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Opportunities and challenges in cardiac tissue engineering from an analysis of two decades of advances

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Engineered human cardiac tissues facilitate progress in regenerative medicine, disease modelling and drug development. In this Perspective, we reflect on the most notable advances in cardiac tissue engineering from the past two decades by analysing pivotal studies and critically examining the most consequential developments. This retrospective analysis led us to identify key milestones and to outline a set of opportunities, along with their associated challenges, for the further advancement of engineered human cardiac tissues.

In the study of cardiovascular disease, increasingly sophisticated systems that can more accurately recapitulate the unique attributes of the human heart are an ever-present need (Fig. 1). Many advances in cardiac tissue engineering originate from studies in animals and cultured cells, yet the limitations of these models have progressively become apparent[1]. In particular, the utility of animal models is constrained by species-specific differences in cardiac physiology, and the usefulness of cell monolayers is limited by their simplicity (with respect to the complexities of myocardial structures). Human engineered cardiac tissues (hECTs) are free from many of these constraints. Enabled by advances in the generation of human pluripotent stem cells (hPSCs), today cardiac tissue engineering allows for the production of hECTs that more closely recapitulate cardiac physiology in humans.

In regenerative medicine, studies in animal models have shown that the engraftment of cardiac tissue improves cardiac function and attenuates remodelling after myocardial infarction[2–4]. In disease modelling, hECTs employing patient-derived cell lines and leveraging genome-editing techniques can be used to recapitulate cardiac-disease phenotypes to elucidate the underlying mechanisms of disease and to develop new therapies[5,6]. Engineered cardiac tissues can also be used to test the safety and efficacy of drugs to efficiently screen for patient-specific therapeutics and assess their off-target effects[7]. However, many knowledge gaps hinder the ability to unlock much of the translational potential of hECTs.

As the scientific questions and hypotheses that can be formulated and answered in cardiac tissue engineering become progressively more sophisticated, it is increasingly important to develop strategies to accurately recapitulate and manipulate the nuanced characteristics of the adult heart. Because the challenges faced today are fairly different from those of even the recent past, reflecting on past progress to map out future directions can be particularly insightful. We therefore set out to analyse progress in cardiac tissue engineering. Starting from the shift towards using human cell sources about 20 years ago, we analysed relevant advances (Table 1) according to three distinct areas of focus: the establishment of cardiac cell populations, the maturation of cardiac cells and engineered tissues, and the control of tissue structure and function (Fig. 2). We then used this structured retrospective analysis to identify and examine the key innovations that furthered progress (Fig. 3), and outline the directions of current and future research. In doing so, we gathered insights into how cardiac tissue engineering has progressed, where it has struggled and how it may push forward.

Establishment of cardiac cell populations

Tissue engineering relies on the robust generation of pure cell populations for the production of tissues that faithfully reflect human physiology, and hPSC technology has provided a means to produce cell populations from the full spectrum of the cardiac cellular landscape[8]. Over the past two decades, the ability to generate cells with increasing efficiency and specificity has led to improvements in the biological fidelity of hECTs (Fig. 2a).

Identity and function of parenchymal cells. The earliest engineered cardiac tissues were made using rodent cells. Technologies for the generation of hPSCs then enabled the production of cardiomyocytes from human sources. Marked by visible spontaneous contractions, clusters of hPSC-derived cardiomyocytes (hPSC-CMs) were first isolated from spontaneously differentiated human embryonic stem cells[9] (hESCs). These populations expressed cardiac structural proteins and ion channels, displayed sarcomeric structures and excitation–contraction coupling, and exhibited cardiac action potentials[10,11]. Early findings showed that hESC-derived cardiomyocytes recapitulated key structural and functional properties of native human cardiomyocytes. This marked the beginnings of hECTs.

In 2007 it was shown that hPSCs could also be reprogrammed from adult somatic sources[12]. As with hESCs, human induced pluripotent stem cells (hiPSCs) are capable of cardiac differentiation, and the resulting cardiomyocytes were shown to be functionally equivalent to those of embryonic origins[13,14]. Unlike hESCs, however, hiPSCs do not come with associated ethical controversies that limit their use. This led to a shift towards hiPSCs as the predominant cell source for cardiac tissue engineering.

The further characterization of the identity of hPSC-CMs showed that their differentiation typically yields heterogeneous cell

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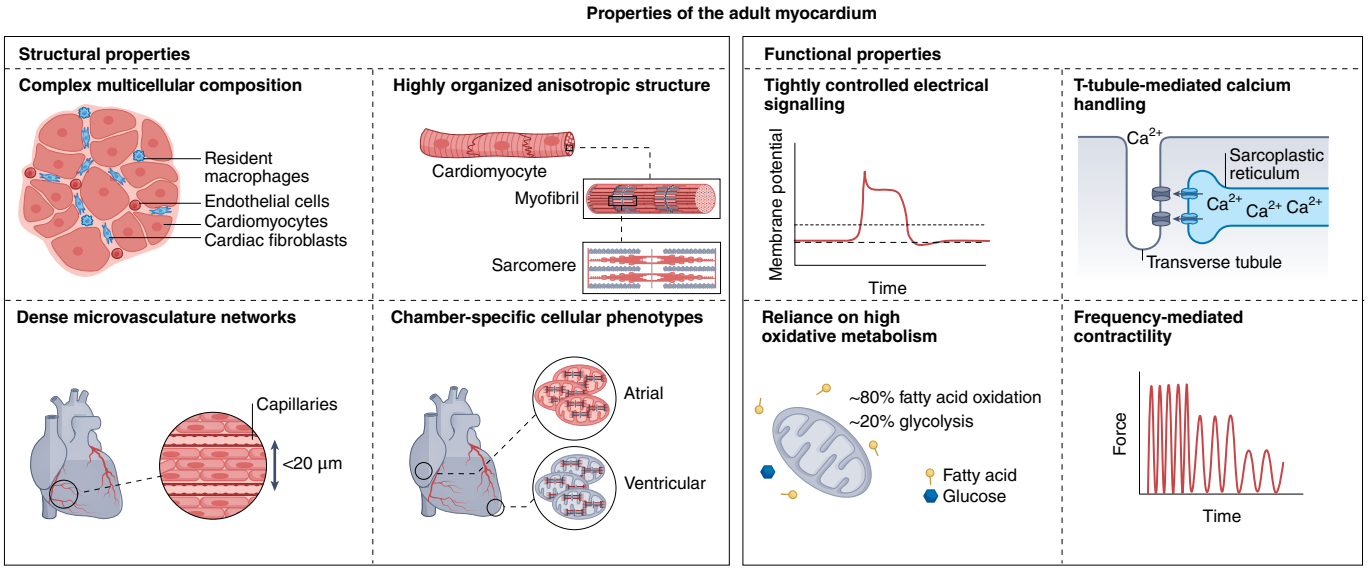

Fig. 1 | Unique characteristics of human heart muscle. The properties of myocardial tissue are location-dependent. For example, cardiac-chamber-specific phenotypes differ in the levels of expression of genes and proteins, in electrophysiology and in responses to external stimuli[15,18,166]. Regardless of the cardiac chamber, adult human myocardial tissue has a highly anisotropic hierarchical structure that allows for the efficient generation of contractile force. Contractility in the adult heart is frequency-mediated, with contractile forces increasing as the frequency of contraction increases, and this is tightly regulated by electrical signalling[166,167]. When the depolarizing action potential propagates through a cardiomyocyte, calcium mediates the excitation–contraction coupling[166,168,169]. The mechanisms governing calcium signalling in the heart are unique and are facilitated by T-tubules that enable the synchronized release of calcium into the cell to generate mechanical contraction[166,168,169]. The continuous cycle of contraction and relaxation relies on mitochondrial oxidative metabolism to produce the necessary high amounts of adenosine triphosphate for proper function[166,168–170]. To fulfil this need, the heart contains highly organized and dense microvascular networks that are composed primarily of endothelial cells and efficiently deliver oxygen and nutrients[132]. Although cardiomyocytes are the main functional cell type in the heart, the cellular landscape of myocardial tissue is heterogeneous and complex. Fibroblasts, endothelial cells, mural cells, immune cells and other supporting cell types are essential for heart homoeostasis; they contribute to intercellular communication and to the support function of the ECM[166,168,169]. Cardiac tissue engineering aims to recapitulate the unique structural and functional characteristics of the adult human heart in hECTs to maximize the utility of the engineered tissues for specific applications.

populations with atrial, ventricular and pacemaker phenotypes[15]. Differentiation protocols for obtaining cardiomyocytes with specific subtype identities have been recently developed[15–17]. The differential activation of bone morphogenetic protein 4 (BMP4) and Activin A pathways during the induction of early mesoderm was found to be critical for atrial–ventricular specification. Specifically, high ratios of Activin A to BMP4 favour ventricular development, whereas atrial specification requires the induction of retinoic acid signalling[15]. Similarly, sinoatrial-node-like cardiomyocytes can be generated via the early manipulation of BMP4 and retinoid signalling, and the inhibition of transforming growth factor β (TGF-β) signalling[16]. These advances for the precise control of the lineage specification of cardiomyocytes are critical for studying specialized cardiomyocyte functions and for engineering cardiac-chamber-specific properties in vitro[17–20].

Differentiation of cardiac stromal cells. Cardiomyocytes make up 70–85% of the heart by volume, yet they only account for 25–35% of the cellular population by number[21]. Non-myocytes with distinct cardiac identities, including endothelial cells, smooth muscle cells and fibroblasts, all play vital roles in maintaining myocardial function[22]. Hence, the robust derivation of hPSC-derived stromal populations is crucial for the engineering of tissues that faithfully recapitulate the cellular composition of the myocardium.

Several cardiac stromal populations originate from pro-epicardial progenitors. Similar to cardiomyocyte differentiation, the temporal modulation of canonical Wnt signalling induces pro-epicardial lineages ($WT1^+$) from hiPSCs, and these can be maintained in long-term culture under TGF-β inhibition[23,24]. Through treatment with platelet-derived growth factor and TGF-β1, pro-epicardial cells differentiate into smooth muscle cells (displaying the protein markers actin alpha 2 and calponin 1) that show physiological contractile responses to vasoconstrictors[23]. Similarly, quiescent cardiac fibroblasts can be generated via the activation of basic fibroblast growth factor (FGF2) and the inhibition of TGF-β in hPSC-derived pro-epicardial cells[25,26]. Transcriptomic and proteomic comparisons between hiPSC-derived and primary cardiac fibroblasts lead to similar levels of expression of fibroblast-specific and cardiac-specific markers[26]. Functional analyses have also shown that hiPSC-cardiac fibroblasts are capable of fibroblast-specific functions, including the production of extracellular matrix (ECM), differentiation into myofibroblasts and contraction[25,26].

As with cardiomyocytes, endothelial cells (ECs) in the heart originate from the lateral plate mesoderm[27]. After cardiac-mesoderm induction, supplementation with vascular endothelial growth factor (VEGF) gives rise to VEC^+ $CD34^+$ $CD31^+$ endothelial populations[28–30]. Gene-expression analysis has shown that hPSC-ECs differentiated from a cardiac lineage cluster closely with human primary cardiac microvascular ECs, and that they express the cardiac-specific gene GATA4, suggesting that their identity is cardiac endothelial[28,29]. Functional characterization revealed that these hiPSC-ECs can also form a lumen[28,30].

Deriving the full spectrum of cardiac stromal populations will enable engineered patient-specific tissues with an organ-specific cellular make-up. Doing so will aid the study of cardiac-specific intercellular crosstalk dynamics, the development of disease models for the study of stromal involvement in pathogenesis and their use for drug screening, and the creation of fully autologous tissues for cardiac regeneration.

Table 1 | Milestones in the engineering of human cardiac tissues

	Milestones	References
Establishment of cardiac cell populations		
Characterization of the identity and function of cardiomyocytes as parenchymal cells	Derivation of cardiomyocytes from hESCs	9–11
	Derivation of cardiomyocytes from hiPSCs	13,14
	Differentiation of cardiomyocytes with distinct subtype identities	15–17,154,155
Differentiation of cardiac stromal cells	Differentiation of cardiac-specific fibroblasts	24–26,156
	Differentiation of cardiac-specific endothelial cells	28–30
	Differentiation of cardiac-specific mural cells	23,24,156
Improvements in the reliability of the cell sources and in the efficiency of cell differentiation and purification	Chemical definition of media compositions for differentiation	31–34,37,157
	Modulation of signalling pathways for guided differentiation	35,36,157,158
	Isolation and purification of cardiomyocytes	11,42–46
Maturation of cardiac cells and engineered tissues		
Shift to 3D culture	Construction of scaffoldless cardiac tissues	55,88,140
	Development of synthetic scaffolds for tissue construction	52,57,58,159,160
	Use of natural scaffolds for tissue construction	18,20,51,60–62,64,86,161
Inclusion of stromal cell populations	Elucidation of the cell-type-specific roles of primary stromal cells	29,53,84,88,89
	Understanding of the roles of organ-specific stromal cells	18,29,88
	Development of isogenic tissues using hPSC-derived stromal cells	29,88–90
Application of external stimuli	Use of uniaxial mechanical stretching for tissue maturation	85,93–95,162–164
	Application of electrical stimulation for tissue maturation	18,86,87,165
	Modulation of biochemical factors for cellular maturation	96–102
Control of tissue structure and function		
Purposeful organization of tissue architecture	Understanding of the factors driving the self-organization of tissue structures	108,109
	Modular assembly of multi-component cardiac tissues	111,112
	3D bioprinting of tissues with physiological structures	78,79,113,114
Dynamic control over tissue functions	Spatiotemporal control of properties of the tissue microenvironment	116–118
	Dynamic manipulation of genetic sequences	122
	Optogenetic control of tissue functions	124–127
Integration of functional subsystems	Integration of functional vasculature	78,79,111,112,114,142
	Autonomic innervation of cardiac tissues	134,135
	Incorporation of tissue-resident immune components	137
	Incorporation of cardiac conduction systems	16

Efficiency, purity and reliability in cell sourcing. Early protocols for the derivation of hPSC-CMs were limited by low yields and high variabilities between differentiations and among cell lines[10,11,31,32]. Improved methods for guiding cells down the myocardial lineage helped increase control over stem-cell fate. A plethora of factors were identified as critical to cardiomyocyte differentiation, including the inhibitive role of insulin on the induction of early cardiac mesoderm and the importance of ascorbic acid on the proliferation of cardiac progenitors[33,34]. The greatest strides were informed by knowledge from cardiac embryology. The temporal modulation of FGF/TGF-β and Wnt signalling allowed for the stage-specific induction of cardiac-mesoderm and cardiac-progenitor cell populations, and proved to be critical for the derivation of cardiomyocytes at high efficiency[35,36].

The earliest protocols yielded less than 10% of hPSC-CMs. Small-molecule-modulated Wnt signalling and defined culture media then allowed for consistent differentiation into cell populations that were more than 80% pure (positive for cardiac troponin T)[36]. A cost-effective chemically defined protocol with comparable yield used media containing only the minimal components essential for cardiogenesis[37]. Recent efforts have improved the scalability of differentiation through the development of expansible bioreactors for cardiomyocyte differentiation and of protocols for cell expansion[38–40]. Notably, GSK-3b inhibition and the removal of cell–cell contacts facilitated the expansion (by up to 250-fold) of hiPSC-derived cardiomyocytes[41].

Alongside improvements in differentiation efficiency, a wide range of methods for cardiomyocyte purification have been developed to eliminate variability among cell populations. Earliest solutions, such as Percoll-based separation and the manual isolation of beating clusters, led to high variability[11,42]. The development of cardiac-specific reporters and drug-resistant hPSC lines enabled the consistent enrichment of hPSC-CMs through drug selection or fluorescence-activated cell sorting[43,44]. However, these methods involve genetic manipulation. In 2011, it was reported that hPSC-CMs express the cell-surface receptor signal regulatory protein alpha[45] (SIRPA); this enabled hPSC-CM selection via anti-SIRPA antibodies. However, because hPSC-CMs have a low innate proliferative capacity, live antibody-mediated selection is costly and time-consuming, thus limiting the production throughput.

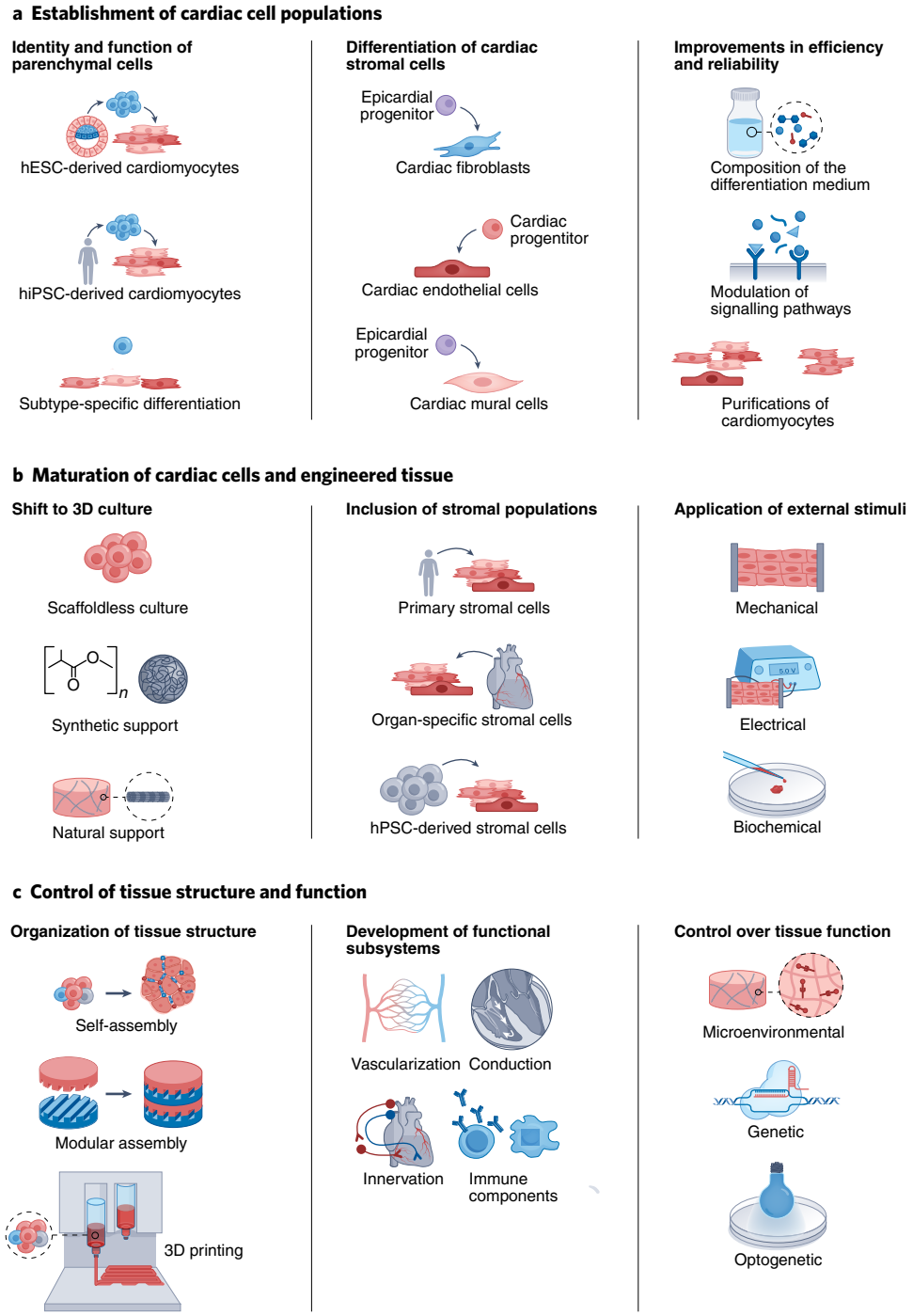

Fig. 2 | Three areas of focus in cardiac tissue engineering. a, Efforts to establish cardiac cell populations began with the characterization of the identity and function of human cardiomyocytes as parenchymal cells. This was followed by the characterization of the identities of cardiac-specific stromal cells: fibroblasts, endothelial cells and mural cells. These efforts relied on continuous improvements in the reliability of the cell sources and in the efficiency of cell differentiation and purification. **b**, Many methods have been devised to promote the functional maturation of cells and tissues. The main advances were moving from 2D monolayer cultures to 3D cultures, which better mimic the native geometry of the myocardium, the integration of the various stromal populations into the tissues so as to mimic native cellular heterogeneity, and the optimized application to in vitro tissues of external forms of stimuli—mechanical, electrical and biochemical—that naturally occur in the native heart. **c**, As methods for the production of hECTs became established, increasing tissue complexity and utility to recapitulate the more nuanced characteristics of the native myocardium became increasingly possible. This can be done by increasing control over the organization of tissue structure, adapting technologies developed in other areas of biological research to exert control over the function of hECTs by manipulating the genomes of the cells or the properties of the tissue microenvironment, and developing and incorporating native functional subsystems—in particular, the vascular, nervous, conduction and immune systems—into hECTs in vitro.

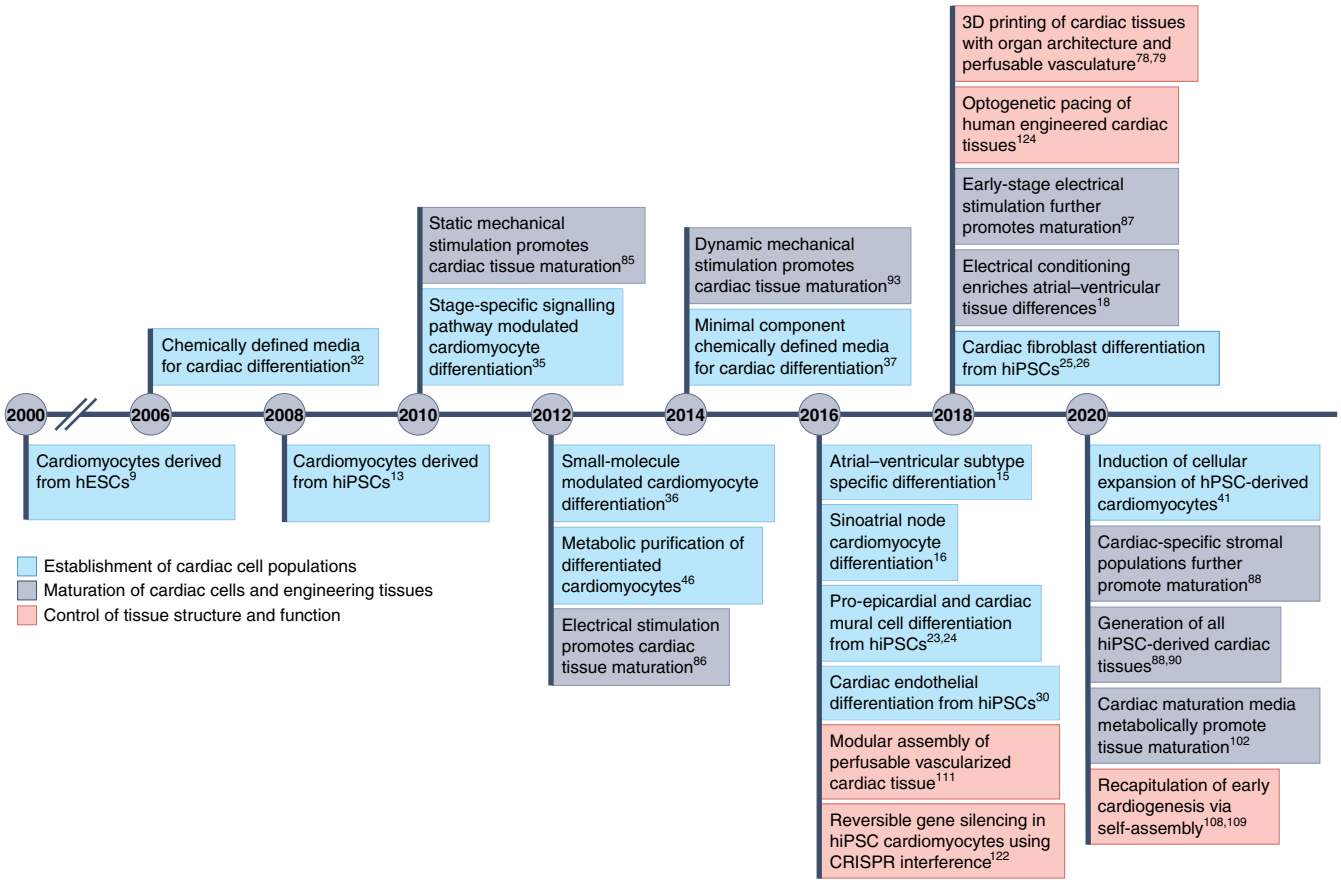

Fig. 3 | A timeline of progress in the development of hECTs. Progress in the development of hECTs is outlined by specific achievements, organized in chronological order into the three areas of focus defined in Fig. 2. In 2000, cardiomyocytes derived from hPSCs marked the beginning of human-based cardiac research in vitro. Work in the establishment of cardiac cell populations continued, and research in the maturation of cardiac cells and engineered tissues soon followed. In 2010, hECTs could be routinely fabricated and methods for maturation began to be more thoroughly investigated. Progress continued simultaneously in both areas while advancements in the control of tissue structure and function began to bear fruit. The timeline also illustrates the substantial progress that has been made since the generation of a basic cell source in the early 2000s. Today it is possible to recapitulate early cardiogenesis and other complex processes of the native heart.

Glucose starvation offers an alternative purification method free of these complications. By leveraging the unique metabolic properties of cardiomyocytes, the culture of differentiated cells in lactose-supplemented glucose-deprived media causes massive cell death in non-myocyte populations, allowing for consistent purification through a simple temporary switch in media composition[46].

Today, hPSC-CMs with over 90% purity can be consistently derived[36,37]. The highly efficient differentiation and purification protocols available are the result of years of optimization. However, recent reports have shown that despite the optimizations, batch-to-batch and cell-line-to-cell-line variabilities remain, even for baseline functions of healthy hiPSC-CMs[47]. Despite the high degree of control of troponin positivity, hECTs can vary significantly in contractile frequency, force and kinetics. Elucidating the underlying causes of such remaining variability will be important for the translatability of research findings from hECTs.

Maturation of cardiac cells and engineered tissues

Following differentiation, hPSC-derived cell populations display an immature phenotype, which limits their utility in biological studies and in translational research. In general, hPSC-CMs more closely resemble foetal-stage cells than their adult counterparts[48–50]. By taking cues from human development, a range of strategies have been devised to drive the functional maturation of hECTs (Fig. 2b).

The shift to 3D culture. The natural geometry of the myocardium is three-dimensional and highly anisotropic. By taking such architectural cues into consideration, a concerted effort to mimic native extracellular environments in vitro for the study of their effects on cell phenotype led to studies that compared monolayers of hPSC-CMs to hPSC-CMs in tissue culture and in various biomaterials. These studies have shown that improved maturation, as indicated by electrophysiology measurements and protein-expression and gene-expression profiles, is possible[51–54].

Even without any extracellular support, hPSC-CMs cultured in 3D spheroids display functionality that is superior to that of monolayers[55]. To better support the cellular environment, a variety of synthetic and natural biomaterial scaffolds can be used for the fabrication of hECTs. Synthetic scaffolds have defined compositions with chemical and mechanical properties that can be finely tuned for the desired application[56]. When fabricated in a highly aligned manner, they can mimic the anisotropic myocardial architecture, and guide cell shape and function[57,58]. However, synthetic structures lack biological cues that are critical for cellular integration. Also, they can be electrically insulating at biologically relevant frequencies and require the incorporation of conductive nanomaterials[59]. Alternatively, natural scaffolds, although not as versatile, can offer appropriate cell-binding sites and an increased capacity for cellular remodelling[54,56,60]. Two commonly used materials are fibrin and

collagen[51,60–63]. Fibrin is used for its biodegradability and its ability to provide temporary structural support while cells deposit their own ECM. Collagen is chosen for its role as a major structural component of the native myocardial ECM. These materials are often supplemented with Matrigel (a mouse-derived surrogate mixture of basement-membrane proteins) to provide further ECM components[18,51,61,63]. Natural scaffolds can also be directly derived from animal myocardial tissue through decellularization. Decellularized scaffolds preserve the composition of the native myocardial ECM and its mechanical properties and fibrous architecture, facilitating hECT culture[64]. However, their sourcing is limited. Composite scaffolds have garnered increasing attention as alternatives to synthetic or natural biomaterials, as they offer high levels of tunability and bioactivity. They often combine synthetic structural materials (such as poly-(lactic-co-glycolic acid) or polycaprolactone) with natural materials (such as collagen, fibronectin or laminin) for higher degrees of biocompatibility and cellular integration[65–69]. Cardiac tissues fabricated from composite scaffolds often show greater functional maturity than those made from their individual counterparts. Alternatively, recombinant-protein engineering allows for peptides with distinct functionalities to be modularly assembled to form biomaterials with adequate cellular adhesion, signalling, crosslinking and growth-factor delivery, and with degradation tailored for a specific application[65,70–72].

Each form of extracellular support comes with its own advantages and limitations. hECTs without extracellular support are simple to fabricate and are easily scalable for high-throughput experiments. This makes them ideal for high-volume applications, such as drug screening. Scaffolds allow for increased control over tissue geometry and the extracellular environment. For example, tunable synthetic scaffolds make it easier to probe how the heart responds to differences in environmental conditions, whereas natural polymers provide a more physiological environment to study healthy and pathological biological processes[73,74]. These options support a variety of hECT designs, such as tissue strips, tubes, pouches and anatomical chamber-like tissues[18,62,75–79]. However, there is currently no definitively superior method for providing extracellular support to hECTs. Yet years of research optimizing materials for extracellular support have resulted in an ever-expanding toolkit for the tailoring of hECTs to the needs of specific applications.

Inclusion of stromal cell populations. In the native heart, different cell types play distinct yet complementary roles in supporting cardiac function. Fibroblasts provide structural integrity in the heart muscle by maintaining the ECM[80,81]. Endothelial cells, besides making up the cardiac vasculature, play a vital role in the regulation of cardiomyocyte metabolism, remodelling and hypertrophy[82,83]. These stromal populations are in constant communication with cardiomyocytes via paracrine and endocrine factors, and it has been postulated that myocyte–stromal-cell interactions are integral to promoting the maturation of cardiac tissue.

Both primary stromal cells and non-myocyte by-products of cardiac differentiation have been included in the stromal populations of hECTs[51,53,84–86]. hECT models have been established using a range of cellular compositions, and typically consist of 50–80% cardiomyocytes and 20–50% stromal populations[18,53,87,88]. Progress in this area has delineated the distinct effects that the individual stromal cell types have on the function and maturity of cardiac tissue. In vitro, fibroblasts enable ECM deposition and tissue remodelling, and increase the generation of myocyte forces[53]. Endothelial cells promote early cardiomyocyte proliferation and, when added along with vascular mural cells, improve sarcomeric structure, cellular alignment and electrophysiology[84,89]. Comparisons of the effects of different compositions of stromal cells on the maturity of cardiac tissue showed that maturation is most enhanced when both fibroblast and endothelial-cell populations are present[88]. However, the understanding of the compositional impacts of individual stromal cell types on tissue structure and function remains limited, and an optimal cell composition for engineered cardiac tissues has not yet been determined.

Cardiac-specific stromal populations can further promote maturation. Co-differentiation of cardiac endothelial-cell populations during cardiomyocyte differentiation has been shown to yield larger cardiomyocytes with a more mature expression of sarcomeric and ion-channel genes[29]. Additionally, cardiac fibroblasts displayed emergent electrophysiological coupling with cardiomyocytes (electrophysiological coupling doesn't occur when non-cardiac fibroblasts are used[88]). hECTs formed with stem-cell-derived cardiac endothelial cells and cardiac fibroblasts developed mature ultrastructural characteristics, such as H-zones, I-bands, M-lines, t-tubule-like structures, improved calcium handling, more mature action-potential profiles and improved metabolism via higher mitochondrial respiration capacity[88]. Overall, the study of the impact of cardiac-specific stromal populations on the phenotypes and functions of hECTs is a fairly new avenue of research that may lead to important steps towards fully isogenic tissue models[90].

Mimicking the heterogeneous cellular landscape of the myocardium significantly enhances the maturity of cardiac tissue. Current protocols for tissue fabrication employ a wide range of cell types, cell sources and cell-type ratios for stromal support. The fabrication of hECTs has shifted towards the use of hPSC-derived and cardiac-specific stromal cell sources for the creation of tissues with defined initial cellular compositions. As hECT models continue to be developed, interrogating how changes to the composition of the tissue impact tissue phenotypes and functions will be vital to advancing their utility.

Application of external stimuli. During development, the heart is exposed to mechanical, electrical and biochemical stimuli. Early attempts to mature cardiomyocytes solely through prolonged culture time yielded limited results[91,92]. As such, research shifted towards the investigation of the maturational effects of applying these stimuli in vitro.

To mimic the physiological loading cycles of the myocardium, early studies applied static and cyclic mechanical stretching to hECTs, and showed that loading leads to structural alignment, the development of ultrastructure and increased force production[85,93–95]. Physical stimulation alone was shown to improve the structural and mechanical aspects of myocardial function. Electrical stimulation also leads to the maturation of these same functions, as well as improved electrophysiological and calcium-handling properties[86]. Moreover, the application of electromechanical stimuli to tissues formed by nascent differentiated cells is important for achieving adult-like phenotypes in vitro. hECTs that undergo early electromechanical frequency-ramped pacing show further maturation of their structural, mechanical, electrophysiological and calcium-handling properties, and display a positive force–frequency relationship, oxidative metabolism and the formation of T-tubules[87]. Interestingly, the maturation of atrial and ventricular hECTs requires different stimulation regimes, and long-term stimulation yields tissues with distinctly different phenotypes that are in line with physiological atrial–ventricular differences[18].

Throughout development, the heart is subject to both transient and persistent changes in its biochemical environment. However, the culture of hPSC-CMs generally relies on glucose-based media formulations that lack these physiological factors. The addition of hormones such as Triiodo-L-thyronine (T3) and glucocorticoids promotes the functional and expressional maturation of hPSC-CMs[96–98]. Supplementing hPSC-CM cultures with fatty acids also drives cardiomyocytes towards more mature phenotypes[99,100]. More recently, the compositions of culture media have been tailored to better replicate the fatty-acid-rich and glucose-deprived metabolic

environment of native adult cardiomyocytes. Cardiac-specific media formulations promote higher oxidative metabolism in hPSC-CMs, and lead to the maturation of mechanical, electrophysiological and calcium-handling properties[98,101,102].

Understanding, replicating and combining physiological stimuli in vitro is vital for tissue maturation. Studies of the effects of mechanical stimuli and electrical stimuli suggest that control over the temporal dynamics of stimulation is integral to cardiomyocyte maturation. Notably, many studies have shown that applying stimuli in a supraphysiological manner or in concert further accelerates and advances maturation[86,87]. Subjecting hECTs to native physiological stimuli via engraftment in vivo has also been shown to lead to profound levels of maturation[103–105]. This suggests that there is a myriad of in vivo signals to be identified that could greatly improve tissue maturation. However, the understanding of the mechanisms underlying many of these processes is incomplete. How these factors affect the phenotypes of hPSC-derived cardiac stromal cells, and whether these cells require maturation, has not yet been explored. Elucidating the processes impacted by external stimuli and optimizing the most beneficial combinations of stimuli will facilitate the development of more comprehensive protocols for driving the maturation of cardiac tissues towards an adult phenotype.

Control of tissue structure and function

The utility of engineered cardiac tissues is dictated by the phenotypes of its individual cells, their collective ability to recapitulate the heart's distinct 3D hierarchical architecture and supporting functional subsystems (vascular, nervous, immune, conductive and others), and whether the engineered tissues allow for dynamic control over their functions. The development and integration of methods to exercise fine levels of control is paramount for advancing the utility of hECTs. In this section, we highlight some of the key innovations that have driven progress towards a more precise control over the complexity, architecture and function of hECTs (Fig. 2*c*).

Purposeful organization of tissue architecture. The hierarchical organization of cellular structure is integral to the heart's function. At the scale of the organ, cardiomyocytes localized in the atria and ventricles have distinctly different properties that support chamber-specific functional demands. At the level of the tissue, cardiac fibroblasts surround myocyte bundles to deposit ECM for mechanical support[106]. Although most current cardiac-tissue models include multiple cell types, recapitulating the precise cellular arrangements of native tissue remains an elusive goal. To this end, various methods of tissue fabrication have been devised to control the spatial heterogeneity of cardiac tissue.

Cellular self-assembly—the intrinsic ability of cells to self-organize into physiological structures—allows for the generation of complex tissues, and requires control only over the initial cellular composition and culture conditions. This approach, although simple, relies on a profound understanding of the factors that impact tissue morphogenesis. At the most basic level, the application of anisotropic stimuli such as uniaxial tension promotes the alignment of cardiomyocytes[86,95].

In vitro models of cardiac organogenesis have recently been applied to human organoid models to create hECTs that resemble physiological tissue architectures[107–109]. For example, cardiac organoids recapitulated aspects of the early development of the heart before heart-tube formation. These organoids underwent progressive morphological stages of physiological development, and displayed myocardial layers lined by endocardial cells as well as vascular-like networks lined by endothelium[108]. Also, the combinatorial modulation of signalling pathways involved in early cardiogenesis allowed for the elucidation of the mechanisms behind cavity formation, and these were used to instruct epicardial spreading, inward migration and differentiation to direct cardiomyocyte–endothelial-cell separation[109].

Complex cardiac tissue structures can also be fabricated by modular assembly. In this approach, the overall structure is created by generating and assembling multiple simpler units[110]. It tends to be more complicated and provides lower throughput than relying on cellular self-assembly. However, it allows for enhanced control over tissue structure. For example, pre-fabricated branched vascular modules can be cast within functional tissues to create cardiac tissues that have a hierarchical vasculature and allow for anastomosis[111]. By simplifying and downsizing the vascular modules, this technology can also be used for high-throughput screening[112].

3D bioprinting using cell-based bioinks allows for control over tissue design down to cellular resolutions. Early uses of 3D bioprinting involved the printing of vascular structures around which cardiac tissues could be cast[113]. Sacrificial inks can also be used to embed vascular structures into preformed cardiac tissues[114]. Recent advances in 3D bioprinting have allowed for the simultaneous re-creation of organ-level, tissue-level and cellular-level structures. Functional hECTs can be printed with structural characteristics of the ventricular chamber, and different vascular structures within tissue can be printed simultaneously[78]. Vascularized multicellular hECTs can be printed with patient-tissue architectures via the simultaneous use of parenchymal and vessel-forming hydrogel bioinks[79]. The printed cardiac tissues had perfusable vascular networks for oxygen and nutrient delivery to overcome the diffusional limits of oxygen transfer. Notably, the bioprinting of a fully cellularized small-scale heart with major blood vessels showcases the potential of bioprinting technology for the faithful re-creation of complex anatomical cardiovascular architectures[79].

Cellular self-assembly, modular assembly and 3D bioprinting allow for varying degrees of control over the structure of hECTs to mimic the architectural properties of the native heart. Complete reliance on self-assembly currently only works at the microscopic level in simple tissue models. However, the intrinsic potential of cells to collectively undergo tissue morphogenesis remains to be fully tapped. At present, fabricating multiscale complex architectures requires precise spatial control via modular assembly or 3D printing. However, progress in the recapitulation of native heart architectures has improved the ability to create hECTs that can structurally integrate in vivo, and such hECTs can also serve as tools for a range of applications (for example, they can be used as models for the study of cardiovascular diseases[79,111]).

Dynamic control over tissue function. One main advantage of tissue engineering over the use of animal models is the degree of precision that can be applied to control genetic, functional and extracellular cues. The continued development of increasingly sophisticated technologies allows for the manipulation of hECT functions with spatiotemporal precision to elucidate the subtle mechanisms that underlie complex phenomena. Dynamic control over tissue behaviour can be implemented using microenvironmental and genetic manipulations.

Exercising control over biochemical factors and ECM properties has long been used to study how extracellular changes affect the behaviour of cardiac tissue. Recently, functional biomaterials have been used to dynamically control microenvironmental cues[115,116]. By using techniques in photochemistry, biomaterials can be functionalized with biochemical factors, small molecules, peptides and proteins[117]. Also, the photoreversible immobilization of proteins in tissue scaffolds allows for the spatiotemporal control of cells[118]. Such functional biomaterials could thus be used to probe the spatiotemporal effects of microenvironmental changes on cardiac function.

Because genome editing allows for the easy interrogation of individual genes, studying genetic cardiac disorders by leveraging genome-editing techniques eliminates the confounding

effects of genetic background. hECTs fabricated using gene-edited hiPSC-CMs have been used to model diseases such as Barth syndrome, dilated cardiomyopathy and hypertrophic cardiomyopathy[119–121]. CRISPR-interference technology, which allows for dynamic control over genetic expression with temporal precision, has been leveraged to edit hiPSCs to knock down developmentally related and disease-related genes at various stages of cardiac differentiation[122]. This is a proof of concept of the utility of temporal genetic control in the study of developmental-stage-specific factors in cardiac genetic disorders.

The manipulation of the genome can also be leveraged to encode for specially designed optogenetic proteins. When expressed, these proteins allow for optical control over cellular processes with high degrees of spatiotemporal precision. The most commonly used methods are optogenetic actuators that can modulate electrical activity with millisecond-scale precision[123,124]. These methods can be used for the assessment of drug cardiotoxicity. In particular, optogenetic pacing of cardiomyocytes has allowed for the frequency-controlled evaluation of drug effects on cardiac action potentials and calcium transients[125]. To study frequency-dependent aspects of drug action and arrhythmia, optogenetically paced cardiac tissues have been used to identify emergent drug-induced cardiac instabilities that are only apparent at certain frequencies[126]. Similarly, arrhythmogenicity can be induced in cardiac tissues via optogenetic pacing as a means for disease modelling[127]. More recent advances in optogenetics allow for the same degree of optical control over intracellular signalling pathways[128–131]. Although these optogenetic techniques have yet to be adopted in cardiac tissue engineering, we anticipate that they will improve the utility of hECTs.

Integration of functional subsystems. The heart is controlled and maintained by vascular, nervous, immune, conductive and other subsystems that require the orchestration of multiple cell types and structures so that they work synchronously. The development and integration of these functional subsystems into the design of hECTs is vital for accurately modelling their contributions to disease progression in vitro, for the evaluation of their roles in drug cardiotoxicity and for creating tissue grafts for transplantation.

The cardiac vasculature provides oxygen and nutrients to support the high metabolic demands of cardiomyocytes. Its functionality is heavily dependent on its hierarchical structure: vessels branch into dense networks of capillaries that have high aspect ratios and wrap around cardiomyocyte bundles[132]. Without functional vasculature, the size of hECTs is limited by the diffusional penetration depth of oxygen. Although early attempts to create vascularized hECTs through the simple inclusion of endothelial cells showed that some level of self-assembly occurs, these attempts did not lead to the formation of the perfusable vessels that are necessary to facilitate mass transport[84]. Modular assembly and 3D printing have allowed for better guidance and control over the structural design of vascularized cardiac tissues capable of surgical anastomosis and vascular functionality on implantation in vivo[78,79,111].

The precise patterns of the electromechanical cardiac cycle necessitate a conduction system that coordinates contractions for cardiac output. Following the hPSC differentiation of sinoatrial-node-like cardiomyocytes, these cells were identified to have electrophysiological properties similar to those of primary pacemaker cells. In fact, they can function as biological pacemakers on implantation. When co-cultured with ventricular iPSC-CMs in vitro, sinoatrial-node-like cardiomyocytes act as stable initiation sites for electrical activity[16]. These findings marked an important first step towards the integration of cardiac conduction systems into hECTs.

The nervous and immune systems are also important to cardiac function, yet they have been studied less. Although the cardiac conduction system coordinates electrical signals within the heart, changes in heart rate are physiologically dictated by the autonomic nervous system[133]. Early studies on the innervation of cardiac tissues have shown functional coupling between hiPSC-CMs and sympathetic neurons in vitro[134–136]. The electrical stimulation of neurons results in transient increases in beat frequency. Recent studies have co-cultured human hiPSC-derived sympathetic neurons with hiPSC-CMs, and showed the formation of cardiac-specific direct synaptic contacts[134–136]. However, how these nervous couplings affect the function of cardiomyocytes has yet to be determined.

Immune components, such as resident cardiac macrophages, are believed to be important both for normal cardiac homeostasis and for pathological responses to injury[137]. However, as with the integration of the nervous system, there has been little research in this area. Although much has been done to categorize the immune regulation of implanted cardiac tissues and biomaterials, the incorporation of resident immune cells into the tissues has been scarcely explored. Progress in the generation of hPSC-derived macrophages provides substantial opportunities for research in this area[138].

Overall, methods to seamlessly integrate fully functional subsystems into hECTs are being established. They will facilitate the study of the roles of cardiac subsystems in homeostasis and disease.

Lessons learned, opportunities and challenges

Engineered human cardiac tissues are the culmination of decades of development in developmental biology, stem-cell technologies, materials science, tissue engineering and the medical sciences. They are built on foundational developments in tissue engineering using cells from rodents and have greatly benefited from advances in hPSC technologies.

Cutting-edge engineered cardiac tissues have commonalities across applications: they are hiPSC-derived, include organ-specific cell types necessary for the maintenance of tissue function, display a degree of maturity necessary for physiological relevance and offer control over their structure or function for the intended application. These common criteria for the generation of hECTs heighten their translational utility.

When used for drug development, cardiac tissues can recapitulate the known therapeutic and cardiotoxic effects of many compounds, and are beginning to be used to screen for drug candidates[139,140] at high-throughput without sacrificing sensitivity and specificity. In disease modelling, functional tissues generated from patient-derived hiPSCs are opening the door to understanding complex genetic contributions to disease phenotypes[18]. Furthermore, state-of-the-art methods in cardiac tissue engineering that allow for precise control over tissue composition and the microenvironment can be used to study functional responses in the complex processes underlying cardiac responses to injury (such cardiomyopathies, myocardial infarction and fibrosis[26,140,141]). In regenerative medicine, engineered cardiac tissue grafts with anatomical structures can be surgically integrated into host myocardium in preclinical models, and will pave the way for first-in-human clinical trials[79,142].

Robust sources of cardiac cells derived from iPSCs are a foundation for engineered cardiac tissues. The translation of physiological developmental processes into precisely defined protocols has led to improvements in the generation of different types of human cardiac cells (myocytes and supporting cell populations), and has facilitated many advances. As differentiation methods become more reproducible and efficient, more research laboratories will be able to easily generate human cardiac cells and tissues. Improved reproducibility in cellular differentiation will also enhance the statistical power of the experimental data. However, batch-to-batch and cell-line-to-cell-line variabilities when differentiating iPSCs into cardiac cells remain major hurdles for the translation of hECT research. It will thus be important to rigorously standardize and optimize methods for cardiac-cell generation. The implementation of lessons learned from quality controls in the manufacture of pharmaceuticals may be helpful in overcoming these barriers. In fact,

some of these challenges are being considered and addressed in recent clinical trials of hPSC-CM-based therapies[143,144].

Building on progress in cell sourcing, the ability to functionally mature hECTs in vitro was central to improving their translational relevance. The most successful methods for maturing hECTs were taken from the cues that naturally occur during development, yet were adapted for in vitro maturation. In particular, the application of certain stimuli in a supraphysiological fashion drives hECT maturity further than just recapitulating physiological stimulation[86,87]. The application of structural and cellular cues in a multimodal manner more comprehensively matures cell phenotypes[62,86]. However, assays for the characterization of the maturation of cardiomyocytes (spanning gene and protein expressions, morphology, ultrastructure, electrophysiology, calcium handling, contractility, metabolism, cell-cycle activity and responses to drugs or to other external stimuli) will need to be standardized. Most studies only characterize maturation in select categories, and objective comparisons between different metrics are rare. This challenge may become easier to address as technologies for the comprehensive characterization of tissues continue to advance.

Alongside the development of hECTs, there have been concomitant advances in tools for the characterization of functional tissues. Live readouts using 3D imaging systems and embedded-sensor technologies enable new insights into functional tissue dynamics (such as contractility, force generation and calcium handling) by providing real-time information in a non-destructive manner[145–148]. Sequencing technologies have become more widespread, and recently developed techniques are available for the profiling of the genome, the epigenome, the transcriptome, the proteome and the metabolome[149–151]. In parallel with the adoption of these tools for analysing cultured cells and tissues, atlases of native tissues allow for comprehensive comparisons between hECTs and native tissues, and between different hECT models across multiple dimensions and at single-cell resolution[152,153]. Comparisons between maturation protocols will reveal the specific advantages of different methods so that they can be combined into an optimized approach for the maturation of hECTs.

The development of tools for cardiac tissue engineering is reaching a point where the clinical impact of hECT technology is no longer just a dream. Bottom-up approaches have driven substantial progress in the development of hECTs. Many unique characteristics of the human heart can be replicated stepwise and subsequently combined to generate sophisticated tissues. The creation of such cardiac tissues with functional capabilities is more widely accessible. Improving the ability to manipulate cellular behaviour and structure within hECTs in a biologically meaningful way and with high levels of precision will help to more effectively translate hECT technologies. Currently established tools for the control of cardiac tissues have been adapted from technologies used in other disciplines: this is the case for genome editing, developed in bacterial research, and 3D bioprinting, adapted from industrial manufacturing technologies. They have proven to be highly useful in isogenic disease modelling and in the fabrication of tissue grafts. Yet comprehensive control of the formation of hECTs, their maturity and their functional behaviour is still in its infancy. Moving from proof-of-concept developments towards the multiplexing of methods for hECT control into physiologically relevant tissue designs will be central to driving further progress.

Our analysis of the past two decades of research in cardiac tissue engineering has helped us to identify some of the knowledge gaps in the development of hECTs and to suggest opportunities for advancement. Other areas of tissue engineering may benefit from similar retrospective analyses. Although the specifics may be different, most approaches are widely applicable, regardless of tissue type. Moreover, the milestones that we identified as key indicators of progress (Table 1) can serve as guidelines for the next steps forward.

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R.Z.Z., R.L., B.L. and G.V.-N. conceptualized, outlined and edited the manuscript. R.Z.Z., R.L. and B.L. surveyed relevant literature and wrote the manuscript.

Competing interests

G.V.-N. co-founded TARA Biosystems, a company that has licensed some of the cardiac tissue-engineering methodologies developed in her laboratory, holds equity in the company and is serving on the Board of Directors. The other authors declare no competing interests.

Additional information

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