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Engineering the niche to differentiate and deploy cardiovascular cells

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Applications for stem cells have ranged from therapeutic interventions to more conventional screening and *in vitro* modeling, but significant limitations to each is due to the lack of maturity from decades old monolayer protocols. While those methods remain the 'gold standard,' newer three-dimensional methods, when combined with engineered niche, stand to significantly improve cell maturity and enable new applications. Here in three parts, we first discuss past methods, and where and why we believe those methods produced suboptimal myocytes. Second, we note how newer methods are moving the field into an era of cell mechanical, electrical, and biological maturity. Finally, we highlight how these improvements will solve issues of scale and engraftment to yield clinical success. It is our conclusion that only through a combination of diverse cell populations and engineered niche will we create an engineered heart tissue with the maturity and vasculature to integrate successfully into a host.

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Introduction

For more than two decades since the initial isolation of human pluripotent stem cells (hPSCs), and even more so with the advent of human induced pluripotent stem cells (hiPSCs), the stem cell and cardiovascular fields have developed protocols to differentiate cells into cardiomyocytes. hPSC-progeny and hiPSC-progeny have been used in a variety of applications ranging from therapeutic applications [1,2] in the failing heart to mechanisms of action studies in disease-in-a-dish models [3]. To develop as many cells as possible for these applications, most protocols have relied on feeder layer-free monolayer culture methods with chemically defined media, which achieve reproducible, near homogeneous cardiomyocyte populations, for example, $\sim10^6$ myocytes/cm^2 or >90% efficiency in a matter of days. More recently, protocols have included negative selection to kill cells not metabolically mature [4] or to express genetically encoded reporters and positive selection to guide cells towards mature subtypes via mechanically and electrically defined niche. These protocols can be massively scaled up and enable labs to create 10^8–10^{11} cells required for therapeutic applications in large mammals [1]. However, they do not create functionally mature atrial or ventricular subtypes as well as the non-cardiac subtypes that can account for up to 70% of all myocardial cells *in vivo*. A combination of three-D maturation with many supporting cell types and further engineering of the niche may more appropriately signal to cells and push myocytes into these subtypes. As the field begins to recognize this problem [5], we briefly review and then provide detailed opinion on: (i) where did the field 'go wrong' when trying to produce mature ventricular myocytes, (ii) how can the field correct itself and produce these cells, and (iii) how can we use these more mature cells for new approaches to improve disease? With this review, we hope that these sections inform but also describe the direction we believe the field should pursue to maximize opportunities for cardiac regeneration.

Where did we go wrong?

hPSCs, including embryonic (hESC) and induced (hiPSC), have a vast potential as a cell replacement source due to their indefinite self-renewal capacity and ability to be reprogrammed or derived from patient cells [4,6••]. Numerous studies have demonstrated that hPSC-derived cardiomyocytes (CMs) have the ability to survive and engraft within host tissue, but these cells are typically a mixed population of immature CMs including atrial, ventricular, and pacemaker-like cells which can lead to altered cardiac physiological behavior such as arrhythmias [1,2,7–9]. Therefore, it is of great importance to understand how to best recapitulate the cardiac cell states present in the heart utilizing the appropriate cellular cues.

Originally, mouse stem cells were differentiated into CMs by generating multicellular aggregates called embryoid bodies (EBs), since they can form all three germ layers, which then produce spontaneously contractile regions consisting of a low number of CMs [10,11]. As more information was uncovered about the process of cardiogenesis, growth factors were utilized to direct the differentiation of stem cells into CMs in a more efficient manner. Currently, two methods predominant how hPSCs are differentiated into CMs — 2D and 3D models. Protocols of both systems are based on heart formation *in vivo*, beginning with the differentiation of pluripotent cells into the mesoderm germ layer, then into the specific cardiac lineages. This process is done by modulating the Wnt pathway using small molecules including Wnt/β-catenin activators such as Gsk3β inhibitors (e.g. CHIR99021, BIO, etc.) and BMPs, and Wnt inhibitors (e.g. IWR1, WNT-C59, XAV939, etc.) [12,13]. The more common system is a 2D monolayer protocol, which gives rise to CMs consistently and efficiently [4,6••]. The 2D system was introduced in Mummery *et al.* where hPSCs were co-cultured with an endodermal cell line, which cleared insulin thereby allowing for cardiac induction [14]. Future studies built on this protocol by utilizing an insulin-free media along with either WNT-driven or BMP-driven differentiations [15]. While these protocols have proved successful in generating CMs, the 2D protocol generates an immature population of CMs that does not adequately mimic the electrical, mechanical, chemical, or organizational properties of the human heart, thus there is vast room for improvement to this model [16–18]. For these reasons, a subset of the field has employed a 3D model to generate CMs [19•,20–22], but these models are not as efficient or reproducible and are more complicated to execute. Despite this, there is growing evidence suggesting that 3D models influence cardiac maturation more than the 2D system; thus, 3D systems have the potential to generate higher proportions of functional and mature CMs [23,24••]. The increased maturity of 3D-derived CMs has been attributed to differences in morphology, spatial organization, biochemical signals, and electrical and mechanical loading due to both direct and indirect interactions between CMs and other cardiac cell types, including endothelial cells and fibroblasts (Figure 1) [25–28]. However, there is still room for improvement in these 3D models as high capacity electrical and mechanical outputs, metabolic maturity markers, and mature cellular structure and morphology are missing that are present for *in vivo* human CMs [16,17]. Thus to recapitulate the maturity of CMs seen *in vivo* and capture the mature aspects of the 3D-based hPSC-CM protocol, there needs to be an emphasis on utilizing cellular interactions, specifically chemical and physical cues, to better mature CMs.

Recent studies have further explored the role of cell–cell communications on cardiac maturation. For instance, signaling between non-CM cell types, for example, endothelial cells and fibroblasts, and CMs has been identified as crucial for cardiac maturation [29,30•]. One study found that the co-culture of hiPSC-CMs and — endothelial cells upregulated cardiac hormones and cardioprotective factors, while also increasing structural maturity and extracellular matrix deposition in hiPSC-CMs [30•]. Another study found that VEGF-VEGFR2 signaling between non-CMs (endothelial cells and fibroblasts) and CMs led to increased expression of gap junction proteins and contractile function [31]. These findings demonstrate that physical and biochemical signaling from distinct cardiac cell types assists in generating mature CMs. While it would be ideal to recapitulate all of the cell types of the human heart, it is infeasible; thus these biochemical and physical cues can be applied to CMs ectopically to induce a more functionally mature population of CMs.

How are we trying to get it right?
Material stiffness

In addition to biochemical cues and paracrine signaling that come with 3D multi-cellular cultures, recent efforts have included the combination of structural and functional stimuli to aid *in vitro* cardiac maturation. ECM stiffness and composition, among other cues, have a direct impact on stem cell fate and hPSC-CM function [32–35]; many groups have utilized biomaterials that recapitulate the stiffness and composition of the native cardiac microenvironment to create cardiac tissue engineering models. Collagen-based scaffolds are attractive due to their biocompatibility, mechanical strength, and the abundance of collagen type I within the heart [36,37] whereas the viscoelasticity and bioactivity of fibrin hydrogels can be equally useful for cardiac tissue constructs [38]. Conversely synthetic polymers, for example, poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), and poly(caprolactone), provide a biocompatible environment with controlled degradation rates and tunable mechanical properties that can be temporally manipulated to match *in vivo* stiffness during development [32]. While material stiffness can provide mechanical cues for 2D cell cultures, stretch of compliant biomaterials, such as polydimethylsiloxane (PDMS), can drive active remodeling in 3D cardiac tissue constructs; uniaxial CM tension can be driven by stretching PDMS and active responses can be modulated by tuning the stiffness and length of PDMS posts to which cells adhere [39]. These posts can even mirror the effects of afterload on engineered heart tissues (EHTs), that is, heart wall stress during ventricular contraction, when they bind to one rigid and one flexible post; moderate afterload increases maturation markers [40]. Further examples of mechanical stimulation methods for cardiac maturation have been described elsewhere [41].

Figure 1
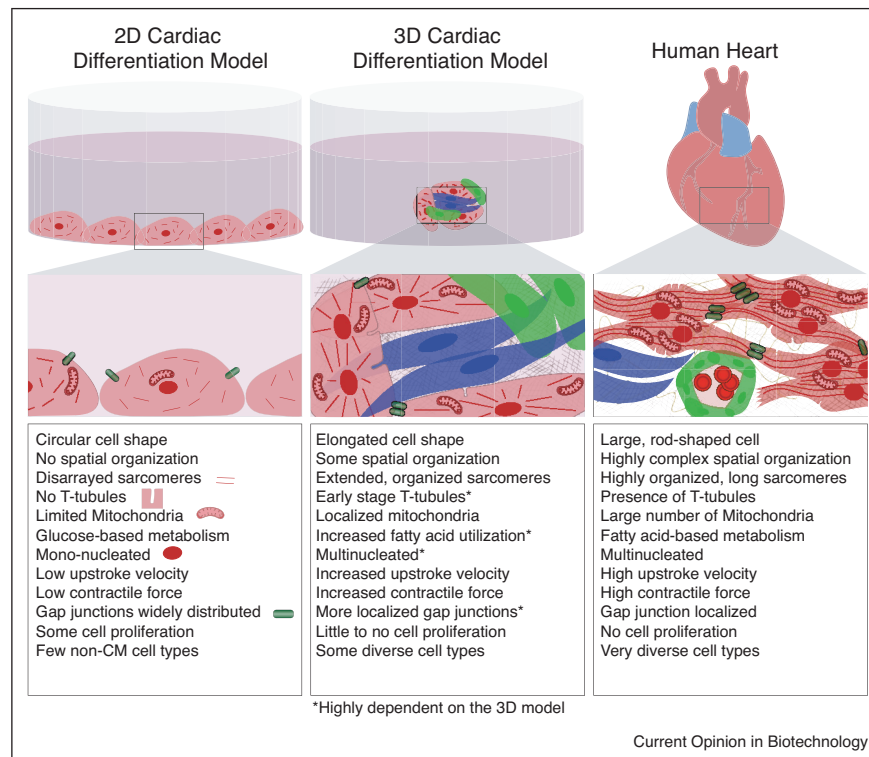
In vitro Differentiation Methods and their comparison to *in vivo* cardiac tissues.
Schematic and lists indicate how the differentiation methods differ, both in approach as well as in cellular outcomes. Both are compared to native myocardium. Cell types and intracellular structures drawn in the figure are noted in the key at bottom with descriptions of the cell state in the models. Note that for the 3D system, * indicate that the cell structure is dependent on model parameters and can vary widely between systems and users.

Electromechanical stimulation and multicellularity

Researchers have also explored the use of conductive materials to potentially improve hPSC-CM electrical maturation even when there is minimal cell–cell contact [42,43[•],44,45]. For example when Ruan *et al.* implemented static stress and electrical stimulation to hPSC-CM/collagen constructs, they found an increase in the overall stress production compared to unconditioned constructs ($\sim$1.34 mN/mm^2 versus $\sim$0.055 mN/mm^2), as well as an increase in *SERCA2* and *RYR2* expression, which are necessary for proper calcium handling and electrical function [46]. Electrical signals can also be propagated within the conductive polymer poly(3,4 ethylenedioxythiophene) (PEDOT), and for hiPSC-CMs, electrical stimulation improves sarcomere formation and contraction velocity and amplitude compared to non-conductive materials without external stimuli [43[•]]. Conductive graphene oxide can also improve expression of cardiac genes related to contractile and electrophysiological function in hiPSC-CMs [42]. Given the importance of electrical signaling in heart development, researchers have explored electrical stimulation for cardiac maturation

[47[•],48]. Radisic *et al.* electrically stimulated neonatal CMs in 2D culture and found improved ultrastructure organization, increased levels cardiac maturation markers such as *cTnI* and *Cx43*, and greater contraction forces compared to non-stimulated cultures; the findings of this work have served as the basis for hPSC-CM electrical maturation [48].

Each of the examples above used complex engineered systems but employed cells derived from monolayers, thus having only a single cell type present. While monolayer methods are still clearly the dominant method, several newer cardiac tissue construct examples employ heterogeneous EB methods, showing further improvements in cell maturity and could be a sign of the field 'correcting' itself. hPSC-CMs dissociated from EBs, combined with conductive Biowire, and trained with increased stimulation frequency over one week led to improved ultrastructure organization, calcium handling, and electrophysiological maturity [47[•]]. Electromechanical intensity training has even further advanced maturity levels [24[••],46,48–50]. For example, Llucia-Valldeperas

et al. investigated the effects of 2D electromechanical stimulation, albeit on adipose-derived and not hiPSC-derived or hPSC-derived CMs [49]. Immature EHTs containing multiple cell types have also been suspended between flexible PDMS posts, stimulated from 2 to 6 Hz and then maintained at 2 Hz. These EHTs formed a significant number of T-tubules for calcium handling and showed the first positive force frequency relationship in an hPSC-derived model, although maximum force was 10-fold lower than *in vivo* ($\sim$4 mN/mm^2 versus $\sim$40 mN/mm^2) [24$^{\bullet\bullet}$]. These works demonstrate the importance of various niche stimuli for hPSC-CM maturation along with requiring a diverse population of supporting cells; this combined approach, which we believe needs to become standard in the field, is summarized in Figure 2.

## How can we translate new approach to humans?

While recent advances in hPSC technology, differentiation methods, and biomaterial applications could push cell maturity, the lack of easy scalability could prevent significant clinical applications in humans and limit hPSC-CM approaches to disease modeling and screening. With 10^8–10^{11} cells required for therapeutic applications [1], producing large quantities of CMs required for transplantation [51,52] and achieving their successful engraftment [53] could prove a significant impediment. Continued improvements in CM differentiation efficiency and maturation, and a greater understanding of other endogenous cell populations could greatly improve regenerative strategies for patients [54$^{\bullet}$,55$^{\bullet\bullet}$].

While 2-dimensional and 3-dimensional protocols have been shown to effectively generate CMs from hPSCs [4,6$^{\bullet\bullet}$,19$^{\bullet}$] the generation of therapeutically relevant numbers of CMs remains challenging. Recent studies have focused on modulating Wnt and Hippo pathways in monolayer cultures [54$^{\bullet}$] to induce large-scale hPSC-CM expansion in bioreactors. Molecules previously identified in promoting CM proliferation [56,57], when applied *in vitro* in combination with cell contact inhibition, can robustly generate 10^9 hPSC-CMs necessary for therapeutic approaches [54$^{\bullet}$], but the tradeoff is cell maturation. Conversely, suspension culture-based strategies [58,59] suffer from low CM efficiency and cell death [60]. To overcome that limitation, hPSCs were formed into aggregates and differentiated via canonical Wnt modulators in an approach that allows for >80% CM purity without the need for further lineage enrichment [60]. While 2D and 3D suspension culture methods can produce large quantities of hPSCs, additional mechanical and electrical stimuli should be employed to ensure the functional maturity of hPSC-CMs.

Delivery and engraftment of these vast numbers of hPSC-CMs remains a second critical issue. Of all the potential solutions, we believe that direct live cell and ECM printing is most promising. 3D bioprinting improves upon the decades-old approach of loading cells onto

Figure 2
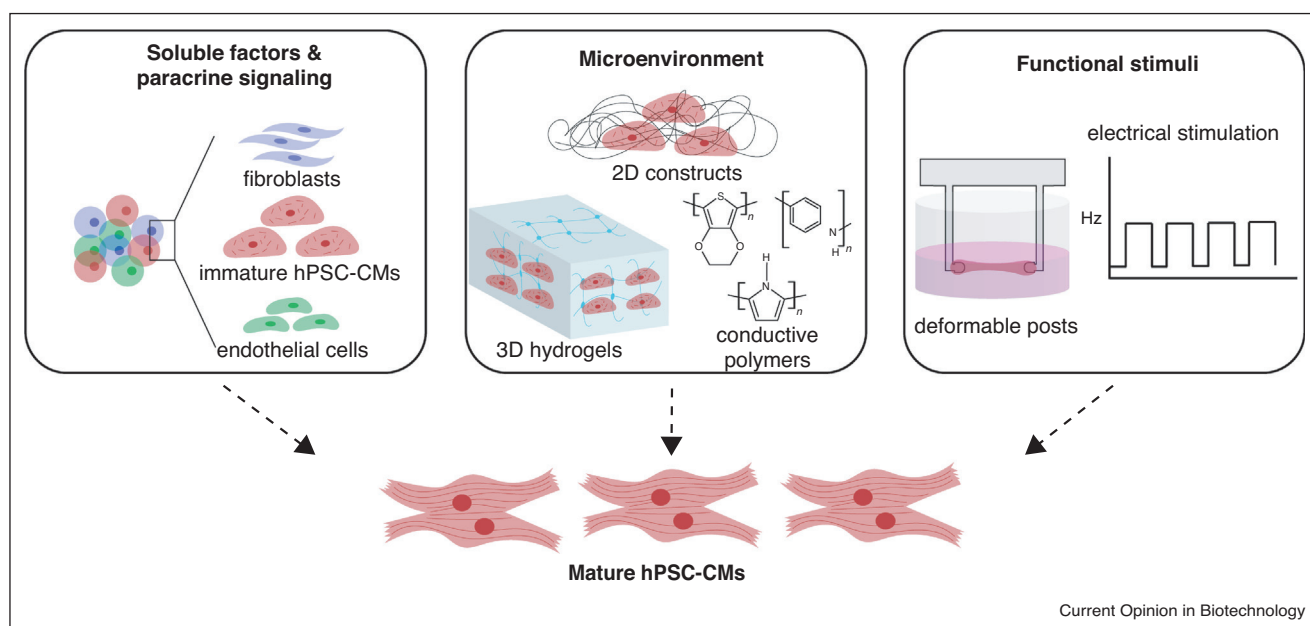
Engineered cues presented to hPSC-derived myocytes.
Schematic indicating diverse cell populations providing soluble and cell–cell-based factors to improve CM maturation (left). This can be augmented by passive (center) and active (right) insoluble extracellular cues, for example, biomaterials, stretch, electrical stimulation, and so on.

biocompatible scaffolds to offer precise control of construct architecture and biological function, as described at greater length by Zhang *et al.* [61]. While it has proved to be a challenge in the past, due to difficulties in maintaining structural support during printing [62,63•], novel methods such as the freeform reversible embedding of suspended hydrogels (FRESH) provides the precise control that could overcome these limitations. FRESH printing provides mechanical support for printed filaments by printing into a bath of spherical gelatin microparticles that can be melted at 37°C after printing [63•]. In addition to printing, perfusable vascularized networks could further promote hPSC-CM engraftment and anastomosis with the host vasculature [37,51], for example, the sacrificial writing in functional tissue (SWIFT) method where organoid bodies are populated throughout matrices through which perfusable channels are introduced via 3D printing [51]. These next generation printing methods may improve engraftment, but as mentioned before, cell populations must recapitulate the cell diversity found in the ventricle wall.

To bridge the gap between current hPSC technologies and cardiac regenerative therapies, robust cellular differentiation protocols and engineered tissues recapitulating the vascular networks and cellular composition of native niches must be achieved. Recent advances in differentiation methodology, orchestration of biomechanical and chemical cues, and 3D bioprinting represent promising pathways for overcoming this challenge [64,65]. However as articulated here, we believe that much is left to do before and if engineered cardiovascular tissues can be deployed in a clinical setting.

Conclusion
One significant goal for the field of stem cell-derived myocytes has been to create enough mature cells for cardiac regeneration. As we noted at the outset in trying to create the most efficient monolayer differentiation protocol, the field lost track of the basic developmental biology that should guide cardiomyocyte differentiation and maturation into adult cardiac phenotypes. By turning to maturation methods where a diverse cell population is present, we can create a CM subpopulation that is more mature and better specified for specific jobs within the myocardium. However, this recent shift in thinking must be coupled to a decades long effort to improve the myocyte cell niche; only through the combination of defined matrix properties and a diverse cell population will sufficiently mature cells be produced. Of course once this process can be scaled up, only then will regenerative therapies for hPSC-based and hiPSC-based CMs be possible.

Author contributions
The manuscript was conceived of and written by all authors equally.

Conflict of interest statement
Nothing declared.

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- • of special interest
- •• of outstanding interest

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