] but it is the most frequent way cancer cells use for metastasis. In this transition, cell’s cytoskeletal arrangement remodeled to enable motion and invasion. In our study, EMT was considered as a possible way for cells to choose. EMT process is active in breast cancer, in the process of cell invasion and metastasis [287]. In this process, the direction and magnitude of intracellular forces are the determinants. Cell-cell connections and connexins in the EMT process have been shown to be regulated by active transcription factors in the EMT process [288-292]. Decreased cell connections cause loss of apical-basal polarity and reduced structural support in cells [293].

The first and most important molecular marker seen in breast cancer cells in the EMT process is the decrease in E-cadherin expression[285]. It is known that transcription factors such as Snail / Slug family factors, Twist, Zeb1 and Zeb2 play an active role in this decrease [275]. Snail and Twist act together to trigger ZEB1 expression [286], and thus with the decrease in E-cadherin expression the cells weaken and lose their binding to neighboring cells [276]. Subsequently, with the morphological changes in the cell skeleton, the cell becomes ready for migration [276]. This change begins with G-actin polymerization, which leads to a tip that will lead to migration in the cell [99]. The increase in the expression of vimentin, a mesenchymal marker, changes the organization of the cortical actin skeleton and forms membrane protrusions. The reorganization of the actin cytoskeleton and the formation of stress fibers also enhances cell contractility [294].

Vimentin plays a role in network deterioration and altered cell stiffness, and in the direction of cell invasion [295]. Mechanical forces outside the cell as well as within the cell have been shown to be effective in this process. The increase in extracellular medium stiffness activates EMT pathways with TWIST1 activation and enhances cell invasion [296].

We hypothesized LMMS would also be sensed and responded by LaminA/C knock down BM-MSCs even if they had an impaired nucleoskeleton. To test this, LMMS was delivered to mesenchymal stem cells which are in the increased adipogenesis state, to restore any loss in mechanoresponsiveness.

Our results show that, in concordance with the literature, in mesenchymal stem cells (MSC), Lamin A/C knock down results in abnormal nuclear shape [403] and an increased basal FAK phosphorylation immediately after mechanical signal. Application of LMMS increased FAK phosphorylation in both siC and siL cells. This suggests that, given that Lamin A/C knock down MSCs are still responsive to mechanical signals, Lamin A/C is not required for generation of a mechanical response.

Lamin A/C is in direct contact with LINC and peripheral heterochromatin [404]. Disruption of this Lamin A/C scaffold in the nucleus, may change the anchoring sites for both LINC and peripheral heterochromatin and therefore may have effect on gene expression profile of the cell. In our study, loss of Lamin A/C in MSC increased the Protein/mRNA levels of adipogenic marker Adiponectin. However, adipogenesis rate was lowered in cells that received LMMS regardless of siRNA treatment.

Recent studies revealed the close relations between Lamin A/C nucleoskeleton with gene expression profiles and cellular differentiation [59]. The link between nucleoskeleton and the chromatin affects expression states of certain genes. In disruption of this nucleoskeleton, certain genes repressed by Lamin A/C links, may be activated or *vice versa* [404, 405]. Lamina-associated domains (LADs) are heterochromatin sections that are repositioned to the nuclear periphery and these domains are transcriptionally inactive [406, 407]. Although lineage-committed cells share an identical genome with embryonic stem cells, the epigenomic landscape differs markedly, with most changes arising from redistribution of transcriptional repressor marks H3K9me3 and H3K27me3 (21). Our model suggests that many of these changes are driven by the increased expression of lamin A during terminal differentiation of myoblasts and other cells [408]. Agrelo et al. reported CpG island promoter hypermethylation resulted in loss of Lamin A/C expression in lymphoma and leukaemia [409]. This phenomenon may explain the

increased rate of adipogenesis in those cells.

While being able to still maintain a mechanical response against LMMS, adipogenesis rate is slowed down but not returned to basal levels in LaminA/C knocked-down cells. This tells us that mechanical response can still be generated by other mechanically responsive elements of the cell such as the cytoskeleton and LINC complex, while differentiation processes are not only mechanically regulated but also needs chromatin regulation. Houben et al. reported that effects of lamin A/C loss, may need several cell cycle to become apparent [410]. They also reported that, Lamin A/C knockdown and knockout cells have similar changes in terms of nuclear organization and shape. LINC elements Sun1 and 2 expressions change similarly, but Nesprin expressions are rather dependent on knockdown/knockout state of the cell. Nesprin localization changes in knockout fibroblasts and vimentin and actin cytoskeletal changes appear in those cells. Lamin B organization remains intact in both types of genetic manipulation state.

Physical exercise, is beneficial for a healthy lifestyle. Beginning with a healthy diet, exercise as in form of mechanical vibrations are used in medical areas to increase life quality of the patients. For lung cancer patients, exercise is recommended for management of the disease [411]. Vibrations are widely used to increase flow rates and trigger secretion in respiratory system [412]. Oscillatory devices are used to clear airways in cystic fibrosis patients [413]. For thoracoscopic lung resection surgery patients' physiotherapy, a vibratory device called Acapella is present in the market [414].

Mechanical vibration levels for pain relief, muscle training and relaxation are characterized and physiotherapists frequently use these methods especially for disabled or elderly. According to the literature, 200Hz for pain relief 50 Hz for muscle relaxation and 300 Hz for muscle training are used in physiotherapy [415].

Other than focal vibrations, whole body vibrations are also used for medical purposes. Together with muscle training [210, 220, 416] and weight loss purposes [417], whole body vibration devices are used for management of pain and prevent neuropathy in cancer patients [418]. A vibratory bed product Evocell is present in the market for post-operation patients [419].

Mechanical vibrations are frequently used for medical purposes, as seen in the examples. The area will be more focused on exact magnitude and frequencies that are beneficial and specific for body regions in the future. So, investigations in this field should expand to obtain optimal therapeutic windows specific for health and disease.

CHAPTER 5

CONCLUSION

In this doctoral dissertation, low density mechanical vibrations, which have been previously demonstrated on tissue such as bone, muscle, tendon, and cells forming these tissues, have been used on breast cancer cell lines and bone marrow derived- Lamin A / C knockdown mesenchymal stem cells, for the first time.

Breast cancer cells are softer than normal mammary epithelial cells and less sensitive to environmental physical signals as stated widely in the literature. The anabolic effect of physical and mechanical signals such as exercise, on the normal tissues and cells has been shown to be a decline in various cancers, which is also summarized in the literature. Our study aimed to test the effect of physical exercise on the cancer cells by replacing the reduced mechanical forces in order to explain the mechanism of the protective and regenerative effect on the cancer.

Our results showed mechanical vibrations acted on aggressive breast cancer cells to reduce proliferation and viability. In these G1-arrested cells, neither cell detachment nor apoptosis was significant. Similarly cells ability of wound closure was not affected. In other type of breast cancer cell line, which was less aggressive, none of the properties that were examined was significant. From the literature, we know that these cells are responsive for environmental texture and mechanical signals such as fluid shear etc, but, mechanical vibrations may need a distinctive window of frequency and/or magnitude for MCF7 cells to respond. As their normal controls, MCF10A cells were not affected.

From this part of the study, we now know that breast cancer cell lines are responsive to; and aggressive types are negatively affected by mechanical vibrations. This information may have translations in medical and physiotherapy treatments for cancer patients. Cancer patients need less harmful treatments and complementary treatments for radio- or chemotherapy to increase their life quality. Mechanical vibrations may lead to a promising solutions for these problems.

In order to reveal another perspective for mechanical vibrations' effects on differentiation and cytoskeleton-nucleus connections, we used bone marrow derived mesenchymal stem cells. These cells were LaminA/C knocked down, which disabled the

nucleoskeleton. In these cells which have increased adipogenesis rate, mechanical vibrations were not enough to stop or reverse this differentiation but they slowed the rate. These results, showed even if the cell's nucleoskeleton was disrupted, they were still responsive to vibrations. It may also suggest that other members, such as cytoskeleton, membrane or the LINC complex had more responsibility in mechanosensation and mechanoresponsiveness.

Our study was designed in 2D environment. The results need to be replicated in a 3D environment to interpret better perspectives. 2D environments have certain limitations for medical purposes. First, the extracellular environment is not simulated. In our study, we applied mechanical vibration directly onto cells to see their pure response, aside from extracellular matrix. In a 3D environment, extracellular matrix will be another element that responds to mechanical forces and this response may also change or contribute cellular responses. This dimension cannot be predicted in a 2D design. But in another perspective, our hypotheses were set on cellular responses and our design let us determine pure cellular responses.

Another limitation is the translation of the results into medical applications. Applying mechanical vibrations directly onto cells in a human body is close to impossible. Surrounding tissues and organs will be somehow affected. Our study presents a starting point in developing a medical application. Our results showed that breast epithelial cell line MCF10A was not affected. These limitation points need to be overcome in following studies.

LaminA/C related diseases are mostly involved with abnormal differentiation (such as progeria, lipodystrophies, muscular dystrophies). With application of LMMS signals, we tried to present a control point between adipogenic differentiation and mechanical responsiveness. All these disease have distinc progression mechanisms and our cellular model does not represent any of the diseases particularly, but physical exercise is recommended as a complementary treatment for most of them, to keep muscle tissues intact. LMMS, may present a harmless physical exercise option for those critical diseases.

Currently, vibrations are used in physiotherapy in different forms and magnitudes. For better and defined results, they should be optimized in magnitude, frequency and application regimen for each specific purpose or disease. For both healthy and diseased, mechanical vibrations hold promise for a better life quality.

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