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Concise Review: Growing Hearts in the Right Place: On the Design of Biomimetic Materials for Cardiac Stem Cell Differentiation

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ABSTRACT

Tissue engineering aims at recapitulating permissive conditions that enable cells to collaborate and form functional tissues. Applications range from human tissue modeling for diagnostic purposes to therapeutic solutions in regenerative medicine and surgery. Across this spectrum, human stem cells are the active ingredient, expandable virtually indefinitely and with the propensity to generate new tissue. Engaging lineage-specific differentiation requires a precise concerto of key spatial and temporal factors, such as soluble molecules and growth factors, but also physical and mechanical stimuli. These stimuli compete to modulate distinct developmental signaling pathways and ultimately affect the differentiation efficiency. The heart is a chemo-mechano-electrical biological system that behaves as both a sensor and an actuator. It can transduce electrical inputs to generate mechanical contraction and electrical wave propagation. Such a complex organ arises from multipart developmental events that interact with one another to self-regulate. Here, we overview the main events of heart development and the role of mechanical forces in modifying the microenvironment of the progenitor cells. We analyze the cascades regulating cardiac gene activation to illustrate how mechanotransduction is already involved in the most popular protocols for stem cell differentiation (SCD) into cardiomyocytes. We then review how forces are transmitted to embryonic stem cells by cell-substrate or cell-cell communications, and how biomaterials can be designed to mimic these interactions and help reproduce key features of the developmental milieu. Putting this back in a clinical perspective, many challenges needs to be overcome before biomaterials-based SCD protocols can be scaled up and marketed. STEM CELLS *2015;33:1021–1035*

INTRODUCTION

Rationale for Cardiomyocyte Production

Beyond chemical signaling, mediated by soluble growth factors, providing the foundation for stem cell differentiation (SCD) protocols [1], there is growing evidence that environmental cues are also of prime importance in guiding differentiation events [2]. Indeed, it appears that the whole stem cell niche is important in determining cell fate. In addition to chemical factors (including transcription factors and other proteins), oxygenation [3], extracellular matrix (ECM) proteins [4–7], innervation, support cells [8, 9], and mechanical loading [10] are some of the key parameters that have been identified. In the heart, cardiac cells form anisotropic layers able to contract in response to electrical signals. Therefore, mechanical properties are thought to contribute to differentiation and further maturation during embryogenesis. Here, we focus on the impact of the mechanical and topographical properties of materials used for cell culture on the differentiation of stem cells into cardiomyocytes. Adequate differentiation of stem cells into cardiomyocytes has significant medical applications offering the aptitude to recreate cardiac-like tissue for patient-specific in vitro drug toxicity assays [11, 12] as well as designing cell-based therapies for treatment of cardiac diseases [13–15]. Indeed, hopes were reinforced after historical observations on the benefits of human embryonic stem cell-derived cardiomyocytes (hESC-CM) on infarcted rat hearts [16] have been recently extended to non-human primate hearts [17, 18].

Mechanical Forces During Cardiac Development, Systems Biology, and the Main Cardiac Differentiation Protocols

With the formation of the four-chambered heart, the cellular arrangement of cells highly evolves from a cardiac crescent to a cardiac tube, followed by two looping events, the

formation of the four chambers, and finally septation (Fig. 1A). During these steps, differential growth occurs as well as increased blood flow and the initiation of electrical signals. Hence, cells are stretched, sheared, thereby resulting in different cell phenotypes at different stages of development [19, 20].

However, in vitro SCD is often realized only by activating signaling cascades mimicking the way they are activated during embryogenesis. This is achieved by using transcription factors [1], small molecules [1], or miRNAs [21, 22], often identified from high-throughput screenings [23–26].

The most used protocols involve modulation of developmental signaling pathway such as the canonical Wnt ([Wint] family of genes related to major developmental pathways. Wnt is a portmanteau word made of int and Wg, for "Wingless-related integration site.") pathway [25, 27–31], the Wnt/planar cell polarity (PCP) (noncanonical) pathway [32–35], TGF-β and bone morphogenetic protein (BMP) pathways [36–39], and all their combinations [40]. These protocols are increasingly efficient and simpler than the original ones [41], and while the first versions relied on reagents that were either difficult to translate to the clinics or simply too expensive, a lot of effort is now made in order to create the simplest cocktails possible [42].

While the two main pathways used in in vitro cardiac differentiation are the Wnt/β-catenin pathway and the BMP pathway, it is becoming clearer that the common denominators are mechanosensing and calcium signaling. The Wnt/PCP pathway is responsible for convergence/extension of the gastrulating embryo [43–45] while the BMP pathway and smad genes activation is promoted by membrane mechanosensors [46] as well as calcium signaling [47]. Calcium is also responsible for stabilization of cell-cell interactions through N-cadherin [48]. As N-cadherins are ultimately coupled to β-catenin [49–51] and α-catenin, overpresence of N-cadherin contacts at the membrane can trigger overactivation of N-cadherin as well as inhibition of β-catenin translocation in the nucleus, which is equivalent to Wnt/β-catenin inhibition or GSK3B overactivation [34, 52]. Another example of the importance of forces and calcium signaling is the establishment of the Left/Right axis, which precedes cardiac looping. Combining two models, it is thought that a right-to-left shear flow induces the generation of a gradient of growth factors, but also induces the bending of the primary cilia. Bending of the primary cilia will lead to the increase in calcium on the left side, cooperating with Nodal and BMP signaling to activate Pitx2 and trigger cardiac looping [20, 53, 54]. Finally, calcium is also one of the main actors of cardiac contraction, re-establishing the link between mechanical forces and calcium signaling (Fig. 1B).

Of importance, the Hippo pathway has also been found to be involved in cardiogenesis through the Yes activation protein (YAP)/TAZ molecules [55], already known to act as mechanical transducers in tissue-growth servo-regulation pathways [56, 57]. Similarly, myocardial differentiation was observed by GSK3β activation (i.e., Wnt/β-Catenin inhibition) in a signaling cascade involving the insulin-like growth factor pathways, under control of the YAP/TAZ pathway [58]. This is no longer surprising under the light of a study by Azzolin et al., where Wnt inhibition is reinforced by the presence of YAP/TAZ in the beta-catenin destruction complex, while Wnt activation triggers both YAP/TAZ and beta-catenin release and nuclear translocation, a phenomenon responsible for the inhibition of mesendodermal differentiation [59]. More details on the relationship between Hippo, Wnt, and SCD can be found in recent reviews by Hao et al. and Varelas [60, 61]. As further discussed later in this review, both the topography and the elasticity of the substrate influenced the fate of adult cardiac progenitor cells (CPC) through a YAP/TAZ-dependent mechanism [62]. Signaling studies on the relationship between the Hippo pathway, mechanotransduction, and cardiogenesis have not been translated to efficient SCD protocols yet, but increased evidence of their intricate relationships can be found in other models, like for mesenchymal stem cell (MSC) differentiation [63] or neuronal differentiation from induced pluripotent stem cells (iPSCs) [64].

In this review, we highlight the mechanotransduction effects in a hierarchical fashion, first by defining briefly how cells can sense forces, and then, zooming out on the physical forces generated by the cell-matrix interactions, the cell-cell interactions, aggregate mechanics, tissue mechanics, and finally the heart development itself. At each scale, we will summarize current knowledge regarding the biophysics of development and maturation of cardiomyocytes, and which techniques are available to recapitulate these environmental properties in a modular bottom-up fashion.

Debate 1: Murine Versus Human Cells. Differentiation studies using murine models are very important in the field of developmental biology. Like other model species (the fruit fly, the xenopus, or the zebra fish), they allowed identifying the main regulatory pathways that control embryonic development, with regard to both biological and mechanical behaviors. However, major differences exist between murine and human pluripotent cells. While murine ESCs (mESCs) can be cultivated on gelatin-coated Petri dishes with the only addition of leukemia inhibitory factor, human ESCs (hESCs) need a far more complex ECM coating (fibroblast feeder layer, Matrigel, or vitronectin for noncellular materials). Another distinction is the timing of development and of expression of membrane proteins. For instance, while induction of mesendodermal differentiation of mESC by BMP2 leads to CD15 negative cells, the same protocol on hESCs will give rise to a mesendodermal population of CD15-positive cells. Hence, the sorting will be reversed [65, 66].

Additionally, the beating rate of mature cardiomyocyte is completely different. While murine cells can be paced at more than 4 Hz (240 bpm), human cells prefer slower paces, of approximately 1–2 Hz (60–120 bpm). These interspecies differences thus need to be cautiously taken into consideration when trying to translate animal data into potential clinical applications. Still, in this review, many animal models will be referenced in an attempt to identify interesting mechanistic results or promising techniques that have not been translated to human cells yet.

Force Transmission: Cell—Cell Interactions and Cell—ECM Interactions

Interactions in the Developing Embryo

Before being able to adapt to different mechanical environments, cells need ways to sense the environment. Before

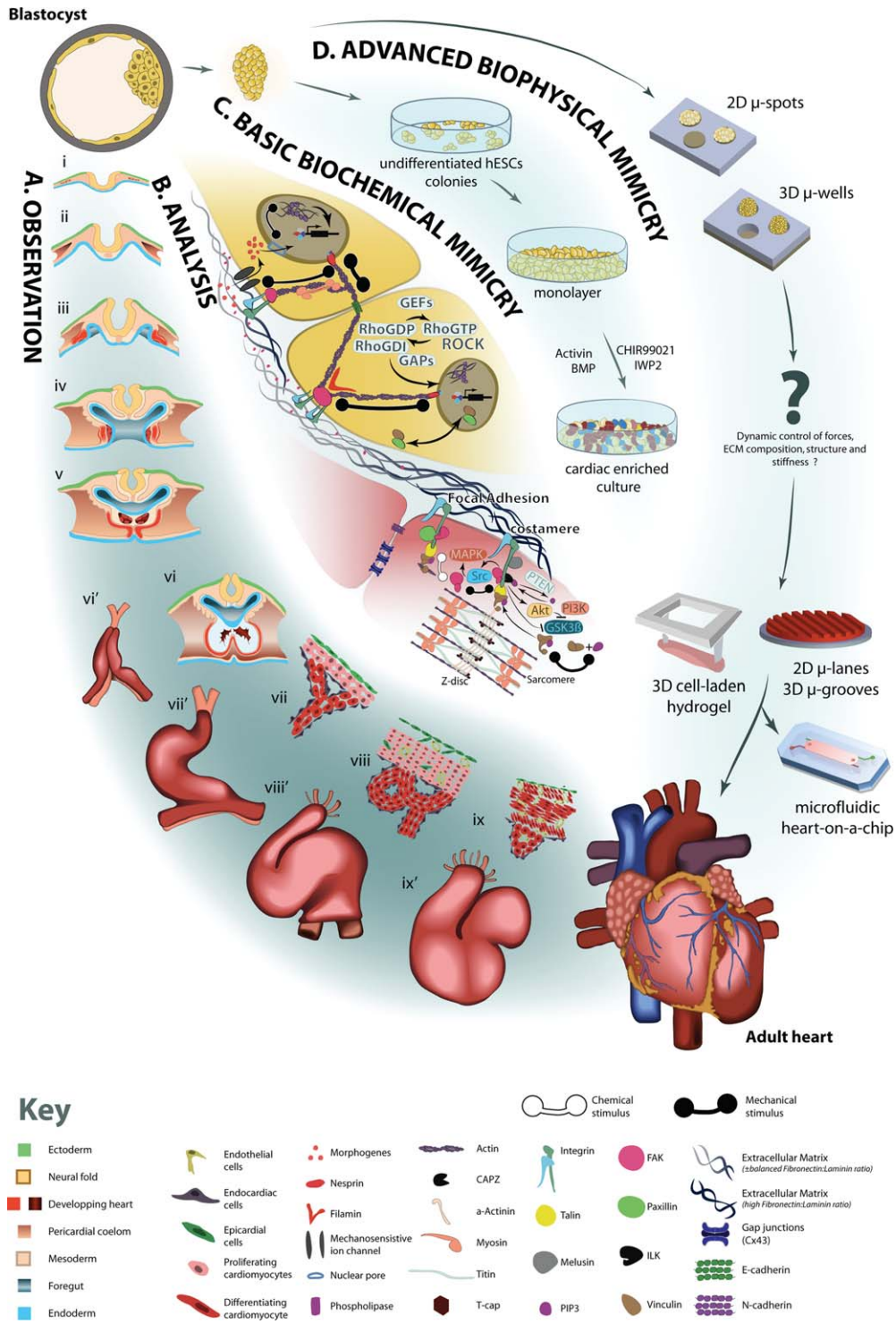

Figure 1. General strategy for differentiating pluripotent stem cells into cardiomyocytes. **(A):** Observation of the heart's developmental stages. After gastrulation and somite formation (i), the foregut arises and folding brings the two branches of splanchic mesoderm together (ii–iv) before eventually merging (v) and forming the heart tube (vi, vi′). Cardiomyocytes are already beating and further differentiating but some keep proliferating (vii), cause the tube to start its C-loop (vii′). The S-loop (viii′) causes the cardiomyocytes to stretch even more (viii) and when the four chambers are finally formed (ix′), they almost completed their elongation and differentiation (ix). **(B):** Analysis of the relationships between external mechanical forces and gene expression. Although the main principles are similar, ESCs (top) and cardiomyocytes (bottom) have different mechanosensing architectures. ESCs connect together using E-cadherins and form round-shaped colonies (cortical actomyosin network), while cardiomyocytes connect through N-cadherins, establish gap junctions for electrical signal transmission and express an important set of proteins for crystallization of actomyosin into highly anisotropic sarcomeric structures. Connections to the extracellular matrix are regulated via chemical (angiotensin II) or mechanical stimuli. Along with differentiation, tissues secrete different kind of extracellular matrix (ECM) protein. For instance, the heart as a higher fibronectin/laminin ratio than the blastocyst. **(C):** Basic biochemical mimicry can be achieved by culturing ESCs on Petri dishes coated with ECM mixtures or mimics. Using sequential addition of cocktails of growth factors and/or small molecules, developmental signaling pathways are triggered and ESCs are directed toward cardiomyocyte differentiation. **(D):** Advanced biophysical mimicry emerges through microfabrication techniques. 2D or 3D patterning of circular shapes allows for mimicking of the blastocyst structure (top) while patterning of lanes allow for mimicking aligned cardiac tissues (bottom). Abbreviations: 2D, two-dimensional; 3D, three-dimensional; hESCs, human embryonic stem cells.

compaction at the eight-cell stage, every single cell is only connected to its neighbor through cell-cell interactions, using proto E-cadherins and a few integrins [67, 68]. Upon cell compaction, cells increasingly express E-Cadherins and start secreting other types of cadherins [69], which can be seen as the first differentiation step before implantation of the embryo in utero, as cells self-sort by cadherin-type affinity. Later, cells start to secrete more and more ECM components, like collagen, vitronectin, tenascin, elastin, fibronectin, hyaluronic acid (HA), or laminin [70]. Cells then bind to these components through different mechanisms, for example, by creating focal adhesion complexes through RGD (argininyl-glycyl aspartic acid. Peptide of sequence L-Arginine (R)-Glycyl (G)-Aspartic acid (D))-integrin interactions, or by other specific receptors (like CD44 for HA) [71].

By the end of development, cells can sense external forces either through cell-cell interactions, cell-ECM interactions, mechanosensitive ion channels, or by directly sensing the force by wave propagation throughout the cell and toward the nucleus [71]. Hence, in order to manipulate the stem cell niche, the surface chemistry at the interface between the cell and the materials must reflect the integration of all the coupled mechanical signals before it reaches the cell membrane. Although not clearly demonstrated in the literature, one can think that if ECM proteins are too weakly bound to the materials, cells will not be able to generate the same amount of force, in similar ways as if it was linked to a soft material. Cells would then behave as if in suspension. Also, if ECM proteins are not abundant enough, integrin clusters and focal adhesions would be insufficient to form as efficiently as in a physiological context [72]. Cadherins and integrins are both linked to the cytosolic network of actomyosin. When the tension of this network is changed, a signaling cascade occurs, involving the Rho pathway, directly deforming the nucleus. These signals are then integrated within the nucleus leading to a differential gene expression and thus activating or repressing various developmental pathways [73, 74] (Fig. 1B).

Strategies for Mimicking Cell-Cell Communication and Cell-ECM Coupling

Providing cell-cell communication capabilities and cell-ECM coupling was the first strategy stem cell researchers have used. In contrast to mESCs, one of the first attempts to culture what we call now hESCs showed that regular Petri dish treatments for cell adhesion allowed expansion but triggered spontaneous differentiation as early as the second passage [75]. These dishes are made adherent by exhibiting positively charged functional groups. Although cells can form adhesions, they do not recapitulate the signals given by different ECM components during embryogenesis. Additionally, the high brand-variability in terms of nanotopography and surface charge can have dramatic effects on cell behavior [76]. Because of their electrostatic properties, most of the ECM proteins can be adsorbed on the surface of tissue-culture-treated plates. Thomson et al. in 1998 [77], followed by Reubinoff et al. in 2000 [78], were the first to demonstrate that hESCs could be cultured for months provided that they were cocultured on a layer of feeder cells. Later, feeder-free conditions were introduced by just coating a mixture of ECM proteins (Matrigel), or laminin on the surface of a Petri dish [67] (Fig. 1C, top). However, it has been observed that ESCs behaved differently depending on the coating protein: vitronectin would enhance self-renewal and proliferation of PSC while fibronectin, collagen IV, or laminin would drive more easily the differentiation of the cells toward various lineages. In addition to ECM proteins, the use of recombinant cadherin coating to mimic cell-cell interactions occurring between feeders and ESCs has been reported [79].

Debate 2: Choice of the Right ECM. The choice of the right ECM proteins for cardiac SCD is intricate. Different ECM proteins at different densities are present in cardiac tissues depending on the developmental stage [70]. Differentiating PSCs on Matrigel by an Activin A/BMP4-based protocol, Chan et al. observed in vitro time-dependent levels of HA and versican in the cell culture [4]. This evolving composition in ECM components not only affects the mechanical properties of the ECM but also many signaling cascades triggered by these two glycosaminoglycans (GAGs). Alternatively, Schenke-Layland et al. [80] made murine embryonic bodies (mEBs) on dishes either coated with collagen I, collagen IV, laminin, or fibronectin, and evaluated mesodermal and cardiovascular markers after culture in medium without any exogenous factor. They found that collagen IV induced significantly more mesendodermal cells (characterized by high Flk1 expression) than other ECM proteins. After sorting these precursors and seeding them again on the four coatings, cardiomyocytes appeared to be significantly more present on fibronectin-coated dishes. Also for mESC, Stary et al. reported that the protein SPARC acted in similar ways as BMP2 by increasing cardiomyogenesis in EB with clear upregulation of Nkx2.5 [81]. These studies highlight the fact that it might be questionable whether or not it is best to let the cells generate their own ECM components or designing biomaterials to use them as source polymers to control simultaneously stiffness dynamics and chemical signaling.

Influence of Substrate Topography

General Overview

There is growing evidence that cell shape is an important parameter during heart development (Fig. 1Avii–1Aix). Although cell proliferation may account for major mechanical events like asymmetric looping, it cannot explain the whole process, such as how the looping direction is controlled or why the growing four chambers are different from one another. Indeed, oriented growth may explain how any of the four chambers grow differently [82]. It has been reported that this kind of growth may be explained by mitotic spindle orientation [83], which can be directly correlated to cellular shape and more generally ECM distribution [84]. Another event, cardiac looping, is also highly influenced by the cell's geometrical shape. Studies in the chick have highlighted the fact that the cardiac tube starts looping not only by differential growth but mainly due to different morphologies of the cardiomyocytes at the outer curvature (elongated cells) as compared to the inner curvature (packed cuboidal cells) [85]. In the mature heart, cardiomyocytes are elongated and contract simultaneously in the direction of elongation. A specific aspect ratio has been correlated with healthy cardiomyocytes, and any

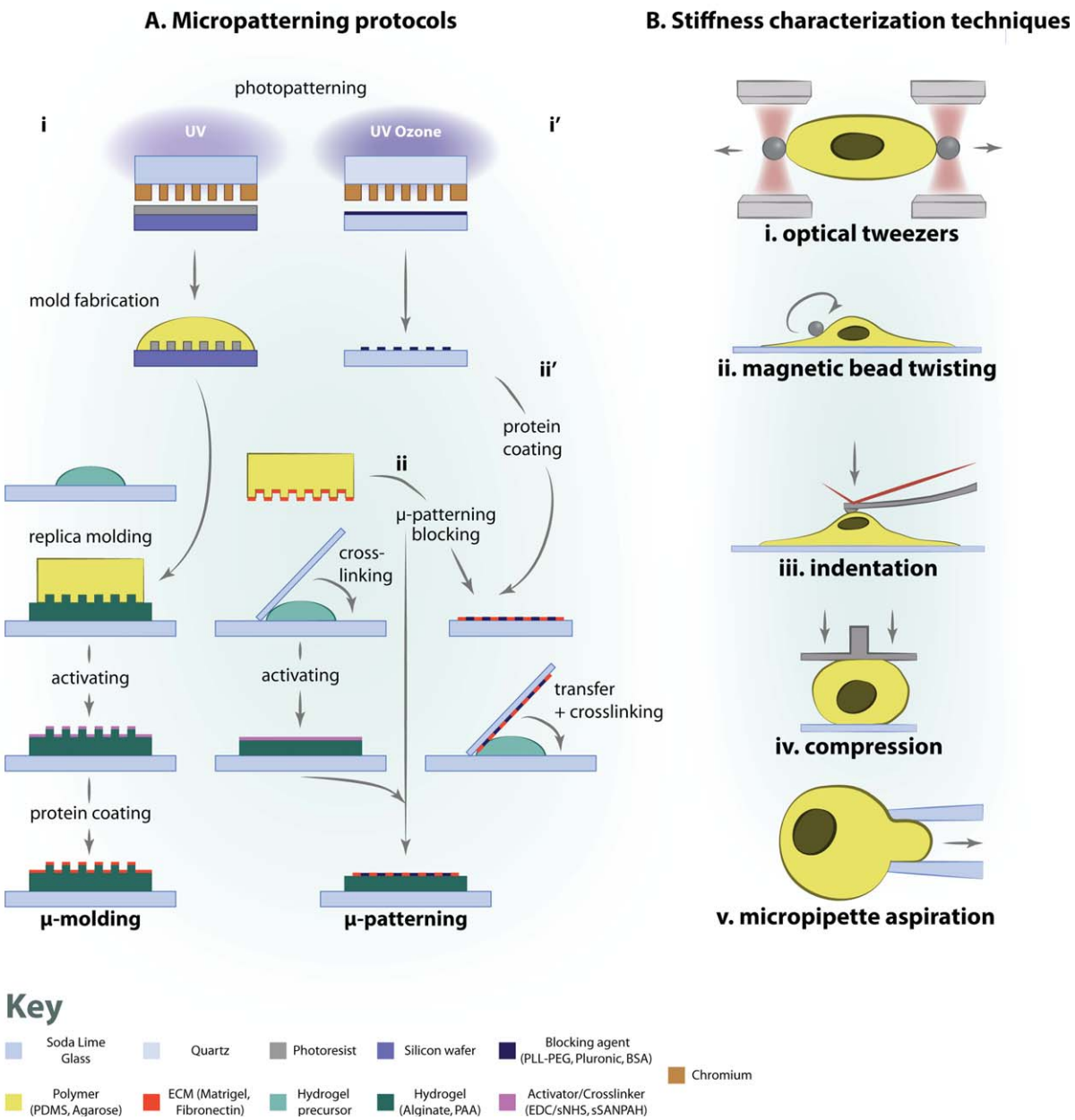

Figure 2. Overview of popular techniques for controlling and characterizing materials and cells at the microscale. **(A):** Microfabrication techniques, **(B)** elasticity measurements (adapted from [89]). See Technique Overviews for details. Abbreviations: ECM, extracellular matrix; PDMS, poly-dimethyl siloxane.

variation to that aspect ratio may mimic features of the failing myocyte [86].

Technique Overview 1: Micropatterning on Flat Hard Substrates. There are multiple ways of controlling the distribution and topography of proteins on a substrate. Many of them have been adapted from Chen et al. first experiment of cell patterning [87] and rely on alternating regions of nonfouling coatings (or naturally nonfouling polymers), and ECM-like proteins. Consequently, cells cannot extend their focal adhesions beyond the regions coated with proteins:

a. Microcontact printing (μCP) offers remarkable spatial control on two-dimensional (2D) surfaces. Soft lithography techniques (Fig. 2Ai, 2Aii) are used to fabricate polydimethyl siloxane (PDMS, a kind of silicon) stamps with microfeatures. Acting as the ink like in a regular post office stamp, protein solutions are rinsed, dried, and then transferred on top of a flat rigid surface (Fig. 2Aii).
b. Surfaces can also be selectively activated by exposure to ultraviolet light (UV)-ozone. If the surface had been previously rendered nonfouling (using poly(L-lysine)-grafted poly-ethylene glycol [PEG] coatings for instance), the UVO will burn the nonfouling molecules and activate the underlying substrate (Fig. 2Ai′), thus providing ways to protein adsorption a posteriori (Fig. 2Aii′). Bypassing the stamping step improves the resolution to submicrometer features and enables standard biology laboratories to develop microfabrication strategies without the need for dedicated

nano-fabrication clean rooms [88]. Nevertheless, one of the limitations might be that UV–ozone lamps still are an important investment and still too few platforms for cell biology provide them.

Technique Overview 2: Micropatterning on Soft Materials and Curable Polymers.

a. Stamps can be fabricated by soft lithography (for micrometer resolution) or by standard machining (micromilling, three-dimensional [3D] printer) for bigger features (Fig. 2Ai). Instead of transferring proteins, they can act as replica molds while a polymer or hydrogel polymerizes (Fig. 2A, left). Depending on the depth and spacing of the features, it provides the materials with quasi-2D microstructures (low spacing and height $<$100 nm) or with real 3D-cues (larger spacing and height $>$500 nm). Proteins are then coated uniformly, and thus cells will not constrain by adapting to changes in chemical signals, but by sensing local variations in topography [90]. If deep enough, these techniques can be adapted for miniaturization of culture wells for high-throughput screening on 3D aggregates [91, 92].
b. Coating proteins chemically on soft material is affected by the structural properties of the material itself, and protein surface density will vary as a function of local porosity. When using polyacrylamide (PAA), rather than using optochemical reactions to bind the proteins as in Engler et al. in 2006 [2], it has been recently suggested that simple transfer of proteins could be achieved by polymerizing a solution of acrylamide/bis-acrylamide sandwiched between a micropatterned surface and a glass coverslip [93] (Fig. 2A, right), or simply using μCP on polymerized PAA rendered adhesive with hydroxyl —OH groups [94] or with coupled streptavidin-acrylamide [95] (in this case, protein should be biotinylated prior to coating) (Fig. 2A, center). Further studies are needed to confirm that these techniques overcome, as claimed, the artifact of the effect on cell mechanosensing of PAA's variation in porosity with regard to elasticity.

Many variations on this theme are proposed depending on the way the hydrogel of interest is polymerized, like photocuration for methacrylated or PEG-DA-based hydrogels [96, 97]. Although many 3D-patterning techniques are emerging, their resolution still does not allow for single cell studies. We will briefly describe them in the Debate 4.

Influence of Topography on SCD into Mesodermal Progenitors and Cardiomyocytes: The Importance of Colony Size

The first step in deriving cardiomyocytes from ESCs in vitro is a step of specification. Like during embryogenesis, cells first differentiate into one of the three germ layers (namely ectoderm, mesoderm, and endoderm, Fig. 1Ai). These three layers are more restricted in their fate and cardiomyocytes can only arise from mesodermal or mesendodermal cells. Peerani et al. showed that colony size mattered in driving this first specification [98]. They microcontact printed Matrigel islands of varying diameters on glass coverslips and seeded them with ESCs without any exogenous inductive signals (Fig. 1D, top). By constraining the size of the aggregates, they showed that, after 2 days in culture, smaller aggregates (200 μm in diameter) expressed more endodermal markers and had higher levels of BMP2 whereas bigger aggregates (1,000–1,200 μm in diameter) expressed more pluripotent markers. This was attributed to the modulation of the ratio of pSmad1 agonists over pSmad1 antagonists. Indeed, while pSmad1 antagonists increased with colony size, no correlation was found for pSmad1 agonists, thus leading to lower levels of agonists in smaller colonies when compared with pSmad1 antagonists. Later, they repeated the same experiment but adding Activin A and BMP2 in the culture medium to force mesendodermal differentiation [99]. This time, cells on smaller spots (200–400 μm in diameter) were found to express more endodermal markers (GSC, Sox17, and Cer1) while bigger patterns (800–1,200 μm) led to more mesodermal cells (T, KDR). To investigate further the hypothesis that colony size influences differentiation by changing cell number and thus local concentrations in proteins and chemical, they used microtiter plates coated with Pluronic F-127 (a poloxamer used to create nonfouling regions on glass) to generate EBs by centrifugal aggregation [100, 101]. After sorting these cells for KDR, c-kit, and cTnt (markers for early to late cardiomyocyte differentiation), they concluded that the largest number of cells was reached at an optimum of approximately 1,000 cells per aggregate. Endodermal markers were predominantly found at the periphery of the aggregates, creating a higher ratio of endodermal cells over other cells for smaller aggregates (when calculating the surface/volume ratio).

Similar conclusions were drawn by Hwang et al. [102] after they cultured ESC on nonfouling micromolded polyethylene glycol (PEG) wells of different diameter (from 150 to 450 μm). Endothelial cells emerged from cells that had been cultured in small aggregates while cardiomyocytes emerged from cells cultured in bigger aggregates. Interestingly, they have been able to correlate these differences with modulation in Wnt5a and Wnt11, two important regulators of the noncanonical Wnt pathway.

Together, these four studies suggest that differential differentiation emerges from size-induced variations in the concentration of modulators of key signaling pathways involved in cardiogenesis (Nodal, BMP, and Wnt). Although qualitatively consistent with each other, these studies were not performed with the same ranges of diameters, and in a similar experiment by Mohr et al., the greater relative number of cardiomyocytes was reached for 300 μm-diameter EBs [103].

An explanation could be that they neglected the impact of mechanotransduction. In these studies, radii of curvature are different depending on the spot size and therefore a surface tension emerges at the periphery of the colony whereas pressure is increasing inside the colony due to cell proliferation, as suggested by Nelson et al. [104]. Also, PEG microwells [102] might have been softer than gold-coated polystyrene wells [103].

At a completely different scale, Myers et al. compared six of the most popular differentiation protocols (all based on exogenous signals modulating either the BMP or the Wnt pathways) while constraining initial colony growth to 2-mm wide spots of Matrigel [105]. Clearly, micropatterning increased homogeneity in the yields of differentiation. Most importantly, this study highlighted the important discrepancies that can affect cardiac differentiation depending on the choice of the modulated pathway. Put together, these data emphasize the importance of controlling the size of cell aggregates to optimize the cardiomyocyte yield.

Influence of Topography on Sarcomere Maturation and Force Generation of Cardiomyocytes: The Importance of Anisotropy

Contact guidance and topographical effects on cardiomyocyte maturation have been studied in many ways. At the single-cell level, in vitro studies on micropatterns [86, 106, 107] and in silico models of sarcomerogenesis [108] conducted by Parker's group suggested that a single cell will form more mature sarcomeres when its shape is constrained to a rectangle with an aspect ratio length/width of roughly 7:1. If the ratio is bigger, interdisc space will become bigger and cells will behave as a hypertrophied cardiomyocyte. If smaller (like in the case of circular patterns), sarcomeres will not be able to align in the same direction. They will be more randomly distributed within the cells, as characterized by orientation factors, and they will hence generate less force. At the multicellular level, they made the same observations regarding force generation (Fig. 1D, bottom). In this case, what mattered was less the aspect ratio than the elongated shape itself. If not all the cells were elongated in the same direction, the gap junctions were less well established and electromechanical coupling was inefficient in the tissue. The force generated was then considerably smaller than for anisotropic tissues [109–111]. These results have also been confirmed in 3D collagen gels encapsulating neonatal rat cardiomyocytes (NRCMs) [112], thus confirming the importance of anisotropy itself. A recent study by Wang et al. highlights that in the case of ESC-derived cardiomyocytes (ESCd-CM), seeding cells on a topographically aligned substrate did not improve the maturation state of the cells. The improvements in term of electrical stability and reduction in induced reentrant arrhythmias were solely due to the spatial organization, as assessed by monophasic action potential measurements [113].

Anisotropy in Early Differentiation and Isotropy in ESCd-CM Studies

If many studies have shown similar results with NRCMs [114–120], ESCd-CMs [114, 119], or extracted CPCs [121], there is little evidence of any effect of anisotropy of the ECM proteins on earlier stages of mesodermal differentiation [122, 123]. It could be interesting to know at which stage of development cells are able to sense lines of proteins and start aligning. This switch could correspond to the loss or the gain of a phenotype, like, one might suggest, the vanishing of the primary cilium, an important mechanosensing feature involved in cardiogenesis [124]. Cardiomyocytes could also start aligning because fibroblasts first aligned and hence anisotropically secreted ECM proteins, driving the orientation of other cells [125–127]. Nevertheless, anisotropy is involved in many morphogenetic events and for instance, researchers recently reported that the culture of human PSCs (hPSCs) on nanogrooved surfaces let to rapid differentiation of the cells into neurons [128]. In a completely different approach, it was found that reprogramming of fibroblasts into iPSCs was enhanced on grooved substrates due to increased acetylation and methylation of histone H3 [129]. This phenomenon could imply that anisotropy would act as a global inducer of epigenetic modifications leading to increased genetic sensitivity to differentiation/reprogramming protocols.

As for culturing beating cardiomyocytes on circular spots, experiments reported above do not really encourage it in that it would rather mimic pathological behaviors than physiological ones. However, in light of the high-throughput drug-screening platform designed by Serena et al. [130], one can argue that circular microtissues of cardiomyocytes might still give precious information on the relative behavior of cells subjected to different kinds of drugs.

Influence of Tissue Elasticity

General Overview

As cells are differentiating, their phenotype considerably changes, that is, not only their cytosolic composition is affected (resulting in a different stiffness by polymerization of the cytoskeleton), but their proliferation rate as well. Regions of differential growth can be observed, leading to different compressive or tensional forces applied to their neighbors. The composite material that is the cell environment considerably changes—the overall stiffness changes as well as the stretching forces. At the same time, cells are rearranged by affinity due to the different expression and amounts of cadherins. Mimicking these dynamic changes of highly nonlinear materials is obviously very challenging but simple models of constant linear elasticity have shown important results on SCD.

Not only the cell-cell interactions but also the cell-ECM interactions play a large role in cardiac differentiation and maturation. Particularly, the cardiac jelly, which interfaces the endocardium and the myocardium, is composed of GAGs (such as HA), proteoglycans, and proteins (such as fibronectin, collagen, or laminin). Based on the properties of GAGs to osmotically attract water, the cardiac jelly acquires a compressive strength and the internal pressure it generates on the myofibrillar architecture of the myocardium is thought to drive cardiac chamber expansion [20].

Technique Overview 3: Measurement of Tissues Elasticity in the Developing and Adult Muscle. In order to mimic the mechanical properties of cardiac tissues, it is important to be able to have precise measurements from tissue samples at different stages of the development. The difficulty relies on defining precisely the "substrate": is the tissue in its whole, only the ECM or the ECM plus the support cells? And if we deplete the tissue from their cells, what are the effects of the decellularization techniques on the mechanical properties of the tissue? Many laboratories have tried doing such measurements, either using atomic force microscopy (AFM) or standard rheology or tensile/compression testing. Moreover, elastic anisotropy (different stiffness in orthogonal directions) has been identified for cardiac tissue but is rarely considered as a requirement for biomimetic cardiac constructs in the literature [131]. Furthermore, measuring the stiffness of a material will not mean much if the bonding force with the coated ECM protein is too weak [132].

In the field of tissue engineering, the measure most reported for characterization a material's elasticity is the Young's modulus. However, depending on the technique used, it can be indirectly derived from more or less complex mathematical extrapolations of other mechanical characteristics.

a. Nanoscale elasticity can be measured by magnetic/optical tweezers. Nanobeads are constrained in a magnetic field and attached to the material. The material is then pulled away from the beads and the force needed to maintain the bead inside the field is measured (Fig. 2Bi). Similar setups can use twisting of the beads to measure shear elasticity (Fig. 2Bii).
b. Nanoscale and microscale elasticity can also be measured by AFM in contact mode (Fig. 2Biii). The cantilever tip is modeled as a pyramidal or spherical tip in Herzian contact with the material. Measuring the deformation upon nanoindentation is directly related to the Young's modulus and the Poisson ratio of the material.
c. Microscale elasticity of cells or materials can be measured by micrograph analysis of deformation upon an applied force. Cells and materials can be squished between plates (Fig. 2Biv), or aspirated through micropipettes (Fig. 2Bv).
d. Mesoscale elasticity (cell aggregates and hydrogel microspheres) can be measured by micropipette aspiration (Fig. 2Bv) as well as microrheology. In microrheology, fluorescent nanobeads displaced inside the tissue or material are optically tracked and statistical analysis of their random motion can be related to a measure of elasticity.
e. Mesoscale indentation can also be performed with simple handmade setups by measuring the deformation of a weight or small tip on the material or by hooking a small weight under the material to measure the implied deformation.
f. Bulk elasticity of macroscale materials is often measured by standard mechanical engineering testing machine, like the Instron. It can be set to apply uniaxial or biaxial strain in tension or compression on the tissues.

Influence of Elasticity on Mesodermal SCD

In cardiac repair, it has initially been thought that the use of striated muscle, be it skeletal muscle or cardiac, would be sufficient for engraftment and contraction inside the infarcted heart. They have quite similar mechanical properties and one can think that the properties of the matrix will feature a roughly similar elasticity. When Engler and Discher first reported that matrix elasticity had a great influence on SCD [2], they showed they had been able to make MSCs differentiate into myoblast-like cells on polyacrylamide gels of about 10 kPa in elasticity. From then on, material design studies for cardiac patches report measurements of the elastic properties of their materials in order to show that they closely match native heart muscle [90, 131, 133–136].

However, no strong evidence has been shown yet during the entire process of cardiac differentiation and experiments are often performed only after exposition to exogenous differentiation chemicals. Culturing preimplantation stage embryos on 2D PDMS substrates of varying stiffness resulted in significantly greater frequency of development from the two-cell stage to the hatching blastocyst stage, as compared to cultures in standard Petri dishes [137]. Sun et al. linked higher levels of Oct4 expression to higher stiffness when seeding hESCs on 2D PDMS micropillars but did not really look at the whole panel of genes expressed by the three germ layers [138].

As explained in Debate 1, many differences can emerge when switching from a mouse model to human cells. But as importantly, researchers recently started to point out that the influence of elasticity on SCD varies depending on the dimensionality (2D or 3D) of the materials [139]. Indeed, the distribution of ECM cues is completely different and affect the response of the membrane receptors (integrins for instance). It has been reported that cells in 2D tend to form more stress fibers than in 3D, which could prevent cardiomyocytes from forming mature sarcomeres [140]. Also, if high stiffness allows cell to generate high traction forces in 2D, it is not true in 3D where high stiffness can prevent the cell from moving around and force it to stay round. Interestingly, Zoldan et al. performed a strong investigation on the effect of elasticity on early specification in the three germ layers by encapsulating hESC into 3D polymers [141]. As suspected before, there is a strong correlation between the material's stiffness and germ layer differentiation. Surprisingly, the ranges of measured elasticity are several orders of magnitude higher than in the other studies afore-mentioned. Also, few details are given on the impact of the surface chemistry of the various polymers used in this study.

One study, nevertheless, has been able to correlate cardiac differentiation to matrix stiffness, through the regulation of YAP/TAZ expression [62]. Using Sca1+ adult CPCs seeded on polyacrylamide gels of controllable stiffness, the authors have showed that the control CPCs differentiated best into cardiomyocytes on gels of 10 kPa in elasticity, while YAP-silenced CPCs would not differentiate at all into cardiomyocytes under the same conditions.

Influence of Elasticity on Cardiomyocyte Sarcomere Maturation

Sarcomere maturation is probably the most studied effect among mechanical properties. By isolating cardiac cells at different developmental stages of the mouse embryo, Engler et al. have been able to establish a relationship between the variation in elasticity and the developmental stage of the heart [133]. Interestingly, it has been shown that embryonic cardiomyocytes beat best on substrates where the rigidity matches the embryonic tissue rigidity. These cardiomyocytes were only able to form mature sarcomeres on PAA gels of approximately 10 kPa. On softer gels, sarcomeres were less spaced and not fully organized whereas on stiffer gels, sarcomeres were not present at all and some sarcomeric proteins were completely diffuse in the cytoplasm. This result was also confirmed for NRCMs [142] as well as for ESCd-CMs [134, 143], although Hazeltine et al. found that the intermediate stiffness supporting best ESCd-CM differentiation from a progenitor stage would be approximately 50 kPa [143]. Previous studies on the differentiation of adipose stem cells into myotubes had already shown that pathological tissue stiffness led to reduced differentiation whereas tissue-like elasticity led to optimal maturation and striation formation of the myotubes [144].

Not surprisingly, it has been shown that depending on the type of extracellular element that was linked to the hydrogel, there could be different cellular responses to stiffness. Cardiomyocytes have been able to grow mature sarcomeres on HA-based gels of less than 500 Pa whereas it was only achieved at around 10 kPa with fibronectin-coated PAA gels [145]. This starts to show the limits of considering the Young's modulus as the principal mechanical parameter influencing cell phenotype. It is thought that the HA signaling cascade could bypass

the mechanotransduction pathway and direct sarcomerogenesis. One explanation could be that in this case, HA does not convey its message through mechanical signaling but rather through chemical interaction with the CD44 receptors [146]. However, it was also shown that the dynamic properties of HA gels are actually important in the cell response. Whereas PAA has a static elastic behavior, HA gels show time-dependent stiffening and thus can go from approximately 1 kPa to almost 10 kPa depending on its fabrication process [147]. This phenomenon was also observed in 3D fibrin cultures of myoblasts, where the fibrin gel, initially softer than 5 kPa, exhibited a stiffness of 15 kPa after several days [139].

This highlights the fact that static mechanical signals alone cannot act as a replacement of chemical induction of differentiation. Both signals are complementary and will act synergistically in a time-dependent manner. Furthermore, the point of introducing mechanical compliance and mechanical stimuli in cell culture is to avoid mixed signals. As cells sense their mechanical environment, adding promyogenic soluble factors can be hindered by the pro-osteogenic mechanical signals of the rigid Petri dish.

Debate 3: Elasticity Versus Viscosity and Porosity. If current techniques of microfabrication are increasingly simple, a few biases arise from the technique itself. Trappmann et al. recently suggested that depending on the polymer (they compared PAA and PDMS), the results shown initially by Engler and Discher could not be reproduced in the case of PDMS [132]. This could be explained by a difference in porosity and tethering of the ECM proteins to the synthetic polymer. Soft PAA gels (that led to neuronal differentiation) were highly porous and the ECM density at their surface was considerably lower than on stiffer gels, but these problems were not observed for PDMS, which has a more constant porosity. To further complexify this issue, a new study addressing the problem of porosity in soft polyacrylamide gels has refuted the hypothesis that tethering had biased the observation of differential fates depending on elasticity [148]. Yet it is unclear whether tethering of ECM is the only source of this difference as very soft PDMS made out of big ratio of the PDMS base and curing agent can exhibit significant viscous behaviors. The balance between the viscous modulus and the elastic modulus of this kind of soft PDMS directly influenced the cell's migration properties [149]. Although other types of PDMS have been reported as good candidates for traction force studies in purely elastic conditions [150, 151], there is no doubt that further studies will underpin the importance of viscosity during guided differentiation of ESCs into cardiomyocytes.

Perspectives

Influence of Stretching and Shearing

In addition to designing biomimetic materials for stem cell culture and differentiation, other mechanical stimuli can be dynamically applied to the cells. While shearing is considered to be of prime importance in vascular remodeling, cyclic stretching and static strains mimic more faithfully the mechanical behavior of muscle tissues. The effect of stretching on signaling pathways has been reported in review by Riehl et al. [152]. When subjected to static stretching, ESCs and iPSCs increased their markers of (cardio-) myogenic differentiation [153]. Although many setups exist for stretching cells (in 2D or 3D), like magnetic stretcher, manual stretcher, or vacuum stretching [152], the results are usually consistent.

As for the cardiac cells, it has not been shown that stretching PSCs would act synergistically with chemical factors for the induction of cardiac differentiation. In this particular case, it has only been shown that either NRCM, endogenous CPC, or stem cell-derived cardiomyocytes (SCd-CM) had better contractility and sarcomere maturation when subjected to cyclic stretching [154, 155]. Cyclic stretching enhanced their alignment and favored gap junction formation for a better electromechanical coupling. Not only can stretching improve the alignment of cells, but also of the 3D matrix that surrounds the cells. When static strain was applied on a polymerizing gel of fibrin, the nanofibers of the mesh tended to align in the stretching direction [139, 156, 157]. A global review on some other physical signals that can affect cardiac differentiation protocols can be found in the review by Ghafar-Zadeh et al. [158].

Influence of Electrical Signals on the Maturation of Cardiomyocytes

Many efforts have been made to develop either conductive scaffolds or electrically stimulated systems for recreating the cardiomyocyte's natural environment [159–162]. In these articles, emphasis is put on trying to mimic a healthy environment to avoid the appearance of diseased phenotypes. The changes that have been demonstrated using these many proof-of-concept materials are more related to phenotypic changes in already mature cardiomyocytes, rather than to specification, differentiation, or maturation events in cardiovascular progenitors or PSCs. One of the main improvements observed by electrical stimulation is an increase in connexin43 (Cx43) expression. This is a phenotypic change that considerably improves cardiac contraction as compared to standard in vitro controls (without electrical stimulation).

The goal of these new materials and systems is either to help implanted cardiomyocytes to integrate better with the host's cardiomyocytes or to drive pathological cardiomyocytes to regain a healthier phenotype by expressing more Cx43. Future studies involving the culture of immature cardiogenic cells or even PSC using these materials will be needed to address questions like the relationship of cardiac progenitor phenotype and the establishment of electrical currents in the developing heart.

Debate 4: 2D Versus 3D Culture. 2D models helped understand the signaling cascades in mechanotransduction, but this is still far from reality and adding a third dimension should be the next step. However, although 2D soft and patterned materials can now be prepared in a relatively high scale, 3D techniques are still cost-prohibitive, time-consuming and poorly resolved. Multi-photon technologies allow for 3D patterning by in situ chemical bonding of proteins to the backbone materials [163] as well as for the localized cell encapsulation by polymerization of photosensitive materials [164]. If these techniques can be quite slow, complex microfluidic systems have been designed for high-throughput and digitally tunable fabrication of 3D-patterned cell-laden fibers

and sheets [165, 166]. Yet the impact on PSC viability, pluripotency, and differentiation potential remains to be addressed.

Another pitfall would be to expect the same effects than previously shown on 2D substrates on 3D substrates from the same material or a material with similar mechanical properties. Indeed, the distribution (and production) of membrane receptors will be completely rearranged. Additionally, embedding cells in a 3D matrix will paradoxically decrease the number of degrees of freedom of the cell system. While 2D substrates allow cells to move freely on the topside, 3D materials constrain the cell in all directions. The stress distribution will be completely different. This is even more true when considering the integration of matrix metalloproteinase-degradable sites inside 3D scaffolds, as it has been shown that it would induce, at least in MSCs, traction forces different from those seen in nondegradable materials, thus leading to different fates [167].

As for in vivo cardiomyogenesis and embryogenesis, cells are arranged either in 3D or in 2D assemblies depending on the developmental stage. At first, cells are clustered in 3D but soon form the three germinal layers, which can be roughly modeled in 2D. Later on, gastrulation leads to the primitive streak formation and the mesodermal cells that will become the heart form 3D structures again. But after cardiac looping and the beginning of the formation of the four chambers, cardiomyocytes are stretched and eventually define the final myocardium. Although three dimensional in theory, ultrasounds and diffusion tensor magnetic resonance imaging studies have highlighted the fact that the myocardium is composed of layers of anisotropic cardiomyocytes oriented with a variable angle from the endocardium to the epicardium. Therefore, the myocardium can also be described as a monolayer of aligned cells wrapped around the ventricle with varying angles. In that case, one could argue that 2D models are better. Nevertheless, the question remains whether the best strategy would be to create a 3D scaffold and push it to self-organize into a multi-layered structure of aligned cells (like what happens during cardiogenesis [168]) or to force the cells to align on multiple layers before stacking them (as proposed by Takahashi [169] et al. for the design of cardiac patches [170]). In both cases though, the limits of oxygen and nutrient diffusion will have to be overcome in order for the construct to be viable, and this is particularly relevant to cells embedded in its core.

Comments on Nanofibers

Nanofibers have been extensively used to show the importance of surface roughness and hydrophilicity in maintaining pluripotency in PSC cultures. They are often cited as a simple way to have mesh-like topographies or substrates with aligned fibers [171]. Also, new techniques demonstrate the possibility to overcome the slow speed, high variability [172, 173] and high cost of electrospinning [174]. However, generating fibers only allows for two kinds of patterns: random fibers and aligned fibers. Whenever different shapes are needed, electrospinning has still quite a poor resolution [175] and needs to be combined with the most popular techniques described here above [176, 177].

Pharmacological and Medical Applications

In this review, we have seen that two parameters, substrate elasticity and ECM protein patterns, had a strong influence on sarcomerogenesis and cardiac maturation. Recent advances in materials design have led to the possibility of studying the synergetic effect of both properties [93–95, 136, 178] and it appears that appropriate stiffness (approximately 10 kPa) and aspect ratio (7:1) enabled optimal sarcomeric organization and optimal contractility both at the single-cell level but also at the cell pair level [136]. This study highlights the fact that cell-cell coupling also results from a balance of forces influenced by the environment.

Whether the end goal of producing striated muscle cells is to develop toxico-pharmacological assays [11, 12, 179–182] or regenerative solutions [18, 183–185] does not change the fact that cells would have to behave like in a healthy muscle. The fabrication of scaffolds for regenerative medicine has the same requirements than when mimicking developmental mechanics, but with additional constraints. First, in the case of surgical applications, structural integrity of the patch is of prime importance. Although cell sheets are a tempting option due to their fully natural composition and their promising results in cardiac failure [169, 186–188], they were found to be quite fragile and hard to handle. Second, to our knowledge, there is still no work reported on the combined influence of the materials properties described here on earlier mesodermal induction and cardiac differentiation. One can suggest that ESCs or iPSCs will behave like MSCs and differentiate into cardiomyocytes at around 10 kPa. However, studies reported above suggest that cell density will have an important impact as well. This complexity needs to be addressed when determining the whole range of materials that need to be used from the extraction, reprogramming, or thawing of stem cells to the patch fabrication. On one hand, scaffold-free techniques would ideally emerge and give rise to bioreactors that act directly on the 3D stem cell aggregate by providing chemical, mechanical, and electrical stimulations to produce highly pure cardiomyocytes. These techniques would naturally overcome additional constraints related to the "memory" a cell can have of its previous mechanical environments, which, as described by Yang et al. [189], can affect cell's fate in a given physical environment. On the other hand, it might be more realistic in a nearer future to develop multiphasic culture systems: one biomaterial-based bioreactor for early commitment of ESC, then a second biomaterial-based bioreactor onto which cells would be transferred for cardiac maturation (in parallel to the production of endothelial and smooth muscle cells) and eventually, the three cell types would be mixed in a third bioreactor prior to implantation onto the failing myocardium [190–192]. Notwithstanding the complexity of streamlining the translational process for adapting these approaches to wide-scale clinical applications, a key and yet unsettled issue is that one still ignores whether highly mature cardiomyocytes integrate better or not than earlier progenitor cells in the host myocardium.

In a nutshell, the ideal scaffold for differentiation and maturation will likely be a scaffold that can diffuse some key growth factors, like BMP2 or Wnt inhibitors, have the appropriate stiffness, and be coated with adhesion molecules that can trigger mechanosensitive events, such as ECM protein or GAGs, or, when considering upscale of these processes, short mimicking peptides. These elements would have to be patterned to direct oriented proliferation and spreading of the differentiating cardiomyocytes. Such materials have been

partially reported by Agarwal, Farouz et al. when they showed that NRCM could adhere, align, and spread on soft micropatterned calcium-alginate scaffolds functionalized with fibronectin. These muscular thin films showed enhanced contraction when stimulated electrically as compared to isotropic equivalents. Thus, using these kinds of scaffold for ESC differentiation could be the beginning of new standardized culturing conditions for cardiac differentiation [90].

Conclusions

Whether mechanical forces arise by activation of pathways resulting in varied cytoskeleton behavior or developmental pathways are modulated in response to cytoskeletal modifications promoted by mechanical forces is unclear. It can be seen as a "chicken and the egg" kind of question and undermining one or the other component can dramatically affect the outcome of the experiments to be performed.

A few efforts have been made toward the establishment of a global interactome of cardiopoiesis [193–196] and this kind of approach keeps being expected by cardiovascular researchers [197]. Additionally, in silico models are increasingly efficient in predicting cardiogenic events during development [198]. Combining the latest high-throughput and high-content technologies [25, 199] to the techniques described here in the design of micropatterned stiffness-controlled materials will eventually unravel many of the mysteries of cardiac development and provide invaluable information for the establishment of robust analytical models [200] and reliable patient-specific therapies.

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Author Contributions

Y.F.: conception and design, manuscript writing, figure organization and drawing, and final approval of manuscript; Y.C.: final approval of manuscript; A.T.: conception and design, manuscript writing, and final approval of manuscript; P.M.: conception and design and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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