Mechanical regulation of cellular phenotype: implications for vascular tissue regeneration

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Abstract  Cells sense a myriad of cues from their surrounding microenvironment to regulate their function. In recent years, it has become clear that physical and mechanical cues are as critical as biochemical factors in regulating cellular function. The geometry of the extracellular matrix (ECM), degree of cell spreading, and ECM rigidity all influence the physical connection between cells and their microenvironment and play a major role in regulating proliferation, differentiation, and migration. Leveraging these findings to promote specific cell behaviours will be paramount to realize the full potential of cellular therapies. In this review, I examine our current understanding of how mechanical cues—specifically, geometric control of cell shape and matrix rigidity—are transduced by stem cells to control their stemness, proliferation, and differentiation. The implications of these findings for vascular smooth muscle cell differentiation and cardiovascular tissue engineering will be highlighted.

Keywords  Stem cells • Vascular smooth muscle • Mechanotransduction • Micropatterning • Rigidity

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1. Introduction

Vascular tissue engineering remains a promising solution for replacing diseased blood vessels with healthy functional tissue.1-3 This approach typically combines cells with a physical scaffold onto which the cells can expand and grow into the appropriate tissue structure.4 However, obtaining an appropriate and available cell source remains a major challenge in the field. Recent developments in stem cell biology have opened up the possibility of using adult and embryonic stem cells as a potential source of cells for tissue engineering applications.4-6 Stem cells are capable of expanding in culture while maintaining the ability to differentiate into multiple distinct lineages. Moreover, inducing pluripotency from adult cell sources may circumvent issues associated with immunogenicity and transplant rejection.7 Stem cells could thus provide an abundant source of endothelial, smooth muscle, and adventitial cells needed to engineer artificial vascular constructs.

In order to make stem cells a therapeutic reality, much needs to be understood about how to regulate their expansion and differentiation. Traditional studies have focused on the role of soluble factors, such as growth factors or cytokines, as well as genetic regulators including transcription factors. However, a growing body of evidence now suggests that physical cues in the cellular microenvironment also play a critical role in regulating stem cell lineage commitment. Lessons can often be learned from examining the mechanical cues present during development, homeostasis, and disease. For example, shear stress and mechanical stretch imparted by pulsating blood flow are sensed by the endothelium and underlying smooth muscle layers and are known regulators of the normal physiological function as well as embryonic development of the cardiovascular system.8-11 In addition, the biophysical properties of the tissue itself contribute to vascular physiology, and disease processes such as atherosclerosis are associated with changes in the physical tissue microenvironment.12-14 In the adult organism, many cell types including vascular smooth muscle cells display remarkable plasticity and can undergo phenotypic changes depending on their environment. In normal, healthy vasculature, smooth muscle cells remain quiescent and highly contractile; however, during vascular injury, smooth muscle cells adopt a proliferative and non-contractile phenotype. Moreover, differentiation of smooth muscle cells from adult stem cells contributes to vascular regeneration. Thus, understanding how biophysical factors regulate differentiation and phenotypic switching of vascular smooth muscle will help define the microenvironmental cues that are necessary to direct stem cell fate. Incorporating mechanical cues along with biochemical stimuli will likely be necessary to achieve optimal cell phenotypes for vascular tissue engineering applications.

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On the cellular scale, mechanotransduction—the transduction of physical forces into biochemical signals—leads to changes in signalling pathways that control a variety of cellular behaviours including proliferation, differentiation, and migration. While force and function have been linked for many decades, the molecular mechanisms underlying these processes are only currently being unravelled. The number of studies investigating how mechanical cues regulate biological function at the cellular and molecular level has swelled enormously in the past several decades, in part due to the numerous technological advances that enable the control and measurement of forces at a scale relevant to cells. Flow chambers, flexible cell culture substrates, micromechanical devices, patterned surfaces, and optical tweezers have been developed to deliver and sense cellular forces at the pico- to nano-Newton range. Using such bioengineered tools, it is now clear that cells are intricately linked to their physical environment through mechanosensitive proteins and that forces are transmitted through cytoskeletal structures, which eventually lead to alterations in intracellular signalling pathways and gene expression patterns.

The focus of this review article will be to critically examine recent findings demonstrating the regulation of stem cell fate by mechanical cues in the cell microenvironment. I will describe work demonstrating how the spatial presentation of adhesive cues as well as the rigidity of the extracellular environment regulate stem cell expansion and commitment to different lineages. Where possible, I will highlight how similar biophysical cues have been shown to regulate the vascular smooth muscle phenotype. While the role of haemodynamic forces is significant, the reader is directed to other excellent reviews that cover this topic. A better understanding of how physical and mechanical cues regulate stem cell biology and vascular smooth muscle phenotype will ultimately aid in the development of new strategies for vascular smooth muscle regeneration and tissue engineering.

2. Regulation of cell phenotype by shape

Cell shape has long been known to play a crucial role in cell physiology. During development, cell shape changes often precede differentiation for a number of different cell types. For smooth muscle cells, it has been observed that the onset of cellular elongation is immediately followed by the production of smooth muscle-specific proteins. Using polycarbonate surfaces with topographies that stimulated cell rounding or elongation, Yang et al. demonstrated that rounded mesenchymal stem cells remained undifferentiated while elongated cells expressed smooth muscle markers and exhibited smooth muscle-like calcium signalling. Treatment with soluble stimulators of smooth muscle differentiation could only occur when cells were permitted to elongate, suggesting that mesenchymal cell shape is a downstream and critical regulator of myogenesis.

Initial studies investigating the multilineage potential of mesenchymal stem cells focused on the effects of soluble mediators, but the differentiation procedures required cells to be cultured in very specific architectures in order to enhance differentiation potential. Stem cells were cultured within sparse, confluent, or pellet cultures, which was a requirement for the efficient commitment of cells to different lineages. It was not until micropatterning tools were applied to specifically control cell adhesion and spreading when it became clear that cell shape itself can influence stem cell commitment. Using surfaces with discrete islands of adhesive proteins to control the degree of spreading, McBeath et al. demonstrated that mesenchymal stem cells that were spread onto large islands of extracellular matrix (ECM) tended to differentiate into bone. In contrast, cells seeded on small islands of ECM which were restricted from spreading tended to differentiate into fat. These effects were dependent on intracellular contractility and RhoA signalling, since expression of a dominant negative mutant of the protein led to fat differentiation while expressing a constitutively active form of RhoA lead to differentiation into bone, independent of the soluble factors present.

These studies suggest that intrinsically generated mechanical forces play a role in the regulation of cell behaviour by cell shape. Such forces were first shown by culturing cells on soft polymer substrates, which wrinkled upon cell contraction. This technique was later extended to include fluorescent beads embedded within the polymer in order to measure cell traction forces. Cellular contraction leads to the bead displacement, which can be measured to calculate the amount of forces generated by cells. Taking a microfabricated approach, Tan et al. demonstrated that cells cultured on top of a micromitos of needles deflect the needles depending on the amount of force generated. Using such bioengineered tools, it has been demonstrated that cell shape, including the degree of spreading as well as isotropy, contributes to intracellular contractile force generation. Increases in the degree of cell spreading correlate directly with an increase in traction forces, and abrogation of cytoskeleton abolishes shape-dependent effects on force.

In addition to the degree of cell spreading, anisotropy and cell curvature has also been shown to provide a geometric cue to direct stem cell differentiation. Two separate groups have recently shown that cells spread on rectangular-shaped islands exhibited a preferential differentiation to bone, while compared with cells spread on squares of the same area. Moreover, cells that were engineered to have more sharp edges in a star shape differentiated into bone, whereas cells with concave edges that were spread to the same degree differentiated into fat. These effects were associated with an increase in myosin II expression and thus intracellular contractility. Genomic analysis suggested that MAPK and Wnt signalling were downstream effectors of cell shape and important regulators of differentiation. This work demonstrated that even subtle changes in cell geometry may have a remarkable impact on the stem cell phenotype.

For the control of smooth muscle cells, it appears that elongation of cell shape is also a critical determinant of the phenotype. Using micropatterned substrates to control the adhesion of vascular smooth muscle cells in vitro, Alford et al. demonstrated that cellular elongation helps to maintain the smooth muscle phenotype. Culture of vascular smooth muscle cells on patterned lines of ECM caused cells to elongate. Cell and nuclear morphology correlate with contractile force output: cells on 20 μm wide lines exhibited the most elongated cell bodies and nuclei and exerted the highest amount of force when compared with cells cultured on wider (up to 180 μm) lines (Figure 1A and B). In a complementary approach, smooth muscle cells cultured on microfabricated grooves also elongate along the direction of the groove. This surface geometry allows the smooth muscle cells to form sheets, similar to what is observed in vivo. In this configuration, smooth muscle cells generated similar amounts of ECM protein, and the failure stress and stiffness of the elongated cells was significantly higher than that of unpatterned cells. Changes in contractile force have been associated with an increase in smoothelin and smooth muscle myosin heavy chain expression, suggesting
a link between cell shape, the cytoskeleton, and the nucleus for the regulation of the vascular smooth muscle phenotype. Another study by Thakar et al. demonstrated that cell and nuclear elongation caused a decrease in vascular smooth muscle cell proliferation; however, these investigators showed that the effects were independent of changes in spread cell area and smooth muscle contractile markers (Figure 1C and D). Instead, a decrease in neuron-derived orphan receptor-1 (NOR-1), a transcription factor known to be involved in vascular remodelling, was involved.

Taken together, these studies suggest that cell shape, and in particular cellular elongation, is a major regulator of stem cell differentiation and the vascular smooth muscle phenotype. Geometric patterning of adhesion or topology of scaffolds may be a powerful way to encourage stem cell differentiation towards vascular smooth muscle lineages. Indeed, a recent study demonstrated that strain in combination with microfabricated grooves together encouraged differentiation of neural crest stem cells to a smooth muscle lineage, as indicated by an increase in expression of smooth muscle myosin. While these initial results are promising, a greater fundamental understanding of how surface topographies and cell shape regulate differentiation towards a smooth muscle lineage will undoubtedly be needed in order to develop successful strategies for engineering vascular tissues.

3. Regulation of cell phenotype by the rigidity of the microenvironment

To study cells in vitro, biologists have traditionally cultured cells on very stiff plastic or glass substrates. However, cells in the body exist within environments of varied mechanical properties, which is now known to be a critical regulator of cellular behaviour. Leveraging a previously developed technique that utilized polyacrylamide gels to modulate the mechanical properties of the underlying substrate, Engler et al. demonstrated in a seminal study that mesenchymal stem cells (MSCs) cultured on substrates of varied rigidities become committed towards different lineages. By examining cell-type-specific markers and genome-wide expression patterns, they found that MSCs cultured on very soft substrates (0.1–1 kPa) tended to differentiate into neurons, whereas cells cultured on very stiff (25–40 kPa) tended to differentiate into bone. Cells cultured on substrates of intermediate stiffness (8–17 kPa) differentiated into muscle cells. Interestingly, the mechanical properties of the substrate recapitulated the properties of the native tissue to which the cells differentiated. Blocking non-muscle myosin II inhibited rigidity-directed cell
commitment, suggesting that the physical microenvironment is linked to stem cell function through mechanisms involving intracellular contractility. Although previous work had demonstrated that matrix rigidity plays a key role in the maintenance of the phenotype for numerous cell types including neurons and muscle cells, this study was the first to show a role for substrate rigidity on pluripotent stem cell lineage commitment.

More recently, these findings were extended from two-dimensions to three-dimensions by Huebsch et al., who cultured stem cells within alginate hydrogels modified with the integrin-binding peptide RGD to foster cell adhesion. In agreement with previous work, this study confirmed that murine mesenchymal stem cells tended to differentiate into fat when cultured within soft hydrogels (2.5–5 kPa) and into bone when cultured within stiff hydrogels (11–30 kPa) in three-dimensional culture. In contrast to the studies performed on two-dimensional flat substrates, which showed a direct correlation between cell shape and matrix rigidity, cells cultured within three-dimensional gels of varied stiffness all exhibited relatively similar cell and nuclear geometries. Rather, the authors demonstrated a biphasic relationship between matrix rigidity and integrin clustering and binding. Moreover, the disruption of integrin binding using blocking antibodies abrogated stiffness-dependent lineage commitment. The clustering of cell adhesion proteins and the strength of integrin-cytoskeletal linkage have indeed been shown to be regulated by mechanical force. While alginate gels modified with the integrin-binding peptide RGD provide a useful model three-dimensional culture system that circumvents issues associated with enzymatic degradation of matrix proteins, translation of these findings to physiologically relevant ECM conditions remains to be examined. Substrate rigidity not only provides signals that regulate stem cell commitment and differentiation, but also their expansion and maintenance of stemness. When muscle stem cells, or adult myogenic precursor cells that reside within muscle tissue, are cultured on hydrogel substrates mimicking the mechanical properties of muscle tissue (≏12 kPa), they exhibit a greater capacity to self-renew when compared with cells cultured on very stiff or very soft hydrogels. Furthermore, the cells expanded on the gels of medium compliance are better able to contribute to muscle regeneration after

Figure 2 Substrate rigidity regulates vascular smooth muscle phenotype. (A) Vascular smooth muscle cells cultured on PEG-fibrinogen gels varying from 448 to 5408 kPa show a modest increase in F-actin with increasing substrate stiffness. Adapted from Peyton et al. with permission from Elsevier. (B) On a stiffer range of polyacrylamide surfaces (19–84 kPa), vascular smooth muscle cells exhibited increased cell area and proliferation with increasing substrate stiffness. Adapted from Brown et al. with permission from John Wiley and Sons.
transplantation into a mouse. This study was the first to demonstrate a therapeutically useful consequence of culturing cells within a defined mechanical environment in an animal model. In a different study, it was shown that the mechanical properties of the underlying substrate regulate the expansion of haematopoietic stem and progenitor cells. Bone marrow-derived stem cells were cultured on tropoelastin, a naturally derived elastic protein, which provided signals for the expansion of haemopoietic stem and progenitor cells leading to the expansion of undifferentiated cells.\(^4^4\)

Thus, matrix rigidity plays a pivotal role in maintaining pluripotent stem cell expansion and commitment to various lineages. Moreover, matrix stiffness can also regulate the phenotype of vascular smooth muscle cells. Peyton et al.\(^4^5\) showed that the regulation of vascular smooth muscle contractility is dependent on substrate rigidity. Using gels composed of polyethylene glycol and fibrinogen, it was demonstrated that smooth muscle cell contractility was directly correlated with the stiffness of their surroundings, as measured by the visualization of F-actin (Figure 2A) as well as the expression of contractile markers including a-actin and calponin. Interestingly, the proliferation of smooth muscle cells appeared to be independent of substrate stiffness. In contrast, other groups have demonstrated that the proliferation of smooth muscle cells increases with the stiffness of the substrate using dextran/gelatin-based hydrogel\(^4^6\) and polyacrylamide gels\(^4^7\) (Figure 2B). While these in vitro studies have suggested...
that a proliferative phenotype and a contractile phenotype are both enhanced by increases in substrate stiffness, it is unclear how these results translate to a physiological role for stiffness as these distinct phenotypes are typically mutually exclusive. Thus, further studies will be needed to better understand how stiffness regulates smooth muscle behaviour in vivo.

Although it is evident that substrate stiffness is critical in the regulation of both stem cell and vascular smooth muscle biology, it has yet to be demonstrated how stiffness might be leveraged to enhance stem cell differentiation towards vascular smooth muscle lineages. Scaffolds designed to have elastic moduli that could enhance stem cell proliferation and then differentiation towards a contractile vascular smooth muscle phenotype will ultimately be required.

4. Molecular signals are transduced from mechanical signals

Based on the growing body of evidence described above, it is clear that biophysical signals present in the cellular microenvironment, including geometric cues that regulate cell shape, ECM rigidity, and applied mechanical forces, all play a major role in modulating the cell phenotype. However, the molecular mediators that regulate how these mechanical cues are transduced to biochemical signalling events and thus changes in cell behaviour are still currently being unravelled. At the surface of the cell, numerous receptors have been shown to exhibit mechanosensitivity (Figure 3A and B). Deformations of the plasma membrane lead to activation of stretch-activated ion channels and changes in ion flux.68 The glycocalyx and growth factor receptors can also be directly impacted by mechanical forces, leading to changes in downstream signalling events.49,50 However, perhaps the most thoroughly investigated cell surface receptors implicated in mechanotransduction are integrins, which cluster to form focal adhesions and tether the intracellular actin cytoskeleton to the ECM. Focal adhesions are not only important for the formation of cohesive tissue structure, but are also critical for cells to sensing their physical environment.51,52

Experiments using micropipettes or magnetic microparticles coated with ECM ligands have shown that deformations of integrins lead to conformational changes that unmask the cytoplasmic tail promoting actin binding and the formation of large multi-protein complexes called focal adhesions.17,40,53,54 These complexes include talin, which binds directly to actin, and vinculin, which binds actin as well as the actin cross-linking protein α-actinin. In addition, integrin-containing focal adhesion complexes also recruit scaffold and signalling proteins including paxillin and focal adhesion kinase (FAK).55,56 Formation of focal adhesions requires mechanical force, and accumulating evidence suggests that the composition, size, and structure of focal adhesion are dependent on the mechanical properties of the matrix as well as the contractility of the cell.35,57 Cells cultured on softer surfaces typically contain smaller and more dynamic adhesions when compared with cells on stiffer surfaces, which have larger and more stable adhesions. In three-dimensional culture, adhesions are more similar to those of cells on soft surfaces.58 Formation of larger adhesions leads to increased actin polymerization, stress fibre formation, and actin–myosin contractility.

In addition to being a structure upon which force can be exerted and sensed, focal adhesions are a major hub for signalling molecules including FAK, MAPK proteins, Src, and Ras and Raf. As such, changes in intracellular signalling pathways are a direct consequence of changes in the adhesive environment. Central to the regulation of both adhesion dynamics and the actin cytoskeleton organization is the Rho GTPase family of signalling proteins, the activity of which modulates cell proliferation, differentiation, and migration.59 Rho signalling modulators including guanine nucleotide exchange factors and GTPase-activating proteins are recruited to and regulated by focal adhesions. Interestingly, manipulation of Rho signalling has been shown to modulate shape and stiffness effects on stem cell commitment.26,60

5. Transmission of mechanical signals to the nucleus

How mechanical forces are transmitted to the nucleus to exert changes in gene expression and thus cell behaviour is currently an intense area of research.61 Changes in nuclear shape induced by microenvironmental cues, such as the reduction of size observed during cell crowding or cellular compaction, have long been observed, but the effects of these changes on nuclear function remain to be understood. The shape of the nucleus appears to directly correlate with the shape of the cell in most instances: as cells become more spread or constrained, their nuclear area and volume also expand and contract accordingly. Structurally, the nucleus is directly connected to the actin cytoskeleton through nestin proteins, which bind to Klarischt-Anc-Sine1 homology (KASH) and Sad1-UNC-B4 homology (SUN) domains present on the nuclear membrane (Figure 3C).62 Moreover, the KASH/SUN complex is connected structurally to proteins within the nucleus. Analogous to the cytoskeleton, the interior of the nucleus contains a meshwork of structural proteins including intermediate filaments and nuclear lamina.63 Dynamic changes in the structure and interactions of these proteins with chromatin may indeed modulate the chromatin structure, unwinding, and interactions with DNA-binding proteins.64

Nuclear shape changes and stresses exerted on nuclear lamins are thought to alter the chromatin structure, thus controlling gene expression patterns. Several recent studies have demonstrated that elongation of the nucleus as induced by geometry or surface topology results in changes in the clustering of intranuclear lamins as well as histone acetylation.65–67 These structural changes were associated modifications in cellular behaviours including cell proliferation. Although the mechanisms are not fully understood, it is likely that the compression or expansion of the nucleus may directly lead to exposure of transcription binding sites or DNA regulatory motifs and thus control the binding of DNA-associated proteins and changes in gene transcription. Moreover, it is thought that compressive physical forces caused by aligned actin cytoskeletal filaments are required. As molecular tools and imaging techniques to probe the cell nucleus become more advanced,68 many of the unanswered questions about how forces are transduced and influence on the nuclear structure and function will begin to be understood.

Recent experiments have begun to elucidate how mechanical signals lead to changes in gene expression. Most strikingly, an important role for the Yorke-homologues YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) in relaying mechanical signals by cell shape and substrate rigidity to the nucleus has been identified.69,70 Changes in cell morphology induced by cell density, micropatterning, or matrix stiffness were associated with the modulation of Yap phosphorylation and localization to the
nucleus as well as its distribution within the nucleus (Figure 3D). In well spread cells and cells cultured on stiff surfaces, transcription of genes downstream of YAP/TAZ including those involved in proliferation and differentiation were observed. While Dupont et al. 59 found that YAP/TAZ signalling was not dependent on the Hippo pathway, Wada et al. 70 demonstrated that Hippo signalling is required. Both studies found that the regulation of YAP/TAZ required RhoA GTPase activity and actin—myosin contractility. Interestingly, Yap1 has been also implicated in the regulation of the phenotypic plasticity of smooth muscle cells, as down-regulation of YAP leads to activation of the contractile phenotype through a complex involving serum response factor and myocardin. 71 How shape and mechanics regulate YAP/TAZ and Hippo signalling in smooth muscle differentiation from stem cell populations will be an important future area of investigation.

6. Future directions: engineering cell phenotype to promote vascular regeneration

Understanding the development of vascular smooth muscle cells from stem cell populations is important not only for achieving a fundamentally biological understanding, but also for engineering new vascular cells and tissues for therapeutic applications. While the notion that mechanics are critical for the regulation of stem and vascular smooth muscle cell behaviour has existed for some time, a full understanding of how mechanical cues are transduced into biochemical signals is far from complete. Future advancement of this field will rely on tools with increasing complexity. For example, the integration of multiple different mechanical cues (tunable adhesive geometry, rigidity, and applied mechanical forces) will be important to elucidate how multiple signals are integrated by cells to elicit a single response. Recently, an organ-on-a-chip model was developed to mimic lung physiology, where endothelial and epithelial cells were cultured on a stretchable membrane on which cells were stretched and fluid flow was applied. 72 Recreating other organ systems including blood vessels or heart tissue will likely also require integration. Ultimately, smooth muscle cell behaviour has existed for some time, a full understanding of how mechanical cues are transduced into biochemical signals is far from complete. Future advancement of this field will rely on tools with increasing complexity. For example, the integration of multiple different mechanical cues (tunable adhesive geometry, rigidity, and applied mechanical forces) will be important to elucidate how multiple signals are integrated by cells to elicit a single response. Recently, an organ-on-a-chip model was developed to mimic lung physiology, where endothelial and epithelial cells were cultured on a stretchable membrane on which cells were stretched and fluid flow was applied. 72 Recreating other organ systems including blood vessels or heart tissue will likely also require integration. Ultimately, tools with high temporal and spatial resolution will be necessary to probe and evaluate how dynamic mechanical cues are integrated by cells and influence their decisions to become certain fates or adopt specific phenotypes.

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