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Mechanical influences on Cardiovascular Differentiation and Disease Modeling

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Abstract

Tissues are continuously exposed to forces in vivo, whether from fluid pressure in an artery from our blood or compressive forces on joints from our body weight. The forces that cells are exposed to arise almost immediately after conception; it is therefore important to understand how forces shape stem cell differentiation into lineage committed cells, how they help organize cells into tissues, and how forces can cause or exacerbate disease. No tissue is exempt, but cardiovascular tissues in particular are exposed to these forces. While animal models have been used extensively in the past, there is growing recognition of their limitations when modeling disease complexity or human genetics. In this mini review, we summarize current understanding of the mechanical influences on the differentiation of cardiovascular progeny, how the transduction of forces influence the onset of disease, and how engineering approaches applied to this problem have yielded systems that create mature-like human tissues in vitro in which to assess the impact of disease on cell function.

Keywords

Stem cells; Extracellular Matrix; Shear Stress; Stiffness

Introduction

When first identified and then isolated nearly two decades ago, the promise of embryonic stem cells rested in their regenerative potential, to assist the body where natural processes either incompletely as with scarring or completely as in heart disease failed to maintain or regenerate function. With their ability to differentiate into many specific cell types, a vast number of chronic diseases were thought to have a new therapeutic option. While expectations have been significantly tempered since then, due to safety and efficiency concerns among others, their ability to mirror complex disease in vitro, especially when patient-specific genetics (using induced pluripotent stem cells or iPSCs) and appropriate

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unique environmental conditions are present, has provided a new avenue of research over the past several years with relatively easy entrance into the field. For example, iPSCs can now be created from easily accessible cell types such as fibroblasts, keratinocytes, and peripheral blood mononuclear cells and numerous companies and core facilities are available to create patient-specific lines for researchers. However, the challenge remains with a second caveat that an appropriate cellular environment should be present to ensure appropriate differentiation and separately to model disease-in-a-dish [1]. Without these conditions, differentiation efficiency and purity may be lower and specific disease-like behaviors may not occur [2]. As such, we focus this mini review on a summary of the state-of-the-art in modeling diseases-in-a-dish [1, 3], first examining how the surrounding niche and its mechanical properties have been used to optimize differentiation and secondly to induce disease-specific conditions and monitor cell outcomes.

Mechanical Influences on Stem Cell Differentiation into Cardiovascular Lineages

Many recent investigations have taken advantage of our current understanding of developmental biology to create protocols for specific cell type differentiation, including cardiovascular progeny such as cardiomyocytes [4], endothelial cells (ECs) [5], and smooth muscle cells (SMCs) [5]. These protocols typically involve the addition of certain key growth factors added to stem cell media to take advantage of the biological and chemical cues involved in embryogenesis to induce mesendoderm differentiation pathways. Differentiation protocols range in duration from weeks to months, depending on cell types. Given that cardiovascular cells have common progenitors [6], purifying techniques including cell sorting and selection media are often necessary to ensure high purity. While typically highly defined, protocols can also involve 3D culture as embryoid bodies (EBs) [7] or in 2D on tissue culture plates coated with Matrigel or other matrix ligands [8] to create cardiovascular progeny. However while stem cells respond to numerous environmental factors, recent evidence has suggested that physical parameters are as important as the chemical ones that have been described above [9, 10]. For reference, we have summarized the common differentiation stages and the markers that describe them in Figure 1 from references [4–8] noting that this is not an exhaustive list either in terms of stages or markers (shown in red). We also include, as known, a summary of where mechanical cues, be they active or passive, influence differentiation for each lineage and the maintenance of PSC/iPSC self-renewal (indicated by blue numbers corresponding to specific mechanical cues in the dashed box). As described below, mechanical cues are often applied throughout differentiation such that they are not specific to a given stage, applied to culture conditions where their influence is less direct, e.g. multicellular EBs, or applied to further enhance chemically defined protocols at their conclusion or to alter the phenotype of a committed cell. Concurrent with growth factor-driven primitive heart field specification through mesoderm induction and cardiac specification, significant changes in mechanics, both matrix deposition and pressure changes [11], drive progenitor cell fate in the early heart in vivo. Subsequently mechanical demands can change frequency- and length-dependent contractility, allowing the mature tissue to accommodate beat-to-beat variability as organism activity changes. Active mechanical stretch, when integrated into differentiation protocols,

however, may be dose and context specific [12–14]. Since beneficial conditions have been less chemically defined, others have examined the influence of passive properties, such as stiffness of the culture substrate, or when these material properties are varied in space or time. In a small range of substrate stiffness that is developmentally appropriate for the heart, nascent cardiac muscle organizes itself better [15, 16]. More recently, dynamic changes in the substrate, which better mirror developmental changes in matrix [11], have been shown to further improve specification of pre-cardiac mesoderm [17]. Despite all of these inductive cues, a chronic problem with cardiomyocytes is their inability to induce functionally mature cells from PSCs or iPSCs, i.e. they are unable to replicate phenotypic and mechanistic characteristics of primary tissue. To combat this maturation problem, it is becoming increasingly popular to differentiate cells in chemically defined cultures for 60+ days [4], and, once at the immature cardiomyocyte stage, to add external stimuli to further enhance maturation. For example, functionally mature cells form gap junctions allowing for the electrical connections between cells, and when exposed to 10% cyclic stretch, immature cells form more robust junctions [18]. Equally common are studies that explore myofibrillogenesis in immature or neonatal cardiomyocytes where mechanical influences drive the assembly of the contractile apparatus [15, 16]. It is important, however, to note that cell origins with these studies are not always PSCs and thus should only be seen as separate from those mentioned above that look at de novo influences of mechanics on stem cells.

In addition to influences on cardiac specification, differentiation, and maturation, vascular progeny are also influenced by mechanical stimuli. Endothelial cells, which line the vessel wall, are a perfect example of these effects. Pluripotent stem cells (PSCs) or their endothelial progenitor cells (EPCs) can take on mature characteristics when placed under physiological shear stress [19]. While additional purification by fluorescence activated cell sorting is required, it is important to note that unlike cardiomyocytes, mechanically-mediated signaling is an important component of EC differentiation in chemically defined conditions, so long as shear remains physiological; under normal flow of 10–20 dynes/cm^2, shear stress is felt by the cytoskeleton as an 'outside-in' signal [19–22]. However when flow becomes pathological in magnitude or disturbed (i.e. not laminar), differentiation of progenitors and critical functions of mature endothelial cells, e.g. tight junction maintenance, become compromised. For example, iPSC-derived microvascular endothelial cells form tight junctions [23], but upon exposure to increasing flow, junctions remodel and EC monolayers become more permeable [21, 24]. Underlying these changes are mechanosensory complexes, which are also implicated in controlling progenitor maturation [25]. Thus when aberrant flow is present in the developing embryo or in microphysiological systems meant to recapitulate differentiation conditions, cells fail to completely mature. While ECs are typically differentiated from iPSCs or PSCs [5] for disease modeling, vessel lumens constantly turn over in vivo, in which case they are created from EPCs. These cells, which are recruited from bone marrow into peripheral blood, circulate and attach to the existing endothelial lumen, transmigrate, and then replace, repair, or create neo-vessels; luminal damage, due in part to excessive shear stress or disturbed flow [21], recruit EPCs, and evidence suggests that as they home to the injury site, EPCs become mechanosensitive [26]; after adhering to the lumen, EPCs upregulate canonical EC markers and reinforce cell-cell and cell-matrix adhesive structures in a shear stress-dependent manner in order to

transmigrate [20]. Whether arising from PSCs or EPCs, EC differentiation is also sensitive to stretch, as in vivo vessels are constantly stretched from cyclic changes in pressure [21]. While its effects are less clear for ECs, mechanisms at the cell membrane most likely differentiate between mechanical inputs and determine the differentiation pathways to be activated. Aside from shear stress, other mechanical cues, including passive matrix stiffness [22, 27], compression [28], and vessel pressure [9] also contribute to final EC specification but evidence of its specific influence at progenitor stages is not clear to date.

Along with ECs, SMCs are also mechanically active vascular cells that are clearly involved in many disease processes. There is extensive literature on chemically defined differentiation protocols to create SMCs from iPSCs [5] and adult stem cells [29] based on developmental pathways [30], but the effects of mechanics on their differentiation from iPSCs or PSCs is less certain. Significant effort in mechanics has been focused on its effects on adult stem cells, e.g. alignment relative to the stretch axis, although a variety of disparate effects have been observed, e.g. perpendicular or parallel alignment, increased SMC gene expression, etc. [29]. Significant effort has also focused on phenotype plasticity once committed to the SMC lineage (hence the mechanical cues noted in Figure 1 do not point to SMC differentiation pathways). This latter focus is due in part to significant changes that SMCs can make in the medial layer of the vessel, which causes them to invade the tunica intima, form plaques, and alter local hemodynamics. As such, we will leave the majority of our discussion of mechanical influences on SMCs for subsequent sections on the impact of disease and the use of iPSCs for disease modeling.

Disease-mediated Mechanotransduction as a basis for In Vitro Modeling

Mechanics influences nearly every major solid organ and its disease processes, but central to most cardiovascular diseases is altered transduction of signals within or between cells or layers of cells; these signals can alter cell phenotypes to exacerbate disease outcomes. As the cardiovascular field has known over more than two decades, ECs and SMCs are able to maintain physiological homeostasis by reciprocally modulating barrier function via junctions and vessel tone via contracting [21, 31]. Indeed, vessels and the heart wall are elastic and capable of dynamically responding to physiological changes in the microenvironment, e.g. variations in shear stress from blood flow (10–20 dynes/cm^2), arterial pressure up to ~20 kiloPascal, and artery wall strains up to ~100 kiloPascal [32]. Should mechanical input begin to fall outside of the range where cooperative mechanical signals–either direct or indirect–can adjust cell function, cells plastically change phenotype, which can induce disease [9, 10, 31, 32]. Atherosclerotic plaque formation is one such example where disturbed flow can cause oxidative stresses and lipoprotein accumulation in ECs, inflammatory cells then home to the site whereupon they take up the lipoprotein and become foam cells, and finally SMCs are recruited to form a fibrous cap on the plaque [33]. For each of these cell types, mechanical signals help in part to recruit cells involved or to create conditions that caused phenotypes to change. One classic example at the cellular level is SMC phenotype; although a relatively small fraction that contributes plaque formation in vivo [34], significant evidence in vitro describes conditions where excessive stretch, compression, and shear will transform SMCs from 'contractile' to 'synthetic' via changes in morphology, proliferation, migration, and marker protein expression [35]. While some

heterogeneity can be beneficial, it is clear that plaque formation among many diseases induce permanent phenotype changes in vascular cells that are detrimental to function. While vessels highlight crosstalk between vascular cell types, cardiomyocyte contributions to heart disease in the myocardium highlight crosstalk between pathways within a cell. Multiple intra- and extra-cardiomyocyte structures, e.g. sarcomeres, intercalated discs, and the extracellular matrix, remodel during disease progression and contribute to pathology to varying degrees. Within each of these structures, multiple protein complexes have been identified [36], but their specific contributions to disease are uncertain; for our purposes however, it is perhaps more helpful to consider the functional consequences that can subsequently be modeled in vitro. For the sarcomere, which serves as point of contractile force generation in cells, mutations disrupt assembly and attenuate contractility. Mutations in titin for example, a protein that helps set sarcomere length, cause dilated cardiomyopathy by impairing sarcomere formation [37] and alignment [36]. For intercalated discs, which electrically connect myocytes together, defects arising from missing components such as the connexins impair conduction and can cause arrhythmogenic cardiomyopathies [38]. Beyond specific mutations, cardiomyocyte hypertrophy, i.e. an increase in cell size, enhances protein synthesis, heightens organization of the sarcomere, but can also lead to specific dysfunction within the myocyte [39]. While these examples focus inside the myocyte, mechanical transductive defects across the heart wall itself, such as wall strain, influence not only lineage commitment [12, 13, 18] but also contribute to cardiomyopathies when excessive [40]. This can activate other myocardial cells, including cardiac fibroblasts, via signals transduced by extracellular matrix. These cardiac fibroblasts, in the event of cardiac dysfunction, begin to secrete ECM proteins, resulting in a stiffening of the localized cardiac injury. Such matrix deposition and remodeling, while possibly salvaging mechanical functionality temporarily, ultimately alters the heart wall mechanical flexibility and stiffness. An increase in mechanical stiffness has multiple negative consequences for the myocardial cell population. Increases in myofibroblast populations of fibroblast lineage increases further matrix deposition, cell motility, and inflammatory signaling such as TNFalpha. The increase in inflammatory signaling recruits more macrophages to the sight of injury which they themselves increase additional inflammatory cytokine release, perpetuating a chronic inflammation cycle. The increase of surrounding stiffness further leads to cardiac dysfunction, such as hypetrophy, as well as increased contraction stress by cardiomyocytes. Thus, while we focus on myocytes specifically, other cell types within the heart play a role in disease progression.

Improvements on Disease Modeling with iPSCs and Mechanics

While mammalian models are closely related to the human condition, there remain key genetic and physiological differences between human and mammalian models, particularly mouse and rat models. For specific genetic questions involving mechanotransduction like those raised above, mammalian systems are inappropriate or fail to recapitulate key hallmarks of human disease [41, 42]. Cadaveric models or primary human cells could be ideal in this situation, but many cell types and systems are difficult to study by the nature of those cell types lacking proliferative phenotypes or source acquiring difficulty, such as cardiac, endothelial, and neural tissue. Thus instead of acting in a confirmatory manner,

iPSC- or PSC-derived cell types are therefore a highly plausible option to investigate these systems and cell types as a reliable and consistent source for in vitro modeling and small scale drug testing.

Patient-specific iPSCs in particular are becoming more accessible and provide a steady source of *in vitro* human cells. They have become a standard means of modeling the genetic underpinnings of disease-in-a-dish [3] with minimal concern for patient variability, reprogramming vector, and differentiation protocol heterogeneity that existed even a few years ago [43, 44]. Recent technological advances in the ease of iPSC derivation, in cardiovascular differentiation protocols [4, 5], and in relatively straightforward genome editing techniques [45] enable one to insert or delete patient-specific variants and create standard, reproducible, and isogenic models. Gene editing techniques now even allow knocking in or out mutations over a range up to several thousand kilobases. This can enable isogenic comparison between two cell lines of the same cell type, providing a statistical comparison difficult to create otherwise. Using this methodology, the field has developed significant understanding about how mutations affect signaling [46] and cytoskeletal architecture [1, 3, 36, 37] in the progression of heart disease. Despite these advances in both technology and understanding of cardiovascular disease, new concerns have arisen about how widely applicable disease-in-a-dish models may be. One current concern raised with the disease-in-a-dish strategy is that the models typically developed now employ monocultures. While many differentiation protocols today aim to achieve as pure and mature a population as possible [4, 5], realistically, there is significant heterogeneity within a complex structure such as the endothelium, vessel, or myocardium, even within a single cell phenotype [35]; single populations are simply less representative. Complex diseases described above cannot arise when crosstalk is needed if only one cell type is present. The beneficial effects of co-culture models have been observed in organoids, so similar combinations in vessel or heart wall models may not be as technically challenging as one might expect. While appropriate tissue striation may not be apparent, initial attempts at multi-lineage cardiovascular tissues indicate some degree of mimicry with human tissue, at least in terms of assessing drug toxicity [47].

A second concern–and one directly applicable to mechanics–is the lack of 3D structure and context specific forces in many current disease models [1–3, 36, 37, 46]; 2D substrates in static cultures remain all too common [2]. Spatial complexity in disease modeling is necessary for understanding of propagational effects across tissue such as mechanical contraction and full construct deformation, disease-response signaling of co-culture in relation to spatial positions and motility of involved cells, and electrical conduction and impedence through sections rather than a sheet of tissue. Bioengineering efforts have attempted to begin addressing this using a variety of systems, including material-patterned cell rings (i.e. "tissue rings") [48], 3D meshes of many types and synthesis methods [49, 50], degradable natural or synthetic materials [51–55], and self-assembled multicellular structures (i.e. "tissue pillars") [56–58] among others. Specifically when using iPSC-derived progeny to create microphysiological systems, however, groups have emphasized the need to develop dynamic systems [59] that reproduce critical aspects of in vivo physiology [60] and electrical conduction [61]. Additional effects of external mechanical stimuli on 3D structure is an area in need of further investigation. When iPSC-derived cells from genetically

characterized diseased patients are used, influence from the genotype should be readily apparent. Similarly, drug responses should reproducibly alter the system's physiology as well [58, 62]. Figure 2 provides a summary of common approaches to building in vitro structures that better model disease.

Along with the need to increase the mechanical complexity of the niche in 3D to better model disease, it is important to note that these systems remain relatively small as they rely primarily on diffusion for nutrient transport. While an engineered vasculature would directly address this third concern, the efforts described above remain focused on constructing larger vessels that could be scaled down. Similarly the engineering challenging of scaling up to larger and more interconnected systems remains largely unsolved. Thus the success of future attempts at disease modeling and understanding disease mechanisms using in vitro systems hinges on solving these concerns.

Conclusion

Stem cells are a promising technology with the potential to be utilized at the bedside in addition to their current role in the lab. New clinical trials continue to investigate how stem cells can be used. Yet given the experiment challenges that are ahead, stem cells remain an excellent tool to investigate difficult to attain human tissue, model disease and genetic conditions, and observe phenotypic changes, e.g. smooth muscle transition from contractile to synthetic. However, one must be aware that while stem cells offer much flexibility and ease of acquisition in comparison to some human tissue samples, the concern of tissue maturity and complexity remains present in studies involving stem cells. With additional advancement in stem cell culture and differentiation technique, which could involve simultaneously biologically, chemically, and mechanical stimulation for differentiation, the perspective on stem cells as a promising clinical and investigative tool for understanding and treating human disease and genetic conditions continues to grow brighter.

Acknowledgements

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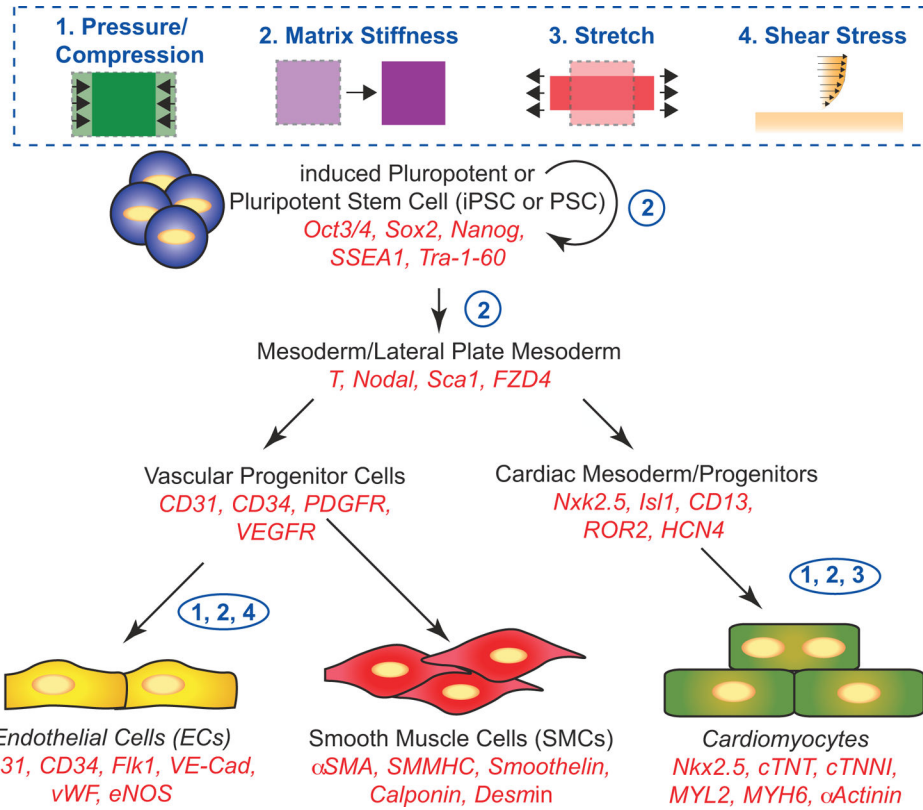
Fig 1: Mechanical Influences on Cardiovascular Progeny.
This schematic depicts stem cells and their key progenitor stages during chemically mediated differentiation protocols; black arrows denote cells' progression through the differentiation process (circular arrow at top denotes self-renewal). Red italics indicate genes characteristic of each stage. Mechanical cues and/or signals are shown in the blue box (top) and circled numbers by each arrow indicate the differentiation stage at which each can have an effect.
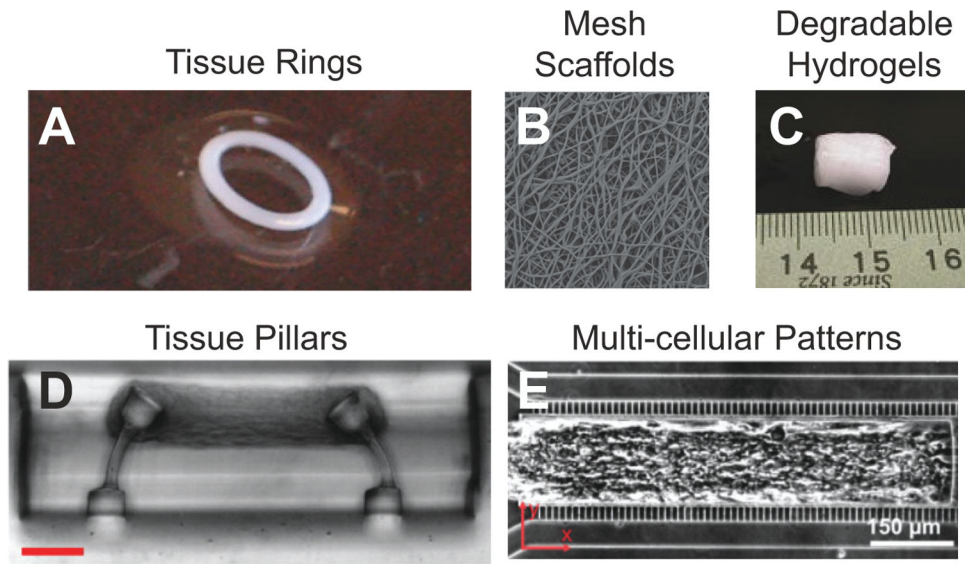
Fig 2: Mechanically active disease models in a dish.
Images illustrate the breadth of fabrication methods employing bioengineering approaches to create systems that mimic cardiovascular mechanics in vitro to better model disease. Specifically labs have created tissue rings where embedded cells contract a matrix polymerized around a central post resulting in a tissue ring (A), electrospun scaffolds where cells migrate throughout the material to create a 3D tissue (B), polymerized hydrogels with cells embedded in it (C), embedded cells in a matrix that are allowed to contract around multiple posts and is often flexible to report contractile force (D), or created chambers where multicellular tissues are exposed to controlled flow (E). Images are reproduced from references [48], [50], [55], [56] and [62], respectively.