Building gyms for cells – development of combinatorial screening platforms for mechanical stimulation

Ravi Sinha

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mechanical stimulation

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PhD Thesis, University of Twente, Enschede, The Netherlands

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**BUILDING GYMS FOR CELLS – DEVELOPMENT OF
COMBINATORIAL SCREENING PLATFORMS FOR
MECHANICAL STIMULATION**

DISSERTATION

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Chapter 1. General introduction and aims

Cells and mechanical stimuli

It is well known today that cells can sense and respond to mechanical stimuli. Enabled by modern tools, interactions between mechanical forces and biology are being studied with increased rigor, but the interest is not all new. The mechanics of cells have been of interest ever since they were observed under the early microscopes over 300 years ago¹. The early observations were about cellular movement and viscosity, followed by studies of tissue mechanics using the material characterization tools developed during the nineteenth century and then with the development of more sophisticated tools such as the atomic force microscope, the mechanics of individual cells could be studied in the twentieth century¹. The realization that mechanical signals can bring about biological changes has also been there for some time. Wolff's law, stating that bones can adapt their shape and architecture in response to mechanical loads, is a famous example and dates back to the late nineteenth century². How the mechanical signals get converted to a biological response at cellular levels needed more advanced tools of applying forces at a single cell level and tracking biochemical entities at molecular levels. In the last few decades, it has been established with the uncovering of mechanisms that cells use specialized structures and molecules to detect and respond to mechanical signals by undergoing or initiating biochemical changes.³

Mechanotransduction

The process of converting mechanical signals to biochemical change is known as mechanotransduction³. Some key players involved in this process include the cytoskeleton, nuclear lamina, cadherins, integrins, focal adhesions, primary cilia and stretch activated ion channels³. Figure 1 schematically shows the locations of these key players in the cells.

The cell cytoskeleton is one of the most important structures in the process of mechanotransduction. It consists of a network of filaments and tubules distributed over the entire cytoplasm⁴. Thus any force transfer inside the cell tends to involve it. The cytoskeleton has been shown to be a tensegrity (tensional integrity) structure with elements under compression (microtubules) held together by elements under tension (microfilaments, intermediate filaments)⁵. This structure enables it not just to bear loads and give the cells their shape, but also to respond quickly to external forces. In addition the cytoskeleton generates contractile forces (actomyosin fibers) which enable the cell, among other things, to move and to assess its environment by tugging at it⁴. Many cells also develop protrusions or surface processes, such as the primary cilia, which are structurally supported by

cytoskeleton molecules but have associated specialized machinery (receptors, channels) that can be activated through the deflection of these processes by external forces⁶. To be sensed by the cells, external forces need to produce displacements and this requires the cells to be constrained in some way. Two kinds of cell constraints exist for adherent cells namely attachment to the extracellular matrix (ECM) and attachment to other cells. These constraints use specialized membrane proteins or molecular assemblies (cadherins⁷ and gap junctions⁸ for cell-cell attachment, integrins and focal adhesions for cell-ECM attachment⁹) which are connected to the cytoskeleton. Biochemical changes resulting from mechanical stimuli can begin at the cell membrane itself via conformational changes in membrane proteins, as illustrated in figure 2, or inside the cell as the forces are transmitted inside through the constraint points. Longer term biochemical changes involve changes in gene expression. This can happen due to downstream reactions resulting from the mechanical stimuli¹⁰, but also by the direct transfer of forces to the nucleus¹¹. The nucleus containing the chromatin (DNA and associated molecules) is surrounded by the nuclear lamina which shares connections with the cytoskeleton, providing a route for transfer of forces to the nucleus¹².

At the molecular scale, mechanotransduction generally involves physical changes to the conformation of large biomolecules such that it leads to further biochemical processes. Some examples are illustrated in figure 2. Forces applied to the membrane or membrane proteins (figure 2A) or forces from the cytoskeleton (figure 2B) can open ion channels, leading to electrochemical changes within the cell¹³. Similarly force application to other molecules can cause conformational changes that restrict their activity (for example inhibition of substrate cleavage by an enzyme, (figure 2C) or expose new reactive sites for reactions not possible before (figure 2D)¹⁴. Such conformational changes do not always need the active application of forces and can also be brought about by the presentation of appropriate geometries, for example using topographical cues¹⁵. Also, besides the force, the rate of force application can be important as well, for example some biomolecules can form catch bonds and become tightly bound to other molecules when pulled apart at a high enough rate¹⁶. And finally, the removal of forces can affect chemistry as well, for example the release of compression on microtubules changes their chemical potential and leads to the addition of monomers and polymer chain growth (figure 2E)¹⁷.

Mediators of Mechanotransduction

Figure 1 – Schematic representation of the key players in the cell mechanotransduction process³.

Figure 2 – Molecular mechanisms used by cells to detect mechanical signals³. Tensile stretch, marked as T, can open ion channels (A,B), inhibit enzymatic activity such as substrate cleavage (C) and reveal hidden reactive sites in molecules (D). Release of compressive force on microtubules can change their chemical potential permitting polymerization and chain growth.

In vitro platforms to apply mechanical stimulation to cells and mechanobiology

As the mechanotransduction mechanisms have been discovered, parallel studies have shown that externally applied forces in the form of strains, flow induced shear stresses, compression and pressure variations can affect cell and tissue morphology and function. Also since cells generate forces internally and tug at their environment to assess it and adapt accordingly, passive mechanical stimuli such as substrate stiffness, surface topography, arrangement of cell attachment areas, confinement and substrate relaxation ability from stretch have also been found to affect cell behavior. Biochemical changes in cells in response to mechanical stimuli are now being widely explored and the studies are being collectively called the field of mechanobiology. Mechanobiology holds a lot of promise in terms of offering new mechanical ways of complementing the field of health and medicine where biochemistry has so far dominated.

Application areas

Two areas where mechanobiology can play an important role are tissue engineering and in vitro platforms for pharmaceutical discovery.

Tissue engineering

Tissue engineering, in a very general sense, involves combining cells and materials in the lab to make tissue substitutes that can repair or replace damaged tissue in the body. The field of tissue engineering, while highly promising, has had only a few clinical successes^{18,19}. Over time several advances have been made, some examples being the use of stem cells as an efficient cell source²⁰, use of decellularized extra cellular matrix realizing the importance of the surroundings of cells²¹, and vascularization of engineered tissue eliminating the nutrient diffusion based tissue size limit²². Nonetheless complete success leading to clinical translation remains elusive. With increasing appreciation of mechanobiology, the need for the inclusion of the correct mechanical stimuli is also realized²³. While mechanical stimulation mimicking the dynamic mechanical situation in vivo has been used in tissue engineering bioreactors for several years now²⁴, newer approaches such as the use of stem cells, where the stimuli required cannot always be obtained from in vivo measurements, necessitate in vitro studies investigating the effects of various stimuli on the cells.

Pharmaceutical testing platforms

The pharmaceutical discovery process currently follows the sequence – testing drug candidates on cells, followed by small animals, followed by large animals and finally in humans. However due to several differences in the biology, results from animal models are

not predictive for effects in humans. Testing on human cells is not sufficient due to the lack of the *in vivo* complexity such as the interaction between various organs. To fill this gap, organs-on-a-chip models were proposed where microfluidic devices with small cell populations representing specific tissues could be connected by a network representing the circulatory system and together give a body-on-a-chip model for pharmaceutical testing²⁵. Such a model would be more predictive of the effects of the tested drugs in humans as well as reduce the burden on animal studies which are ethically questionable. What some recent studies have shown is that such models could significantly benefit from physiological mechanical stimuli to the cells^{26,27}.

Thus, *in vitro* studies in both application areas described above are increasingly looking at including mechanical stimuli to cells to bring cell behavior closer to *in vivo* conditions.

Thesis outline and aims

The several ways of presenting mechanical stimuli to cells *in vitro* are inspired from the *in vivo* conditions of the cells. However, while many of these stimuli are presented to the cells simultaneously *in vivo*, early *in vitro* studies have looked at them separately to understand the effect of each. *In vitro* studies presenting combinations of the mechanical stimuli are now starting to emerge (chapter 2). Optimizing the variations (magnitude, direction, frequency, dosage) of each mechanical stimulus becomes complicated in such studies as the independent variations of each stimulus rapidly increase the number of test conditions needed. This could be addressed by combinatorial high throughput platforms that can apply combinations of several variations of each stimulus in parallel. The aim of this thesis was to develop such an enhanced throughput platform to combinatorially apply strain and shear stress simultaneously to cells. The platform was desired to be of general use for stimulating cells, but a primary application of interest was vascularized bone tissue engineering from co-culture of mesenchymal stem cells and endothelial cells. Thus, a design requirement was sufficiently large test areas to study up to a few hundred cells. In terms of mechanical signals, the desired strains and shear stresses were to be within the physiological ranges which both mesenchymal stem cells and endothelial cells could withstand (up to 10% strains and 0.1-1.0 Pa shear stresses were selected based on previously reported studies), although an ability to apply supraphysiological levels of these stimuli was considered an additional advantage for applying pathological stimuli to cells. The stepwise goals were: (1) to develop a device to apply various combinations of equibiaxial strains and shear stresses to cells and validate it with a model cell type (chapter 3), (2) to develop a variant of the device capable of applying anisotropic biaxial strains in combination with shear stresses and validate it as well with a model cell type (chapter 4), (3) to apply various combined mechanical stimulations to mesenchymal stem cell – endothelial cell co-cultures using the

developed devices (chapter 5) and (4) to demonstrate the flexibility of the platforms by using them for another application, for which studying the osteogenic-adipogenic differentiation balance of mesenchymal stem cells in combined differentiation medium was chosen (chapter 6). The expected outcomes were (1&2) fabrication and characterization of devices to apply the desired mechanical stimuli and insights on the effects of the combined mechanical stimuli on the model cell types as well as a comparison with the effects of each stimulus applied alone, (3) insights on how the mechanical stimuli affect the spontaneous assembly of tube like structures in the co-cultures, and (4) a distribution of the extents of osteogenic and adipogenic differentiations of mesenchymal stem cells as a function of the applied mechanical stimuli.

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Chapter 2. Tuning cell and tissue development by combining multiple mechanical signals

Abstract – Mechanical signals are important for cells. Cells use specialized mechanisms to detect and actively adapt to their mechanical microenvironment, including external forces and the mechanical properties and shapes of their surroundings. How mechanical signals are translated to cellular actions is not completely understood, but several structures and molecules have been recognized and their roles in the cell's response to the mechanical stimuli have been demonstrated. A wide variety of approaches have been developed to apply mechanical stimuli to cells in vitro to further understand biomechanical processes in cells, but they remain lower in complexity compared to the in vivo conditions. Since a mechanical microenvironment is the result of a combination of multiple mechanical signals, it is unlikely that the complete link between the environment and cellular responses can be unraveled by investigating the effect of individual mechanical signals on cells. Hence combinations of mechanical stimuli have been applied to cells in vitro. While a few such studies have been reported over the past three decades, a large number of systematic approaches of applying combinations of mechanical stimuli are now starting to emerge. A review of such approaches, the new insights gained from them, the challenges and the future prospects are presented here.

Introduction

Recent years have seen an increasing interest in the effects of mechanical stimuli on cells. This has been fueled, among other reasons, by the technology enabled improved understanding of cellular mechanotransduction¹ and the promise showed by the addition of appropriate mechanical stimuli in tissue engineering approaches² and better predictive in vitro test platforms for pharmaceutical discovery^{3,4}. The main ways in which mechanical stimuli are applied to cells in vitro include stretching the substrate to which cells are attached, fluid flow over cells applying shear stress to the cells, compressive forces such as hydrostatic pressure, varying the substrate stiffness, varying the substrate topography and controlling the cell attachment area using patterning of the substrate. These stimuli are primarily inspired by the in vivo environment of cells and are applied to whole cells or cell populations. Other techniques such as magnetic tweezers, optical tweezers and atomic force microscopy have been employed for subcellular mechanical stimulation along with force quantification to understand mechanotransduction mechanisms⁵. And yet more techniques, such as traction force microscopy⁶ and micropillar arrays⁷ have been used to measure the

forces exerted by cells on their surroundings. However in all the techniques used, simplifications were used to study the effects of a particular stimulus. The application areas, namely tissue engineering and predictive cell cultures for pharmaceutical discovery, require providing an in vivo like environment for cells to elicit in vivo like behavior. In terms of mechanical stimuli, the in vivo situation is often complex with multiple stimuli being simultaneously present. In the absence of a complete understanding of cellular mechanotransduction mechanisms, it is difficult to predict the behavior of cells in a complex mechanical environment. Applying combinations of mechanical stimuli in vitro offers a way to further investigate the mechanotransduction mechanisms as well as find optimal in vitro stimuli for the application areas. While macroscale mechanical bioreactors have been explored in the past to apply tightly controlled combined mechanical stimulation to tissue constructs prepared from cells and materials⁸, most studies now employ micro or meso scale systems that not only allow for good control on the stimuli but also permit constant monitoring of the cells inside. Micro and meso scale platforms to apply mechanical stimuli to cells have been reviewed before⁹ but we will focus here on those that look at a combination of multiple stimuli.

Figure 1 – A schematic representation of various methods of applying mechanical stimuli to cells in vitro is shown here (middle column). Also shown are the schematic representations of the combinations of those mechanical stimuli covered in this review (side columns).

Figure 2 – The application of combinations of mechanical stimuli to cells in vitro has been put to several uses. (i) Aligning cardiomyocytes using attachment area patterning combined with the application of pathological strains has been used to develop cardiovascular disease models¹⁰. (ii) A combination of substrate stiffness and attachment area patterning has been applied to control the generation of neural networks in vitro¹¹. Strain – shear stress combinations have been utilized in (iii) lung-on-a-chip³ and (iv) gut-on-a-chip⁴ platforms where they elicit more in vivo like behavior from the respective cell types and provide a useful way for pharmaceutical testing.

Figure 3 – Stimulating cells in vitro with combinations of mechanical stimuli has also provided several insights. A few examples include: (i) Topography combined with strains has been used to show that basal actin responds to topography while the actin cap, that goes over the nucleus, responds to strains¹², (ii) Topography modulated cell attachment area and the corresponding modulation of cell contractility have been shown to have the potential to regulate MSC differentiation in the presence of flow induced shear stress¹³, and (iii) Strains applied to a hydrogel providing a physiological stiffness for cells revealed that the hydrodynamic forces from the fluid movement caused by the strains can have significant effects on cells¹⁴.

Application of combinations of mechanical stimuli

Cyclic substrate strain and flow induced shear stress

The cyclic substrate strain and flow induced shear stress combination has been the most widely studied combined mechanical stimulation for cells. It represents the dynamic stimuli present inside blood vessels and hence has been applied most often to endothelial cells¹⁵⁻²². One way in which such stimulation was initially achieved was by seeding cells on the inner lining of cylindrical elastomeric constructs and applying flow with a pressure pulse similar to blood flow^{15,16}. Most approaches developed later have used uniaxially stretched flat membranes which allow for easier microscopic imaging, the uniaxial strains in such

systems representing the circumferential strains in blood vessels^{17-19,22}. The flow is usually applied to the cells on these membranes perpendicular to the strain direction, also as in blood vessels^{17,18,22}. More recently, the application of these two dynamic stimuli has been achieved using micro^{3,4,22} and meso^{20,21,23} scale platforms. The small size and low reagent requirement advantages of such platforms have also been put to use in increasing the throughput of the experimental platforms^{20,21,23}.

The two stimuli have been found to have synergistic effects on endothelial cells – for example the simultaneous application of the two stimuli have been found to improve the endothelium morphology, leading to elongated cells and long, aligned stress fibers^{15,16}. However, the effects of the two stimuli are not simply additive, as was shown by Breen et al by comparing the regulation of Intracellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1) by the two stimuli alone and in combination¹⁸. More examples where strains and shear stresses have been found to have beneficial synergistic effects include a study where the stimuli were effective in increasing the intracellular calcium in tenocytes²⁴ and another study where they enhanced the differentiation of mesenchymal stem cells into endothelial cells with the aim of blood vessel tissue engineering²⁵. Very interestingly, it has been shown in microfluidic platforms called lung-on-a-chip³ (figure 2(iii)) and gut-on-a-chip⁴ (figure 2(iv)) that the combined strain and shear stress stimuli can enable cells to assemble into in vivo like structures as well as better predict the effects of pharmaceuticals. Combined flow-strain stimulation platforms have been used to study pathological conditions as well – Amaya et al found an upregulation of atherogenic genes in endothelial cells when the cyclic substrate strains and the pulsatile flow were not synchronized as they are in vivo¹⁹. Combining complex variations of the two stimuli have also shown interesting results, for example combined application of anisotropic biaxial strains and shear stress at various orientations to the strains showed that a 0.55 Pa shear stress dominated over cyclic substrate strains in terms of endothelial cell alignment response²⁰.

A challenge that is often encountered in designing test platforms for combined strain-flow stimulation is the interaction between the stimuli. When applying only strains to cells, a reactive shear stress is always applied to the cells due to the relative motion between the substrate and the culture medium²⁶. When strain and shear stress are applied simultaneously, the strain actuation generally also changes the confining volume of the flowing fluid, thus adding to the unintended shear stress. Computational modeling has been used to design systems to reduce these unintended effects of the strain actuation on the shear stress, or at least to quantify and characterize them well²³.

Cyclic substrate strain and hydrogel stiffness

Many cell types respond to cyclic substrate strain by aligning along a preferred direction, generally the lowest strain direction. This effect has been attributed to the cells' attempt to minimize the stress, strain or elastic energy²⁷. However these studies were done on substrates much stiffer than natural tissue, such as lung or vasculature, which experience strains *in vivo*. It is known that the substrate stiffness is extremely important for cells as they constantly gauge their environment²⁸. Gradients in stiffness are also known to determine the direction of cell migration, a process known as durotaxis²⁹. Hence applying tissue specific stiffness and strain stimuli simultaneously to cells is important. Applying strains to cells attached to substrates closer in stiffness to natural soft tissue has been achieved in general by placing cells on or in hydrogels of appropriate stiffness attached to an elastic substrate. For cells cultured on hydrogels, ECM coated polyacrylamide hydrogels have been commonly used^{14,30,31}, while for culture within hydrogels, ECM hydrogels such as collagen³²⁻³⁴ or fibrin³⁵ gels have been generally used. When cultured within hydrogels, cells see a significantly different attachment milieu compared to a substrate surface and this has been suggested to be responsible for the observed significant differences in cell behavior³⁶. The nutrient transport to the cells from the culture media is also modified³⁶. However, it is difficult to separate the effects of the stiffness and the additional factors. For simplicity, the studies where cells were cultured inside hydrogels and a strain was applied are considered in this section. For strain-stimulating cells within hydrogels, increasing the throughput of the experimental platform has also been reported³⁷⁻³⁹.

For cells cultured on hydrogels and stimulated with cyclic substrate strains, the general observation in multiple studies has been that cells respond quite differently to the strains when compared to cells cultured on much stiffer silicone surfaces^{31,40,41}. While on stiff silicone membranes, cells usually readily align perpendicular to the major strain direction, on hydrogels the response is less prominent for a low stiffness^{31,41} and even contrary in instances (cells align along the major strain direction)⁴⁰. It has also been shown that strain stimulation can make cells display a more spread morphology on low stiffness hydrogels where they show low spreading under static conditions³¹. As hydrogels are stretched and relaxed, water moves out and back into the hydrogel network. The forces caused by this movement have been shown to be responsible for the rupture of epithelial cell layers, which was earlier thought to be due to the breaking of cell-cell or cell-ECM bonds as a result of the stretching¹⁴ (figure 3(iii)).

In the case of cells cultured within hydrogels, the application of strains generally lead to better tissue specific phenotypes and arrangements. For example, osteogenic differentiation was shown to be induced in mesenchymal stem cells⁴², valve interstitial cells were found to respond to anisotropies of strain and take on an active myofibroblast phenotype at high

strain anisotropies³³, contractility of smooth muscle cells was found to increase³⁴, endothelial cells were found to form structures with lumen at the cost of sprout formation³⁵ and tendon/ligament markers were shown to be upregulated in mesenchymal stem cells⁴³. Another effect commonly observed was the higher mechanical strength of strained cell hydrogel constructs^{34,39}. When non-physiological strains were applied, such as equibiaxial strains applied to smooth muscle cells that reside in an anisotropically strained environment *in vivo*, the loss of the healthy phenotype was observed³². In the studies with cells within hydrogels, the experiments were designed around providing a three dimensional environment around the cells, resembling their *in vivo* environment. The stiffness was not really controlled, although the stiffness probably played a much more significant role as evidenced from the high interaction observed between the cells and the hydrogels in the form of hydrogel compaction⁴⁴ and rearrangement³³ in several studies. This would not have been possible in a three dimensional porous scaffold of a much higher stiffness.

The studies combining hydrogel stiffness and strain show that cell response to strain on hydrogels can vary significantly from response on stiff elastomeric substrates which were normally used previously. New forces from fluid squeezing in and out of the hydrogel network due to strains need to be considered as well¹⁴. Since cells are able to deform the hydrogels, techniques such as traction force microscopy offer a way to quantify the cell-substrate interactions¹⁴. Putting cells within the hydrogels permits even greater interaction with the ECM network. When comparing effects of strains on cells on hydrogel with those on stiff silicone sheets, an important consideration is the transfer of the strains to the cells³⁰, although it is not always reported.

Cyclic substrate strain and surface topography

Substrate surface topography has long been known to affect cell attachment. Surface topography can increase the surface area for better cell interaction and attachment⁴⁵ or can prevent microorganisms from attaching (anti fouling)⁴⁶, organized structures such as grooves can orient cells a certain way (contact guidance)⁴⁷, or topography can direct stem cell differentiation^{48,49}. Even though surface topography has many applications for the control of cellular behavior, not enough is known to always be able to predict how a topography would affect certain cells. High throughput techniques are now being used to explore the effects of a large number of topographies. By accumulating large quantities of data, hopes are that a predictive link between topography and cellular action can be made⁴⁹. When combined with strains, topography can affect the way substrate strains are transferred to the cells and in the case of contact guidance of alignment, oppose the alignment response of cells to the strain. To test for these effects, topographical features have been created on stretchable silicone sheets.

The general observation has been that microgrooves (1-20 μm width) can keep cells aligned along them even when strain is along the groove direction and hence trying to make the cells align perpendicular to the grooves⁵⁰. This has in fact been used to apply tensile strains to cells such as ligament fibroblasts in the direction of alignment, which resembles their in vivo condition, but is otherwise not attainable in vitro since the strains direct them to align perpendicularly⁵¹. The strain along alignment direction stimulus also resembles the in vivo condition of smooth muscle cells and has been found to increase smooth muscle cell markers in mesenchymal⁵² and neural crest⁵³ stem cells. However, it has been suggested that there is a strain threshold after which the strains dominate the alignment response – for example, Loesberg et al found this threshold to be at 8% strains⁵⁴. In contrast, nanogrooves (300 nm – 600 nm width), which can also guide cells to align along them, could not sustain the alignment if the strain was applied along the grooves and directed the cells to align perpendicular to the grooves^{55,56}. The strain threshold for dominating topography directed alignment along nanogrooves was found to be much lower (3%) than for microgrooves⁵⁵. Wang et al showed that as the nanogroove sizes increase, they are able to resist the strain stimulus for a longer duration, but given sufficient time, strains can always dominate topography driven alignment⁵⁷. In all the studies, if strain and topography were applied perpendicular to each other, they had a synergistic effect on the alignment of the cells. A mechanistic understanding of the competition between strain and contact guidance has only recently started to emerge with the demonstration that the basal actin responds to the contact guidance and the perinuclear actin cap, which is directly connected to the nucleus, follows the strain avoidance response to strain and guides the nucleus alignment with it¹² (figure 3(i)).

Few studies have combined strain and topography where the topography was not used to get contact guided alignment. In one such example, when the topographical cue was micropillars, the addition of strain changed how the cells attached to pillars and regions between pillars⁵⁸. The strain also caused larger changes in the expression levels of genes related to the cellular mechanotransduction machinery than the presence of the pillars alone⁵⁸. In another example, the topography presented to cells was micropillars and the strain was applied only to the pillars by magnetically actuating them. It was shown that the pillar strains slowed down the cell migration⁵⁹.

Cyclic substrate strain and attachment area patterning

In addition to topography, another method that has been used to guide cell attachment is the chemical patterning of the substrate surface area into parts where cells can attach and parts where they cannot attach. This has generally been achieved by microcontact printing, where a silicone stamp of the pattern is obtained by soft lithography microfabrication and the pattern is stamped on the substrate after adsorption of the patterning molecule (usually an

extracellular matrix protein) on the stamp⁶⁰. By patterning the attachment area for individual cells, it has been shown how the pattern shape determined cell shape can determine cell death⁶¹ or differentiation of stem cells⁶². Arrays of cell adhesive islands smaller than cells have been used to differentiate between the effects of attachment area and cell shape⁶¹ and also to determine the limits of attachment point size and distance between them needed for cells to attach and spread⁶³. Rows of attachment areas have also been used to align cells in a way similar to contact guidance by microgroove topographies⁶⁴. And gradients in availability of attachment areas have been used to study the corresponding determination of cell migration, a process known as haptotaxis⁶⁵. The number of studies stimulating cells with both attachment area patterning and cyclic substrate strain is few. Two of these studies used parallel patterned lines along which cells showed alignment. On the application of strains at various angles to the patterned lines (0°, 45° and 90°), the cells mostly ignored the alignment stimulus of the strain and remained aligned along the patterned lines^{64,66}. Although in one of these studies, when the strains were applied along the patterned lines, the cells aligned along an intermediate angle between the dictated alignment by the strains and the surface patterning⁶⁴. Two other studies used patterning to align and assemble cells into arrangements resembling their in vivo condition and then applied strains either along the same direction as the in vivo strain direction for them or along the perpendicular direction, which represented a pathological condition^{10,67} (figure 2(i)). In one study the cells used were valvular endothelial cells and it was shown that the application of a pathological condition of 20% strain perpendicular to the cell alignment direction drove the cells to a mesenchymal phenotype (endothelial to mesenchymal transition, EMT)⁶⁷. In the other study, using cardiomyocytes, the application of pathological strains perpendicular to the alignment direction lead to a disruption of the cell layer architecture¹⁰. And lastly, in a study using patterning to control individual cell shapes, on the application of strains⁶⁸, it was found that strain increases the amount of stress fibers and focal adhesions in radially symmetric cell shapes (circular, square) while in bipolar (rectangular) cells there is a higher rearrangement of the stress fibers and focal adhesions⁶⁸.

Flow induced shear stress and hydrogel stiffness

This combination of stimuli, just like strain-flow combinations, is very relevant for blood vessels and has mostly been applied to endothelial cells. The effects of shear stress have been widely studied for endothelial cells but these studies were generally performed on cells attached to very stiff substrates such as glass⁶⁹. It is known that endothelial cells are affected by the stiffness of their environment⁷⁰. It is also known that blood vessels stiffen with age and in some pathological conditions⁷¹. Hence, it is likely that substrate stiffness will have an effect on the response of endothelial cells to shear stress. This was found to be the case from multiple studies where shear stress was applied to endothelial cells cultured on substrates having a more tissue like stiffness. Galie et al cultured endothelial cells on

polyacrylamide gels of various stiffnesses and applied various shear stresses to them⁷². They observed that on lower stiffness hydrogels, the cells were more sensitive to shear stress in terms of spreading, although less sensitive in terms of alignment⁷². They also observed that the cells could better use shear stress to modulate the effect of the inflammatory cytokine TNF α on a lower stiffness hydrogel⁷². Kohn et al observed that endothelial cells responded to shear stress with a more elongated morphology and tight cell-cell contacts on substrates with lower stiffness when compared to a glass substrate⁷³.

Flow induced shear stress and substrate surface topography

This combination of stimuli has been applied to cells in vitro to study various useful processes, such as the competition between the alignment responses to each stimulus, achieving tightly bound cell layers that resist detachment under high shear flows, directed cell migration, preparing cell layers predictive of the in vivo condition and uncovering mechanotransduction pathways.

In a way similar to the topography-strain competition in aligning cells, a larger sized groove topography (>800 nm pitch) was found to be able to retain endothelial cell alignment when opposed by shear stress⁷⁴. However, unlike in the strain-topography case, smaller topographies were not completely dominated by the shear stress and a loss of alignment to either stimulus resulted⁷⁴. When the two stimuli directed the cells to align in the same direction, the effects were synergistic⁷⁴. Tightly bound endothelial cell layers resisting detachment by shear stress are of utility for cardiovascular implants such as vascular grafts^{75,76} or devices with an antithrombotic surface^{77,78}. Topography induced alignment along the flow^{75,78} and even just the presence of topographical features aligned along the flow direction⁷⁶ have been shown to achieve this, although topography aligning cells perpendicular to the flow were found more useful in withstanding very high shear stresses (8 Pa)⁷⁷. Aligned topographies can be used to provide direction to migrating endothelial cells irrespective of the flow direction⁷⁹ and such directionality can be used to advantage in enhancing wound healing⁸⁰. Combined topography and shear stimulation has also been used to improve the alignment and tight junction formation in kidney epithelial cells, thus providing a better in vitro model of renal tissue⁸¹. And lastly, using topography cues to control the cell shape modulated contractility of MSCs and applying shear stress to further affect their contractility, the regulation of the differentiation of MSCs has been shown¹³ (figure 3(ii)).

A challenge in combined topography-shear stress stimulation is the estimation of the actual shear stresses experienced by the cells since topographical features can potentially disturb the flow. Hence, computational modeling has been used to study shear stress variations caused by topography as well as by the experimentally determined surface of a cell layer resting on the topographical features⁸².

Flow induced shear stress and attachment area patterning

Only a few studies have been reported where combinations of these two stimuli were applied to cells. You et al studied the intracellular calcium signal of osteoblast cells attached to an array of adhesive islands in response to applied shear stress. Based on the calcium signals they observed that the cells attached to the islands were more sensitive to the shear stress than those attached to non-patterned substrates and that increasing the cell spreading area by moving the islands apart reduced this sensitivity to shear stress⁸³. Hsu et al showed using a combination of collagen patterning and shear stress that up to a certain shear stress, cells move in the direction of higher collagen density (haptotaxis) even if it is against the flow, but at higher shear stress mechanotaxis takes over and cells migrate in the direction of the flow ignoring the collagen densities⁸⁴. In addition, shear stress has been used to determine the strength of binding between cells and patterned adhesive islands^{85,86}.

Hydrogel stiffness and substrate surface topography

The number of studies reported for this combination of stimuli is quite small as well. Schulte et al varied stiffness and topography for a polyethylene glycol hydrogel and found that the presence of microgroove topography turns the normally cell non-adhesive hydrogel slightly cell-adhesive and this adhesion is stronger for hydrogels of lower stiffness⁸⁷. Lu et al explored various topography and stiffness combinations to find an optimum substrate for maintaining the stemness of mouse embryonic stem cells⁸⁸. Wong et al⁸⁹, Wu et al⁹⁰ and Jeon et al⁹¹ also looked at stiffness and topography combinations but they used materials of non-physiological stiffness. Still, differences in the cellular responses were observed.

Hydrogel stiffness and attachment area patterning

Combined stiffness and attachment patterning has been used to identify the forces generated by cells on spreading⁹² and on being exposed to a stiffness gradient⁹³, both using the detection of hydrogel deformations produced. The combination of these two stimuli have also been investigated for application in neural tissue engineering, where the patterning of islands and lines was used to organize neurons into networks (with cell bodies localizing to the islands and the dendrites to the lines) and the stiffness was found to control the speed and quality of network formation (faster arrangement on a lower stiffness substrate, but more synapses and action potentials on a stiffer substrate)¹¹ (figure 2(ii)). Another application of the combined control of stiffness and patterning was shown in assembling breast adenocarcinoma cells into hollow structures resembling epithelial structures in vivo⁹⁴. This was achieved by comparing the combined effects of adhesive islands and substrate stiffness, which showed that lower substrate stiffness and far apart adhesive islands reduced the cell-ECM interaction⁹⁴.

Combinations of more than two stimuli

Since many mechanical stimuli are coupled, it is not always possible to apply a particular mechanical stimulus without applying another – for example when strain is applied to cells attached on a substrate surface, reactive shear stress is applied to them as well caused by the relative motion between the substrate and the medium. So, when applying combinations of two mechanical stimuli, more stimuli can actually be present. However it is not always straightforward to vary or control these unintended stimuli. A platform has been reported where multiple mechanical stimuli can be intentionally and controllably varied. Galie et al reported a platform to apply interstitial flow induced shear stresses and cyclic substrate strains simultaneously to cells cultured in a compliant hydrogel (stiffness stimulus)⁹⁵. They later used it to study the effects of the stimuli on cardiac fibroblast activation⁹⁶.

Discussion and conclusion

The application of combinations of mechanical stimuli to cells has provided a number of insights on how opposing stimuli compete, how supporting stimuli produce synergistic effects which might not be directly additive, how one stimulus can sensitize cells towards another stimulus, how unintended stimuli might appear as side effects of the intended ones and how the combined stimuli can produce more in vivo like behavior from cells in vitro. These effects would have been impossible to predict due to our incomplete understanding of the mechanical workings of cells. In addition, the combinations of stimuli have been used to better understand the workings of the mechanotransduction machinery in complex mechanical environments. In the coming time many more new insights can be expected as systematic studies combining multiple stimuli are continued to test the effects of various magnitudes, directionality, frequencies and dosage of the stimuli. Increased application of optimized combined mechanical stimuli for tissue engineering and pharmaceutical discovery can also be expected. High throughput platforms can speed up this discovery process and hence more of them could be expected to be employed. With increasing knowledge, the simplest in vitro systems for each application could be identified, for example if a topography and substrate stiffness can elicit the same response from cells as putting the cells within a hydrogel and applying strains, the former stimulation could be selected for its ease of application. A better understanding of mechanotransduction mechanisms is also likely to emerge from such studies. Mathematical and computational modeling have already been used to predict the mechanical stimuli in complex conditions and to predict cell response to simple stimuli. With the advanced knowledge from combined stimulation studies, the models can be updated to provide them with higher predictive ability. Thus, we conclude that in vitro stimulation of cells with combinations of mechanical stimuli is an useful approach and systematic studies should continue.

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Chapter 3. A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells

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Abstract - We report a medium throughput device to study the effects of combinations of two mechanical stimuli – surface strains and fluid flow shear stresses, on cells. The first generation prototype can screen combinations of five strain and five shear stress levels. Computational modeling and empirical measurements were used to determine the generated strains and flows. Uniform equibiaxial strains up to 20% and shear stresses up to 0.3 Pa can be generated. Compatibility of the device with cell culture and end point fixation, staining and imaging is shown using C2C12 mouse myoblast cells.

Introduction

It is well known that cells sense and respond to local mechanical signals and geometry.¹ These physical factors can influence cell behaviors such as differentiation, proliferation, alignment and apoptosis.¹ Hence the physical factors are of great significance for tissue engineering and regenerative medicine, where often the goal is to control local tissue development. Physical factors are especially relevant for the development of complex, multi-cellular tissues, such as a pre-vascularized tissue. In such tissue, chemical factors,

which are difficult to localize, are not optimal for controlling the development of all tissue components. Pre-vascularized tissue development is a promising strategy to overcome a nutrition-diffusion based size limit on engineered tissue.² The successful integration of a pre-vascular network has been shown amongst others for engineered muscle³ and bone⁴ tissues but the integration of this pre-vascular network with the in-vivo vasculature following implantation is not always optimal in the case of pre-vascularized bone. Since both bone and blood vessel formation in-vitro are known to be improved by mechanical stimulation,^{5,6} we wish to investigate the effect of mechanical stimulation on pre-vascularized bone development and improve models predicting tissue formation in scaffolds based on local mechanical stimuli.⁷

Mechanical signals can be applied to cells through resistance to their internal forces (for example substrate stiffness) or in the form of external mechanical stimuli (for example substrate strain, fluid flow shear stress, hydrostatic pressure). However, in order to use these signals to control cellular behavior, we first need to know in detail how cells actually respond to such signals. The effects of two mechanical stimuli - substrate strains and fluid flow shear stresses have been widely studied for a variety of cell types.⁸ These mechanical stimuli are commonly present in the body, for example in blood vessels, lungs, heart and gut; and they can be applied to cells in a controlled way in the lab. Cell response is influenced by variations in these stimuli, such as in the magnitude, frequency and duration of stimulus application.^{9,10} Therefore, many experiments are required to find the optimum conditions for the cells. Therefore, high throughput techniques have been developed to study several conditions simultaneously. High throughput platforms have been reported that look at multiple fluid flow shear stresses^{11,12} and others that look at multiple substrate strains.¹³⁻¹⁵ Since in-vivo the two mechanical stimuli are present simultaneously, the effects of a combination of the two stimuli have also been studied,¹⁶⁻¹⁹ but the reported systems can look at only a single combination or at most a few combinations of the two stimuli at a time. In this paper, we report a platform to increase the throughput for studying the effects of combinations of substrate strains and fluid flow shear stresses.

A device has been developed to look at the effects on cells of combinations of five substrate strains and five fluid flow shear stresses, each with four replicates (giving a total of 100 units). Each unit has a region where a combination of a uniform equibiaxial surface strain and a fluid flow shear stress can be applied to cells. The generation of the various equibiaxial strains as well as the various flow regions has been shown in computational models and verified empirically. Furthermore, the variations in the fluid flow (and hence the generated shear stresses) caused by the strain actuation mechanism have been minimized. Compatibility of the device with cell culture and fluorescence microscopy to quantify cellular responses has been shown using C2C12 cells.

Materials and Methods

Device design and fabrication

The device (figure 1) consists of an array of strain units producing a variety of strains, overlaid with a varying width fluid flow channel producing a variety of shear stresses. Strain in a unit is produced by deforming a thin flexible membrane over a stiff cylindrical pillar using a pressure drop, and is varied between units by varying the size of the pressure actuation cavity (hereafter referred to as the trench) around the pillar. The trenches are connected to the pressure source and to each other by air-channels so that the pressure drop can be driven from a single point.

The device assembly steps, shown schematically in figure 1(b), were as follows: (1) The device layers were fabricated using a casting of polydimethylsiloxane (PDMS) (Sylgard 184, 10:1 base:curing agent ratio) in molds. Three molds (figure 1(c)) were prepared by milling aluminum blocks: (i) a pillar array to generate a PDMS negative for pillars; (ii) a mold for a trench-air-channel layer; and (iii) a mold for fluid flow channels. After filling with PDMS, the molds were covered with a polymethylmethacrylate (PMMA) slab to acquire a flat surface. (2) A PDMS membrane was prepared by spin coating PDMS containing dyed polystyrene beads (6 μm , red, Polysciences Inc.), on an 8-inch silicon wafer (Siegert Wafer GmbH) rendered non-stick by a vapor deposited perfluorodecyltrichlorosilane (FDTS) coating. The spinning program used ran for 5 seconds at 100 rpm followed by 40 seconds at 1000 rpm. (3) The trench-air-channel layer was bonded on the side without the air-channels to the PDMS membrane using uncured PDMS as glue. The glue application was achieved by spin coating (2000 rpm for 2 minutes) a thin layer of PDMS on a silicon wafer and stamping it on the trench-air-channel layer. (4) A pillar array made using a NOA81 (UV curable glue, Norland Products Inc.) casting into the PDMS pillar array negative,²⁰ was assembled on a borosilicate glass base. (5) The tops of the pillars were covered with silicone oil (Griffon HR260 siliconespray) lubricant by manually placing drops of the lubricant and then the trench-air-channel layer was attached on the air-channel side to the pillar array using NOA81 as glue. The glue was applied to the trench-air-channel layer by placing drops of glue and manually spreading. To prevent glue from entering the trenches and the air-channels, a physical blocking unit was prepared by filling the cavity formed by these features with NOA81 and curing it. The blocking unit was placed in the cavity during glue application and removed later. Both surfaces were plasma treated before binding using NOA81 to achieve a stronger bond. (6) Finally the fluid flow channel structure was bonded on top using NOA81 as glue. The glue was applied to the fluid flow channel layer by placing drops and manually spreading. In this step too, both surfaces were plasma treated before binding using NOA81 to achieve a stronger bond. PDMS was always cured at room temperature for 48 hours (except for the membrane,

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which was cured at 60°C for ~2 hours) to avoid mismatched shrinkages in the various layers.

Figure 1 – The device schematic (a) shows the device design and the mechanism of strain generation. The device is fabricated in layers made by casting PDMS in aluminum molds (c). The device assembly is shown schematically for a single unit (b, steps numbered corresponding to description in the main text) and a finished prototype is shown (d). The various strain regions (s1, s2, s3...) and the various flow regions (f1, f2, f3...) are marked on the schematic and the prototype. For cell studies, the device is operated while stored inside a 37°C, 5%CO₂ incubator and connected to two pumps, one controlling the strain actuation air pressure and another controlling the fluid flow (e).

The key dimensions of the device are: radius of pillars 0.75 mm; height of pillars 1 mm; distance between pillar centers 7mm (along device width) and 11mm (along device length);

trench-air-channel layer thickness 1 mm; trench height 1 mm; air channel height 0.5 mm; air channel width 0.5 mm; trench radii – 1 mm, 1.25 mm, 1.5 mm, 1.75 mm and 2 mm; fluid flow channel height 2 mm; fluid flow channel regions' widths 6 mm, 7 mm, 8 mm, 9 mm and 10 mm; membrane thickness 80 μm . The membrane thickness was measured by imaging the cross-section of a small strip of the membrane, while the other dimensions were treated as accurate within the precision limit of the milling machine.

Computational Modeling

Computational models were developed using ANSYS 13.0 Workbench (ANSYS Inc.) to simulate the operation of the device. Three models were developed : a single strain unit, the complete fluid flow channel and a single unit with combined strain and fluid flow.

For the strain unit model, a quarter of the unit was modeled under symmetry conditions. The PDMS parts were modeled as flexible bodies with an elastic modulus of 1.84 MPa²¹ and the pillar was modeled as a rigid body (since NOA81 is much stiffer than PDMS and unlikely to deform under the applied conditions). PDMS-PDMS contact was modeled as bonded and PDMS-pillar contact as frictional. A coefficient of friction of 0.01 was used between pillar and PDMS, based on a pin-on-disk (NOA81 pin, PDMS disk) experiment with silicone oil lubrication (data not shown). The base of the unit was constrained as fixed and a linearly increasing pressure drop was applied to the bottom surface of the PDMS membrane. The strains generated on the top surface of the membrane were analyzed.

The fluid flow channel geometry was drawn in SolidWorks (Dassault Systemes) and the model was solved using ANSYS FLUENT (ANSYS Inc.). A simple laminar flow model was used with a velocity inlet and a pressure outlet. The effects of changing flow velocity, fluid viscosity and channel height on the wall shear stress were analyzed.

The effect of strain actuation on fluid flow generated wall shear stress was analyzed by means of a two-way fluid structure interaction (FSI) analysis of a device unit with both strain and fluid flow. The variation of wall shear stress was monitored over a strain cycle both with and without fluid flow and the same was repeated for a lower height flow channel of 0.5 mm. A 1 second cycle was modeled at a constant fluid flow and a linearly varying pressure drop varying between 0 and 40 kPa (fig 2(f)). For the conditions under which flow was present, the model was solved for two cycles, so as to include the dynamics of the system at the start of a cycle.

To isolate the contribution of the geometry change during strain actuation on the fluid flow, the flow was modeled for several fixed geometries with the membrane in various deformed states. The deformation of the membrane was modeled as a circular arc with various maximum dips from the surface.

A 20 ml/min volumetric flow rate, 0.001 Pa-s viscosity (viscosity of water at 20°C), 1000 kg/m³ density (density of water) and 2 mm channel height were used for the fluid dynamics models, except when studying the effects of variations in those parameters. The 20 ml/min volumetric flow rate translated to a 0.1 m/sec inlet velocity for the complete fluid channel array (2 mm diameter circular inlet) and a 0.03 m/sec inlet velocity for the section used for the FSI analysis (6 mm x 2 mm rectangular inlet), both values rounded to one significant figure.

Device characterization

The strain array and the fluid flow channels were characterized by tracking 6 µm beads (red, Polysciences Inc.) embedded in a membrane and suspended in fluid respectively.

A strain array was assembled and actuated using an Elveflow® AF1 dual pressure-vacuum generator and controller combination (Elvesys® Innovation Center). For strain measurements, images were taken at various stretch positions, the bead co-ordinates were extracted, the center of deformation was approximated and radial strains were determined from them. Bead co-ordinates were extracted using a maxima/minima finding command in ImageJ (NIH). To eliminate points incorrectly identified as beads and to map bead positions in images for various strains, a custom-written MATLAB® (MathWorks Inc.) script was used that checked that a bead and its two nearest neighbors were present in all images, displaced and changed in relative orientation within specified limits. Centre approximation and strain calculations were also done using custom MATLAB® (MathWorks Inc.) scripts.

As has been noted previously,¹³ a setup like this is unsuitable for circumferential strain measurements. Moraes et.al. used an arbitrary reference line and measured circumferential movements with respect to that line, and found that this method of circumferential strain determination was not sufficiently robust due to sensitivity to errors in center determination as well as errors in the location of the reference line (measurements close to the arbitrary line were less accurate). Therefore, to show the equibiaxial nature of the strain, we calculated the linear strains between pairs of beads and plotted them against the angle between the line connecting the beads and the horizontal direction in the images (orientation angle of bead pair). This will result in the same strain values for all orientation angles for a perfect equibiaxial system.

The strain measurements were performed for all 100 units (n=20 per strain condition). Also, strains of five units were determined at various intervals after continuous use to see variations in generated strains over a long duration of use.

For fluid flow measurements, fluid flow channels were attached to a PDMS-coated glass base and water with suspended beads (6 µm, red, Polysciences Inc.) was flown through

them using a syringe pump. Fluid velocities in the various flow regions were measured by tracking the beads. 10 beads were tracked for each region to get an average velocity. Images were taken in the centers of the five flow regions in a plane at approximately half the channel height. Bead tracking and flow velocity calculations were done from these images using the ImageJ (NIH) manual tracking plugin.

Screening for the effect of mechanical stimulation on C2C12 cells

To validate the compatibility of the device with cell culture and to investigate the response of cells to various mechanical stimuli, cells were seeded on devices coated with fibronectin (Sigma-Aldrich F1141).

The flow channels were assembled just before the cell experiments so that the plasma treatment and the UV treatment involved in this step sterilized the device for cell culture. The flow channels, right after assembly, were filled with a 0.5 μ g/ml fibronectin solution and incubated for ~1 hour at room temperature. ~10,000 C2C12 cells/cm² in C2C12 medium: Dulbecco's Modified Eagle's Medium(DMEM; Invitrogen) supplemented with 10% FBS(Invitrogen), 1% penicillin and streptomycin (Invitrogen), 1% L-glutamin (Invitrogen) & 0.1% fungizone (Invitrogen), were seeded in the device directly after removing the fibronectin solution. While filling an empty device with fluid, it was tilted so that the fluid front moved ahead against gravity. This prevented any bubbles from being trapped as the bubbles rose against gravity and went to the fluid front and were finally pushed out of the device.

After cell seeding, the device was incubated in a 37°C, 5% CO₂ incubator. The cells were allowed to attach and grow under static conditions for 18 hours and then mechanical stimulation was applied for 6 hours. Three mechanical stimulation conditions were applied on separate devices – flow only, strain only and flow-strain combination. 20ml/min flow (~0.07-0.13 Pa shear stress range) and a sinusoidal pressure cycle with 0-400mbar (pressure drop) range and 1 second period (~2-20% strain range) were applied. Cells were also cultured on another device for 24 hours without any mechanical stimulation as a control. The pumps for applying the flow and the strains were stored outside the incubator and connected to the device through a side hole (figure 1(e)). A two-way syringe pump was set up for applying continuous flow using one way check valves (Value Plastics). The syringes filled from and emptied (after flowing through the device) into a common reservoir (100ml flask, Elvexys). The flow entered the device through a bubble trap (10ml tube). A three-way connector with a stop-cock and one port with a swabable valve was used at the device inlet, which allowed for cell seeding without opening the flow circuit.

The experiments were stopped at 24 hours and the cells were fixed with 4% paraformaldehyde for 10 minutes and stored under PBS at 4°C. Fixed cells were stained

with Phalloidin (Alexa Fluor 488) and DAPI after permeabilization with 1% Triton X-100 and blocking with 5% bovine serum albumin (BSA). After staining, the PDMS over the flow channels was removed to allow for better microscopy access. Imaging was done using BD Pathway with a 4x objective which captured a frame of size 2.1mmx1.6mm. A macro was used to focus and capture images throughout the device in an automated fashion. Images were captured over each pillar (area over a pillar hereafter referred to as region of interest (ROI)) and the unstrained region between each pair of ROIs along the flow channel. The images were corrected for non-uniform illumination by applying a 10 pixel rolling ball radius background subtraction using ImageJ. For ROI images, the area outside the circular ROIs in the rectangular images were cleared by manual selection, also using ImageJ. The images thus processed were analyzed using a CellProfiler²² pipeline – the nuclei were identified as primary objects (adaptive background thresholding and intensity based separation of clumped objects were used) from the DAPI images, the cells were identified from the phalloidin images as secondary objects around the nuclei (using the Distance-B method on phalloidin images with adaptive background thresholding) and shape and size parameters were measured for both. Orientation of cells measured by CellProfiler was converted to radial/circumferential orientation with respect to the ROI centers (angle between cell fit ellipse major axis and line joining cell center and ROI center) using a custom MATLAB script. The data was averaged per image (ROI or region between ROIs) and then the ROI averages were grouped by condition (strain, flow, strain-flow combination) and analysis of variance was performed using MATLAB functions (anova1 or anova2 depending on whether the effects of one or two conditions were considered). Also, all ROI averages and all regions between ROI averages were grouped and compared using a T-test (MATLAB ttest2 function).

Results

Computational Modeling

Analysis of the strain unit showed that uniform equibiaxial strains can be generated at the top surface of the membrane over the pillar. The strain is higher at a higher pressure drop, and at a given pressure drop a higher radius trench can generate a higher strain. The radial strain generated is uniform over the central region above the pillar and increases near the edge. The region of uniform strain becomes bigger with increasing pressure – at 5kPa a central region of radius ~0.3mm has a uniform strain (figure 2(a)) while at 20kPa the uniform strain region spreads to a ~0.6mm radius (figure 2(b)).

Analysis of the fluid flow showed the generation of various shear stress regions corresponding to the various channel widths (figure 2(c)). Figure 2(d) shows - for each flow

region – the average shear stress in the ROIs. The large standard deviation error bars for the 6mm and 10mm regions are caused by these regions lying next to bends and areas of sudden large dimension change, which were allowed in the design due to geometric constraints.

Figure 2 – Computational modeling was done using ANSYS. The strain generation was modeled for a quarter strain unit with symmetry boundary conditions and showed the generation of uniform strain regions in the ROIs. The uniform strain regions became bigger with increasing pressure drop as shown in the radial strain vs. radius plots at 5 kPa (a) and 20 kPa (b). Fluid flow was modeled for the complete flow channel setup (c) and showed the generation of the various shear stress regions. The shear stresses generated can be varied by changing the fluid viscosity, flow rate or channel height (d). A fluid structure interaction (FSI) model was used to study the effects of the strain actuation mechanism on the fluid flow shear stress in the ROI (e) and showed that using a high channel reduces the effect (f). The results shown are for the 2nd cycle for the conditions under which flow was present.

Figure 3 – Strains were empirically determined by tracking beads embedded in the stretching membrane in images taken at varying strains. A customized program was used to extract bead co-ordinates in images and identify beads that were detected in all images.

Center of deformation was determined from the bead paths as well as visually approximated from an overlay of bead positions at varying strains (d). Radial strains were then calculated and plotted against radius (a). The linear strain between each pair of detected beads is plotted against the orientation angle (O.A.) of the beads to check if the generated strains are equibiaxial (b) or have directional variations (c) due to fabrication effects such as misalignment. The average radial strains determined empirically for all units at multiple pressure drops were averaged for all the units with the same trench size ($n=20$ per trench size; standard deviations shown as error bars) and compared with model-determined values (e).

This effect can be minimized by increasing the fluid viscosity, as shown by the smaller error bars. Increasing the viscosity has the added advantage of increasing the shear stress. Higher shear stresses can also be achieved by using a higher flow rate, but this will increase the effects of the bending and the sudden dimension change as was shown by a doubling of the flow rate (figure 2(d)). An additional method to achieve higher shear stresses without increasing the bending and sudden dimensional change effects is to use a lower height flow channel (figure 2(d)).

Fluid structure interaction (FSI) analysis enabled visualization of the changes in the flow and the generated shear stress due to the strain-producing membrane deformation. In the case of a 2mm high channel and a ~20ml/min flow rate (0.03m/sec inlet velocity), there was an increase in the average shear stress in the ROI during the strain cycle, while in the case of a 0.5mm high channel with the same inlet velocity the average shear stress in the ROI decreased during the strain cycle. The variations in the average shear stress in the ROI over a strain cycle were higher for the 0.5mm channel (maximum 40% decrease from initial value for the 0.5mm channel and maximum 17% increase from the initial value for the 2mm channel). The shear stress distribution variability in the ROI was also higher for the 0.5mm channel (maximum standard deviation to average value ratio 0.11 for 2mm channel and 0.17 for 0.5mm channel). The shear stress variations were higher at the ROI edge and choosing a smaller ROI (the central 50% area of the original ROI) reduced the shear stress distribution variability (maximum standard deviation to average value ratio 0.06 for 2mm channel and 0.07 for 0.5mm channel). For the smaller ROI the variation in average shear stress over a strain cycle was lower than the original ROI for the 2mm channel and higher than the original ROI for the 0.5mm channel (maximum 47% decrease from initial value for the 0.5mm channel and maximum 11% increase from the initial value for the 2mm channel). FSI analysis under the stationary fluid conditions showed shear stresses an order of magnitude smaller than the shear stresses generated under the applied flow conditions for both channel heights used.

The fluid flow models with the fixed geometries showed a ROI shear stress variation in response to increasing membrane deformation, which is very similar to that observed in the FSI analysis (Supplementary figure 1). This demonstrates that the membrane motion is slow enough for the flow to be primarily determined by the local channel geometry. Thus at this device operation frequency, the fixed geometry models provide a computationally easier way to investigate the device behavior.

Figure 4 – Flow velocities were measured in the center of each flow region at half channel height by tracking suspended beads. Three measurements were taken in each region and for two flow rates, namely 1ml/min (a) and 2ml/min (b). Flow velocities in both cases showed good agreement with values determined by modeling.

Device characterization

The membrane thickness was found to be $80.9 \pm 3.9 \mu\text{m}$ based on single measurements on 25 membranes.

Radial strains determined by tracking embedded beads were plotted against radii for all 100 strain units on a device. For each unit, over 100 points were identified as beads initially. After the mapping between the image sets, on average 18 beads were detected and used to determine the strains. No centering was applied for bead detection, which gives a maximum possible error equal to the bead radius ($3\mu\text{m}$, which corresponds to 5 pixels in the images used) in the detection of the bead co-ordinates. Figure 3(a) shows a typical plot with a higher spread in the strain near the smaller radii, possibly due to the errors in bead location and center determination being comparable to the distances determined from them. The plots of the strains between pairs of beads against the bead pair orientation typically showed that the strains were the same in all directions (equibiaxial strain, figure 3(b)). The bead pairs for which the beads were close together were generally responsible for the very low or very high strain values, an effect similar to the strains for small radii, explainable by the errors and distances being similar in magnitude. Misalignment of pillars with respect to the trenches was sometimes observed, which led to the centers of deformation not coinciding with the pillar centers. Due to this, anisotropies in the biaxial strain were observed (figure 3(c)). The effects of the misalignment on the strains generated and the equibiaxial nature of the strains generated were more prominent in the case of the small trench sizes.

When grouped by trench size, the averages of the average radial strains for each unit showed the expected pattern, namely increasing radial strain with increasing trench size. The results compared well with those of the computational models, except for the two smallest trench sizes. However this difference could be explained by the increased sensitivity of the strain measurements to misalignments in those cases, since small misalignments brought the pillar to one corner of the trench.

Strain measurements after continuous cyclic straining at 1 Hz showed a decrease in the strains to 80% of initial strains after 24 hours of operation. The results were similar when testing in air at room temperature or under cell culture media at 37°C. The lubricant layer showed thinning after the 24 hour testing but did not show any signs of evaporation, and strains generated were similar before and after a ~4 month storage period.

Fluid velocity measurements by bead tracking showed good agreement with the predicted fluid flows of the computational models (figure 4).

Screening for the effect of mechanical stimulation on C2C12 cells

Cells remained attached and exhibited a spread out morphology for the experiment duration of 24 hours, indicating that cells remained viable on all tested devices. Imaging was shown to be possible with a 4x objective having the whole ROI in a frame as well as using a 20x long working distance objective for capturing more details, for example actin stress fibers. Images could be segmented to detect the cells and the nuclei and measure various shape and size descriptors.

C2C12 cells showed significant overlap, as has also been observed previously,¹⁵ making segmentation harder and cell shape/size descriptors less reliable. As examples, averages of measured cell area, cell form factor($4\pi\text{Area}/\text{Perimeter}^2$) and cell major axis to minor axis ratio are shown for the 25 conditions on the device subjected to combined flow-strain stimulation (figure 5(g)-(j)). Statistical analysis was performed on this data (supplementary table 1). For the C2C12 cells under the applied mechanical stimuli, differences were observed between the various strain conditions but not the various flow conditions. Regions exposed to higher strains showed fewer, smaller and more rounded cells. C2C12 cells are known to align perpendicular to uniaxial strains, but no specific alignment was observed in response to strains in our experiments. This could be expected since the strains were equibiaxial and it has been previously observed that C2C12 do not align when exposed to equibiaxial strains.¹⁴ Cell alignment was also not observed in response to flow, which could be a cell specific result or because of the shear stress magnitude and duration not being high enough to elicit an alignment response.

Figure 5 – Cell studies were done with C2C12 cells. Cells were cultured on the device statically for 18 hours and then stimulated with flow only, strain only, or flow-strain combination for 6 hours. Cells were then fixed with paraformaldehyde and stained for filamentous actin (phalloidin, green) and cell nuclei (DAPI, blue). Significant differences were observed between the cells subjected to the various strains (a:1.5%, b:4%, c:7%, d:13% and e:20% strain, scale bar 1mm) but no significant differences were observed between the various flows. The system is compatible with higher magnification imaging, which makes it fit for the screening of intracellular responses (f-h, scale bar 200 μ m). Cell and nuclei images were segmented and measured using CellProfiler. Measured cell shape and size descriptors were grouped by condition and compared (i-l, mean values are depicted including standard deviations (n=4)).

Discussion

Mechanical stimuli are important for tissue engineering approaches. In particular, they offer an important method of controlling tissue development for complex, multi-cellular tissues, where other approaches such as chemical factors encounter localization problems. However, in order to use mechanical signals to control tissue development, researchers must know in detail how cells respond to single and combinations of mechanical signals. Also, a quantitative optimization of the mechanical stimuli is needed for tissue engineering. The reported device is a tool developed to enable this optimization with increased

efficiency by using a high throughput approach. Even though devices have been previously developed to study the effect of surface strains,^{13,14} or fluid shear stresses^{11,12} in high throughput, or the combination of the two stimuli with multiple strain conditions but a single fluid flow shear stress condition,²³ to our knowledge, this is the first reported device to study combinations of multiple substrate strains and multiple fluid flow shear stresses in high throughput.

A design requirement that was set for this device was having the ROIs big enough to study a few hundred cells, so that co-cultures of cells could be studied. Millimeter scale features were therefore chosen. While this had a few disadvantages in comparison to smaller microfabricated features, such as the need for large cell numbers and a requirement for more culture media, there were also some advantages. One advantage of working with this feature size is that the molds for casting device parts can be easily made using an automated milling machine as compared to the complex mask making and lithography setups required to make microfluidic molds. Also, features of different heights can be made relatively easily, which in microfabrication would need to be done in separate layers demanding fine alignment. Also, the height of features that can be made using the commonly used microfabrication technique of making SU-8 molds is limited by the depth of SU-8 that can be UV-cured.

The device's external dimensions are the same as a standard 96-well plate, allowing for ease of imaging using a bio-imager such as the BD PathwayTM (BD Biosciences). The transparency of the device allows imaging using inverted as well as upright microscopes. The pillars are all of the same size so that the surface area covered with cells and exposed to the mechanical stimuli is the same for all the units, allowing for a better comparison. For simplicity, all strain units are connected to a single opening for pressure control and there is a single pair of inlet and outlet points for fluid flow. A common fluid flow between units was chosen under the assumption that any paracrine signaling will be washed away by the continuously flowing media.

The pressure drop based strain actuation, besides its self-evident advantages of producing planar uniform equibiaxial surface strains and keeping the cells in a plane allowing for real-time imaging, has an additional advantage over pressure increase based strain actuation,^{24,25} which is that there is a lower risk of device layers delaminating.

Estimating the effect of the strain generating mechanism on the fluid flow and minimizing it was an important part of the device design. From parallel plate fluid flow chamber studies it is well known that fluid flow shear stress depends on the flow rate, fluid viscosity and channel dimensions ($\tau=6Q\mu/(wh^2)$, where τ is the shear stress, Q is the flow rate, μ is the dynamic viscosity, w is the width of the channel and h is the channel height). The expected cause of disturbance in the shear stresses experienced by the cells placed in the ROIs in the

device was the flow initiated by the fluid displaced during the strain actuation. A higher flow rate compared to these disturbed flows would therefore mask their effects. However the high flow rates would increase the shear stresses generated, an effect which could be compensated for by increasing the channel height. This simplistic analysis was used to help choose the channel height for the device. The FSI analysis further served as a useful tool to estimate the shear stresses generated and the variations caused by the strain actuation for the actual device geometry, which was more complex than for the parallel plate flow chambers. The directional fluid flow made it hard to find any symmetric conditions to reduce the size and complexity of the FSI computational model, as Brown et al²⁶ were able to do. Therefore only the area having the highest impact of strain actuation on fluid flow was chosen for the FSI analysis. The FSI analysis was done for the region with the biggest trench and the smallest fluid flow channel region. This region had the biggest membrane motion and the lowest total volume of fluid, making the displaced fluid volume to total fluid volume ratio the highest. The FSI analysis showed that a 2mm high channel gives a less disturbed flow than a 0.5mm channel (measured in terms of variation of mean shear stress in ROI over a strain cycle and the variability in distribution of shear stress in ROI at any given point). Despite the reduction in shear stress variation by increasing the channel height, this variation cannot be completely eliminated. At any given point, the variability in shear stress in the ROI is primarily near the edges and selecting a smaller area in the center of the current ROI as the new ROI can reduce this shear variability. The shear stress variations over a cycle are small for the high channel and comparable with effects of pulsatile flow shear stresses on the cells of interest, but must still not be ignored.

For device strain characterization, embedded beads were tracked. Previously, similar studies have made use of fluorescent beads deposited on the membrane¹³ or a patterned membrane with small traceable features¹⁴. In both these approaches, the cells are likely to respond to these features. Since it is hard to wash off the beads or remove the features after measuring of the strains, the characterized strain membrane could not be used for the actual cell studies. Embedding beads in the membrane has the advantage of allowing the cell studies to be performed on the characterized membrane. To ensure that the bead embedding did not significantly change the mechanical properties of the membrane, the beads used were several times smaller than the membrane thickness (bead diameter 6 μm and membrane thickness 80 μm) and a low number of beads (total bead volume $< 1/10000$ th of the total PDMS volume) were embedded. The low number of beads also ensures that they do not block imaging of cells later.

In the used configuration, the flow was switched between syringes (one syringe driving the flow while the other filled) every 1 minute. As per the manufacturer, the syringe pump switched direction in 0.25 seconds and during this time the built up pressure in the bubble

trap drove the flow. Hence the flow never stopped or switched direction, but only slowed down for ~1-2 seconds per minute.

Misalignment between device layers during assembly, the presence of bends and sudden dimensional changes in the fluid flow channel, and the drop in strains generated over long duration use are some limitations that will be addressed in designing the next-generation prototype. The strain variation over a long period of use is most likely due to thinning of the lubricant. Other similar systems have reported a lower variation in strains over long use^{13,14} when using different lubricants and so this will also be evaluated in future generation systems. Apart from that, pre-conditioning of the device may also stabilize the lubricant layer that remains and hence reduce further variations in the strains. The shear stresses generated in the current prototype are on the lower side of commonly tested ranges and hence the effects of higher shear stresses, which can be obtained by increasing the media viscosity (for example by addition of dextran²⁷), will also be studied.

The developed system has been used to test the (combined) effect of surface strains and fluid flow shear stresses on C2C12 cells in order to demonstrate the applicability of the device. These experiments were successful in showing that differences in cell shape, cell size, and number of cells can be imaged and analyzed. It should be noted that the variance in the acquired data is relatively high. This is in part due to the overlap of the C2C12 cells, making segmentation harder and cell shape/size descriptors less reliable. Apart from that, cell shape and cell size are dependent on other factors that are not directly related to surface strains or fluid flow shear stresses, such as the proliferative state of cells, where cells that are in the process of division are often rounder and less spread.²⁸ By using fluorescent immunohistochemistry and focusing on output parameters that are directly influenced by mechanical signals, such as the presence and density of focal adhesion sites, it is likely that the output of the system can be enhanced, resulting in the improved distinction between cellular responses to different levels of mechanical stimulation.

As it has been shown that anisotropic strains can have different effects on cells compared to the equibiaxial strains such as those produced in the current device,²⁹ future generation prototypes will also include pillars of varying shapes to produce anisotropic strain profiles. The current device, operating at pressure drop as high as 40kPa, can generate strains in the range of 2-20% and with a 0.003 Pa-s viscosity fluid flowing at ~20ml/min can produce shear stresses in the range 0.18-0.33Pa. These ranges can be easily varied by operating the device under different conditions, namely a different strain range by changing the pressure drop and a different shear stress range by changing the flow rate. However as a starting point the two ranges provide relevant test conditions as cells have been shown to detect and respond to these levels of mechanical stimuli as well as showing variations within these ranges^{11,14}.

Conclusions

A medium throughput device has been developed to study the effects of combinations of surface strains and fluid flow shear stresses on cells by using units that can be individually characterized. The fact that operation of the device does not have an effect on the plane where the cells are located makes this system highly promising for the real-time investigation of cellular behavior. The design considered the interaction of the two stimuli and attempted to minimize their impact on each other so that they could be independently varied. Straightforward fabrication and ease of use are strengths of the device. All in all, this provides us with an easily adaptable and flexible system to screen for the effects of mechanical signals on cells.

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Supplementary figure 1 – Fluid structure interaction (FSI) analysis was used to study the variation of the fluid flow in response to the strain actuating membrane deformation. Fluid flow was also modeled for fixed deformed geometries and compared with the FSI results.

The wall shear stress contours are shown here for the un-deformed and maximally deformed states of the membrane for a 2mm high channel (a-d) and a 0.5mm high channel (e-h). An inlet velocity of 0.03 m/sec and a zero pressure outlet was used in all cases. The flow direction is marked by arrows in all figures. The variations in the average shear stress in the ROI corresponding to membrane deformation are shown for both methods (i-l). The variations in the average shear stress in the ROI are similar for the FSI and the fixed geometry analyses, suggesting that the variations are primarily a result of the geometry variations.

Supplementary table 1 – Two-way ANOVA was used on cell data from ROIs grouped by the strain-flow combination condition (25 conditions with 4 replicates each) and one-way ANOVA was used on the cell data from ROIs grouped by either strain or flow condition (5 conditions with 20 replicates each). Both analyses returned p-values, which if less than 0.01 (shown in red in the table), were taken to negate the null hypothesis that there was no significant difference between the various conditions. The analyses were carried out for 4 devices – flow only (FO), flow and strain (FS), strain only (SO) and static control (St). The two ANOVA analyses were carried out even for cases where the number of conditions present and number of conditions compared did not match (for example two way ANOVA for the strain only device) in order to check whether any observed differences were effects of the position on the device. Significant differences ($p < 0.01$) were found mainly between the various strain conditions. Some factors showed differences between other conditions, but the differences were smaller, as also seen by the relatively higher p values. T-tests were done to compare data from ROIs with data from regions between ROIs. The t-test returned a 1 when the two conditions were significantly different ($p < 0.05$), a 0 otherwise. On devices where no strain was applied (FO and St), differences between ROIs and non-ROI regions were expected to be insignificant, but found to be significant for factors other than cell number. This suggested that using the non-ROI regions as unstrained controls needs careful consideration.

A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells

2-way ANOVA

		Cell # (normalized)	Area	Form Factor	Major axis/Minor axis
2-way ANOVA	FO	flow	0.263213325	0.091201	0.052799793
		strain	0.013252454	0.171383	6.57E-08
		interaction	0.041103131	0.482364	0.43319145
	FS	flow	0.356026947	0.32859	0.202778108
		strain	1.39E-14	2.00E-10	4.78E-14
		interaction	0.627780101	0.933926	0.355938506
	SO	flow	0.058291705	0.161963	0.598837836
		strain	6.43E-23	4.50E-13	3.51E-16
		interaction	0.008131175	0.129575	0.788479979
2-way ANOVA	St	flow	0.140654525	0.450029	0.007681472
		strain	0.087907369	0.456048	0.152737632
		interaction	0.185109819	0.127023	0.368383033

1-way ANOVA

	Cell # (normalized)	Area	Form Factor	Major axis/Minor axis
FO - flow	0.371555918	0.09571	0.169892616	0.410157909
FO-strain	0.033530264	0.130339	8.15E-08	0.00469338
FS - flow	0.723187556	0.581433	0.582993143	0.740649714
FS-strain	6.07E-16	2.14E-11	3.93E-14	5.73E-06
SO - flow	0.638943789	0.521241	0.879676821	0.576318542
SO-strain	2.86E-21	4.39E-13	3.34E-19	0.005119711
St - flow	0.177585145	0.488578	0.009073113	0.115100237
St-strain	0.099771061	0.567622	0.294709284	0.226614697

T-test

	Cell number	Area	Form Factor	Major axis/Minor axis
FO	0	1	1	1
FS	1	1	1	1
SO	1	1	1	1
St	0	1	1	1

Chapter 4. Endothelial cell alignment as a result of anisotropic strain and flow induced shear stress combinations

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Abstract - Endothelial cells (ECs) are continuously exposed *in vivo* to cyclic strain and shear stress from pulsatile blood flow. When these stimuli are applied *in vitro*, ECs adopt an appearance resembling their *in vivo* state, most apparent in their alignment (perpendicular to uniaxial strain and along the flow). Uniaxial strain and flow perpendicular to the strain, used in most *in vitro* studies, only represent the *in vivo* conditions in straight parts of vessels. The conditions present over large fractions of the vasculature can be better represented by anisotropic biaxial strains at various orientations to flow. To emulate these biological complexities *in vitro*, we have developed a medium-throughput device to screen for the effects on cells of variously oriented anisotropic biaxial strains and flow combinations. Upon the application of only strains for 24 h, ECs (HUVECs) aligned perpendicular to the maximum principal strain and the alignment was stronger for a higher maximum: minimum principal strain ratio. A 0.55 Pa shear stress, when applied alone or with strain for 24 h, caused cells to align along the flow. Studying EC response to such combined physiological mechanical stimuli was not possible with existing platforms and to our best knowledge, has not been reported before.

Introduction

Endothelial cells reside in a mechanically active environment and are known to be highly responsive to mechanical stimuli. They are continuously exposed to cyclic circumferential strain and shear stress from the pulsatile blood flow. *In vitro* studies have shown that when exposed to these mechanical stimuli, endothelial cells distinctly change in morphology as compared to static cultures. They align perpendicular to the strain direction when uniaxial strain¹ is applied and along the flow direction in response to flow induced shear stresses², which resembles the morphology found *in vivo* in the presence of these stimuli³. The mechanical stimuli also affect the cellular function. Both strains⁴ and shear stresses⁵ are known to regulate the permeability of endothelial cell monolayers, thus affecting one of their major functions of serving as a barrier between blood and surrounding tissue. Besides existing blood vessels, strains⁶ and shear stresses⁷ also play an important role in the formation of new vessels (angiogenesis). More importantly, disturbed mechanical stimuli can lead to endothelial dysfunction. Disturbed flows near bends or branching in blood vessels leading to low and/or oscillating shear stresses are widely recognized as a cause leading to atherosclerosis⁸. Similarly, strains have been linked to atherosclerosis⁹.

In vitro platforms to apply well defined mechanical stimuli to cells have played a critical role in understanding how strains and shear stresses affect endothelial and other cells. Early research has been performed using macro scale platforms such as the commercially available Flexcell and several other custom built devices¹⁰. However these devices are fast being replaced by micro^{11,12} and meso¹³⁻¹⁵ scale devices that offer several advantages such as better control on the physical environment of cells, lower cell and reagent requirements, in situ imaging capabilities, high throughput potential and flexibility in design and fabrication permitting increasingly complex systems¹⁶. For endothelial cells, devices applying uniaxial strains in a flat substrate have been the most common as they mimic the circumferential strains found in blood vessels¹⁷. However, this simplification ignores the axial strains in blood vessels, which are usually small compared to the circumferential strains for straight parts of the vasculature in physiological conditions. However clinical observations and biomechanical studies have found that in disease states such as hypertension¹⁸, atherosclerosis¹⁹ or aneurysms²⁰, the vascular wall changes in shape and mechanical properties, which in turn can lead to changes in the strains. A flat substrate undergoing anisotropic biaxial strains is a closer model to such a situation. For complex geometries, such as vessel branching regions, the anisotropic strains can better represent even the physiological conditions¹⁷. In many cases where strain anisotropies are present, the flow might not necessarily be perpendicular to the maximum strain direction and hence the strain and the shear stress do not direct the cells to align in the same direction. Hence, a combined anisotropic biaxial strain and shear stress producing system, which can also test for various orientations between the two stimuli, is essential to better address the biological

complexity. The knowledge of the cell response to varying degrees of strain anisotropy, in combination with flow induced shear stress oriented at various angles to the strain, is relevant in understanding physiological or pathological cell behavior *in vivo*, or in predicting cell fate in a strain- and perfusion-stimulated cell seeded tissue engineering scaffold.

In this study, we have developed a medium throughput device to screen for the effects on cells of various anisotropic strains, each oriented at various angles to flow applied in combination with the strains. The device was developed using methods previously employed by us to design a separate device which could screen for the effects of various equibiaxial strain and various shear stress combinations{Sinha, 2015, A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells}. The current device is similar in dimensions and the number of test units to the previous device but very distinct in the mechanical stimulation conditions that each device can test for (various anisotropic strains and flow at various orientations versus various equibiaxial strains and various shear stresses). The developed system has been used to study the effect of combined anisotropic surface strains and fluid flow shear stresses on human umbilical vein endothelial cells (HUVECs). Previously, combinations of anisotropic (uniaxial) strains and shear stresses have been only applied in situations where they complement each other in their alignment response, i.e. flow is applied perpendicular to the uniaxial/circumferential strain direction²¹⁻²⁴, resembling flow and strain in straight parts of vasculature *in vivo*. To our knowledge, this is the first study investigating the alignment response of HUVECs to strains and shear stresses in complementary as well as non-complementary combinations.

Results

A device offering uniform anisotropic strains with varying degrees of anisotropy has been developed

The developed device can screen for four degrees of anisotropy (maximum : minimum principal strain ratio) and five orientations of each anisotropic strain with respect to flow (total 20 conditions). The device can also screen for five equibiaxial strain magnitudes. Each of the 25 strain conditions is present in four replicates, giving a total of 100 test units. For ease of identification in the rest of the paper, the various conditions are assigned condition numbers, as listed in table 1. The device consists of an array of 100 strain producing areas overlaid with a continuous winding flow channel with a single inlet and a single outlet. Strains are produced by pulling a thin elastic membrane over a circular pillar into an ellipse (anisotropic biaxial strain) or a circular (equibiaxial strain) trench using a

pressure drop, as illustrated schematically in figure1(a). The device has the base dimensions of standard well plates, permitting imaging automation using commercial bio-imagers such as the BD Pathway.

Table 1 – The developed device can apply 25 separate mechanical stimulation conditions to cells. The conditions are listed here and assigned a condition number each for ease of identification. In the absence of flow, the variously oriented strain conditions are identical and hence a strain condition number is also defined to collectively refer to these identical strain conditions.

Condition number	Strain condition number	Trench shape	Trench dimensions	Orientation angle of trench major axis with respect to flow
1	1	circular	radius 1.25 mm	-
2	2	circular	radius 1.5 mm	-
3	3	circular	radius 1.75 mm	-
4	4	circular	radius 2 mm	-
5	5	circular	radius 2.5 mm	-
6	6	ellipse	half minor axis 1.55mm half major axis 2.3mm	0°
7				30°
8				45°
9				60°
10				90°
11	7	ellipse	half minor axis 1.7mm half major axis 2.3mm	0°
12				30°
13				45°
14				60°
15				90°
16	8	ellipse	half minor axis 1.9mm half major axis 2.3mm	0°
17				30°
18				45°
19				60°
20				90°
21	9	ellipse	half minor axis 2.1mm half major axis 2.3mm	0°
22				30°
23				45°
24				60°
25				90°

Figure 1 – A schematic representation of the reported device (a) illustrates how strains are generated by stretching a membrane over a pillar and how these strains are combined with shear stresses using an overlaid flow channel. The schematic also shows how varying degrees of anisotropic strains ($a1$, $a2$, $a3$) are generated by using various aspect ratios of the strain actuating trenches, and that by using various orientations ($o1$, $o2$, $o3$) of the trench with respect to the flow channel, various orientations of the anisotropic strains with

respect to the shear stresses can be tested. The developed prototype (b) and the layout of the various trench shapes (c) demonstrate how the various conditions are distributed randomly on the device to avoid location specific effects, while keeping a replicate of each condition in four regions that can be expected to differ. These four regions that the device is divided into are depicted in different colors – gray (near inlet), yellow (near outlet), blue (near bends) and red (mid-channel).

Computational modeling was used to design the device. Models of the strain production demonstrated that regions of uniform anisotropic surface strains can be produced by stretching a thin elastic membrane into an elliptical trench over a circular pillar using a pressure drop. At a given pressure drop, the degree of anisotropy could be varied by altering the ellipse major axis to minor axis ratio. The maximum and the minimum principal surface strains in the region over the pillar were directed along the ellipse trench major and minor axis directions respectively. In the central 50% surface area over the pillar, both the maximum and the minimum principal strain were largely uniform and hence this region was selected as the region of interest (ROI).

Figure 2(a) shows the ANSYS modeling results for the highest degree of anisotropy tested. Since the model was three dimensional, three principal strains (maximum, middle and minimum) were returned by ANSYS, where the minimum principal strain is perpendicular to the membrane plane and therefore not sensed by cells attached on the membrane surface. The maximum and the middle principal strain distributions in the membrane over the pillar and the trench regions, and the principal strain direction vectors over the pillar are plotted for an applied pressure drop of 20kPa. In the ROI, the maximum and the middle principal strains from the 3D model lie along the membrane surface and correspond to the maximum and the minimum surface strains in the membrane respectively. The latter terminology will be used in the rest of the paper.

Based on these modeling results, geometries were selected to apply anisotropic strains with maximum : minimum principal strain ratios of 1.2, 1.6, 2.5 and 4.4 for a 20kPa pressure drop; each with a maximum strain of ~9%. For the geometries selected for the equibiaxial strains, the model predicted strains were ~1, 3, 5, 7 and 11%. The modeling predicted principal strains and the principal strain ratios for all tested conditions are shown in figures 2(b) and 2(c) respectively.

Strains that were empirically measured in the developed prototype by tracking beads embedded in the membrane, displayed the same trend as the model data but the strain values were higher than those predicted by the models. Similarly, the anisotropy ratios were affected as well. For a 20 kPa pressure drop, the empirically determined maximum

principal strain for the anisotropic units was $\sim 15\%$ (figure3(b)) and the average maximum : minimum principal strain ratios for the various types of units were $\sim 1.1, 1.5, 1.9$ and 2.7 (figure3(c)). The equibiaxial strains were $\sim 2, 5, 9, 13$ and 17% (figure3(b)) with maximum: minimum principal strain ratios between ~ 0.9 and ~ 1.1 (figure3(c)).

Figure 2 – Finite element modeling of strain units showed that stretching a membrane over a circular pillar into an ellipse trench can generate uniform maximum and middle principal strains in the central 50% area over the pillar (ROI) (a). In the ROI the maximum and the middle principal strains were along the membrane surface and corresponded to the maximum and the minimum principal surface strains respectively. They were aligned along the trench major and minor axis respectively (a). The maximum and the minimum principal surface strains were averaged for the ROIs (b, bar plots displaying average $\pm$ standard deviations) and their ratios were calculated from the averages (c).

Multiple factors can account for the mismatch between the models and the actual device, such as the use of inaccurate values for the friction coefficient between the pillar and the membrane, the membrane thickness, or the elastic modulus of PDMS. Varying these factors

in one of the models suggested that the elastic modulus value used is most likely causing the differences. Changing the elastic modulus in the model to a lower value of 1 MPa, which is on the lower side of the reported range in literature²⁵, results in strain values close to the empirical results (data not shown).

Figure 3 – Empirical strain measurements were performed by tracking 6μm beads embedded in the membrane. The beads were identified and mapped between strained and unstrained images of the membrane (a). Strains were calculated for all pairs of identified beads ($\Delta d/d$) and plotted against the angle that the bead pair made with the trench major axis (θ) (a). The strains for bead pairs oriented within 2.5° angles along the trench major and minor axis were averaged (strain 1 and strain 2 respectively) and used as approximations for the maximum and the minimum principal strains in the membrane. For the equibiaxial strain units, strain 1 and strain 2 were the averages along and perpendicular to the image horizontal direction respectively. The principal strains thus calculated (strain 1 and strain 2) were then averaged for each strain condition (b) (average $\pm$ standard deviations, $n=20$ for the anisotropic strains and $n=4$ for the equibiaxial strains). The principal strain ratios were also calculated and averaged for each strain condition (c) (average $\pm$ standard deviations, $n=20$ for the anisotropic strains and $n=4$ for the equibiaxial strains).

Flow induced shear stress in the device and its variation due to the strain actuating membrane dip were analyzed using computational fluid dynamics (CFD) modeling

CFD results demonstrated that when cell culture media (viscosity 0.001Pa.s) is flown through the flow channel, ROIs in the middle of the channel experience uniform shear stresses. However, those near the channel bends are exposed to disturbed shear stresses. The disturbances near the bends become small compared to the applied shear stress if the viscosity of the fluid is increased. These results are similar to our previous findings for a slightly different channel geometry¹⁵. To limit fluid flow variations and increase fluid flow shear stresses, media with 5% (wt/wt) dextran (viscosity ~0.0037 Pa.s) was used for the cell experiments. Figure 4(a) shows the shear stress contours for media alone and with added dextran, both for two inlet velocities – 0.1m/s and 0.2m/s (corresponding to 20ml/min and 40ml/min volumetric flow rates). The 40ml/min flow rate was used in experiments with the high viscosity media to keep the inflow higher than the total device volume change produced by the strain actuating membrane dips. For low viscosity media, however, the 20ml/min flow rate was preferred in order to avoid reversing flows near bends, at the cost of flow reversal for ROIs near the outlet due to inflow not being able to fill up the increased device volume during strain actuation. Figure 4(b) shows the average shear stress in all ROIs for the two flow conditions tested in the experiments. The average shear stress values in the ROIs are ~0.08 Pa for the media without dextran at 20 ml/min flow and ~0.55 Pa for the media with 5% dextran at 40 ml/min flow.

Shear stress variations in the ROIs due to the membrane deformations were estimated using various deformed membrane geometries. The membrane deformations used were obtained from the strain models. Such an approach was chosen based on our previous observation that flow modeling with fixed geometries for the deformed membrane in a similar system¹⁵ predicted shear stress variations comparable to those predicted by more computationally expensive fluid structure interaction models. Figure 4(c) shows the shear stress contours for sample geometries and figure 4(d) shows the average shear stresses in the ROIs of the various test units for membrane deformations corresponding to five different pressure drops. Over half a strain cycle (i.e. 0 to 20kPa pressure drop / 0 to maximum strain) the average shear stress in the ROIs was found to increase with increasing membrane deformation (except for the 2.5mm radius circular trench, where the shear stress in the ROI plateaued after an initial increase). For circular trenches, the increase in shear stress was higher for a bigger trench and for ellipse trenches, the shear stress increase was higher for the orientation with the bigger membrane deformation along the flow path before reaching the ROI (i.e. the increase was highest for the trench major axis aligned along the flow and lowest for the trench major axis aligned perpendicular to the flow). The biggest variation

was observed for the condition number 6 where the average shear stress changed by 15.7% over a strain cycle.

Figure 4 – CFD models of the entire channel network were used to get the overall distribution of shear stress in the channel (a). Disturbances in the shear stress were observed in the regions near the flow channel bends when media alone was used. At a higher flow rate of 0.2 m/sec (corresponding to 40 ml/min), reversing flows were found near bends with media alone. However, when 5% (wt/wt) dextran added media was used, the disturbances near the bends were reduced and flow reversal near bends was avoided at the higher flow rate. The average shear stress in all ROIs was calculated from these models (b, average $\pm$ standard deviation). Local deformed geometries were used to assess shear stress variations due to the membrane dips during the strain application for an applied flow rate of 40 ml/min and a 0.0037 Pa-s viscosity (c, arrows indicate the flow direction). The shear stress was averaged for the ROIs for all deformed geometries (d, average $\pm$ standard deviation).

A ~0.55 Pa shear stress dominates surface strain in alignment response of HUVECs

In experiments conducted with HUVECs cultured on the device under media containing 5% wt/wt dextran, it was found that the cells aligned perpendicular to the maximum principal strain direction when strain alone was applied and along the flow direction when flow alone was applied. No alignment occurred in response to equibiaxial strains or in the static control, and the alignment in response to strain alone was significantly noticeable only for the higher degree of anisotropy strains. Interestingly, when flow and strain were simultaneously applied, cells aligned along the flow direction. Figure 5 shows the cell morphology for one replicate of each of the 25 conditions tested for the strain only and the flow and strain devices. Figure 6 shows the cell orientation angles for all conditions as beeswarm plots, where overlapping points in the distribution are spread apart. Thus the plots, which are a scatter of each individual cell's orientation angle, are thicker (have a big spread) for the angles along which a large number of cells are aligned. For each condition, the replicates are pooled together but depicted in separate colors. The cell orientation angle distributions were multimodal, so medians were used to determine the general cell orientation direction. Figure 6 shows all cell orientation angles with respect to the respective trench major axis. The medians are marked as blue lines, a dotted red line marks the orientation direction in the case of perfect alignment along the flow and a dotted green line marks the orientation direction in the case of perfect alignment perpendicular to the maximum principal strain direction. The blue lines were generally close to a red or a green line. Some cases where this did not happen were (i) in low density plots, i.e. conditions where few cells were present, (ii) in cases where there was no significant alignment (no prominent bulge in the distribution observed) and (iii) in cases where two lines were almost 180° apart, which was not a real difference since 0° and 180° angles represent the same alignment direction. A fourth case of a big difference between the red and the blue lines was observed for the flow and strain stimulation conditions with high degree of anisotropy strains aligned at intermediate angles (30°, 45° or 60°) to the flow. This indicates a mixed response of the cells to the two stimuli, where the alignment due to one signal is dominant (fluid flow shear stress in the case of high viscosity medium and strain anisotropy in the case of low viscosity medium), but a clear contribution of the other signal is present. At more extreme angles, this turned to a complete dominance of one stimulus when they opposed the other's alignment response.

In experiments using media without dextran and a lower flow rate, cells aligned perpendicular to the maximum principal strain direction in response to simultaneous flow and strain stimulation. Flow only and strain only stimulation experiments performed with the low viscosity media had a shorter stimulation time (17 h, for the other experiments it was 24 h) and the data is not included here. However it was observed that the cells

displayed feeble alignment along flow for the flow only stimulation and alignment perpendicular to the maximum principal strain for the strain only stimulation. No alignment was observed in the static control (data not shown).

Figure 5 – Fluorescent images of HUVECs stained with Alexa 488 phalloidin were taken using a long working distance 20x objective with BD Pathway for multiple (an)isotropic

strain regimes at multiple angles to the fluid flow direction. The cells are shown after a 24 hour strain only (a) or a 24 hour flow and strain (b) stimulation under media containing 5% dextran (high viscosity). Scale bar - 100 μ m. In each image set, the conditions are arranged by the degree of anisotropy in the rows and by the orientation of the trench major axis in the columns. Ellipses of various aspect ratios drawn on the left depict the various degrees of anisotropy in the respective rows and the circles of various sizes drawn at the bottom depict the various equibiaxial strains in the last row. The ellipses drawn on the top of each image set represent the trench orientations while the red lines represent the flow direction or just the orientation of the flow channel (in the case of strain only stimulation).

Figure 6 – Images were also taken using a 4x objective with BD Pathway. 3x3 montages were captured for cells and nuclei stained with Alexa 488 Phalloidin and DAPI

respectively. The montages captured complete pillars and trenches. The images were cropped to the regions above the pillars and these cropped images were used for segmenting cells using CellProfiler. For analysis, only the cells with centers within the ROIs were considered. Cellular orientation for cells in the ROIs was plotted as beeswarm plots for each mechanical stimulation condition (replicates pooled together but depicted in separate colors). In the beeswarm plots, the orientation of individual cells are plotted as a scatter with added spread where points overlap, making the plots appear thicker for angles along which a large number of cells are oriented. The beeswarm plots display the distributions of cell orientation angles with respect to the trench major axis. Thus, preferential alignment perpendicular to the maximum strain direction results in a bulge in the plot at 90° while preferential alignment along the flow produces a bulge in the plot at the angle that the trench major axis makes with the flow. Median values are marked with a blue line and a central circle. Dotted red lines plus vertical lines in the left corner and green lines plus asterisks in the left corner mark the expected medians if the alignment was perfectly along the flow or perpendicular to the maximum strain respectively. Medians were calculated for orientation angles with respect to the reference line that would put the perfect alignment case at 90° , and were shifted appropriately if the reference line was not along the trench major axis.

Discussion

Using a medium throughput device to apply various anisotropic strains at various orientations to flow induced shear stress, it was found that (1) when stimulated with strain alone, the alignment response of HUVECs is stronger for a higher degree of anisotropy, and (2) when combined strain and flow stimulation is applied, at a low shear stress of 0.08 Pa, alignment response is dominated by strain while at a higher shear stress of 0.55 Pa, alignment response is dominated by the shear stress. Enabled by the device developed here, these results demonstrate for the first time how endothelial cell alignment response is affected by anisotropic strain and shear stress combinations that do not stimulate alignment in the same direction. Since cell behavior and function often depends on the cell shape and orientation, this information can be important to better understand biological processes in complex mechanical environments.

The enhanced throughput ability of the reported device is essential for the testing of a large number of conditions in a few experiments. The ROIs on the device can be exposed to uniform anisotropic surface strains and shear stresses and have an area of 0.88 mm^2 which allows for up to a few hundred attached cells to be studied. This is sufficient for statistical analysis and also makes the device suitable for co-cultures experiments in the future. These features put the developed device in a unique position. Previously reported macroscale

devices²¹ that can apply strains and flows simultaneously to cells are limited in throughput while in microscale¹² devices the culture area and thus the number of cells per replicate is limited. Suspended membrane devices²⁶, where strain actuation is provided by pulling attached walls perpendicular to the membrane, are restricted in the orientations between strain anisotropy and flow that can be tested due to the wall coming in the way. Shear stresses applied using cone and plate mechanisms²⁷ or flows in tubes^{22,24} can be challenging to apply at varying orientations to strain. And out of plane membrane stretching to produce anisotropic strains is not straightforward to integrate with flow²⁸.

Several aspects of the current device can be improved, including flow disturbances due to the strain actuating membrane dips, media pooling between dissimilar conditions, disturbed shear stresses near channel bends, limits on shear stress magnitude, and the relatively large amounts of reagents required compared to microscale devices. We demonstrated previously by comparing 0.5 mm and 2 mm high channels that a higher channel can reduce the variations in shear stresses due to the membrane dips during strain application¹⁵. Hence 2 mm high channels were used. However, still the deformed channel geometry models predicted shear stress variations of up to 15.7% from the undeformed state, making the flow effectively pulsatile in the ROIs. Since the variations could be quantified, an effectively pulsatile flow was not considered a major drawback. Apart from that, pulsatility can be theoretically diminished by varying the inflow over time to compensate for the volume increase induced by the moving membrane during the deformation cycle. The flow velocity change due to the extra volume generated by the membrane dips was minimized by keeping the overall flow rate high. Non negligible flow velocity differences could still persist along the channel and hence replicates were kept in regions near inlet and near outlet (figure1(c)) to detect any significant differences. Similarly, the near bends and mid-channel regions (figure1(c)) housed replicates that could check for significant differences introduced by the shear stress disturbances near the bends. Pooling of the culture medium was tolerated assuming that in the primary mode of operation, i.e. combining strain and flow, the paracrine signaling molecules would be continuously washed off and highly diluted by the flowing medium. The need for large amounts of reagents such as cell culture medium cannot be avoided, but for reagents such as the staining solutions, the solutions could be re-used for multiple devices.

Overall, the developed device provides unprecedented possibilities to study the underexplored effects of anisotropic strain and flow combinations on cells. The device is easy to use and its flexible design allows for future innovations in overcoming existing limitations and addition of functionality such as surface patterning to selectively allow cell attachment in ROIs. The data generated with this device, as shown here for the alignment of HUVECs to combinations of anisotropic surface strains and fluid flow shear stresses, is

likely to provide unique insight into the importance of combined mechanical signals for cell behavior and tissue development both *in vivo* and during *in vitro* cell culture.

Methods

Device fabrication

The device was fabricated by gluing together layers which were produced by casting polydimethylsiloxane (PDMS) (Sylgard 184, 10:1 base : curing agent) in molds and curing at room temperature for 48 h. Three PDMS layers were formed in this way – (i) a pillar array, (ii) fluid flow channels and (iii) a trench-air-channel layer. The molds for the pillar array and the fluid flow channels were made by laser cutting transparent polymethylmethacrylate (PMMA) sheets (Snijlab). The trench-air-channel mold was prepared by milling aluminum.

The fabrication steps, as illustrated in supplementary figure 1, were as follows. A PDMS membrane was prepared by spin coating (5 s at 100 rpm followed by 40 s at 1000 rpm) a mixture of PDMS and 6 μ m polystyrene beads (Polysciences Inc.) on a perfluorodecyltrichlorosilane (FDTS) coated silicon wafer (Siebert Wafer GmbH). The membrane was cured at 60°C for 2 h and then the trench-air-channel layer was attached to it using uncured PDMS as glue, which was spin coated on a silicon wafer (120 s at 1500 rpm) and stamped on the non-air-channel side of the trench-air-channel layer. The pillar array was plasma bonded to a 1mm thick glass base plate and all pillar tops were covered with drops of silicone oil lubricant (Griffon HR260 silicone spray). The trench-air-channel with the membrane was taken off of the silicon wafer, aligned with the lubricated pillar array, and attached to it without a glue. The device parts were stored at this point. Just before the cell studies, the top of the membrane and the inside of the flow channels were plasma treated and glued together using NOA81 (UV curable glue, Norland Products Inc.). The plasma treatment and the UV curing of the NOA81 served an extra purpose of sterilization along with making the surface hydrophilic and bonding the layers. The tubing was attached inside a sterile laminar flow cabinet and the device was clamped between a laser cut steel plate on top and a 2mm thick glass plate at the bottom to avoid leaks during operation. The top steel plate contained openings above the channels to allow for gas exchange through the PDMS. Once the clamped device was ready, it was filled with a fibronectin solution (0.5 μ g/ml) in phosphate buffered saline (PBS) and incubated at room temperature for at least 1 h. Fluid was filled in the device using a syringe and with a small tilt to the device to keep the fluid front moving against gravity, such that any bubbles moved to the fluid front and were finally pushed out of the outlet. The fibronectin solution was removed just before filling the device with a cell suspension.

The important dimensions of the developed prototype are as follows: (i) base 127.8 mm x 85.5 mm (same as standard well plate base dimensions), (ii) pillar and trench height 1mm, (iii) air channel height 0.5mm, (iv) fluid flow channel height 2mm, (v) pillar radius 0.75mm, (vi) membrane thickness 80 μm , (vii) circular trench radii 1.25, 1.5, 1.75, 2 and 2.5mm and (viii) ellipse trench dimensions (half of minor axis length – half of major axis length) 1.55-2.3, 1.7-2.3, 1.9-2.3 and 2.1-2.3 mm.

Computational modeling of strains and fluid flow

Computational models of the strain unit and the flow channels were analyzed using ANSYS 13.0 Workbench (ANSYS Inc). For the strain, a quarter unit was modelled with symmetry boundary conditions. To analyze the overall shear stress distribution, the complete flow channel network was modelled. To analyze the local changes in shear stress due to the strain actuating deformation of the membrane, flow was modelled in deformed channel geometries for the various unit types at various applied pressure drops.

The strain models consisted of three parts – a circular pillar (1 mm high, 0.75mm radius), a membrane (80 μm thickness) and a part of the trench-air-channel layer with various trench dimensions. All parts were modelled as PDMS with isotropic material properties. The elastic modulus used was 1.84 MPa and the poisson's ratio used was 0.499²⁹. All contacts were modelled as bonded contacts except the pillar-membrane contact, which was modelled as a frictional contact with a coefficient of friction of 0.01¹⁵. Fixed contact boundary condition was applied to the base of the pillar and the trench-air-channel layer and a pressure drop was applied to the bottom of the membrane. A linear pressure profile going from 0 to 20 kPa over 1 s was used. The principal strain vectors were exported and averaged for the ROIs.

For the flow models, a velocity inlet, a pressure outlet and a no-slip wall were used as boundary conditions. The fluid properties used were – 1000 kg/m³ density and a 0.001 Pa.s (cell culture media) or a 0.0037 Pa.s viscosity (viscosity of cell culture media with 5% wt/wt dextran). The viscosity for the media alone was taken from literature³⁰ and the viscosity of dextran containing media was determined by comparing the time taken by it to flow across a tubing with a fixed applied pressure drop with the time taken by media without dextran. Inlet velocities used were 0.1 m/s or 0.2 m/s for the full channel (corresponding with 20ml/min or 40 ml/min respectively) and 0.04 m/s for the deformed geometries (corresponding with 40 ml/min). A 0 Pa pressure was applied to the outlet in all cases. Once the models were solved, wall shear stress values were exported and averaged over the ROIs.

The deformed channel geometries were created using the modeling results. The maximum dip of the membrane for the circular trench or the maximum dips along the trench major

and minor axes were calculated and used to create the deformed geometries using SolidWorks (Dassault Systemes). For the circular trenches, the deformed membrane geometry was created by approximating the membrane deformation as a circular arc with the calculated dip. For the ellipse trenches, two circular arcs with the calculated dips were drawn along the trench axes and a loft feature passing through them was created.

Device characterization

Strains were empirically determined by tracking the beads embedded in the membrane. Strains were applied using an Elveflow AF1 dual pressure-vacuum generator and controller (Elvesys Innovation Center, France). Images of the membrane over the pillars were taken at 0, 5, 10 and 20 kPa pressure drops. Bead co-ordinates in the images were determined by using a local maxima/minima detection following a Gaussian blur (2 pixel radius) in ImageJ (NIH). The detected beads were mapped between images using a custom Matlab (MathWorks Inc.) script to assign the same number to beads in all images. Strains were determined for all pairs of beads as the ratio of the change in distance between a bead pair to the initial distance between the bead pair. The bead pair strains were plotted against the angle that the line joining the bead pair made with the trench major axis (bead pair orientation angle). Strains were averaged for bead pairs with orientation angles within 2.5° on either side of the major axis and the minor axis and these averaged strains were used as approximations for the maximum and the minimum principal strains respectively.

Cell studies

The tubing setup during cell studies was similar as reported previously¹⁵. Primary HUVECs under passage 10 were used for the cell studies. The cells were expanded in T75 flasks using endothelial growth media from a bullet kit (EGM2, Lonza). Cells were seeded at a high density ($>20,000$ cells/cm²) to get a confluent cell layer on attachment. After filling the device with the cell suspension, the device along with the tubing was moved to a 37°C , 5% CO₂ incubator and left there for 18-20 h allowing the cells to attach. The cells were then stained with CellTracker Green CMFDA (ThermoFisher Scientific) using the manufacturer's protocol and imaged in the ROIs. Mechanical stimulation was then applied for 24 h total, with one break after ~6 h of stimulation, to check the cells. Separate devices were used to apply flow only, strain only, flow and strain, or no stimuli (static control) to cells. Strains were applied using an Elveflow AF1 system – a sinusoidal pressure profile going between 0 and 20 kPa with a 1 s period was used. Flow was applied using a Legato 270P syringe pump (KD Scientific) – a 20 ml/min or a 40 ml/min volumetric flow rate was applied. The syringe pump used two 50 ml syringes (BD Plastic), one pushing fluid into the device while the other filled, and one way check valves ensuring that fluid was filled from the reservoir and pushed into the device by both syringes. Thus a continuous flow was maintained, except for the brief interval (0.25 s according to pump manufacturer) when the

pump switched directions. This occurred at 1 minute intervals for the 20 ml/min flow and at 30 s intervals for the 40 ml/min flow. The syringes were replaced after every 12 h of use in order to avoid piston failure due to friction build up. After 24 h of stimulation, the cells were fixed with 4% paraformaldehyde. They were then stored at 4°C under PBS until the cells could be stained. For staining the cells, they were first washed with PBS, followed by 1% Triton X-100, then again by PBS and then blocked for 1 h with a 5% bovine serum albumin (BSA) solution in PBS to prevent non-specific staining. Staining for filamentous actin was done using Alexa 488 Phalloidin (solution in 5% BSA) for 1 h followed by staining for DNA using DAPI (solution in 5% BSA) for 20 minutes. The cells were then washed with PBS and water and stored dry until imaging. Automated imaging was performed for all ROIs using BD Pathway (BD Biosciences).

Image analysis for cell alignment quantification

Images captured were 3x3 montages acquired using a 4x objective. The montages covered the entire pillar and trench area around a ROI. Two montages were taken per ROI, one each with the fluorescence excitation and emission filter settings for Alexa 488 (cell image) and DAPI (nuclei image). A custom Matlab script was used to crop images to the regions above the pillars by detecting the pillar edge in the nuclei images. The pillar edge was always darker than the rest of the image, allowing the Matlab script to search for the center of a circle with the same radius as the pillar edge that had the lowest cumulative intensity along its circumference. An image mask was then applied to black out all area outside the pillar area. Background subtraction was performed to remove effects of non-uniform illumination and contrast enhancement using ImageJ. CellProfiler³¹ was then used to segment the nuclei and the cells and measure their shape and size. The CellProfiler pipeline first used thresholded nuclei images to identify the nuclei as primary objects and then the cells were segmented in the cell images as secondary objects for each nucleus using the propagation algorithm. The shape and size of the segmented cells were exported from CellProfiler and analyzed for the cells whose center was within the ROI. Artifacts falsely identified as cells were removed using lower and upper size filters combined with a filter for eccentricity.

Cell orientation angles were plotted as beeswarm plots and the median values were marked. For determining the medians, cell orientation angles with respect to the image horizontal direction (perpendicular to flow) were used when cells aligned to flow and with respect to the trench major axis when cells aligned to strain. This ensured that the bulge in the beeswarm plots occurred near the middle of the distribution and the medians represented the majority alignment direction.

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Author contributions

JR designed the research. RS carried out the experiments and data analysis. RS and JR wrote the manuscript. SLG provided inputs on the fabrication methods. JR,NV and BK supervised the research and provided the facilities and software required for the project. AvdB provided access to lab space, reagents and other materials. All authors reviewed and provided inputs on the manuscript.

Competing interests

The authors declare no competing financial interests.

Supplementary figure 1 – The fabrication process is presented schematically (a). The device layers were made from three molds – pillar array (b), flow channel (c) and trench-air-channel (d) molds. After channels were attached, the device was clamped before filling and using (e).

Chapter 5. Vascularized bone tissue engineering: a possible effect of mechanical stimulation

Abstract - Vascularization is a useful strategy in tissue engineering for creating tissue constructs that are not limited in size by nutrient diffusion. A simple way to achieve vascularization of engineered tissue is by co-culturing the desired tissue's cells with endothelial cells (ECs), where the ECs can spontaneously assemble into a vascular network. Vascularized bone tissue engineering has previously been achieved by co-culturing mesenchymal stem cells (MSCs) with ECs¹. The osteogenic differentiation of the MSCs and the vascular network assembly of the ECs proceed simultaneously in this case. Evidence from previous monoculture studies with MSCs and ECs suggests that mechanical stimuli can play an important role in vascularized bone tissue engineering. For example, cyclic strains and flow induced shear stresses have been found to promote osteogenic differentiation of MSCs while for ECs the two stimuli are known to regulate several functional responses such as alignment^{2,3}, barrier function^{4,5} and angiogenic sprouting^{6,7}. However, the effect of mechanical stimuli in co-cultures of both cell types has not been well explored. Hence we used our previously developed medium throughput platforms^{8,9} (chapters 3 and 4) to screen for the effects of a variety of combined strain and shear stress mechanical stimuli on EC-MSC co-cultures. It was found that endothelial cells do not display a strong alignment response to highly anisotropic strains or a 0.55 Pa.s shear stress in the co-cultures, while our previous experiments showed that these mechanical stimuli resulted in a prominent cell alignment response in endothelial mono-cultures. It was also found that the addition of dextran to increase the applied shear stress has an additional effect of enhancing endothelial cell proliferation leading to the formation of sheet-like clusters at the cost of tubule formation. Further experiments building on the insights gained from the reported experiments are required to comment more conclusively on the effects of mechanical signals on MSC-EC co-cultures for vascularized bone tissue engineering.

Introduction

Implantable engineered tissues currently have a maximum size limit based on the diffusion controlled nutrient supply. The diffusion limit is generally considered to be around 200 μm ¹⁰ and while it can be overcome by applying flow perfusion to engineered tissue constructs in vitro¹¹, such an approach is not sustainable after implantation. In vivo, the diffusion limit to nutrients is overcome by a vascular network supplying the nutrients and hence vascularization of engineered tissue is an important strategy¹². One way to achieve

vascularization of engineered tissue is to include endothelial cells in the tissue culture, which under the correct conditions can spontaneously assemble into a vascular network of tubular structures¹³. These structures can then anastomose with the body's vasculature upon implantation¹³. Due to their high promise, vascularization strategies are under intense investigation. However, the roles of mechanical signals, which are known to regulate vascular developmental processes, remain relatively underexplored in vascularized tissue engineering approaches. The effects of mechanical signals can be especially important when they also have a beneficial effect in the development of the engineered tissue being vascularized, such as bone.

Engineering vascularized bone tissue is a highly complex problem. A recent review by Liu et al lists the large number of parameters that need to be considered in approaching the problem¹⁴. As they point out, the effects of mechanical signals are underexplored. Mechanical signals such as cyclic strains and flow-induced shear stresses are known to significantly affect the cells commonly used for vascularized bone tissue engineering, such as osteoblasts and mesenchymal stem cells for the bone tissue and mature endothelial and endothelial progenitor cells for the vasculature. Both cyclic tensile strain^{15,16} and shear stress^{17,18} have been found to enhance the bone forming potential of MSCs. Strains¹⁹ and shear stresses²⁰ have also been shown to affect proliferation and bone formation from osteoblasts. The effects of strains and shear stresses on endothelial cells have been more widely studied in the context of their function as the inner lining of blood vessels^{21,22}, but studies have also looked at the effects of the mechanical stimuli in the vascular network formation process. Large strains have been found to stimulate an endothelial cell monolayer to form a branched network of cells²³ and uniaxial strains have been shown to cause alignment and prevent branching of tubules formed from endothelial cells in a 3d network⁶. In another study, however, strains were found to disrupt tubule formation²⁴. Shear stresses are important for angiogenic sprouting into a 3d space from endothelial cell layers^{7,25} but have also been reported to reduce the tube forming ability of endothelial cells²⁶. From these studies it is hard to conclude how mechanical stimuli would affect co-cultures for vascularized bone tissue engineering but there is a possibility that the effects can be beneficial.

In this study, the effects of mechanical signals on 2d co-cultures of endothelial cells and MSCs were investigated. Medium throughput platforms, previously developed by us (chapters 3 and 4)^{8,9} were used for this purpose. The platforms could either apply various equibiaxial strains in combinations with various shear stresses (D1, chapter 3)⁸ or various anisotropic strains at various orientations to a flow induced shear stress (D2, chapter 4)⁹. Systemically investigating the effect of these different signals could lead to the optimization of mechanical stimulation regimen in prevascularized bone tissue engineering.

Materials and Methods

Cell and media ratio optimization on PDMS culture surface

Mesenchymal stem cells and endothelial cells are generally cultured in separate specialized media. A combination of the two media can be suitable for providing the right nutrients to both cell types. However, optimization of the cell and media ratios based on the desired outcome is important²⁷. Therefore, three media ratios (hMSC:HUVEC media ratio 75:25, 50:50 and 25:75) and four cell ratios (hMSCs:HUVECs 95:5, 90:10, 80:20 and 50:50) were tested to find the optimum conditions for the formation of tube like structures. hMSC medium consist of α MEM supplemented with 10% fetal bovine serum, 2mM L glutamine, 0.2mM ascorbic acid, 100U/ml penicillin + 100mg/ml streptomycin, 10^{-8} M dexamethasone and 0.01M β glycerol phosphate. HUVEC medium consists of EGM2 medium (Lonza).

The ratio optimization experiments were performed on a PDMS surface which is similar to the culture surface of the mechanical stimulation platforms. The wells of two 24 well plates were covered with PDMS, plasma-treated and coated with fibronectin before cell seeding. The co-cultures were then seeded in triplicate per condition in the 24 well plates at a seeding density of $\sim 20,000$ cells/cm² and cultured for 9 days with media changes every 2-3 days. At the end of the experiment, cells were fixed with 70% ethanol and immunostaining was performed for CD31 to specifically stain endothelial cells. For the immunostaining, first the cells were incubated under 5% bovine serum albumin for 1 h to block non-specific staining, then primary antibody (Dako M0823, 1:50 dilution) solution in 5% BSA was added for 2 h, followed by secondary antibody (Thermo Fisher Scientific A-21125, 1:500 dilution) solution in 5% BSA for 1 h. The plates were then imaged and the formation of thin, long, tube-like structures by endothelial cells was accepted as a positive outcome.

Co-culture on test platforms using optimized cell and media ratios – troubleshooting

It proved non-trivial to translate the co-culture with the optimum cell and media ratios in well plate conditions to the mechanical stimulation device. The troubleshooting steps carried out are described in this section. These experiments were done mainly using the equibiaxial strain device (D1). 90% hMSCs – 10% HUVECs in 75% hMSC osteogenic – 25% EGM2 media were used in all cases for the co-cultures. The various experimental conditions, seeding densities, the controls and the experimental durations used are listed in table 1.

Table 1 - Experimental conditions

No.	Experiment	Cultured on	Cell seeding density	Applied flow	Applied strain	Duration of experiment
1	Long term culture under dynamic stimulation	2x D1 devices – one stimulated with combined flow and strain and the other static	~14,000 cells/cm ² (90% hMSC – 10% HUVEC)	Continuous 20 ml/min flow switching syringe every 1 minute (starting 18-20 h after seeding)	2-10% strain at 0.5 Hz (0 to 20 kPa sinusoidally varying pressure drop) - 2 h per day, starting 3 h after starting flow	Till device failure for the flow and strain stimulated device. 6 days for the static device.
2	Tracking endothelial cells (CD31)	(a) 2x D1 devices – one stimulated with combined flow and strain and the other static (b) 6 well plate with PDMS-coated wells	~20,000 cells/cm ² (90% hMSC – 10% HUVEC)	(a) Continuous 20 ml/min flow switching syringe every 1 minute (starting 18-20 h after seeding) (b) not applicable	(a) 2-10% strain at 0.5 Hz (0 to 20 kPa sinusoidally varying pressure drop) - 2 h per day, starting 6 h after starting flow (b) not applicable	4 days
3	Material effects	12 well plate	~15,000 cells/cm ² (90% hMSC – 10% HUVEC)	-	-	5 days

Table 1 Experimental conditions (continued)

No.	Experiment	Cultured on	Cell seeding density	Applied flow	Applied strain	Duration of experiment
4	Tracking endothelial cells (CellTracker red DiI and CD31)	(a) 2x D1 device channels assembled on a PDMS-coated glass plate (no strain array) – one stimulated with flow and the other static (b) 2x small strain arrays (10 strain units, no flow channel) – one stimulated with strain and the other static	~15,000 cells/cm ² (90% hMSC – 10% HUVEC)	(a) 20 ml/min flow switching syringe every 1 minute (starting 18-20 h after seeding) (b) none	(a) none (b) uncharacterized strains at 0.5 Hz (0 to 20 kPa sinusoidal pressure drop cycle) - 2 h per day, starting 18-20 h after seeding	5 days
5	Oxygen distribution	1x D1 device channels assembled on a PDMS-coated glass plate (no strain array) and 1x D1 device	~20,000 cells/cm ² (90% hMSC – 10% HUVEC)	-	-	2 days
6	VE cadherin staining	D2 device	>20,000 cells/cm ² (HUVEC)	40 ml/min switching syringe every 30 s	0 to 20 kPa sinusoidal pressure drop cycle at 1 Hz	2 days

1. Long term culture under dynamic mechanical stimulation

The medium throughput platforms for mechanical stimulation of cells were first tested for long term use (stimulation for >24 h) for the co-culture studies. Previously they were validated with shorter cell studies of stimulations for up to 24 h. In the initial experiments, 2 h per day strain stimulation and continuous shear stress stimulation were applied starting 18-20 h after seeding the cells. A static and a combined flow and strain device were seeded with cells and tested. For the combined stimulation device, the experiment was carried out till a leak developed in the flow channel. For the static device, the experiment was carried out till 6 days from seeding. Cell distribution and cell morphology on the device were then analyzed after a methylene blue staining.

2. Tracking endothelial cells at end point

CD31 immunostaining was utilized to detect endothelial cells in the co-cultures at the experimental end points. The immunostaining protocol used was the same as that used for the cell and media ratio optimization experiment.

3. Material effects

The cell and media ratios were optimized on PDMS surfaces in polystyrene wells. Both these materials are known to be biocompatible when coming in contact with the media and cells. However on the device, there was a possibility of contact with NOA81, the glue used to bond the flow channel on top of the strain array, and silicone oil lubricant or its solvents diffusing from between the pillars and the membrane to the cell culture side of the membrane. To test for any adverse effects of these materials, HUVECs were cultured in a 12 well plate on (i) a PDMS membrane resting on top of a NOA81 pillar covered with silicone oil lubricant and (ii) a PDMS membrane with NOA81 that came in contact with the culture medium. To account for any other differences in culturing cells on the devices, media stored on a D1 device at 37°C for 4 days was also used to culture cells on the various surfaces mentioned above. For the gas exchange to be similar to the devices, the 12 well-plate was covered with a 2 mm thick PDMS sheet instead of the plate cover. The cells were also cultured in a 6 well plate in fresh and device incubated media as controls.

4. Tracking endothelial cells over the course of the experiment

HUVECs were pre-stained with CellTracker CM DiI (live cell stain) (Thermo Fisher Scientific) and monitored over the course of the experiment. At the end point cells were immunostained for CD31 to visualize HUVECs alone and phalloidin and DAPI staining were used to visualize filamentous actin and nuclei in all cells.

5. Oxygen distribution in device

Ibidi MM2 nanoparticles were used to detect the oxygen available to cells on the device. These nanoparticles get taken up by cells and give a fluorescent signal proportional in intensity to the intracellular oxygen concentration. When excited by 400 nm light, they emit a higher intensity at 650 nm for low intracellular oxygen and a low intensity for high oxygen concentration. HUVECs were stained with CellTracker CMFDA (Thermo Fisher Scientific). They were then combined with MSCs and 1.36 $\mu\text{g/ml}$ MM2 nanoparticles were added. The co-cultures were seeded on a D1 device and in D1 device channels on a PDMS covered glass plate. The fluorescence of the nanoparticles was observed over several regions on the device as well as over a strain cycle for a single area above a pillar. Observations were made 18-20 h after seeding and then more nanoparticles were added (media in devices replaced with media containing 2.14 $\mu\text{g/ml}$ MM2 particles) and observations were made after another 24 h. Imaging was done with BD Pathway using a 377/50 filter for excitation and a 692/40 filter for emission.

6. VE cadherin staining

VE cadherin immunostaining was done on a D2 device which had previously been seeded with HUVECs alone and mechanically stimulated for 24 h (chapter 4). 4% paraformaldehyde fixed cells were incubated under 5% bovine serum albumin for 1 h to block non-specific staining, then primary antibody (Santa Cruz Biotechnology sc-9989 , 1:75 dilution) solution in 5% BSA was added for 2 h, followed by secondary antibody (Thermo Fisher Scientific A-21125, 1:300 dilution) solution in 5% BSA for 1 h.

Application of mechanical stimuli

D2 devices, that can deliver various anisotropic strains in combination with and at various orientations to a flow induced shear stress, were used to mechanically stimulate the co-cultures. D1 devices were not used for these studies past the troubleshooting stage for two reasons: (i) The various shear stresses on the D1 devices did not cover a large magnitude range, making them act as similar conditions. Thus, D1 devices only provided variations in equibiaxial strains, which were also provided by D2 devices, and (ii) As a result of the anisotropic strains on the D2 devices, an easy readout was expected in the form of the alignment response of cells to the various stimuli, based on the results in chapter 4. Also, 5% dextran (w/w) was added to the media to increase its viscosity and hence get shear stresses to levels that were earlier found to elicit alignment response from endothelial cells²⁸ (chapter 4). Since the cell and media ratios were optimized in media without dextran, controls without dextran were also cultured in PDMS covered wells of a 12 well plate. Several other test conditions were included on the control well plate, namely the co-culture

in 5% dextran media and both dextran and no-dextran conditions on the polystyrene base of the plate wells. The PDMS culture surfaces in the devices as well as the well plates were plasma treated and covered with fibronectin before seeding cells. 90% hMSCs – 10% HUVECs in 75% hMSC – 25% HUVEC medium were seeded on the device at a seeding density $\sim 18,000$ cells/cm². Cells were allowed to attach for 18-20 h and then mechanically stimulated with flow and strain or strain only for 6 h/day for 5 days. A 40 ml/min flow rate switching between syringes every 30 s (shear stress of 0.55 Pa) was used for the flow and a pressure drop cycle sinusoidally varying between 0 and 20 kPa with a 1 s period was used to apply the strains. For the strain only experiment, the media was changed in the device every 2 days. After 5-6 days in culture, the cell monolayers started detaching in various spots, primarily in the membrane dip regions and hence the experiment was stopped. At this time cells were fixed with 4% paraformaldehyde and endothelial cells were specifically stained by immunostaining for VE cadherin.

Imaging and image analysis

Imaging was done using BD Pathway. Montages were captured for large areas of interest. The image analysis was generally qualitative. Quantitative image analysis was attempted on the mechanically stimulated co-cultures. CellProfiler²⁹ was used to segment the nuclei as primary objects from DAPI images and VE cadherin stained endothelial cells as secondary objects around the nuclei. Various segmentation algorithms were tested but none were found to give reliable results over the entire image set.

Results

Cell and media ratio optimization

A low percentage of both endothelial cells and endothelial cell medium were found favorable for tubule formation. At higher endothelial cell or media fractions, the tendency of endothelial cells to form sheets instead of tubules was higher, as can be seen in figure 1. A 90% hMSC – 10% HUVEC cell ratio and a 75% hMSC osteogenic medium – 25% EGM2 medium were used for the mechanical stimulation experiments. This cell and media ratio choice was made in order to obtain the highest number of tubular structures without getting too many endothelial cell sheets.

Figure 1 – Three medium ratios and four cell ratios were tested to find the optimum co-culture conditions for hMSCs and HUVECs such that the highest number of tubular structures are obtained and HUVEC clusters that do not form the tubular structures are low in number. The selected condition which was used for further experiments is marked by a surrounding red boundary. Scale bar 500 μm .

Co-culture on test platforms using optimized cell and media ratios – troubleshooting

1. Long term culture under dynamic mechanical stimulation

Long term culture was not found to be an issue for cell viability on the device. However, long term flow stimulation was found unsustainable without frequently changing the syringes used to apply the flow. The rubber piston in the syringes failed on the 4th day of the experiment (after 3 days of continuous flow application) due to friction build up. The images of the cells after methylene blue staining showed large amounts of rubber debris (figure 2) in the flow channels, suggesting a slow failure. Cell detachment was also observed in some areas, although remaining attached cells still looked viable at the time of the device failure based on their morphology.

Figure 2 – Long term culture on the device was not found to be an issue for cell viability, but continuous flow was not sustainable without frequent syringe changes. The rubber debris (large dark spots) in the flow channels is shown alongside methylene blue stained cells (small dark spots) that stayed attached at the time of device failure for a combined flow and strain stimulated device. Images were acquired using BD Pathway, capturing montages covering 5 strain units at a time. 20 montages covering the 100 test units are displayed.

2. Tracking endothelial cells at end point

CD31 immunohistochemistry did not result in positive staining for cells seeded on the mechanical stimulation devices, irrespective of whether any mechanical stimulation was applied or not (figure 3). When the same staining solutions were used with the control on a PDMS surface in a 6 well plate, the staining worked well (figure 3), ruling out issues with the antibodies.

Overall cell viability, based on cell morphology, appeared good on the device. A possible cause of the CD31 staining not working could therefore be the selective death or detachment of HUVECs. Since the static control in a well plate does show positive staining, this could be due to the mechanical stimuli. Alternatively, the loss of HUVEC could be due to contact with extra materials or due to reduced gas exchange in the devices, being permitted only by the relatively slow diffusion through the PDMS, when compared to the controls.

Figure 3 – CD31 immunohistochemistry did not result in positive staining for co-cultures seeded in the mechanical stimulation devices irrespective of whether any stimulation was applied or not. The same antibodies gave good staining results with the control in a 6 well plate.

3. Material effects

The effects of extra materials on HUVECs were explored using cultures of HUVECs in contact with those materials in ways similar to that on the devices. Culturing on surfaces in contact with NOA81 (figure 4) or NOA81 and silicone oil lubricant (figure 4) did not affect the cells in terms of viability (cells attaching to the culture surface were considered viable). The cell viability suggested that the gas exchange through PDMS was also sufficient. However the cells showed a lower viability and slower growth in conditioned media that was incubated in a device at 37°C for 4 days previously (figure 4). A possible cause could be the enhanced partitioning, compared to PDMS surface in well plates, of important factors into the large PDMS surface in contact with media on the device. Partitioning of compounds from the fluid in surrounding PDMS^{30,31} is a known issue and needs to be taken into account. Pre-incubating devices with media could alleviate this issue.

Figure 4 – Culturing HUVECs on PDMS surfaces in contact with NOA81 or NOA81 and silicone oil did not affect cell viability. However previous media incubation in a mechanical stimulation device caused lower viability and proliferation of cells in that media. A possible

cause could be the partitioning of important factors into the large PDMS surface (all walls) surrounding the media on the devices. Control cultures of HUVECs on the polystyrene surface of a 6 well plate confirmed that the HUVECs had no issues and also that culturing in media pre-incubated on a mechanical stimulation device showed lower cell viability.

Scale bar 1 mm.

4. Tracking endothelial cells over the course of the experiment

To further confirm that the HUVECs were viable in the co-cultures on the devices as well, they were stained before combining with MSCs and monitored over 5 days (figure 5). Flow channels without the strain arrays were used for the ease of imaging (figure 6). However, to include the effect of the strain array, if any, small strain arrays with 10 strain units were used (figure 7). The HUVECs were found viable throughout the experiment and some proliferation was also observed. At the end, the cells were also immunostained for CD31, but due to the CellTracker CM DiI and the CD31 secondary being fluorescent in the same (red) channel, it was difficult to distinguish the CD31 staining. It seemed that some CD31 staining did occur in all cases but was weaker in the mechanically stimulated devices.

Figure 5 – HUVECs were stained with CellTracker CM DiI before mixing with the MSCs and culturing on the devices. The HUVECs were tracked over the experiment duration of 5

days and appeared viable and proliferating, shown here for the D1 channels assembled on a PDMS coated glass plate without the strain array. Scale bar 1 mm.

Figure 6 – HUVECs were stained with CellTracker CM DiI for tracing during the culture period and were immunostained using CD31 antibodies after fixation at the experimental end point. The combined staining (red channel) can be seen here for the D1 flow channels with and without flow stimulation. The staining looks weaker in the flow stimulated device.

Figure 7 – HUVECs were stained with CellTracker CM DiI for tracing during the culture period and were immunostained using CD31 antibodies after fixation at the experimental

end point. The CD31-CellTracker combined staining as well as phalloidin and DAPI staining can be seen here for three strain units each with and without strain stimulation. The CD31-CellTracker staining appears weaker in the strain stimulated device. Scale bar 2 mm.

5. Oxygen distribution in device

Even though cell viability on the devices was shown for HUVECs with CellTracker staining and from well-spread morphology for the MSCs, it was considered useful to check whether the cells get sufficient oxygen when cultured on the device. To separate the effects on the two cell types present, the HUVECs were stained with CellTracker CMFDA prior to the co-culture. Ibidi MM2 nanoparticles were used for the oxygen quantification. The MM2 nanoparticle fluorescence looked similar in various regions on the device as well as the channels alone, suggesting that there were no location specific effects. The fluorescence signal was detected only in rounded up cells (red channel, figure 8) and mostly coincided with the CellTracker CMFDA signal (green channel, figure 8). The green channel showed few spread cells as well but the MM2 signal was not seen in these cells. No change in the signal was observed during a strain cycle on the tested device (figure 8). Addition of more MM2 particles did not change the result, only rounded cells showed fluorescence. These results suggest two possibilities. One possibility is that the HUVECs died, and hence did not attach and spread, due to some damage during the CellTracker loading or due to toxicity from the nanoparticles or a combination of both. In this case, the dead cells somehow had low intracellular oxygen and hence appeared bright while the remaining cells had high oxygen and their fluorescence signal was below the detection limit. The other possibility is that the signal in the red as well as the green channel was from the CellTracker and the MM2 concentration was too low to be detected. The manufacturer recommended concentration of the nanoparticles was 2-20 $\mu\text{g/ml}$. Even after the second addition, these experiments cumulatively had only around 3.5 $\mu\text{g/ml}$ of the particles. Controls to verify the toxicity of the nanoparticles for the HUVECs, alone or in combination with CellTracker CMFDA could provide some answers but were not possible due to limited stock of the MM2 particles.

Figure 8 – Ibidi MM2 nanoparticles were used to detect intracellular oxygen levels on devices. They were expected to be taken up by cells and show a fluorescent signal with intensity inversely proportional to the intracellular oxygen concentration. Fluorescence signal (red channel) was observed only for rounded up (probably dead) HUVECs. The rounded up cells were identified as HUVECs from a CellTracker CMFDA staining (green channel). Application of uncharacterized strains (manually applied, strain 2 > strain 1) using a syringe showed no change in the MM2 particles' fluorescence signal.

6. VE cadherin staining

Since CD31 immunohistochemistry did not result in positive staining on the mechanical stimulation devices, VE cadherin staining was tried as an alternative. It was initially tried on a D2 device which had been seeded with HUVECs alone and mechanically stimulated for 24 h. The cells had also been fixed in paraformaldehyde and stained with phalloidin and DAPI previously. The VE cadherin immunostaining worked well (figure 9) and was used in later experiments to detect endothelial cells in co-cultures on the mechanical stimulation device.

Figure 9 – VE cadherin immunostaining worked well for HUVECs cultured in the mechanical stimulation device. It was therefore used in later experiments to selectively stain HUVECs in co-cultures on the device.

Effects of mechanical stimuli

Endothelial cells are known to align along the flow and perpendicular to the maximum principal strain²¹. However in the experiments with the co-culture, no consistent alignment in response to flow or strain was observed (figures 10 and 11). Although inconsistent among replicates, some alignment perpendicular to the maximum strain was found in a few units in both strain only (figure 11) and combined flow-strain (figure 10) experiments. Quantification of cell shape and alignment was not possible since VE cadherin staining was found insufficient to perform automated segmentation of the cells using CellProfiler.

More importantly, tubule formation was not observed. The endothelial cells formed sheet like clusters which resembled the clusters seen with higher fractions of the endothelial cells or endothelial cell medium in the ratio optimization experiment. The endothelial cell clusters covered a large fraction of the area in the flow-strain device (figure 10) and almost the entire culture area in the strain only device (figure 11). Comparison with the controls suggested that the clusters were an effect of the dextran addition, possibly by causing an increase in the proliferation of the endothelial cells. By the experimental end point, the cell

layer in the dextran media on PDMS had detached and could not be observed. The well with the co-culture in dextran containing media on polystyrene showed endothelial cells forming clusters, almost covering the entire culture area (figure 12). The control wells without dextran on PDMS as well as polystyrene showed the formation of tubules (figure 12).

Figure 10 – Combined flow and strain: 90% hMSCs and 10% HUVECs were cultured in 75% hMSC osteogenic – 25% EGM2 media containing 5% (wt/wt) dextran. Starting 18-20 h after seeding, combined flow and strain stimulation was applied to the cells for 6 h/day for 5 days. The cells were then fixed and immunostained for VE cadherin to detect only the endothelial cells. The results are shown here for a single representative replicate of each of the mechanical stimulation conditions tested. The endothelial cells did not form the intended tubular structures but instead formed sheet like clusters. No consistent alignment to either flow or strain was observed although some units showed alignment in response to the strain (i.e. perpendicular to the maximum strain direction). The type of the strains is depicted by the trench shapes drawn on the left and the bottom of the image. The figures on the top of the image represent the directionality of the strains with respect to the flow direction (red lines). Scale bar 500 μ m.

Figure 11 – Strain only: 90% hMSCs and 10% HUVECs were cultured in 75% hMSC osteogenic – 25% EGM2 media containing 5% (wt/wt) dextran. Starting 18-20 h after seeding, strain only stimulation was applied to the cells for 6 h/day for 5 days. The cells were then fixed and immunostained for VE cadherin to detect only the endothelial cells. The results are shown here for a single representative replicate of each of the mechanical stimulation conditions tested. The endothelial cells did not form the intended tubular structures and instead formed sheet like clusters, covering up almost the entire culture area. No consistent alignment to the strains was observed although some units did show alignment perpendicular to the maximum strain direction. The type of the strains is depicted by the trench shapes drawn on the left and the bottom of the image. The figures on the top of the image represent the directionality of the strains with respect to the flow direction (red lines). Scale bar 500 μ m.

Figure 12 – Controls were cultured in a 12 well plate and the conditions tested included the co-cultures on treated PDMS surfaces and the well plate surface (polystyrene), both done in the presence and absence of dextran. The cell layer in the culture with dextran media on PDMS detached before the end point and could not be observed. The images of the remaining control wells after fixation and VE cadherin immunostaining are shown here. The addition of dextran seems to enhance endothelial cell proliferation at the cost of the formation of tubular structures of interest.

Discussion

Vascularization is an important tissue engineering strategy and is likely to be improved with the application of optimum mechanical stimuli. Co-cultures with endothelial cells offer a simple approach of achieving this. Medium throughput platforms that could apply various mechanical stimuli were used to stimulate endothelial cell – mesenchymal stem cell (driven towards osteogenic differentiation) co-cultures and gain insights into possible improvements in pre vascularized bone tissue engineering. While a seemingly simple set of experiments, setting up the co-cultures on the mechanical stimulation devices proved challenging. After optimizing the cell and media ratios for the co-culture, the aim was to reproduce the conditions on the device leading to the formation of tube-like structures by the endothelial cells. The effects of the mechanical signals could then be observed on the formation of these structures. Several challenges were encountered on the way to this last step.

- Firstly, long term cultures on the device were difficult to stimulate with flow using a syringe pump. The rubber pistons in the plastic syringes used for this purpose failed over extended use due to friction. This was easy to resolve by frequently replacing the syringes, but since it is hard to predict when a syringe would fail, roller pumps could be considered for future experiments.
- Secondly, the troubleshooting experiments showed that there might be significant differences in the culture conditions on the device compared to the well plate controls with an identical PDMS culture surface. In initial experiments, CD31

immunostaining for endothelial cells was undetectable for cells on the device, while it worked in the control, ruling out issues with the antibodies or the cells. A selective endothelial toxicity from additional materials on the devices was ruled out and so was the lack of oxygen available by diffusion through PDMS. The actual effect of the device on oxygen levels could not be well characterized using oxygen sensing nanoparticles due to insufficient controls. For experiments, a switch to VE cadherin immunostaining was made as an alternative to CD31 staining. Culturing endothelial cells in medium that was previously stored in a mechanical stimulation device at 37°C for 4 days, showed a slower cell growth than in fresh medium. A possible reason for this might be the partitioning of important growth factors or other molecules into PDMS, which is a known issue. Since the medium is in contact with a larger PDMS surface area on the device (cell culture surface plus flow channel inside surface) than on a PDMS-coated well plate (control), this might account for the differences between the two despite the use of the same culture surface. This needs to be verified, but if it turns out to be the case, initial incubation of the relevant media in the device before the seeding of cells in fresh media is likely to mitigate this issue.

- Thirdly, addition of dextran to the media made the endothelial cells proliferate into sheet like clusters instead of forming tubules. Dextran was added to the media to increase the flow induced shear stress to the levels that were known to elicit an alignment response of the endothelial cells. Without dextran, shear stress was low due to the use of high flow channels, used in order to minimize the effects of volume changes produced by the strain actuation mechanism.
- Fourthly, it was hard to retain the cell layer on the device for the duration of the experiment. The cell layer started peeling off after about 5 days in culture, primarily in the membrane dip region. In a separate experiment, the co-culture was seeded at a higher initial cell density (~ 27000 cells/cm²) to reduce stress in the cell layer from cellular contractility and applied strains, thus alleviating the peeling. To get tubule formation, dextran was not added and since shear stress would be quite low without dextran, only strain stimulation was applied. This experiment was carried out for 10 days, applying strains for 6 h/day, starting 18-20 h after seeding. Peeling of the cell sheet was still observed in the membrane dip regions, starting around 5 days. However, after the initial peeling, cells stayed attached in the regions above the pillars (region of interest) for most units. No tubule formation was observed in the device or the control in a well plate. The probable cause was the use of hMSCs that had been cultured beyond confluence for a few days and had quite likely differentiated. The data from this experiment is not included.

Further optimization is required to fully utilize the medium throughput device to study the effects of mechanical stimuli on hMSC-HUVEC co-cultures. The cell and media ratios

need to be optimized in the presence of dextran. If the presence of dextran limits the formation of tubules by increasing the proliferation of endothelial cells, seeding a lower initial fraction of endothelial cells may still result in the formation of tubules. The cell layer peeling might not be a major issue if the cells stay attached in the regions of interest after partial peeling in the regions of large deformations, releasing some stress. However, peeling is likely to be further reduced if the cells are not attached in the regions of large deformation. This can be achieved by coating the membrane dip regions with a reagent that prevents cell binding (for example Pluronic) or by masking those regions during plasma treatment and fibronectin coating, thereby retaining the intrinsic non-binding (for cells) surface of PDMS. And lastly, the possible partitioning of important molecules should be kept in mind and quantified or its effects should be minimized by perfusion and/or pre incubation of media in device before adding cells.

In the current studies, only 6 h of stimulation per day were used to allow enough time in between stimulations for cells to move around and form the desired tubular structures. In the early stages of cellular assembly, the mechanically stimulated biochemical activation of cells can be countered by physical disruption, as has been shown in-vivo for fracture healing³² and in vitro for tubule formation²⁴. The initiation time and the duration of stimulation are therefore important parameters whose effects should be investigated in future studies. The result that consistent alignment to flow or strain was not observed could be because of the stimulation times being too short and the effects of the stimuli not being cumulative when there are large periods of no stimulation in between. The finding that there was some alignment based on the strain stimulus is consistent with previous observations that short durations of strain (~6 h) are sufficient to get a strong alignment response²¹, while for flow, 12-24 h of stimulation is needed³³. However the alignment based on strain in flow-strain combined stimulation experiment suggested a role of the surrounding hMSCs, since when endothelial cells alone were cultured previously under the same conditions, they aligned along the flow when the strain based and the flow based alignment directions were not the same (chapter 4).

Once optimized, mechanical stimulation studies with the co-cultures are likely to provide insights into the effects of mechanical stimuli on parameters such as the directionality and length of tubule formation, the extent of branching and possibly also about the directionality of cell division as has been observed with embryonic stem cells. The current experiments served to show that a fine balance of various experimental conditions has to be reached before even starting to explore a complex process such as vascularization. Observations about the effects of dextran addition in media, the alignment response of the cells, cell sheet peeling and effects of the modified culture conditions, all provide useful areas to recognize and build upon for future investigations.

Conclusion

A medium throughput device was used to study the effects of strains and shear stress on hMSC-HUVEC co-cultures. The aim was to probe the effects of the stimuli on endothelial cell assembly into tube like structures and gain insights for improving pre vascularized bone tissue engineering. This knowledge would need further optimization of the experiments. However the reported experiments provided useful information about effects of dextran addition to media, alignment of cells, peeling of cells in areas of large deformations and effects of modified culture conditions in the device, which can be used for the optimization of future experiments.

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Chapter 6. Osteogenic – adipogenic differentiation in mesenchymal stem cells: a possible effect of mechanical stimulation

Abstract - Mesenchymal stem cells (MSCs) are an important cell type for tissue engineering due to their ease of autologous extraction and ability to be differentiated into various tissue types. Mechanical stimuli are known to play an important role in the differentiation of MSCs. For example, compressive loads promote chondrogenic differentiation¹, tensile loads² and shear stress^{3,4} enhance osteogenic differentiation and reduce adipogenic differentiation, and uniaxial tensile loads can be helpful for tenogenic⁵ and myogenic^{6,7} differentiation. While there are a large number of studies reporting the effects of certain specific mechanical stimuli⁸, their utilization for tissue engineering applications remains limited due to the lack of systematic studies characterizing the effects of various magnitudes, frequencies and combinations of the mechanical stimuli on the MSCs. Medium throughput platforms developed previously by us^{9,10} (chapters 3 and 4) can be used to apply a large number of combined strain and flow induced shear stress stimuli to cells in parallel, thus making the missing systematic studies faster and easier. With this aim, the devices were used to apply various mechanical stimuli to mesenchymal stem cells cultured in a combined osteogenic-adipogenic differentiation medium. Culturing in combined differentiation medium provides the biochemical stimuli for both differentiation pathways so that the effects of physical factors on shifting the balance between the two differentiations can be studied. Alkaline phosphatase (ALP) expression, detected using fast blue colorimetric staining was used as a marker for osteogenic differentiation and Oil red O staining of lipid droplets was used as a marker of adipogenic differentiation. It was found that on mechanically stimulated devices, MSCs seeded at a low density showed low numbers of attached cells at the end of the experiments. At higher seeding densities, MSCs predominant displayed ALP expression with no detectable Oil red O staining. However, Oil red O staining was also minimal for cells cultured on devices in adipogenic medium, indicating that further optimization is needed to actually achieve a mixed differentiation response on the mechanical stimulation devices. Only then the effects of the mechanical stimuli in determining the differentiation direction can be studied. Apart from that, colorimetric staining was found unsuitable for quantification by automated image analysis due to high overlap of cells and indistinguishable boundaries. In future experiments, colorimetric staining should be complemented with or replaced by fluorescent staining which enables automated analysis, thus maintaining the enhanced throughput nature of the experiments.

Introduction

Mesenchymal stem cells (MSCs) are one of the most widely studied cell types for tissue engineering and cell based therapy applications. The properties that make them so interesting include ease of autologous extraction from bone marrow and other sources, in vitro expansion ability and differentiation into various tissue specific cell types, including osteoblasts, adipocytes and chondrocytes^{11,12}. MSCs are also highly responsive to mechanical stimuli⁸. Hence the effects of various mechanical stimuli on MSCs have been studied. Most significantly, tensile strains² and flow induced shear stresses^{3,4} are known to enhance the osteogenic differentiation while suppressing adipogenic differentiation of MSCs. Apart from that, compressive loading has been shown to improve chondrogenic differentiation¹. The simultaneous increase in osteogenic differentiation and reduction in adipogenic differentiation of MSCs in response to mechanical stimuli resembles the shifting of the in vivo balance between fat and bone tissues in response to exercise, where exercise is linked to an increase in bone and decrease in fat tissue formation¹³. In addition to the dynamic mechanical stimuli, such as stretch, compression and shear stress, MSCs are also affected by their static mechanical environment. Substrate stiffness has been shown to be a major regulator of the lineage MSCs differentiate towards¹⁴. When cultured on hydrogels of stiffness resembling various tissue types, the MSCs showed differentiation towards the respective tissue – for example 25-40 kPa stiffness substrate directed MSCs towards bone and 8-17 kPa stiffness substrate directed them towards muscle¹⁴. In regards to stiffness related control, it has been demonstrated that a controlling factor is the amount of tethering of extracellular matrix proteins to the substrate of a particular stiffness¹⁵. Although, in another study the tethering has been found to be not important by comparing various degrees of tethering for the same stiffness substrate¹⁶. Substrate surface topography also has an effect on MSCs - disordered nanotopographies have been shown to make MSCs differentiate towards bone¹⁷ and topographies at the micro scale have been identified as well to direct MSCs towards osteogenic lineage¹⁸. The cell spreading on a substrate can also affect MSCs, a larger spread area directs them towards osteogenic differentiation while a smaller spread area directs them towards adipogenic differentiation¹⁹. The viscoelastic properties of the substrate have also been shown to of importance – a faster relaxing hydrogel was found to enhance spreading, proliferation and osteogenic differentiation of MSCs²⁰. Thus mechanical stimulation is of great interest for studying MSCs and their differentiation.

In the absence of mechanical stimuli, osteogenic and adipogenic differentiations of MSCs are achieved in vitro by culturing the MSCs in specialized media for each purpose. These media differ from the media used to expand the MSCs where the goal is to minimize cell differentiation. To study the role of mechanical stimuli in shifting the adipogenic-osteogenic differentiation balance, an approach that has been used in the past is to culture

MSCs in a combined differentiation medium^{19,21}, such that biochemically the MSCs have the factors present permitting either differentiation. We have used our previously developed medium throughput platforms^{9,10} to study how strain and shear stress combinations shift the osteogenic-adipogenic differentiation balance in a combined differentiation medium culture of MSCs. The knowledge gained from such a study is likely to improve the understanding about mechanical stimuli that can be applied to tissue engineering constructs in order to get the desired differentiation of MSCs.²²

Materials and Methods

Techniques used for all experiments

Cell seeding and mechanical stimulation

Primary bone marrow MSCs from a human donor were expanded in MSC proliferation medium and then seeded on mechanical stimulation devices in a 50:50 mixture of osteogenic and adipogenic induction media. Cells were also seeded in 6 well plates or 12 well plates as controls on normal and PDMS covered well bases. The PDMS covered wells provided a culture surface similar to the mechanical stimulation device. All PDMS surfaces (devices or well plates) were plasma treated and fibronectin coated before seeding cells. Media was refreshed every 2-3 days in the well plates and the strain only devices. The media got refreshed from the reservoir in the experiments where flow was applied and hence was not changed over the course of the experiment. Table 1 lists the various media compositions used and table 2 lists the various test conditions.

Strain stimulation was applied using an Elveflow AF1 pressure-vacuum pump and controller combination (Elvesys Innovation Center, France) to control the strain actuating pressure drops. Flow was applied using a Legato 270P syringe pump (KD Scientific). The stimulations applied are listed in table 2.

Two types of mechanical stimulation devices were used – D1, which could apply various equibiaxial strains in combination with various shear stresses (chapter 3) and D2, which could apply various anisotropic biaxial strains in combination with and at various orientations to a flow induced shear stress (chapter 4).

ALP and Oil red O staining

At the end of the experiments, cells were fixed with citrate fixative solution (6.25 ml citrate + 16.25 ml acetone + 4 ml 37% formaldehyde) for a few minutes and then stained for alkaline phosphatase (ALP) and lipid droplets using a Leukocyte Alkaline Phosphatase kit (Sigma Aldrich 86C) and Oil Red O (Sigma Aldrich O0625) respectively. For the ALP staining, the staining solution was prepared by mixing 1 ml sodium nitrite with 1 ml Fast Blue BB alkaline solution, diluting this solution with 45 ml DI water and then adding 1 ml Naphthol AS-BI alkaline solution, as per the manufacturer's protocol. The fixed cells were washed with water and incubated with the ALP staining solution for 15 minutes. The cells were washed again with water after the ALP staining and then with 60% isopropanol. A freshly prepared and filtered 30 mg/ml Oil Red O solution in 60% isopropanol was then added for 10 minutes. The cells were then rinsed with water and imaged. Imaging was done using a Leica upright microscope with episcopic or diascopic illumination, whichever gave better contrast, and white balance applied to a region without cells.

The intended readout was cell numbers showing ALP and Oil red O staining respectively. In the controls, it was expected that cells would show ALP staining when cultured in osteogenic or combined media, Oil red O staining when cultured in adipogenic or combined media and neither when cultured in basic or proliferation media.

Experiments performed - optimizing culture conditions

Testing cell culture on mechanical stimulation device and staining protocols

To test the mechanically stimulated MSC culture in combined differentiation media, ~5000 cell/cm² were seeded on two D1 devices and in control wells on a 6 well plate. The devices were stimulated starting 18-20 h after seeding – one device with 2 h/day strain and the other with continuous flow. The experiment was carried out for 7 days, after which the cells were stained with the ALP stain and Oil Red O. The experiment was repeated with two D1 devices (one stimulated with strain only and another with flow and strain) and controls on a 12 well plate. A lower cyclic strain frequency was used in order to apply a gentler stimulation to the MSCs. The detailed experimental conditions for both experiments are listed in table 2.

Application of anisotropic strains and addition of dextran to increase shear stress

The application of anisotropic strains to MSCs in combined media was tested using D2 devices. It was found in previous experiments with endothelial cells (chapter 4), that a higher shear stress (achieved by adding dextran to the culture medium) elicited a distinct alignment response from cells which was absent for a low shear stress. Hence to see if

MSCs gave a similar response, dextran was added to the media. 5% wt/wt dextran was added to the media in the devices as well as in the controls. A slightly higher seeding density was used to avoid low cell numbers on the devices at the experimental end points. The mechanical stimulation used was 6 h/day of strain only or combined flow and strain, starting 18-20 h after the cell seeding. More details are listed in table 2.

Increasing seeding density

It has been reported previously by others that seeding density also has an effect on the osteogenic-adipogenic differentiation balance¹⁹ (REF). Hence a higher seeding density was also tested. The test was performed on a D2 device and strain only stimulation was used. Dextran was not added to the media in the mechanical stimulation device but controls were tested with and without dextran. The experimental details are listed in table 2.

Table 1 – Media compositions

Media	Components
Basic medium	<ul style="list-style-type: none"> • α-MEM (Thermo Fisher Scientific, 22-571-020) • 10% foetal bovine serum (Thermo Fisher Scientific, 26140-079) • 2 mM L-glutamin (Thermo Fisher Scientific, 25030) • 0.2 mM ascorbic acid (Sigma Aldrich, A8960) • 100 U/ml penicillin + 100 mg/ml streptomycin (Thermo Fisher Scientific, 15140-122)
Proliferation medium	<ul style="list-style-type: none"> • Basic medium • 1 ng/ml bFGF (Thermo Fisher Scientific, 13256-029)
Osteogenic differentiation medium	<ul style="list-style-type: none"> • Basic medium • 10^{-8} M dexamethasone (Sigma Aldrich, D8893) • 0.01 M β-glycerol phosphate (Sigma Aldrich, 50020)
Adipogenic differentiation medium	<ul style="list-style-type: none"> • Bullet kit (Lonza, PT3004) for the first experiment OR, for remaining experiments, <ul style="list-style-type: none"> • DMEM (Thermo Fisher Scientific, 41-965-039) • 100 U/ml penicillin +100 mg/ml streptomycin (Thermo Fisher Scientific, 15140-122) • 10% foetal bovine serum (Thermo Fisher Scientific, 26140-079) • 0.2 mM Indomethacin (Sigma Aldrich, I7378) • 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich, I5879) • 10^{-6} M dexamethasone (Sigma Aldrich, D8893) • 10 mg/ml Insulin (Sigma Aldrich, I9278)

Table 2 – Experimental conditions

No.	Experiment	Cultured on	Cell seeding density	Applied flow	Applied strain	Duration of experiment
1-1	Testing cell culture on mechanical stimulation device and staining protocols	2 x D1 devices - strain only (SO) and flow only (FO) stimulated	5000 cells/cm ²	FO device - continuous 20 ml/min flow applied for 6 days, starting 18-20 h after seeding. Syringes were changed every 2-3 days.	SO device – 2-10% equibiaxial strains (0 to 20 kPa pressure drop sinusoidal cycles with 1 s period) applied for 2 h/day for 6 days, starting 18-20 h after seeding.	7 days
		2 x 6-well-plates – normal and PDMS covered wells	5000 cells/cm ²	-	-	7 days
1-2	Testing cell culture on mechanical stimulation device and staining protocols	2 x D1 devices - strain only (SO) and combined flow and strain (FS) stimulated	5000-6000 cells/cm ²	FS device - continuous 20 ml/min flow applied for 6 days, starting 18-20 h after seeding. Syringes were changed every 2-3 days.	SO and FS devices – 2-10% equibiaxial strains (0 to 20 kPa pressure drop sinusoidal cycles with 2 s period) applied for 2 h/day for 6 days, starting 18-20 h after seeding (SO) or an hour after starting flow (FS).	7 days
		1 x 12-well-plate – normal and PDMS covered wells	5000-6000 cells/cm ²	-	-	7 days

Table 2 – Experimental conditions (continued)

No.	Experiment	Cultured on	Cell seeding density	Applied flow	Applied strain	Duration of experiment
2	Application of anisotropic strains and addition of dextran to increase shear stress	2 x D2 devices - strain only (SO) and combined flow and strain (FS) stimulated	8400 cells/cm ²	FS device - 40 ml/min flow applied for 6 h/day for 7 days, starting 18 h after seeding. Syringes were changed every day after the stimulation.	SO and FS devices – various anisotropic strains (0 to 20 kPa pressure drop sinusoidal cycles with 1 s period) applied for 6 h/day for 7 days, starting 24 h after seeding (SO) or together with flow (FS).	8 days
		1 x 12-well-plate – normal and PDMS covered wells	10000 cells/cm ²	-	-	8 days
3	Increasing seeding density	1 x D2 device - strain only (SO) stimulated	24400 cells/cm ²	-	SO device – various anisotropic strains (0 to 20 kPa pressure drop sinusoidal cycles with 1 s period) applied for 6 h/day for 9 days, starting 18-20 h after seeding (SO).	10 days
		2 x 12-well-plate – normal and PDMS covered wells	20100 cells/cm ²	-	-	10 days

Results

Testing cell culture on mechanical stimulation device and staining protocols

The initial experiments showed that after 7 days in culture, most of the MSCs cultured in osteogenic or in combined medium, stained strongly for ALP on control PDMS and polystyrene surfaces (figure 1). The Oil red O staining solution used had been freshly prepared and was clear and hence was not filtered. It deposited precipitates (figure 1) when added on the cell coated culture surfaces, emphasizing the need to filter it in future experiments. The Oil red O also stained the PDMS surface to some extent making detection of the staining in images difficult. The Oil red O staining was also not strong and not easily detectable in low magnification images even on polystyrene surfaces where the surface itself did not take up the stain.

On the devices, a lower number of attached cells were seen at the end of the experiment (figure 2), when compared to the controls. A large number of areas above the pillars showed no attached cells on both devices. The cells on the devices did not seem to stain for either ALP or with Oil red O.

When the experiment was repeated, the controls again showed strong ALP staining of a large fraction of the cells cultured in osteogenic medium as well as in the combined medium (figure 3). A few cells cultured in proliferation or adipogenic media also showed ALP staining (figure 3). The polystyrene controls in adipogenic medium and in combined medium also showed significant Oil red O staining. On the devices, the number of attached cells at the end of the experiments was still low compared to the controls, but attached cells were found in regions above the pillars for most of the strain units (figure 4). This could have been a result of the gentler mechanical stimulation. The cells did not stain for ALP or with Oil red O on the devices (figure 4).

PDMS surface

Polystyrene surface

Figure 1 – In the first study with MSC culture in combined differentiation media, it was found that the cells stain strongly for ALP in controls with osteogenic and combined

medium on both PDMS and polystyrene surfaces. The Oil red O staining solution precipitated large amounts of residue emphasizing the need to filter the staining solution before staining in future experiments. Oil red O also stained PDMS to some extent making detection of lipid staining in images harder. The Oil red O staining of cells was weak and not apparent in low magnification images. Scale bar 500 μm .

Figure 2 – In the first study with cells on the mechanical stimulation devices (D1), a low number of attached cells was detected in all regions on the devices at the end of the experiment. A large number of units on both devices showed no attached cells in the regions over the pillars. The ALP and Oil red O staining were not apparent on either device. Scale bar 500 μm .

PDMS surface

Polystyrene surface

Figure 3 – The controls in various media showed a similar result of high ALP staining in osteogenic and combined media, when repeating the experiments. Significant Oil red O staining was also seen in the polystyrene controls in adipogenic and combined media. Also,

a few cells each in proliferation medium and adipogenic medium stained for ALP. Scale bar 500 μm .

Flow and Strain – replicate 1

Strain only – replicate 1

Figure 4 – A low number of attached cells were found in mechanically stimulated devices. In contrast to previous mechanical stimulation experiments, cells stayed attached in the

stretched regions over the pillars. This could have been the effect of a gentler applied mechanical stimulation. No ALP or Oil red O staining was detected on the stimulated devices. Scale bar 500 μm .

Application of anisotropic strains and addition of dextran to increase shear stress

For the anisotropic strain application using a D2 device, the conditions applied were similar in terms of strains and shear stress magnitudes to those used with endothelial cells in chapter 4, but different in terms of stimulation duration. At the end of the experiment, cells stained strongly for ALP all over the mechanically stimulated devices but Oil red O staining was not perceivable (figures 6 and 7). Alignment perpendicular to the maximum principal strain was observed when strain alone was applied (figure 7). In the device stimulated with combined flow and strain, observations could not be made in all regions due to the presence of debris. Figure 6 shows a region on the flow and strain stimulated device where both cell and debris were visible. The debris could have been from a syringe, although they were changed frequently and none of them were observed to fail before the time of replacement. The other possibility is that the debris was from the cells.

In the controls, some Oil red O staining was observed in wells with adipogenic or combined media (figure 5). ALP staining was absent in all conditions except in adipogenic medium on polystyrene surface (figure 5). This strange behavior could have been caused by the addition of dextran to the media or due to problems with reagents. However this is unlikely since the staining worked for one control well and for the mechanically stimulated devices, which also contained dextran in the media.

The cell densities on the devices and the controls were comparable to each other at the end of the experiment, in contrast to the previous experiments with the combined media, which is possibly an effect of a higher initial seeding density (8400 cell/cm² vs 5000-6000 cells/cm²).

PDMS surface **Polystyrene surface**

Figure 5 – Controls with media containing dextran did not stain for ALP as is usual in the presence of osteogenic or combined media. Instead ALP staining was only observed for

cells in adipogenic medium on a polystyrene surface. Oil red O staining was observed in a few cells each in adipogenic and combined media on both the surfaces (PDMS and polystyrene). Scale bar 500 μ m.

Figure 6 – Flow and strain stimulated cells on a D2 device showed a high ALP staining. However, the presence of debris on the device at the end of the experiment made it hard to observe the cells in most areas. A representative image shows here the cells and the debris (black spots).

Figure 7 – When strain only stimulation was applied to cells cultured on a D2 device in combined medium containing dextran, the cells aligned perpendicular to the maximum principal surface strain in the case of anisotropic strains. A large fraction of the cells stained for ALP in all regions on the device. Oil red O staining was not detectable in the images. The type of the strains is depicted by the trench shapes drawn on the left and the bottom of the image. The figures on the top of the image represent the directionality of the strains with respect to the flow direction (red lines). Scale bar 200 μm .

Increasing seeding density

The originally intended readout for the experiments with MSCs cultured in a combined differentiation medium was the counts of the cells stained positive with ALP and Oil red O. However in the initial experiments neither staining was observed in the mechanically stimulated regions and in the experiment with anisotropic strains and media with dextran, only ALP staining was observed. In the latter experiment, prior to the ALP-Oil red O staining, a brief exploration on the device under a phase contrast microscope had shown distinguishable lipid droplets only in a few non-strained regions. Thus, the Oil red O staining not being detected was not just an effect of the PDMS background being stained by Oil red O.

Since a low initial cell seeding density is known to promote osteogenic differentiation of MSCs¹⁹, perhaps the low seeding density shifted the balance towards osteogenic differentiation in the experiments so far. Hence to shift the initial balance towards adipogenic differentiation, a much higher initial seeding density was used. The cells were seeded on a D2 device and stimulated with strain only. Since shear stress was not being applied, dextran was not added to the culture medium. The usual controls in the various media and on the two surfaces were used, but all controls in media containing dextran were added to check whether the addition of dextran under static conditions perhaps prevents ALP staining.

In the mechanical stimulation experiment, lipid droplets and Oil red O staining were still not detected. ALP staining was still observed for a large fraction of the cells but on a visual comparison, the fraction of cells stained for ALP seemed lower than in the experiment with a lower seeding density and media with dextran. Alignment in response to anisotropic strains (perpendicular to the maximum principal surface strain) was also observed.

The various controls showed a mixed response. ALP staining was found for at least a few cells in all the conditions. In some cases (PDMS surface, media without dextran) a large number of cells were ALP stained in osteogenic and combined media, as expected, while in other cases (adipogenic medium, polystyrene surface, media without dextran) a large number of cells showed ALP staining where it was not expected. The controls in media with dextran showed ALP staining, although to a lesser extent than without dextran, thereby confirming that the addition of dextran was not affecting the staining.

Oil red O staining was prominent only in the adipogenic medium, polystyrene surface, media without dextran control.

PDMS surface **Polystyrene surface**

Figure 8 – ALP staining was observed to some extent in all controls in media without dextran. A stronger ALP staining in adipogenic medium on PDMS and in proliferation and adipogenic media on polystyrene were unexpected. Similarly, a weak ALP staining seen in the osteogenic medium on the polystyrene surface was not expected. Significant Oil red O staining was observed only in the adipogenic medium on the polystyrene surface. Scale bar 200 µm.

PDMS surface – media with dextran

Polystyrene surface – media with dextran

Figure 9 – Controls in media with dextran also showed at least some ALP stained cells in all conditions, though the ALP staining was weaker and in fewer cells than in the same conditions in media without dextran. Unexpected ALP staining in proliferation and adipogenic media and unexpected lack of strong ALP staining in the other conditions were also observed. Oil red O staining was not detected in any condition. Scale bar 200 µm.

investigated. The cell seeding density and perhaps also the culture surface and inert additives to the media (dextran) could also affect the differentiation balance. In further studies, all these factors need to be accounted for.

A few insights were also gained on the experimental methodologies.

- First, it is important to filter the Oil red O solution to avoid precipitates during the staining.
- Second, color based stainings like the ALP stain used and Oil red O are not very suitable for automated image analysis. Overlapping cell boundaries, nuclei locations that were not detectable, and the presence of debris and condensation on the underside of the membrane were some things that made it hard to tell the cells apart. While it was still possible to visually estimate cell numbers stained with a particular stain, it was not possible to use a clear set of instructions to identify the cells and hence the process could not be automated. The automated image analysis previously implemented in chapters 3 and 4 with fluorescently stained cells could not be implemented with ALP and Oil red O stained cell images. Thus, in further experiments fluorescent staining should be used to complement or replace the color based staining used here.
- Another reason to look into other markers of MSC differentiation instead of ALP is that ALP expression is not specific for osteogenic differentiation, as was also observed in some of the controls in our experiments.²³

Thus, the experiments can be significantly improved. In addition, it would also be very interesting for the future experiments to study the effects of the mechanical stimuli on other differentiations of MSCs as well. This becomes especially relevant when anisotropic strains are applied since it has been shown previously that anisotropic and isotropic strains affect MSCs differently.⁷

More experiments are needed to pinpoint the cause of the unexpected ALP staining results, but there could be a donor specific effect since the cells used in experiments with D1 devices and those used with the D2 devices were from separate donors.

Conclusions

MSC culture in a combined osteogenic-adipogenic differentiation media is a useful way to study the effects of physical factors on the differentiation balance while making both differentiations equally likely biochemically. However, it was shown in the current study that to achieve this, the physical factors not being investigated (cell seeding density, culture surface, dextran addition) also need to be balanced so as to start with some degree of both

differentiations. Further studies need to be performed to better understand the effects of various mechanical stimuli on MSC differentiation and the medium throughput devices developed by us are promising tools to study these processes.

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Chapter 7. Conclusions and future directions

Over the past decade, mechanobiology has significantly captured the interest of biomedical engineers in search of new ways to manipulate biology with the aim of improving human health¹⁻³. The ability of mechanical signals alone (without the addition of biochemical reagents) to determine stem cell differentiation fate^{4,5}, organization of cells into tissue like structures⁶ and the display of in vivo like behavior in vitro⁷ have highlighted their importance and offered ways of their exploitation. On the other hand, the increasing understanding of mechanotransduction mechanisms has raised hope that when the molecular machinery involved in sensing and responding to mechanical stimuli is not working properly, biochemical intervention can be designed based on the immense existing knowledge base⁸. However the list of unknowns is huge. The current understanding of cellular mechanotransduction is that forces modify/activate biomolecules, such as proteins, or a structure made up of several molecules, such as focal adhesions, leading to further biochemical changes^{9,10}. Although several key regulators of mechanotransduction have been identified, in principle almost all biomolecules can undergo such changes. Similarly all cells can respond to mechanical stimuli. Where then to focus – which cells, which molecules? The several ways that mechanical stimuli are applied to cells, usually taking inspiration from their in vivo environments – stretch, shear stress, substrate stiffness, topography – all can be varied in magnitude, directionality and dosage. How then to decide which stimulation works best? On one hand the studies have been application driven – mechanical stimulation to make in vitro cell culture more predictive of in vivo behavior with pharmaceutical discovery applications in mind¹¹ or driving tissue development in vitro for implantation (tissue engineering)¹². On the other hand the studies have been directed at understanding how the known components of the cellular mechanotransduction machinery orchestrate their working^{13,14}. In both areas, enabling technologies have played an important role. These include novel materials, fabrication techniques, imaging platforms and computational tools, all coming together to apply various mechanical stimuli to cells and study how cells respond. Powered by the enabling technologies, increasingly complex studies have been undertaken, integrating multiple mechanical stimuli^{15,16} or enhancing the experimental throughput^{17,18} or both^{19,20}. The aim of this thesis was to work at this cutting edge and push it further by combining two dynamic mechanical stimuli and screening for their effects on cells in a combinatorial manner with more conditions simultaneously tested than reported before.

Chapter 1 of the thesis provided a brief introduction to the concepts of mechanotransduction, mechanobiology, mechanical stimuli studied in vitro and relevant application areas, along with presenting the general thesis outline. In chapter 2, the focus

was slightly narrowed and background was provided on studies applying combinations of mechanical stimuli to cells, thereby making the case that it is important to study the effects of combinations of stimuli. Chapter 3 described the development and validation of a first prototype to apply all combinations of five equibiaxial strains and five shear stresses to cells, each with four replicates (total 100 test units). The device was designed with the help of computational modeling and characterized using image based empirical strain and flow measurements. The device was validated using C2C12 mouse myoblast cells and showed that with increasing strains (in the range of 2-20%), there were fewer attached cells and they had a smaller spread area, while there was no detectable effect of the shear stresses (0.18 – 0.33 Pa). Since the *in vivo* strains that cells experience are often anisotropic, a second device to apply anisotropic biaxial strains in combination with shear stress was developed and is described in chapter 4. This second test platform was similarly designed with the help of computational modeling and was characterized using empirical measurements. The conditions that could be tested for included four anisotropic biaxial strains with the same maximum principal strain (9%) but different minimum principal strains, each strain combined with flow applied at five separate orientations with respect to the principal strain direction and each combined condition with four replicates. In addition this device could test for five equibiaxial strain magnitudes in combination with shear stress, also with four replicates each, hence a total of 100 test units. It was shown using this device that the well-known endothelial cell alignment response along the minimum principal strain direction became more prominent with increasing maximum : minimum principal strain ratio and when the same strains capable of aligning cells were applied in combination with a 0.55 Pa shear stress, the cells aligned along the flow direction, almost ignoring the strains. While the experiments with C2C12 cells and endothelial cells validated the devices and gave insights on the effects of the various combined mechanical stimuli on these cells, experiments with a single cell type did not make full use of the complexity the devices were designed to handle. The developed devices were designed to have large enough test areas to culture up to a few hundred cells, allowing for studying co-cultures. This was done with the aim of studying co-cultures of cells with endothelial cells which are used for vascularized tissue engineering. Thus, as a next step, co-cultures of mesenchymal stem cells (primed towards osteogenic differentiation) and endothelial cells were studied, as described in chapter 5. The aim of this study was to find how the various mechanical stimuli affected the spontaneous assembly of endothelial cells into tube like structures. However, the addition of dextran in the culture medium to increase its viscosity lead to enhanced endothelial cell proliferation at the cost of the expected tubule formation. Hence this study stayed inconclusive in terms of tubule formation under the effect of mechanical stimuli, but provided insights for designing future experiments. Mesenchymal stem cells themselves are a very interesting cell type for probing with mechanical stimuli, since mechanical stimuli are known to affect their differentiation^{4,21-23}. Hence the effects of the various mechanical stimuli from the devices were also studied for mesenchymal stem cells

and specifically the balance between osteogenic and adipogenic differentiation was investigated. This study is described in chapter 6 of this thesis. In the presence of mechanical stimuli, adipogenic differentiation was not detectable, which is similar to previous reports that showed that strain and shear stress enhance osteogenic differentiation and suppress adipogenic differentiation²⁴⁻²⁶. However, technical issues leading to unexpected results in control studies prevented accepting the experimental outcomes as conclusive. Nonetheless these experiments also provided useful insights for future experiments.

In the future, there is scope for improvement in the device design, as well as for addition of functionality. Apart from that, the developed platforms will also be more valuable by designing and streamlining good biological assays. These areas for future work will be shortly discussed here.

Besides the above mentioned areas, a question that remains is whether the pooling of media between the several conditions can have an effect on the interpretation of the results. When designing the system, there was a trade-off to be made between the functional aspect of pooling for the separate samples and the practical aspect of the complexity in tubing and connections needed if each unit was to be given a separate media supply. The pooling was chosen since under flow conditions, paracrine signaling was expected to be washed out and highly diluted. Two ways that the effects, if any, due to the pooling can be controlled for may be – (1) by comparing secretome data, if available, between the various conditions (for example, the secretome of endothelial cells in response to various kinds of shear stresses has been reported²⁷) and (2) by using already available macroscale devices for applying one of the conditions of the devices or using a scaled down version of the current devices with a single condition for comparison with the same condition in pooled media.

Future work

Improvements in device design/fabrication/operation

The developed devices perform their desired function of applying well defined mechanical stimuli to cells. However, a few simple adaptations can further improve them. Firstly, using a continuous winding channel, while simplifying the flow circuit, introduced shear stress variations near bends. This effect can be reduced by using a separate inlet and outlet for each straight part of the flow channel, thus eliminating bends. If a continuous channel is retained, all strain units can be scaled down in size and moved closer together such that all regions of interest are outside the non-uniform shear stress regions near the bends.

Secondly, having pillars and trenches in separate layers introduces a need for careful alignment and empirical strain characterization for all devices. The pillars and trenches could be made into a single layer by suitably designing a mold, but such a fabrication method would eliminate the space for air flow channels. A separate layer can be introduced for the air channels as was done by Simmons et al²⁸, though adding layers means increasing the working distance for microscopy. Another approach that may be suitable is vacuum forming the pillars and trenches from a milled mold. Vacuum forming uses a vacuum to emboss the topography of a mold on a heat softened polymer sheet²⁹. Since this would result in a hollow structure, holes can be drilled on the side walls of trenches for air pressure actuation of the strains. Thus there will be no need for additional layers. Also, this method can speed up device fabrication and reduce the amount of material needed. This method can also be useful for replicating milled molds at a higher speed and lower cost than milling additional molds. Thus, several device layers can be prepared in parallel by casting PDMS in several vacuum-formed molds. There may be limits on the depth / aspect ratio of structures that can be vacuum-formed³⁰ and the pressure drops the finished structures can withstand without significantly deforming (based on the elastic modulus of the material used). These will need to be optimized but are likely to not be an issue for the requirements of the reported devices using readily available materials such as polystyrene.

Thirdly, the pump driving the flow should be switched to a roller or a pressure controlled pump. The syringe pump was chosen because of its ability to deliver a steady, pulse free flow. A continuous flow was achieved with the syringe pump using one way valves and a pair of syringes filling and infusing alternatively. Since a high channel was used to minimize shear stress disturbances, a high flow rate was required to obtain physiological shear stresses. In the two syringe system used, a higher flow rate requires a faster switching between the syringes and hence the advantage of steady flow is slowly lost with increasing flow rates.

Finally, a chemical treatment that can render the PDMS surface hydrophilic irreversibly can be very useful. Plasma treatment followed by fibronectin coating works well for cell attachment, but these steps are required to be performed close to the cell seeding time, since plasma treated PDMS, when stored in air, quickly loses its hydrophilicity due to hydrophobic recovery. A possible treatment can be the attachment of poly acrylic acid groups to the PDMS polymer chains³¹. The reaction can be UV controlled, which permits the patterning of the hydrophilic spots and can thus be used to restrict cells to ROIs.

Adding functionality

Due to the flexible device design, it should be possible to add functionality such as grooves on top of the membrane for contact guidance of cells or providing a three dimensional environment to cells by encapsulating them in a extracellular matrix network. A simple way

to introduce grooves (or other topographies) could be using ultraviolet light exposure through a mask to selectively break PDMS polymer chains and then dissolving the depolymerized material³². Such a method would not need a modification of the device fabrication and offers a flexible way to produce features (up to 10 μm deep and 5 μm lateral

Suggestions for future design improvements and functionality addition to the developed devices

Figure 1 – Schematic representation of the suggestions for future design improvements and functionality addition to the developed devices.

resolution³²) at any location and orientation simply by varying the position of the mask. For providing a 3d environment to cells, it will be important that the cell encapsulating gel bonds well with the membrane and transmits the strains to the cells. Several approaches have been reported in the past for achieving this. For example Butcher et al obtained good attachment of a collagen gel containing cells on a silicone membrane by etching the silicone membrane with sulfuric acid and then coating a layer of Cell-Tak cell and tissue adhesive³³.

The possible role of mechanical stimuli in stem cell differentiation to cardiomyocytes has been generating some interest^{34,35}. Addition of electrodes to get electrical readouts from cells can make the developed devices more suitable for this application³⁶.

Biological assays

To keep them easy to use and maintain the throughput advantage, the developed platforms are best suited for optical readouts of biological responses, the acquisition and analysis of which can be automated using existing technology. Staining for filamentous actin using phalloidin and for DNA using DAPI provides a good starting point to study changes in cell and nuclear morphology and orientation as was demonstrated for the developed platforms. Immunostaining based monitoring of proteins can provide additional useful information. For example nuclear translocation events that are known to be affected by mechanical stimuli can be studied¹⁷. Since cells on the devices can be imaged in real time during the mechanical stimulation, fluorescent reporter cell lines³⁷ can be used to monitor biochemical reactions as affected by mechanical stimuli. Similarly, real time fluorescent calcium imaging can be quite useful. Calcium levels within the cell are important regulators of functions such as cell contractility and cell motility³⁸. In response to mechanical stimuli, the modulation of stretch activated calcium channels leading to variations in intracellular calcium is often an early response and hence of interest for our test platforms³⁹. Some biological processes of interest whose mechanical regulation can be investigated using the developed platforms include vascularization, stem cell differentiation and tissue injury by large forces. By introducing a 3d environment such as a collagen gel, mechanically stimulated angiogenesis into the gel from a surface endothelial cell layer can be studied⁴⁰.

Thus, the test platforms developed in this thesis can be used to answer a large number of biologically relevant questions, two examples of which were described in chapters 5 and 6. In addition, the simple and flexible design leaves ample room for functionality addition. Technological advances such as imaging large areas using flatbed scanners⁴¹ or imaging simultaneously with multiple imaging units (for example Optics11 Senza) can enable simultaneously monitoring more units in real time, further adding to the enhanced throughput ability. The current capabilities of enhanced throughput mechanical stimulation and studying flow and strain combinations, already puts the devices ahead of existing

platforms in utility for applications such as rapidly finding the optimum mechanical signals for tissue engineering or exploring the effects of variations in the stimuli on various types of cells.

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Summary

Mechanical signals from the surroundings of cells can significantly affect their function. Cells achieve this sensing and responding to mechanical signals through specialized molecules and structures. The knowledge of the cellular responses to various mechanical stimuli holds the promise of exploiting this understanding to direct cells to a desired fate by applying the correct mechanical signals. Thus, the field of mechanobiology which studies these responses of cells to mechanical signals has been highly active since the past few decades. This thesis details the development of experimental platforms for mechanobiological studies. More specifically, platforms were developed that could apply several combinations of substrate strains and flow-induced shear stresses simultaneously to cells. These mechanical stimuli are present for various cell types within the body, such as in blood vessels, in lungs and in the gut. However, they were not applied simultaneously to cells very often in past studies. A possible reason could have been the large number of experiments needed to study various combinations of variations in each stimulus. Hence the developed platforms were designed to have enhanced throughput by carrying out several studies in parallel.

Chapter 1 of this thesis presents a brief background of the field, introduces the important terminology and outlines the potential applications that drive the research.

Chapter 2 highlights past studies where applying combinations of mechanical stimuli to cells in the lab provided insights about the functioning of cells which could not have been otherwise estimated. Thus, this chapter makes a case for the need to apply combinations of mechanical stimuli to cells.

In chapter 3, the development of the first experimental platform is described. This device could test for combinations of various equibiaxial strains and flow induced shear stresses. A basic design was selected, computational modeling was used to estimate the strains and shear stresses that could be applied to cells, the device was fabricated and empirically characterized, and finally cell studies were performed. It was shown for C2C12 mouse myoblast cells that higher substrate strains result in fewer attached cells and with a smaller attached area. The applied shear stresses were not found to elicit a significant response in terms of cell morphology in this study.

The platform described in chapter 3 applies equibiaxial strains to cells. However, this is not always the case in vivo. Hence, a second platform was developed that can generate anisotropic substrate strains with varying degrees of anisotropy (ratio of principal strains). Flow induced shear stress could simultaneously be applied in this device as well and

various orientations between the anisotropic strains and the shear stresses were included. The results are reported in chapter 4. It was found that human umbilical vein endothelial cells (HUVECs) align along the flow direction and along the minimum strain direction when these stimuli were applied separately. This effect was already known from several past studies. It was also found that the alignment of the HUVECs was stronger for a higher degree of anisotropy. Interestingly, when the stimuli were simultaneously applied, at a high enough shear stress, the shear stress dominated the alignment response of the cells over the substrate strains.

The developed platforms were further used to study the effects of the mechanical stimuli on cells in other biological contexts. Chapter 5 describes one of these studies, where the device was used to apply the stimuli to endothelial cell – mesenchymal stem cell co-cultures with the aim of understanding the effects of the mechanical signals on the spontaneous assembly of the endothelial cells into tubular structures. In chapter 6, the effects of the mechanical signals were studied on mesenchymal stem cells cultured in a combination of adipogenic and osteogenic differentiation media. The aim in this study was to investigate how various combinations of the strains and shear stresses affect the differentiation balance. On the main questions, both these studies remained inconclusive. However, useful insights were gained for designing future experiments.

Finally, in chapter 7, suggestions are provided for modifying the device design and cell studies of interest which could be facilitated by the developed platforms.

Samenvatting

Mechanische prikkels uit de leefomgeving van cellen kunnen een groot effect hebben op diens gedrag. Cellen gebruiken gespecialiseerde moleculen en structuren om zulke prikkels op te vangen en vervolgens daarop te reageren. Tot op heden is het nog niet precies bekend hoe cellen op omgevingsfactoren reageren, en welke mechanismes hierachter schuilgaan. Deze kennis is echter wel belangrijk om materialen te creëren waarmee cellen naar onze wens gemanipuleerd kunnen worden. Daarom is het veld van 'mechanobiologie' - wat de interactie tussen cellen en de mechanische component van materialen bestudeert - de laatste decennia erg actief. In dit proefschrift wordt een experimenteel platform ontwikkeld om het effect van mechanische signalen op cellen te bestuderen. Dit platform maakt het mogelijk om combinaties van oppervlakte rekken en schuifkrachten veroorzaakt door vloeistof stroming simultaan op cellen te testen. Zulke krachten spelen een rol in verschillende weefsels in het lichaam, zoals in bloedvaten, longen en de darmen. Er was tot op heden echter nog geen experimentele opstelling die de combinatie van de verschillende krachten tegelijk kan bestuderen. Het door ons ontwikkelde platform lost dit probleem op door vele verschillende combinaties van de eerder genoemde krachten parallel uit te voeren in één enkele test opstelling.

Hoofdstuk 1 van dit proefschrift geeft een bondig overzicht van het onderzoeksveld, introduceert de belangrijkste terminologie en schetst een mogelijke toepassing waardoor dit onderzoek gedreven wordt.

Hoofdstuk 2 eerder gepubliceerde studies samen waarin combinaties van mechanische prikkels aan cellen zijn toegediend. De kennis vergaard door dergelijke studies bleek onmisbaar, aangezien hiermee volledig onverwachte inzichten verkregen zijn. De toegevoegde waarde van een platform zoals beschreven in dit proefschrift wordt door deze literatuur studie onderschreven.

In hoofdstuk 3 wordt de ontwikkeling van het eerste experimentele platform beschreven. Hierin kunnen gelijkwaardige-biaxiale rekken worden overgebracht op cellen, gecombineerd met door vloeistof stroming geïnduceerde schuifkrachten. We beschrijven de selectie van het ontwerp, een computer model van de mechanische signalen waaraan de cellen blootgesteld zullen worden, de fabricage en karakterisatie van het systeem, en de eerste experimenten waarin cellen worden gebruikt. Hierin laten we zien dat C2C12 muis spiercellen slechter gehecht blijven aan membranen die cyclisch uitgerekt worden. De invloed van vloeistofstromen op de vorm van cellen is in dit systeem verwaarloosbaar klein.

Het in hoofdstuk 3 beschreven platform stelt cellen bloot aan rekken die de cellen in alle richtingen even veel vervormen. In het lichaam is dit echter niet altijd het geval. Daarom hebben we in hoofdstuk 4 een platform ontwikkeld waarin we rekken kunnen genereren met een verschillende grootte in verschillende richtingen. Dit wordt wederom gecombineerd met schuifkrachten veroorzaakt door vloeistofstromingen, zodat er vele combinaties van mechanische signalen onder verschillende richtingen mogelijk zijn. In hoofdstuk 4 beschrijven we de reactie van endotheel cellen (afkomstig van de menselijke navelstreng ader) (HUVECS) in dit systeem. We observeren dat deze cellen zich oriënteren in de richting van de vloeistofstromen en haaks op de richting van de grootste rekken. Dit gedrag komt overeen met observaties in eerder beschreven studies. Verder observeren we dat de oriëntatie van cellen sterker wordt naarmate de rekken meer anisotroop worden. Interessant om op te merken is de observatie dat de schuifkracht van de vloeistofstroom dominant is voor de oriëntatie van cellen in vergelijking met de oppervlakterekken.

De ontwikkelde platformen zijn vervolgens gebruikt om de invloed van mechanische prikkels op het gedrag van cellen te bestuderen in meerdere biologische contexten. Hoofdstuk 5 beschrijft een studie waarin we een celkweek van 2 celtypen (endotheel cellen en mesenchymale stamcellen) bestuderen in ons platform. In deze experimentele opzet is het mogelijk om de invloed van de krachten te bestuderen op de formatie van vaatachtige structuren. In hoofdstuk 6 zijn combinaties van mechanische- en chemische differentiatie prikkels gebruikt om differentiatie van mesenchymale stamcellen te beïnvloeden. De centrale onderzoeksvragen in deze twee hoofdstukken hebben we niet met zekerheid kunnen beantwoorden, maar de opgedane kennis kan wel direct worden toegepast voor het ontwikkelen van toekomstige experimenten.

Tot slot is er in hoofdstuk 7 een uiteenzetting gemaakt van mogelijkheden om het door ons ontwikkelde platform te verbeteren.

Publications related to this thesis

Journal articles

1. Sinha, R., Le Gac, S., Verdonschot, N., van den Berg, A., Koopman, B., and Rouwkema, J. A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells. *Lab on a Chip* 15, 429, 2015.
2. Sinha, R., Le Gac, S., Verdonschot, N., van den Berg, A., Koopman, B., and Rouwkema, J. Endothelial cell alignment as a result of anisotropic strain and flow induced shear stress combinations. *Scientific Reports* 6, 29510, 2016.
3. Sinha, R., Verdonschot, N., Koopman, B., and Rouwkema, J. Tuning cell and tissue development by combining multiple mechanical signals (Submitted to *Tissue Engineering B*).

Conference abstracts

1. Oral: Semi high throughput mechanobiological screening of MSCs and ECs for vascularized bone tissue engineering; Ravi Sinha, Séverine Le Gac, Albert van den Berg, H.F.J.M. Koopman, Jeroen Rouwkema; 9th World Biomaterials Congress in Chengdu, China, June 1-5, 2012
2. Oral: Lab-on-a-chip Device to Screen for Mechanical Signals on Cells, R. Sinha, S. Le Gac, A. van den Berg, H.F.J.M. Koopman and J. Rouwkema, Netherlands Society for Biomaterials and Tissue Engineering, 20th Annual Meeting, Lunteren, Netherlands, December 1-2, 2011
3. Poster: Semi high throughput mechanobiological screening of MSCs and ECs for vascularized bone tissue engineering, R. Sinha, S. Le Gac, A. van den Berg, B. Koopman, J. Rouwkema, 3rd TERMIS World Congress “Tissue Engineering and Regenerative Medicine”, Vienna, Austria, September 5-8, 2012
4. Oral: Lab-on-a-chip Device to Screen for Mechanical Signals on Cells, R. Sinha, S. Le Gac, A. van den Berg, H.F.J.M. Koopman and J. Rouwkema, Netherlands Society for Biomaterials and Tissue Engineering, 21st Annual Meeting, Lunteren, Netherlands, December 6-7, 2012
5. Poster: ‘Lab-on-a-chip device to rapidly search for optimal combinations of surface strains and fluid flow shears for stimulating cells in tissue regeneration strategies’; Ravi Sinha, Séverine Le Gac, Albert van den Berg, H.F.J.M. Koopman, Jeroen Rouwkema; Advances in Tissue Regeneration 2013 Conference, Lattrop, Netherlands, 14-15 November, 2013
6. Poster: ‘Lab-on-a-chip Device to Screen for the effects of Mechanical Signals on Cells’; Ravi Sinha, Séverine Le Gac, Albert van den Berg, H.F.J.M. Koopman, Jeroen

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7. Poster: 'A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells', Ravi Sinha, Séverine Le Gac, Nico Verdonschot, Albert van den Berg, Bart Koopman, Jeroen Rouwkema, TERMIS EU, 10-13 June 2014, Genoa, Italy
 8. Poster: 'Lab-on-a-chip Device to Screen for the effects of Mechanical Signals on Cells'; Ravi Sinha, Séverine Le Gac, Nico Verdonschot, Albert van den Berg, Bart Koopman, Jeroen Rouwkema; Netherlands Society for Biomaterials and Tissue Engineering 23rd Annual Meeting, 2014, Lunteren, Netherlands, 4-5 December, 2014
 9. Poster: 'A Medium Throughput Device to Study the Effects of Combinations of Surface Strains and Fluid-Flow Shear Stresses on Cells'; Ravi Sinha, Séverine Le Gac, Nico Verdonschot, Albert van den Berg, Bart Koopman, Jeroen Rouwkema; EMBS Micro and Nanotechnology in Medicine Conference; Oahu, Hawaii, USA, 8-12 December, 2014
 10. Oral: 'A medium throughput device to study the effects of combinations of surface strains and fluid-flow shear stresses on cells'; Ravi Sinha, Séverine Le Gac, Nico Verdonschot, Albert van den Berg, Bart Koopman and Jeroen Rouwkema; 5th Dutch Bio-medical Engineering Conference 2015; Egmond aan Zee, Netherlands; 22-23 January 2015
 11. Oral: 'Effects of varying degrees of surface strain anisotropies on endothelial cells'; Sinha R, Le Gac S, Verdonschot N, van den Berg A, Koopman B, Rouwkema J; 10th World Biomaterials Congress; Montreal, QC Canada; 17-22 May 2016
 12. Oral: 'Effects of varying degrees of surface strain anisotropies in combination with fluid flow shear stresses on endothelial cells', Sinha R, Le Gac S, Verdonschot N, van den Berg A, Koopman B, Rouwkema J; TERMIS EU, 28 June - 1 July, 2016, Uppsala, Sweden
 13. Oral: 'The Effect of Anisotropic Strain and Flow Induced Shear Stress Combinations on Endothelial Cell Alignment', Ravi Sinha, Séverine Le Gac, Nico Verdonschot, Albert van den Berg, Bart Koopman, Jeroen Rouwkema, TERMIS AP, 3-6 September 2016, Taipei, Taiwan.

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Curriculum vitae

Ravi Sinha was born on 23 May 1985 in Allahabad, India. He graduated high school from St. Joseph's College, Allahabad. He got a bachelor's degree in Ceramic Engineering from IT-BHU, India in 2007. During the bachelor degree course, he did research internships at IIT Kanpur, India where he characterized the mechanical properties of ceramic-metal composites and prepared TEM samples of bioceramics in the lab of Prof. Bikramjit Basu. He then obtained a Master of Science degree in Materials Science and Engineering from Purdue University, USA in 2009 and a Master of Engineering degree in Biomedical Engineering from Cornell University, USA in 2010. The first masters degree course involved a research project with Prof. Lia Stanciu on the development of an enzyme based electrochemical biosensor for the detection of pesticides (Sinha, R., Ganesana, M., Andreescu, S. & Stanciu, L. AChE biosensor based on zinc oxide sol-gel for the detection of pesticides. *Analytica Chimica Acta* 661, 195-199, doi:10.1016/j.aca.2009.12.020 (2010).). In the second masters degree course, he carried out a research project with Prof. Jonathan Butcher which involved the testing of a mechanical stretcher platform developed in the lab for testing the mechanical properties of soft tissue from small animal disease models. In the project, the device was validated with murine models of marfan syndrome (Gould, R. A., Sinha, R., Aziz, H., Rouf, R., Dietz, H. C., Judge, D. P., & Butcher, J. (2012). Multi-Scale Biomechanical Remodeling in Aging and Genetic Mutant Murine Mitral Valve Leaflets: Insights into Marfan Syndrome. *Plos One*, 7(9). doi:10.1371/journal.pone.0044639). In 2011, he worked for a short duration as a .NET developer at Elixir CT, India. From August 2011 to January 2016, he worked in the Biomechanical Engineering group at the University of Twente on the work reported in this thesis. Since April 2016, he has been working as a post doc with Prof. Lorenzo Moroni at Maastricht University, The Netherlands where he is carrying out computational modeling for the development of a new additive manufacturing platform.