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Role of Extracellular Matrix Signaling Cues in Modulating Cell Fate Commitment for Cardiovascular Tissue Engineering

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Abstract

It is generally agreed that engineered cardiovascular tissues require cellular interactions with the local milieu. Within the microenvironment, the extracellular matrix (ECM) is an important support structure that provides dynamic signaling cues in part through its chemical, physical, and mechanical properties. In response to ECM factors, cells activate biochemical and mechanotransduction pathways that modulate their survival, growth, migration, differentiation, and function. This review describes the role of ECM chemical composition, spatial patterning, and mechanical stimulation in the specification of cardiovascular lineages, with a focus on stem cell differentiation, direct transdifferentiation, and endothelial-to-mesenchymal transition. The translational application of ECMs will be discussed in the context of cardiovascular tissue engineering and regenerative medicine.

Keywords

extracellular matrix; cardiovascular; tissue engineering; cell fate; stem cells

1. Introduction

Cardiovascular disease (CVD) affects one out of every three adults in the US, and the economic impact is staggering with an estimated annual health care cost of \$444 billion.^[1] CVD remains the leading cause of death and it is predicted that by 2030 the total direct cost of this disease will triple.^[2] Although current treatments of CVD such as angioplasty with stenting and bypass grafting can be beneficial, there is a high morbidity associated with these standard treatments. Often patients require multiple surgeries, which dramatically decreases the quality of life and adds to the cost expenditure.

Emerging regenerative strategies are being developed and refined to engineer patient-specific cardiovascular tissues as sustainable and long-term treatment options for CVD. One promising bioengineering approach are engineered tissue substitutes composed of stem cells cultured within a scaffolding structure known as the extracellular matrix (ECM). To more effectively engineer functional vascular grafts and cardiac tissues to treat CVDs, a better

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understanding of the synergistic biochemical and biomechanical interactions created between cells and their matrix substrates is needed.

Cells are extremely sensitive to the chemical, physical, and mechanical properties of their surrounding environment. Stem cells possess plasticity and multi-lineage differentiation potential, making them an attractive source for understanding how cellular interactions with ECMs lead to downstream regulation of cell survival, proliferation, differentiation and function. Therefore, the focus of this review will be on pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), along with multipotent stem cells such as mesenchymal stem cells (MSCs).

Mechanotransduction is a process by which the mechanical properties of the extracellular environment induce an adaptive intracellular response within the cytoplasm and nucleus, leading to changes in cell behavior, function, or cell fate specification. This review will summarize the properties of ECMs that induce cytoskeletal reorganization and mechanotransduction of forces to the nucleus to stimulate mechanosensitive pathways that lead to adaptive cellular remodeling and differentiation, including endothelial-to-mesenchymal transformation and transdifferentiation shown in Figure 1. Although not described here, the molecular basis of iPSC mechanobiology^[3] and comprehensive reviews for engineering functional vascular networks can be found elsewhere.^{[4] [5]}

2. Chemical Aspects of ECMs that Modulate Cell Behavior

The ECM is an important component of the cellular microenvironment and provides signaling cues that regulate cellular behavior, including proliferation, migration, adhesion, and differentiation.^[6] Despite the fact that all cells *in vivo* are in close contact with the ECM, there are spatial differences in the way they interact with the ECM proteins. For example, endothelial and epithelial cells contact the basement membrane matrix proteins on the basal but not apical surface. Mesenchymal cells in connective tissues (ie fibroblasts and chondrocytes) are encapsulated in the surrounding ECM. The anchoring junctions between cells and ECM also vary in different cell types. For example, epithelial cells anchor to the ECM by hemidesmosomes, while mesenchymal cells attach to the surrounding ECM by adherens junctions. A number of cell surface receptors are involved in cell-ECM interactions, and the primary class of receptors that have been intensively studied over the last twenty years are integrins. Cells can sense and respond to mechanical forces from the surrounding ECM through conformational changes and modifications in the binding affinities of integrins.^[7] In addition, ECM proteins can differentially promote or inhibit stem cell differentiation into specific cell lineages.

The ECM is composed of numerous components. For example, the basement membrane is mainly composed of laminins and collagen (type IV) (other components include nidogen, perlecan, and type XV/XVIII collagen); while connective tissue is rich in fibrillar collagens (type I and III). ECM plays an important role in determining cell behavior, including those of human pluripotent stem cells. The ECM proteins surrounding a stem cell can provide signaling cues to either maintain pluripotency or differentiate into a specific cell lineage. For example, it has been reported that pluripotency of embryonic stem cells can be maintained

when they are plated on type I or type IV collagen. However, when the culture substrate is laminin or fibronectin, differentiation was induced.^[8,9] In addition, different laminin subtypes have been implicated for modulation of the differentiation process and lineage specification. For example, laminin-322 favors osteogenic differentiation, while laminin-111 can stimulate neural differentiation.^[10] Fibronectin, on the other hand, has been shown to increase integrin $\alpha 5\beta 1$ expression and promote differentiation of meso-endodermal lineages such as skeletal lineages.^[11]

Induction of pluripotent stem cells with ECMs has been shown to promote generation of cardiovascular and hematopoietic cell types, including cardiovascular progenitors, endothelial cells (ECs), smooth muscle cells (SMCs), and cardiomyocytes in various animal models.^[12] When differentiated on collagen IV-coated dishes, murine ESCs and iPSCs generated FLK1+ mesodermal progenitor cells that could then be differentiated into ECs that express characteristic EC-associated markers, including CD31 and vascular endothelial-cadherin (VE-cadherin), and demonstrated functional incorporation of acetylated low-density lipoprotein.^[12,13] These same FLK1+ progenitors showed the capacity to differentiate into cardiomyocytes based on the presence of sarcomeric myosin and troponin C, as well as SMCs based on functional contraction patterns.^[12] A similar approach was applied to obtain human ECs from ESCs *in vitro*.^[14] Further evidence of ECM induction was shown by the differentiation of murine ESCs into trophoblasts using collagen IV but not laminin, fibronectin or collagen I.^[15] However, despite the expression of many typical mature EC markers such as VE-cadherin and CD31, McCloskey et al. reported that ECs derived by ECM induction retain markers of immature endothelium such as hematopoietic stem cell marker, CD34.^[16]

Fibronectin has been shown to increase EC differentiation. Wijelath et al.^[17] reported a five-fold increase in the number of EC colonies when human endothelial progenitor cells (EPCs) were cultured with fibronectin and vascular endothelial growth factor (VEGF). The $\alpha 5\beta 1$ integrin was believed to play a key role in the enhancement of VEGF activity, which enabled an increase in EC differentiation. Kanayasu-Toyoda et al.^[18] also isolated CD133+ cells from peripheral blood on fibronectin-coated dishes. Other ECMs such as gelatin were also used in combination with VEGF to obtain ECs from human and mouse ESC differentiation.^[19] Another important basement membrane glycoprotein is nidogen, which is expressed by human ESC.^[20] It was shown that induction of human ESCs using laminin-511 and nidogen-1 promotes and restores ESC assembly without the need for culture on traditional murine embryonic fibroblasts or soluble factors. Elucidation of ECM-based mechanisms for cell aggregation has important implications in biology and applications for engineering three-dimensional tissues.

The mechanisms by which ECMs modulate stem cell differentiation are in part due to integrin receptors that transduce a cascade of signals from the external ECM substrates into the cell. When integrins bind to their respective ECM ligands, they undergo conformational changes in both their external and cytoplasmic domains. This is followed by the accumulation of cytoplasmic molecules and adaptor proteins that activate downstream signaling pathways. For example, cardiac differentiation can be modulated by interaction of collagen type I and $\beta 1$ integrin. When integrin $\beta 1$ was blocked, embryoid bodies (EB)

became defective with decreased size and lack of shell like layer of endoderm cells. Moreover, these EBs had decreased beating activity and less cardiac gene expression.^[21] Similarly, fibronectin has been shown to promote mesodermal differentiation via $\beta 1$ integrin through activating Wnt/ β -catenin pathway.^[9] Mesoderm induction was inhibited when fibronectin was knocked down *in vitro* and *in vivo*, and can be rescued by activating β -catenin signaling. Fibronectin not only serves to promote the interaction of $\beta 1$ integrin and caveolin-1, but also increases the phosphorylation of integrins that affect downstream signaling.^[22] The downstream signaling pathway was demonstrated to be RhoA-PI3K/Akt-ERK1/2 pathway in mouse ES cells.^[23] Activation of different signaling pathways, such as Ras/Raf/Mek/Erk and Ca²⁺/CaMKII, were also shown in other cell lines.^[24] Rho activation has been suggested important in fibronectin stimulated cell proliferation.^[25] Inhibition of ROCK, the Rho associated protein kinase, was shown to suppress stem cell proliferation stimulated by fibronectin.

Besides binding to adhesion receptors such as integrins, ECM proteins can also bind to soluble growth factors, thus regulating their presentation, distribution, and activation. ECM-bound growth factors create spatial and temporal gradients, which play a key role in development and cell patterning. For example, one ECM surface protein, heparan sulfate proteoglycans bind to a number of growth factors, such as fibroblast growth factor (FGF) and VEGF. Binding to integral membrane proteoglycans is required for activation of transforming growth factor- β (TGF- β) signaling pathways.^[26] Hence, the ECM modulates cell biochemical pathways through the interaction of integrins with specific ECM substrates and soluble factors.

3. Role of Nanotopography and Micropatterning in Modulating Cell Behavior

Cells in native tissues naturally reside in well-structured microenvironments that consist of precise spatial patterns of various ECM proteins. When cells are grown in culture on polystyrene (PS) dishes, their physiological spatial organization is lost. Bioengineering approaches to recapitulate the native two-dimensional (2D) and three-dimensional (3D) cell-ECM organization enable greater control over cell behavior and ultimately cell differentiation and fate. Some common effective nano/micropatterning techniques include soft lithography such as microcontact printing, dip pen nanolithography (DPN), and electrospinning. Briefly, soft lithography uses elastomeric materials such as polydimethylsiloxane (PDMS) as a physical stamp containing relief structures, to deliver a variety of molecules and materials in a pattern with features that range from 30–100 μm .^[27] DPN uses an atomic force microscope to directly pattern surfaces and is preferred over microcontact printing when numerous patterns are desired for a single substrate. A technique that has the possibility for more 3D substrate designs is electrospinning, which relies on an electric charge to pull fine fibers from a liquid that transitions into a solid as the fibers dry in the zone near the target substrate.

3.1. Tensegrity Model

The idea of biological tensional integrity or “tensegrity” as a cellular cytoskeletal design was introduced by Donald Ingber as a model to explain how the filaments of the cytoskeleton interact and integrate to form one cohesive yet dynamic architecture.^[28] The concept is that contractile forces generated by the inward pull from actin and myosin filaments in the cytoskeleton are countered by the outward forces created from integrins tethering to the ECM. The extracellular forces created through the interaction of the cell with the ECM are mechanically transmitted through the cell’s cytoskeletal structure to the nucleus. In this way, the ECM can modulate mechanosensitive molecular pathways that stimulate changes in cellular chemistry and regulation of gene expression.

3.2. Using Substrate Patterning to Modulate Cell Shape, Distortion, and Migration

Because cells rely on integrins to adhere to their extracellular environment, individual cell shape can be modulated by the pattern and availability of integrin binding domains. When human and bovine capillary endothelial cells were allowed to adhere to 2D ECM substrates that were micropatterned in the shape of squares, circles, and triangles, the cells conformed to the shape of the substrate island. They also preferentially extend lamellipodia from the angled corners of the substrates to initiate migration.^[29–31] The mechanism of cellular orientation lies in the reorganization of the cytoskeleton and concentration of tensional stresses. When cultured on the square micropatterns, for example, cells orient their actin stress fibers and focal adhesion proteins at the corners of the square shapes that promote formation of lamellipodia, filopodia, and microspikes to re-direct the cell’s leading edge and motility away from the cell’s center.^[30] Spatial patterning of cells is involved in numerous biological processes such as embryonic development, tissue formation, and vasculogenesis and provides new insights into engineering artificial ECM environments and utilization of geometric cues to guide cell shape and migration.

Cells are also extremely sensitive to topographical cues and respond by altering their cytoskeleton, morphology, and ultimately their behavior. Micropatterning techniques can guide the orientation of cells and their cytoskeleton, which is a key component in modulating cell organization and differentiation for tissue engineering applications. Topographical orientation of ECM nanofibrils can be used as a tool to align cells along a specific axis for bioengineering applications in which improved mechanical and biochemical function is desired as shown in Figure 2a-b. To mimic the native organization of vascular endothelial cells, which align longitudinally along the direction of laminar flow, recent tissue engineering approaches have incorporated endothelial cell patterning in vascular grafts. Lai et al.^[32] generated a collagen graft and demonstrated the ability of the ECM nanotopography to modulate cell alignment as well as atheroprotective function. ECs on the aligned nanofibrillar scaffolds exhibited alignment of F-actin along the direction of the fibrils, and the cells were more resistant to monocyte adhesion compared to ECs grown on randomly oriented fibrils. Subcutaneous and ischemic hindlimb implantation on aligned nanofibrillar scaffolds demonstrated greater EC as well as iPSC-derived EC viability compared to non-patterned constructs.^[32,33]

Besides 2D substrates, cells can also be spatially patterned within 3D substrates. ECs seeded onto micropatterned substrates with 10 μm wide lanes showed down-regulation of both growth and apoptotic genes within 72 hours and formed 3D capillary-like structures containing lumens.^[34] Tube formation was not recapitulated when wider channels of 30 μm were used. Whereas both sized channels promoted alignment of their actin cytoskeleton, only in the wider channels did cells proliferate and have greater spreading. This study demonstrates how substrate patterning by the creation of spatial restrictions can modulate cellular morphology, organization, and function.

Cell alignment not only plays a role in the native vasculature, but it is also a key component for signal transduction in cardiac tissues. Many cardiac arrhythmias are characterized by abnormal activity of the heart. Conditions that interfere with cell-cell coupling can impact electrical stability. The alignment of cardiac cells *in vitro* was shown to affect multiple electrical properties including action potential duration and transient calcium direction.^[35] Electrospun nanopatterned substrates or gelatin methacrylate (GelMA) hydrogels produced improved alignment of cells that correlated with cell and nuclear elongation, which is known to associate with increased cellular differentiation and DNA synthesis, respectively.^[36] The application of nanopatterned substrates to align cells is a useful tool for *in vitro* engineering of the vasculature and cardiac tissues in which specific spatial orientation of cells directly translates to improved functional properties.

3.3. Cell Spreading Effect on Cell Survival, Proliferation, Differentiation

In response to the ECM, mechanotransduction cues are relayed to the cytoskeleton and to the nucleus to modulate cell survival and actin assembly. A strong connection between cell spreading and apoptosis was shown using fibronectin-coated beads of varying diameters^[31]. Cells on smaller beads that were forced into a rounded shape underwent apoptosis at a much higher frequency compared to cells allowed to spread on larger diameter beads. This demonstrates how survival pathways are stimulated through tensional forces that are transmitted through the cytoskeleton of a cell to specify maintenance or death. Increases in cell spreading have been correlated to increases in stress fiber formation at focal adhesions^[37]. When SMCs were stimulated to contract with lysophosphatidic acid, only cells that were allowed to sufficiently spread their cytoplasm responded with contractile forces. Cells that were restricted from spreading did not respond to stimulation, suggesting that cytoskeletal signaling conveyed via cell shape controls contractile response.

Besides modulating cell survival and contractility, cell shape is also a regulator of lineage commitment. For example, MSC adipogenesis versus osteogenesis was linked to cell spreading. Adipogenesis was associated with cells confined to round geometries of small areas (1000 μm^2), which allows for greater accommodation of spherical lipids. In contrast, MSC osteogenesis was preferentially observed in cells with greater spreading area (10,000 μm^2), which promotes more contact with the underlying substrate for matrix remodeling and calcium deposition.^[38] The switch between these two cell fates was associated with the activity of RhoA, a GTPase that regulates the actin cytoskeleton. The concentration of active RhoA is lower during adipogenesis but higher during osteogenesis, thereby, demonstrating that direct manipulation of RhoA activity can directly induce differentiation.

Modulating cell shape by substrate geometry is also effective on a multi-cellular level. Geometry-based mediation of stem cell differentiation was shown by culturing human adipose stem cells on fibronectin-coated shapes of rectangles or rings with varying dimensions.^[39] Cells at the inner edge of rings and interior regions of small sized rectangles were morphologically small and compact in cell shape; these cells showed the lowest levels of cell proliferation and had enhanced differentiation. In contrast, cells at the outer regions of rings and along the short edges of rectangles had greater cell spreading, which corresponded with higher proliferation. Ruiz et al.^[40] showed a comparable trend with human MSCs cultured in a 1:1 adipogenic:osteogenic medium. Cells at the center of circles and rings contained lipids implicating adipogenic differentiation, whereas cells on the outer edges stained for alkaline phosphatase, which indicated osteogenic differentiation. In both studies, pharmacological treatment to disrupt the cytoskeleton ablated the geometric control of differentiation, which indicates that cytoskeletal cues are required for shape-based lineage specification. Regardless of whether the substrate geometry was presented on a single versus multi-cellular level, the determining factor of cell fate specification was whether the substrate geometries restricted or encouraged spreading of the cell and its cytoskeleton.

The mechanism behind cytoskeletal spreading and differentiation may be partly elucidated by observations of human ESC culture, in which there is a fine balance of biophysical cues to maintain pluripotency. The first cells that begin to undergo spontaneous differentiation are located at either the center or border of the colony. These observations may point to the existence of defined tensional cues responsible for inducing stem cell differentiation. Micropatterned restriction of human ESC colony growth not surprisingly showed differentiation at the periphery and formation of multiple layers in the center that also underwent differentiation. A correlation between expression of β -catenin and Oct-4 expression in human ESC has been shown.^[41] In undifferentiated human ESC, β -catenin mediates cell-cell adhesion and decreased at cell-cell junctions during differentiation, suggesting that localized decreases in β -catenin modulate human ESC spatial patterning and differentiation.

Taken together, geometric patterning of ECMs alters cell shape, cell-cell contacts, and spreading. These biological responses are linked to differential tensional forces that are transmitted through the cytoskeleton and to the nucleus to induce changes in cell morphology, behavior and fate.

4. Role of Matrix Stiffness in Modulating Mechanotransduction

A major modulator of mechanosensing pathways is ECM rigidity. The stiffness of the ECM substrate affects formation of focal adhesions and consequently causes remodeling of the cell cytoskeleton, cell spreading, and differentiation shown in Figure 2c-d. A typical cell culture dish made of polystyrene (PS) has a rigidity near 10^7 kPa, whereas most soft tissues exhibit rigidities between 1 to 100 kPa. Therefore, routine cell culture on conventional PS dishes may have limited translational relevance. A closer matching of the *in vitro* substrate rigidity to the native rigidity is thought to provide cells with a mechanical environment better suited for effective culture and differentiation of specific cell types. The ability to

control stem cell differentiation through the substrate stiffness has useful implications for *in vitro* stem cell culture and combinatorial cell/biomaterial approaches to tissue engineering.

4.1. Effect of Rigidity on Cell Fate

By modulating the interaction between the cytoskeleton and integrins, substrate rigidity induces cell fate determination.^[42] For example, MSC cultured on soft substrates (1 kPa) demonstrated decreased cell spreading, stress fibers, and proliferation rate compared to MSC grown on stiffer substrates (15 kPa).^[43] Fibroblasts cultured on collagen-coated polyacrylamide cells with transitional soft-to-stiff rigidity (140–300 kdyn/cm²) exhibited greater cell spreading (1005 μm^2) on the softer rigidities compared to stiffer rigidities (833 μm^2).^[44] Additionally, cells showed preferential migration in the direction of stiffer rigidities. Cells on the soft substrate migrated onto the stiffer substrate region, whereas cells cultured on the stiffer region remained on the stiff substrate. When cells encountered the transitional region, they turned back towards the stiff matrix side. Furthermore, it was shown that cell movement could be guided by differential substrate stiffness experienced by the front and rear of the cell to control cell polarity and migration.^[44] By deforming the substrate, cells exerted “pulling” or “pushing” forces at either the leading or trailing cell edges. When the leading edge experienced a pushing force, thereby reducing the amount of mechanical input required by the cell at that edge, the cell retracted and migrated away from the deformation. Conversely, when a pulling force was applied to the trailing end, the cell reversed its direction and migrated towards the deformation.

The transmission of force from focal adhesions through the ECM activates a cascade of mechanotransduction pathways capable of not only directing cells in the direction of a preferred stiffness but can determine lineage specification. Engler et al.^[42,45] showed that cells differentiate optimally on substrates of physiological rigidity. MSCs cultured on soft matrix microenvironments with elastic moduli that mimic that of brain tissue (0.1–1 kPa) exhibited neurogenic properties including branched morphology, staining for neurogenic markers (ie nestin, an early commitment marker and B3 tubulin) and gene expression of early and mature neurogenic transcripts (ie neurofilament light chain). In contrast, cells that were cultured on substrate rigidities mimicking collagenous bone (25–40 kPa) underwent osteogenesis, and MSCs on intermediate stiffnesses (8–17 kPa) underwent myogenesis.^[42] Similarly, Murphy et. al.^[46] reported that stiffness-based control of osteogenic versus chondrogenic differentiation could be achieved without use of inductive media. Moreover, the use of hyaluronic acid (HA) instead of chondroitin sulfate (CS) enhanced chondrogenic differentiation, showing that alteration of the chemical composition of the scaffold has a combinatorial effect with stiffness to enhance differentiation.

A cell type whose phenotype is highly dependent on substrate rigidity are pluripotent stem cells. Maintaining ESC in an undifferentiated state is a challenge that can be partially mediated by substrate stiffness. It has been shown that certain mechanical conditions are able to more effectively maintain human ESC in an undifferentiated state. Human ESC show increased cytoskeletal contraction with increasing rigidity, and maintenance of human ESC pluripotency was also correlated with stiffer rigidities.^[47] In addition, ESC phenotypic commitment was also shown to be influenced by substrate mechanics. Higher proliferation

rates were associated with softer fibrin hydrogel matrices compared to stiffer gels. When ESC were allowed to undergo spontaneous differentiation, mesoderm and ectoderm markers were not sensitive to substrate stiffness. However, cells cultured on softer gels (13 Pa) had more pronounced upregulation of endoderm-associated genes, Sox17, Afp, and Hnf4, compared to relatively stiffer substrates of 171 Pa.^[48] When cultured on stiffer substrates, however, the cells demonstrated lower tendency towards endoderm-specification based on the expression of endodermal genes. Although, substrate rigidity is not the only mechanical player in mechanosensitive pathways, it is a property that plays a key role in mechano-responsive lineage-specification.

In addition to modulation of differentiation of stem cells, substrate stiffness has been shown to affect the behavior and function of cardiovascular cells. For example, cardiomyocytes prefer substrates of physiological rigidity.^[45] A clue for this preference can be found in the pathology. Myocardial infarction leads to the formation of fibrous scar tissue that has higher stiffness (35–70 kPa) compared to healthy tissue (10 kPa for striated muscle). It is not surprising that cardiomyocytes prefer less stiff substrates because they are associated with injury instead of normal development or maintenance. This rationale was reinforced in a study in which embryonic cardiomyocytes were allowed to contract on collagen-coated polyacrylamide gels of varying stiffness.^[45] The cells demonstrated either balance or dystrophy between intracellular and extracellular deformation and forces in a rigidity dependent manner. Cells on soft matrices (1 kPa) transmitted the majority of their strain directly to the matrix, which minimized intracellular forces. Cells on stiff matrices (34 kPa) had high intracellular forces because they could not deform the matrix, and eventually lost their ability to contract. For cells on intermediate ranges of stiffness (11 kPa), the strains associated with the cell and the ECM are balanced. It is suspected that the stiffer matrices cause intracellular remodeling involving forced unfolding of key force-generating myosin into unfavorable conformations that impact overall cell contractility.

While cells can lose their phenotype or function by being cultured on incompatible matrix stiffness, culture on the appropriate matrix rigidity can create a gain of function. For example, multipotent cardiosphere-derived cells cultured on fibronectin-coated polyacrylamide gels formed well-organized cell networks when cultured on rigidities that mimicked that of the myocardium (12–16 kPa). These cells expressed greater levels of endothelial markers (CD31 and FLK1) and demonstrated enhanced survival and vascular integration in a rat model of myocardial infarction.^[49] Matrices composed of varying collagen to fibrin ratios were seeded with EC and MSC to examine the effect of ECM composition-related stiffness on vasculogenesis. Improved vasculogenesis corresponded to matrices with greater fibrin content.^[50] Increasing the protein concentration of a set ratio of collagen to fibrin in the matrices was used to increase the matrix stiffness without changing the ECM composition. The matrices with the highest protein concentration, which had the greatest stiffness, were associated with markedly lower vessel formation.

Matrix stiffness transmits important mechanical cues to influence many cell phenotypes including morphology, polarity, migration, differentiation, and maturation. Pronounced lineage-specific differentiation can be accomplished with closely matching the native ECM rigidity to that of the substrate, such that cells cultured in softer or more rigid environments

more readily differentiate towards cells that typically compose tissues of similar rigidities. These trends implicate substrate rigidity as a powerful modulator of cell fate and promising tool for enhancing the efficiency of differentiation and generation of tissue-specific cell types.

4.2. Activation of Nuclear Mechanotransduction Pathways

Rigidity and other mechanical forms of stimulation and cellular interaction translate to cellular changes via complex mechanotransduction pathways. Mechanosensing of rigidity has been linked to two nuclear transcription factors: Yorkie homologous Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). Function of these two factors is required to convey the effects of stiff ECM, while inactivation of YAP/TAZ is associated with soft ECMs.^[51] Specifically, the transcriptional activity of YAP/TAZ was inhibited when human epithelial cells were grown on softer (0.7–1 kPa) acrylamide hydrogels compared to stiffer gels (15–40 kPa). MSC do not normally differentiate towards adipogenic lineages on stiff ECM; however, it was shown that knockdown of YAP/TAZ mimics the effect of soft ECM and enabled adipogenic differentiation of MSC on stiff substrates. Conversely, overexpression of YAP/TAZ activity overrode the geometric control on HMVEC fate and rescued osteogenic differentiation of MSC cultured on soft ECM.

An essential intermediate step in the mechanotransduction cascade is the interaction of cytoskeletal elements with the nucleus. In addition to the many molecular/chemical signaling pathways that are stimulated in the process of mechanotransduction, the cytoskeleton itself can directly act on the nucleus through an efficient tensegrity network. This network functions through the coordination of actin and microfilament elements that respond differentially to varying degrees of strain. The nuclear lamina provides structural support to the nucleus through nucleoskeletal intermediate filaments called lamins.^[52] Long distance force propagation from the cell surface to the nucleus is described by Wang et al.^[53] using a model of prestressed inhomogeneous tensegrity. In this model, cytoskeletal filaments respond to surface forces by direct propagation of mechanical force from the plasma membrane to the nucleus. Mechanical signals are transferred to the nucleus (approximately 5 μ s) faster than diffusion-based molecular signaling (approximately 5s), allowing for more efficient transduction of surface forces through the cell. It has also been shown that the mechanical coupling of surface forces to the nucleus can be accomplished through the cytoskeleton network in the absence of diffusion-based molecular mechanisms. Mechanical stresses were applied to cell surfaces using integrin-bound beads to pull on cells that contained no membranes or cytosolic components. The nucleus distorted and elongated along the direction of the pulling force indicating that the coupling could be achieved independent of membranes, chemical diffusion, and ATP.^[54]

There are several possible mechanisms to explain the mechanical coupling of the ECM and integrins to the nucleus. Changes in the actin-myosin cytoskeleton may be mechanically linked to mechanosensitive ion channels located in the nuclear membrane.^[54] Distortions in cell shape may pull on the membrane and distort the channel, thereby stimulating ion influx. Another mechanism may be the direct transfer of force to the DNA backbone through

specific matrix attachment regions (MARs) and hence expose DNA binding sites that are susceptible to transcriptional regulation.^[55]

A similar mechanism of nuclear mechanotransduction is the selective repositioning of chromosomes to control proximity to silencing factors. Through promotion of contact guidance using microgrooved channels, subcellular changes in the nucleoskeleton, nucleolar morphology, and chromosomal positioning were induced.^[56] Cells in grooved channels had nuclei with aligned chromosomes, whereas non-confined cells on planar substrates had rounded nuclei with randomly oriented chromosomes. Topographical alignment of cells induces nuclear elongation that results in the repositioning of chromosomes. Larger chromosomes, in general, have longer telomeres and therefore, may experience greater mechanical forces and are pulled closer to the lamina to be acted on by silencing factors. The largest chromosomes were repositioned to the nuclear periphery and had the most downregulated genes. In contrast, the most upregulated genes were observed in a smaller chromosome that was not repositioned.

In summary, there are many forms of mechanosensors that translate mechanical cues from the cellular microenvironment through the cytoskeleton to either act directly on the nucleus or function to instruct activation or suppression of mechanosensitive pathways.

5. Synergistic Effects of ECMs with Mechanical Stimulation

There are three major forms of active extracellular physical stimulation that are powerful modulators of cardiovascular cell behavior: 1) shear stress, 2) cyclic tension/stretch, and 3) contraction/compression. These mechanical tools are often coupled with ECM-based materials to assist differentiation and enhance downstream functional properties of mechanosensitive cell types such as vascular and cardiac cells. Under native *in vivo* conditions, these cell types experience constant physiological mechanical forces, particularly shear from blood flow through veins and capillaries and rhythmic contractions and filling of the heart. Common *in vitro* approaches to creating these three forces are 1) forced fluid flow across a monolayer of cells to generate shear; 2) straining of deformable substrates in at least one direction/dimension to induce tension; and 3) electrical stimulation to induce cellular contraction.

5.1. Shear Stress and the Vasculature: Fabrication Using Shear and the Effect of Physiological Shear on Long-term Patency

The native vasculature experiences constant shear from the continual blood flow across the endothelial-lined luminal surface. Studies have shown that shear modulates EC morphology, alignment along the direction of flow, and even induction of vessel formation and angiogenesis.^[57] Physiological levels of shear stress applied to human ESC-derived ECs caused cells to elongate in the direction of flow and their responsive gene expression profiles closely resembled that of primary human microvascular ECs (HMVECs) and human umbilical vein ECs (HUVECs).^[58] However, expression of TGF- β , a paracrine factor known to act as an inhibitor of SMC proliferation during vascular remodeling, was not comparable to primary ECs, suggesting that shear alone may not be capable of mediating negative remodeling of engineered vascular tissues.

Since shear is a potent regulator of vascular phenotype, it has been applied towards cardiovascular differentiation of stem cells. Cell surface HSPG was shown to modulate shear-induced differentiation of mouse embryonic stem cells into ECs.^[59] Application of shear stress in a closed loop parallel plate flow chamber for 8 hours induced expression of EC-specific genes including Von Willebrand factor (vWF), vascular endothelial-cadherin, CD31, as well as tight junction proteins, zonula occludens (ZO-1) and claudin 5 (CLD-5) and vasodilatory genes, endothelial nitric oxide synthase (eNOS) and Cyclooxygenase 2 (COX-2).^[59] Selective disruption of HSPG by heparinase III followed by the same application of shear did not produce the same upregulation of most of the shear-induced genes suggesting that HSPG is a mechanoregulator of shear-induced human ESC to EC differentiation.

5.2. Cyclic Tension/Stretch on Differentiation

Besides shear stress, the cells of the native heart experience continual physiological cyclic strain due to the rhythmic beating of the tissue. During diastole, the right atrium is stretched to accommodate the increased fluid volume due to filling of this chamber. Application of mechanical strain to cells can reorganize cellular alignment and improve cardiomyogenic differentiation. HUVECs cultured on polyacrylamide gels reorientated in response to periodic uniaxial stretches of 10% strain.^[60] This study applied pulsatile strain to cells and utilized Fourier transform tractional microscopy to assess tractional fields and forces to record realignment of tractional fields. Reorientation of the tractional field preceded cell body reorientation. As strain was applied, there was attenuation of the tractional field as the cytoskeleton fluidized and then reassembled in the new orientation as traction is recovered. EC reorientation was attenuated when the same strain frequency was applied to constructs but with half the amplitude. Interestingly, no reorientation was observed when periods between stretches were long. This was likely due to sufficient time for full recovery of tensional forces in the cytoskeleton. Their findings tie into the earlier tensegrity models of the cell-cytoskeleton lattice and the cellular response to rapidly attenuate cytoskeletal stiffness to fluidize the cytoskeleton for reorientation and resolidification perpendicular to the direction of strain. Reorientation of the cell body is a complex process and involves not only stretch-induced reorientation of cytoskeletal stress fibers, but also focal adhesion turnover, sliding, and microtubule dynamic.^[61]

The stretch-induced reorganization of the cellular cytoskeleton can also activate differentiation pathways. For example, cyclic stretching of MSC-seeded collagen gels revealed matrix remodeling that promoted tenogenesis, the differentiation of MSC into tendon or ligament fibroblasts.^[62] Uniaxial cyclic strain alone was shown to induce cardiomyogenic differentiation of rat bone marrow-derived MSC grown on flexible elastic silicone membranes indicated by increased cardiac markers and proteins, cardiac troponin T (cTnT), myocyte enhancer factor 2c (MEF-2c), connexin (Cx43) as well as improved functional response to calcium addition.^[63] Mechanical stimulation by shear forces using a parallel plate flow chamber did not induce the same level of cardiomyocyte-related gene expression compared to cyclic strain, indicating the specificity of the kind of mechanical stimulation and contribution of tensile stretching to MSC-cardiomyocyte differentiation. The adaptive rearrangement of the cell cytoskeleton depended on the type of physical force.

Cells aligned parallel to the direction of shear stress whereas they aligned perpendicular to the applied force under cyclic strain. Furthermore, the combination of mechanical stimulation with addition of 5-azacytidine, a biochemical agent known to induce differentiation of MSC into cardiomyocytes,^[64] created a synergistic enhancement of cardiomyogenic differentiation.^[63]

Synergistic benefits from combining mechanical stretch with molecular or chemical agents are also seen when combined with an appropriate ECM. SMCs cultured on fibronectin-coated PGA scaffolds subjected to cyclic strain demonstrated stimulation-dependent improved growth and production of ECM proteins, elastin and collagen.^[65] Interestingly, this same mechanical-dependence was not observed for SMCs cultured on type I collagen, suggesting that some benefits of cyclic strain may be dependent on a combinatorial ECM-specific effect. The effects of long-term cyclic strain showed that constant application of strain over 10 weeks improved cell density, promoted cell alignment in the direction perpendicular to cyclic strain, and constructs contained almost 50% more elastin than control non-strained constructs.

Although methods to produce most forms of *in vitro* stretches are achieved by physical deformation of the underlying substrate, cells may also be stretched by application of a pulling force on the cell surface. Saldana et al.^[66] utilized an indirect method of cyclic stretch on human MSC were cultured on either rough or smooth metal disks. In addition, small collagen-coated metal beads were allowed to attach to the cell's dorsal surface and a ceramic magnet generated constant tensile force by pulling on the beads to stretch the cells vertically. Without mechanical stimulation cells expressed VEGF to a greater extent on rough surfaces; however, the surface preference switched when cells were tensed and greater VEGF secretion was observed on smooth surfaces. Mechanical stimulation increased total focal adhesion kinase (FAK) as well as FAK phosphorylation on rough surfaces but no such effect was seen on smooth surfaces. This study highlights the impact of combinatorial surface topography and mechanical stimulation on modulating cell behavior.

5.3. Compression/Contraction for Differentiation of Cardiac Cells and Tissues

During systole, the heart tissue contracts in response to electrochemical signals. Although cardiomyocytes are known to spontaneously contract in culture, enhanced engineering of cardiac constructs can be achieved by preconditioning with an electrical stimulus. Biowires, created from collagen-soaked sutures were seeded with cardiomyocytes derived from human pluripotent cells and electrically stimulated.^[67] Improved sarcomeric organization and maturity was noted in the stimulated biowires as well as improved electrical properties including decreased excitation threshold and increased maximum capture rate. Combined mechanical and electrical stimulation is a common approach for engineering cardiac tissue constructs in a bioreactor environment. Perfusion culture coupled with electrical stimulation of neonatal rat cardiac cells in a porous channeled elastomer scaffold demonstrated improved contraction amplitudes, cardiac protein expression, and cell and tissue morphology and organization compared to unstimulated groups.^[68] Similarly, differentiation of MSC towards cardiomyocyte phenotypes was achieved with coordinated mechanical strain and electrical stimulation.^[69]

Since cardiac tissue requires propagation of electrical signals, use of electroconductive biomaterials such as carbon nanotubes and fibers (CNT and CNF, respectively) enables nanoscale control of the electrical field. Incorporation of these CNTs into composite scaffolds creates conductive matrices, which permit more efficient transmission of external electrical stimulation as well as serves as a platform to enhance the spontaneous conduction between cardiac cells. MSC grown in medium containing CNTs or on composite CNT/PLA scaffolds showed increased cellular alignment when an electrical stimulus was applied such that cells re-orient perpendicular to the direction of the current.^[70] In addition, after 14 days of electrical stimulation the cells acquired a cardioprogenitor phenotype characterized by gene and protein expression of cardiac myosin heavy chain (cMHC), cTNT, and Cx43.

5.4. Combinatorial Approaches to Engineering Vessels and Cardiac Tissue

Ultimate engineering of functional cardiovascular tissue will likely involve a combinatorial approach that incorporates multiple cell types and forms of biomechanical stimulation. Engineered cardiac tissue must be well vascularized and withstand a physiological load of both stretch and compression. Mechanical uniaxial stress applied to collagen constructs seeded with human ESC- and iPSC-cardiomyocytes enhanced cell and fiber alignment as well as promoted myofibrillogenesis and sarcomeric banding.^[71] Combination with ECs increased cardiomyocyte proliferation and addition of stromal cells improved vasculogenesis 10-fold. Combination of nanotopography with cyclic stretching was used to improve the biomechanical properties and organization of cardiac cells.^[72] Dynamic tensile stretch of microporous chitosan-collagen scaffolds engineered with 200 μm parallel channels seeded with neonatal rat cardiac cells produced constructs with high tensile strength, aligned cells, and expression of cx43, the primary cardiac connexin common in the ventricular myocardium. Pairing of perfusion bioreactors with electrical stimulation that allows for unconstrained (non-isometric) contractions are proving to be an essential strategy for optimizing functional outcomes. A recurring theme in tissue engineering continues to be that the more closely the *in vitro* culture system mimics the native physiological conditions, including the molecular and mechanical environment, the more closely the engineering tissues recapitulate native tissues in morphology, behavior, and function.

6. Applications of ECMs in Cardiovascular Tissue Engineering

Ultimately, the translational goal of modulating cardiovascular cell fate through nanotopography, ECM composition, substrate rigidity, and mechanical and electrical stimulation is to engineer cardiac patches and vascular grafts that mimic the structure and function of native tissues shown in Figure 3.

6.1. Cardiac Patch

Recent advances in cell biology and biomaterials have progressed the concept of regenerating cardiac tissue close to reality. As a proof-of-concept, Zimmermann et al.^[73] created cardiac tissue that was 4 mm in thickness and 15 mm in diameter using cells from a neonatal rat heart. This engineered tissue was able to generate contractile forces and also couple to the native myocardium *in vivo* after transplantation into rats suffering myocardial infarctions. Caspi et al.^[74] constructed a 3D muscle composed of cardiomyocytes, ECs, and

embryonic fibroblasts using human ESCs. Combinatorial use of soluble factors with an ECM framework has been shown to enhance generation of cardiac patches. Dvir et al.^[75] first seeded neonatal cardiac cells in an alginate scaffold that was able to bind to pro-survival and angiogenic factors, and vascularized this cardiac patch on omentum before transplanting it into infarcted rat heart. The results showed significant improvement in cardiac function *in vivo*. Recent efforts were made to apply biomaterials in order to increase the viability and improve the function of cardiac patches. Huang et al.^[76] developed a cell sheet culture system, in which EB-derived cells can be uniformly cultivated in 3D scaffold that is coated with collagen. After a series of *in vitro* culture, thick cell sheets can be generated with significant high mechanical strength.

Gaebel et al.^[77] generated cardiac patches using laser printing technique. They seeded human umbilical vein endothelial cells (HUVEC) and human mesenchymal stem cells (hMSC) and evaluated the patch in infarcted rat hearts. Left ventricular catheterization was performed 8 weeks afterward and a significant functional improvement was observed, with enhanced capillary density and integration of human cells into murine vascular system. A similar study was performed by Kim et al.^[78] using nanopatterned cardiac patches of cardiosphere-derived cells. Higher cell viability and collagen organization were reported with increased thickness of ventricle walls. More recently, Bursac's group successfully developed a 3D fibrin cardiac patch that contained aligned human ESC-CMs.^[79] A conduction velocity of 25 cm/s was reported for these patches, reaching similar levels of 2D monolayer of cardiomyocytes. Moreover, contractile forces were also improved with enhanced gene expression of cTnT, α MHC, CASQ2 and SERCA2.

When bioengineered tissues exceed the thickness that permits diffusion, the vasculature becomes extremely important in providing efficient nutrition supply and waste removal. Stem cell-derived ECs are believed to be an ideal candidate for vascularization of thick tissue constructs. ECs interact with other cell types *in vivo* to regulate cell proliferation, survival, and migration. It is generally agreed that tissue constructs consisting only of cardiomyocytes survive poorly after transplantation. However, when ECs and stromal cells are included in engineered cardiac constructs, these cells were able to organize into endothelial networks and integrate into the host circulation^[74]. Later studies confirmed that pre-vascularization of constructs improves cell survival and function after transplantation.^[75,80] Thereby, the combination of multiple cell types have additive or synergistic effects in improving the survival or function of engineered tissues, likely through cell-cell interactions or paracrine factors.

6.2. Vascular Graft

Autologous grafts from the saphenous vein are the standard of care for patients in need of vascular grafts. However, these vessels are often limited in supply and/or contain unhealthy endothelium. Therefore, bioengineered vessels may be a good alternative for providing a long-term patient-specific graft with a healthy endothelium.

There are a number of commercially available acellular vascular grafts including polytetrafluoroethylene (ePTFE) grafts (Propaten®) grafts with bioactive heparin conjugation for reduced thrombosis, Intergraft® which resists kinking and compression, and

LifeSpan® which also utilizes ePTFE to construction grafts with a high burst strength and for peripheral vascular reconstructions and AV access. Although these and similar ePTFE grafts have been used clinically, challenges remain with small diameter grafts (< 6mm diameter) which face thrombosis, likely caused by the lack of an autologous endothelial lining. While current *in vitro* bioengineering approaches contain a cellular component to their grafts, a persistent issue is long-term patency and graft integration at the sutured edges.

Huang and colleagues engineered aligned vascular grafts that mimic physiological EC orientation with the goal of enhancing long-term patency.^[32,33] Atherosclerotic plaques and obstructions build up at the bends and bifurcations of blood vessels, which are also the regions that experience disturbed flow conditions. In these areas of disturbed flow, the ECs are randomly oriented; in contrast to endothelium in the straight segments of vessels, which is aligned along the direction of laminar flow. Parallel aligned collagen fibrils were shown to direct the nanofibrillar alignment of EC along the ECM micropattern, resulting in reduced monocyte and platelet adhesion compared to randomly oriented fibrils. The use of matrix nanotopography for modulating EC function, coupled with application of iPSC-derived cell types, is a useful combinatorial approach for generating patient-specific vascular grafts with improved patency.

Like many other tissues and organs, application of decellularization methods to obtain an acellular ECM scaffold holds promise including resistance to dilatation and calcification in addition to adequate patency.^[81] Bourget et al.^[82] developed a vascular matrix from the rolled ECM of decellularized dermal fibroblasts or saphenous vein fibroblasts seeded with SMCs to demonstrate improved mechanical loading and vascular reactivity compared to self-assembled SMCs rolled sheets. There is an extensive range of studies that have utilized either decellularized ECM derived from native tissues, or secreted ECM by cultured cells. For a more extensive scope of decellularized tissues for cardiovascular engineering, please refer to studies by Ott et al.^[83] for heart regenerative approaches and the review by Song et al.^[84]

A different approach to creating alternative vascular grafts for patients is the *in situ* capture of circulating endothelial progenitors. Both CD34 and VE-cadherin antibody-bound stents have been reported to capture ECs from the arterial bloodstream.^[85,86] Although cell attachment to these stents resulted in close to full coverage by cells, the possibility of non-specific binding of non-EPCs is likely. Recently, VEGF was explored as an alternative for selective adhesion EPCs. Co-stimulation of circulating peripheral blood mononuclear cells with arterial levels of laminar shear stress (15 dyn/cm²) on VEGF-bound surfaces had a synergistic effect to augment differentiation of endothelial progenitors towards mature ECs.^[87] Specifically, markers of EPCs and venous ECs were downregulated while markers of arterial ECs were upregulated. However, the limitations associated with this type of approach are the non-uniformity of cell attachment, neointimal coverage with SMCs and the eventual infiltration of inflammatory cell types and monocyte adhesion.^[86] While the concept of drug-eluting stents is far from new, application of this technology to current ECM-based bioengineered vascular constructs has yet to be fully explored and may be an interesting alternative approach for *in vivo* implantation and immune integration.

7. Role of ECMs for Modulating Cell Plasticity

Cell plasticity, characterized by the conversion from one to another cell type, is useful for understanding the pathology of healthy and diseased states in CVD. This transformative ability is also a potential tool for generating specific cell populations from more readily available and easily cultured pluripotent and multipotent cell sources. Cell plasticity occurs under physiological as well as pathological conditions, and the ECM environment can recapitulate those same transformations in a controlled manner to generate a desired cell phenotype.

7.1. Endothelial to Mesenchymal Transition (EndoMT) in Cardiac Fibrosis

Similar to the well-known cellular program of epithelial-mesenchymal transition (EMT) that plays a role in development, tissue repair and disease, is the process of endothelial-mesenchymal transition (EndoMT). Vascular ECs possess a characteristic squamous morphology and are tightly bound to the ECM and each other. During the process of EndoMT, cells lose expression of endothelial phenotypic markers such as VE-cadherin, CD31, and von Willebrand factor (vWF) and gain expression of mesenchymal markers including fibroblast-specific protein-1 (FSP-1), α -SMA, collagens types I and III, and vimentin.^[88] In terms of function, cells lose their cell-cell adhesions, become highly migratory, and reorganize their cytoskeletal into an elongated, spindle-like morphology. The mechanism of EndoMT is thought to act primarily through the TGF- β pathway as well as the notch signaling pathway.^[89] Ten Dijke et al.^[90] showed that shear forces can activate TGF- β signaling pathway in EC through cell cilia, which act as mechanosensors. For a detailed review of the molecular mechanisms of EndoMT please see Van Meeteren et al.^[91]

EC plasticity is key in the formation of the endocardial cushion in the heart during embryonic development.^[92] During this process, cells from the endothelial lining undergo EndoMT and migrate into the underlying ECM to form the septa and valves.^[93] Subsequently, EndoMT is also implicated in several cardiovascular disease states including cardiac fibrosis, atherosclerosis, and pulmonary hypertension.^[94] Cardiac fibrosis often results after myocardial infarction and is characterized by the development of scar tissue. Fibrosis develops from fibroblasts that excessively proliferate and deposit unfavorable ECM. In a landmark study by Zeisberg et al.^[95] it was shown via lineage tracing that 27–33% of cardiac fibroblasts in a murine model of cardiac fibrosis were endothelial in origin, which suggests that EndoMT plays a key role in cardiac fibrosis. Many dynamic changes occur to the ECM during EndoMT. Matrix metalloproteinases (MMPs) digest the basal lamina, which is replaced with ECM of a different composition, consisting mostly of Collagen types I and III as well as fibronectin.^[96] The transformation to a mesenchymal phenotype is paralleled by an upregulation of genes encoding the more traditionally stiff ECM proteins such as collagen type Ia1 (COLA1), which has implications in scar formation and fibrosis.

The role of EndoMT is further broadened by recent findings that vascular ECs that undergo EndoMT display multipotent stem cell-like properties. These cells demonstrated the ability to differentiate along the osteogenic, chondrogenic, and adipogenic lineages similar to bone marrow MSC.^[89] The ability to acquire stem-like properties during EndoMT has important

implication for use in tissue engineering but may also explain heterotopic ossification in which chondrocytes and osteoblasts in calcified tissues were shown to be endothelial in origin.^[89] Harnessing the stem-like potential of ECs by exploiting this EndoMT program may provide a useful tool for obtaining an autologous population of cells that are less abundant such as SMCs.

A major cell component of small diameter blood vessels (3–4mm in diameter) are SMCs, which surround the endothelium to provide mechanical structure and strength. A challenge of engineering vascular grafts is isolation of a pure population of SMCs as well as expansion to a sufficient quantity required for a clinically relevant tissue construct. Generation of SMCs via EndoMT may circumvent these limitations. Krenning et al.^[97] transdifferentiated neonatal ECs into cells that expressed smooth muscle proteins, SM22 α and α -SMA, and contracted the collagen gels on which they were cultured. Similar EndoMT transdifferentiation was performed in 3D collagen sponges, which establishes the foundations for differentiation of ECs into SMCs directly in a vascular scaffold.

7.2. Applications of ECMs for Wound Healing: Differentiation of Fibroblasts to Myofibroblasts

During the physiological process of wound healing, fibroblasts in nearby connective tissue are activated to become myofibroblasts, cells that are phenotypically similar to SMCs. This process is well documented and necessary for beneficial tissue remodeling and reconstruction; however, the presence of myofibroblasts over an extended period of time can lead to many diseased states of tissue fibrosis.^[98] In response to changes in the composition and mechanical properties of the ECM, fibroblasts respond by taking on migratory properties and form contraction bundles composed of small cytoskeletal actins. This enables initial and fast repopulation of the damaged tissue. As a consequence of the increased tractional forces generated during this first migratory phase, these proto-myofibroblasts transition into differentiated fibroblasts that express α -SMA. Myofibroblasts display increased ECM deposition and contractile properties and are removed by apoptosis after tissue repair. However, the persistence of myofibroblasts is responsible for a number of fibrotic tissue states and the transdifferentiation of fibroblasts to myofibroblasts is a hallmark process seen during the acute proliferative phase following myocardial infarction.^[99] It is therefore of interest to understand the mechanism of the fibroblast to myofibroblast conversion as well as what microenvironmental cues promote maintenance of the myofibroblast phenotype in order to reverse the process and mitigate fibrosis.^[100]

Matrix stiffness has been shown to modulate differentiation of fibroblasts into myofibroblasts. Increased stiffness is consistently associated with increased transdifferentiation towards a myofibroblast phenotype. Valvular interstitial cells were activated to become α -SMA expressing myofibroblasts when cultured on photodegradable hydrogels with high (32 kPa) elastic moduli, but remained inactivated when cultured on gels with low (7 kPa) elastic moduli.^[101] Decreasing the elastic moduli of the stiffer hydrogel by irradiation, led to deactivation of the myofibroblasts, which demonstrated a decrease in expression of α -SMA in the myofibroblasts. These cells never recovered expression of α -SMA and would likely require transfer back to stiffer substrates to induce complete re-

activation to α -SMA⁺ myofibroblasts.^[102] It is thought that the mechanism through which matrix stiffness regulates myofibroblast differentiation is by modulating actin polymerization, which in turn, causes the nuclear translocation of megakaryoblastic leukemia factor-1 (MLK-1), a cofactor that plays a role in regulating expression of fibrotic genes.^[103]

Other modulators of myofibroblast transdifferentiation are the ECM composition and matricellular proteins. The extracellular matrix is a potent modulator of fibroblast phenotype and function in the infarcted myocardium. Nanofibrillar collagens such as collagen type IV have been shown to induce myofibroblast transdifferentiation and its disruption attenuated fibrosis.^[104] Artificial extracellular matrix composed of collagen I and highly sulfated hyaluronan was shown to interfere with TGF β 1 signaling by blocking the receptor binding site and thereby prevent TGF β 1-induced myofibroblast differentiation.^[105] Matricellular proteins such as thrombospondin (TSP) are non-structural ECM macromolecules that serve as links between the matrix and the cell body. They are not present in the normal heart but are expressed during cardiac remodeling. TSP-1 loss correlates to reduced myofibroblast transdifferentiation as well as collagen IV deposition. Although this protein may elucidate a potential molecular pathway to target to modulate transdifferentiation, any clinical translation of a cell-matrix pathway interference approach for myocardial infarction will be challenging due to the complexity and extreme heterogeneity of the pathological response between patients.^[99]

7.3. Potential Role of ECMs for Nuclear Reprogramming and Transdifferentiation

An intriguing approach for generation of cardiovascular cell types is the reprogramming of fibroblast to cardiomyocyte, endothelial, and smooth muscle types. By generation of partially induced pluripotent stem cells by reprogramming with the four factors (OCT4, SOX2, KLF4, and c-MYC) for 4 days followed by culture with endothelial specification medium.^[106] These cells expressed endothelial markers such as VE-cadherin and improved neovascularization in a hindlimb ischemia model. This same potential for transdifferentiation was shown could be accomplished with only 2 reprogramming factors.^[107] It therefore stands to reason that since we have seen that ECMs can regulate endothelial-mesenchymal transitions, they may be potentially useful tools to enhance the differentiation and efficiency of reprogramming and transdifferentiation.

8. Future Direction/Limitations

In summary, modulation of ECM physical, biological, and mechanical properties provides unique microenvironmental cues and stimulation of mechanotransductory pathways that direct cell phenotype, function, and fate. Although ECMs are extremely promising tools for engineering cardiovascular tissues there are several limitations that must be overcome for effective application as tissue-engineered therapies. Many cell types required for cardiovascular engineering are not readily available or are not highly proliferative, making scalability of cell requirements a major challenge. Therefore, novel strategies that enhance proliferation and efficiency of differentiation of stem and progenitor cells towards cardiovascular phenotypes are needed. Furthermore, the nanoscale benefits of micro- and nanopatterned substrates as well as rigidity-based approaches will be difficult to scale up to

larger constructs in which cells would need to exist as 3D multilayered constructs and would therefore be less sensitive to ECM properties that are most effective on a monolayer of cells. Understanding the science underlying substrate-based cell fate specification will provide the necessary insight to generate cardiovascular cells and tissues.

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Biography

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Dr. Huang is an assistant professor of Cardiothoracic Surgery at Stanford University and Principle investigator at the Center for Tissue Regeneration, Repair, and Restoration at Veterans Affairs Palo Alto Health Care System. Her laboratory aims to understand the chemical and mechanical interactions between the extracellular matrix proteins and pluripotent stem cells that regulate cardiovascular and myogenic differentiation. The knowledge gained from understanding cell-ECM interactions are applied towards engineering prevascularized skeletal or cardiac muscle constructs.

Figure 1. Microenvironmental modulation of cell morphology and fate

The chemical, physical, and mechanical properties of the ECM influence cell shape and behavior.

Figure 2. Influence of substrate nanotopography or stiffness on cell morphology
Scanning electron microscopy (SEM) of ECs cultured on aligned microfibers and align in the direction of the fibrils, a) scale bar= 100 μm or b) scale bar= 10 μm . c) Human ECs cultured on a hydrogel with a rigidity of c) 12 kPa or d) 50 kPa, scale bar= 50 μm .

Figure 3. Cardiovascular engineering applications for ECM-induced cell fate determination
 Stem cells or dermal fibroblasts, obtained from a patient biopsy, can be cultured in an ideal microenvironment (ie appropriate chemical, spatial, and mechanical properties) to promote lineage specific differentiation into cardiovascular cell populations. These cells have therapeutic utility in the engineering of cardiac patches for damaged heart tissue, or vascular grafts to address for repair of the vasculature.