STEM CELLS

Exercising engineered heart muscle to maturity

Donghui Zhang and William T. Pu

The immaturity of stem cell-derived cardiomyocytes has impeded their use for in vitro disease modelling, cardiotoxicity assays, and cell-replacement therapy. Ronaldson-Bouchard and colleagues report unparalleled in vitro maturation of stem cell-derived cardiomyocytes. This advance promises to unlock the translational potential of these cells.

Refers to Ronaldson-Bouchard, K. et al. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* **556**, 239–243 (2018).

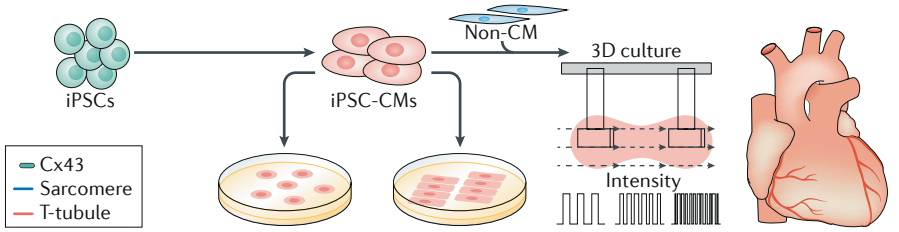
The development of induced pluripotent stem cells (iPSCs) and of robust protocols to differentiate these cells into cardiomyocyte-like cells has provided exciting opportunities for disease modelling and cardiac regeneration. However, the inability to produce mature iPSC-derived cardiomyocytes (iPSC-CMs) with adult cardiomyocyte-specific adaptations, which are essential for their function, is a major barrier to progress in these areas[1,2]. Hallmarks of adult cardiomyocytes include oxidative metabolism, large size and rod-like shape, formation of intercalated discs (characteristic cell–cell junctions at the extremes of cardiomyocytes), highly organized sarcomeres, membrane invaginations known as transverse (T)-tubules that are organized into dyads (structural elements formed by the juxtaposition of the T-tubules, which contain voltage-gated Ca^{2+} channels ($Ca_V1.2$), to the sarcoplasmic reticulum, which contains ryanodine receptor 2, the major Ca^{2+}-induced Ca^{2+}-release channel), and efficient and adaptive Ca^{2+}-handling machinery that enables the characteristic physiological responses of cardiomyocytes such as a positive force–frequency relationship.

Implantation of stem cell-derived cardiomyocytes into hearts fosters their maturation and the acquisition of these adult cardiomyocyte features, indicating that these cells possess the capacity to mature when placed in the correct environment[3,4]. Extensive efforts in multiple laboratories have uncovered some of the ingredients of this nurturing cardiac environment, which include the extracellular environment (extracellular matrix patterning, physiological substrate stiffness, or 3D culture in hydrogels), inclusion of non-cardiomyocyte cell types (endothelial cells and fibroblasts), growth factors and hormones (such as insulin-like growth factor 1 (IGF1), thyroid hormone, and cortisol analogues), and mechanical and electrical stimulation-based protocols that promote maturation of stem cell-derived cardiomyocytes (Supplementary Table 1). However, until now, these efforts have not recapitulated the formation of T-tubules or dyads, which are required for mature Ca^{2+} handling in stem cell-derived cardiomyocytes. A new study by Ronaldson-Bouchard and colleagues seems to resolve this impasse[5]. By combining 3D culture with the early application of a protocol based on electrical and isotonic mechanical stimulations, Ronaldson-Bouchard and colleagues generated human engineered cardiac tissue that recapitulates the hallmarks of adult myocardium, including iPSC-CMs with extensive T-tubule networks and mature Ca^{2+}-handling functional properties[5] (FIG. 1).

> “stem cell-derived cardiomyocytes can be coaxed to differentiate into mature adult cardiomyocyte-like cells in vitro”

Consistent with the findings of this study, another recent study by Parikh and colleagues showed that stimulation with a combination of cortisol and thyroid hormones, together with culture on a matrigel mattress, promotes T-tubulation and mature Ca^{2+} handling in iPSC-CMs[6].

Ronaldson-Bouchard and colleagues studied human cardiac muscle bundles formed from a 3:1 mixture of iPSC-CMs and human dermal fibroblasts in a fibrin hydrogel and cast around elastomeric pillars that provided a dynamic mechanical load[5]. From day 7 to day 28 after muscle bundle formation, one of the three following training regimens was applied: control (no stimulation), constant frequency (2 Hz), or intensity training (gradual frequency ramp from 2 Hz on day 7 to 6 Hz on day 21, then constant frequency of 2 Hz until day 28). Additionally, the investigators compared training of iPSC-CMs that had just initiated spontaneous contractions (early-stage iPSC-CMs) with training of iPSC-CMs that had been matured in regular 2D culture on matrigel-coated rigid plastic plates for 2 weeks (late-stage iPSC-CMs). Early-stage iPSC-CMs subjected to the intensity training regimen showed structural, metabolic, and functional hallmarks of maturation, including large size, high mitochondrial content and capacity for oxidative phosphorylation, organized sarcomeres, and gap junction proteins at the cell–cell junctions at the poles of iPSC-CMs (FIG. 1; Supplementary Table 1). The most remarkable observation was the robust and well-organized T-tubule network of the cardiac tissues that underwent early-stage intensity training. Although the extent and consistency of this finding is not reported, the images suggest that the intensity training protocol resulted in an unprecedented level of maturity of the Ca^{2+}-handling machinery in engineered muscle bundles of iPSC-CMs. Consistent with this highly organized structure, Ca^{2+}-handling function also showed mature characteristics, as demonstrated by the positive force–frequency relationship and post-rest potentiation. Importantly, constant stimulation at a physiological rate did not achieve these maturation end points, which suggests that the ramped protocol to a final, supraphysiological rate is essential. Moreover, the effect of intensity training was observed in early-stage but not late-stage iPSC-CMs,



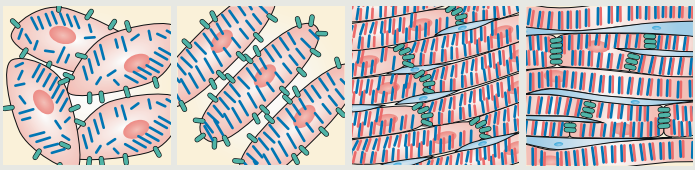
Setting	2D isotropic	2D patterned	Intensity exercise training	Adult heart
Cell structure				
Cell morphology				
Cell size	+	++	+++	+++
Transverse tubules	–	–	++	+++
Intercalated discs	+	+	++	+++
Sarcomere length	+	++	++	+++
Mitochondria	+	+	++	+++
Physiology				
Membrane potential	++	++	+++	+++
Conduction velocity	ND	ND	++	+++
Contractility	ND	ND	++	+++

Fig. 1 | **In vitro maturation of pluripotent stem cell-derived cardiomyocytes induced by ramped intensity training.** Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were cultured in 2D or 3D format. Early-stage or late-stage iPSC-CMs and supporting fibroblasts were encapsulated in fibrin hydrogel to form tissues stretched between two elastic pillars and made to contract by electrical stimulation. Addition of early intensity training to 3D culture conditions, which consisted of ramped electrical stimulation and isotonic mechanical stress, induced remarkably mature cardiac tissue with aligned cardiomyocytes, well-organized transverse (T)-tubules, and polarized connexin 43 (Cx43; also known as gap junction-α1 protein), which was structurally similar to adult myocardium. ND, no data.

which suggests that early-stage iPSC-CMs have a greater developmental plasticity than late-stage iPSC-CMs, which allows them to respond to the training regimen.

The studies by Ronaldson-Bouchard and colleagues[5] and Parikh and colleagues[6] demonstrate that stem cell-derived cardiomyocytes can be coaxed to differentiate into mature adult cardiomyocyte-like cells in vitro through refinements in culture conditions and bioengineered environments. For developmental biologists, these studies raise interesting questions about the molecular signalling pathways and transcriptional networks that promote and coordinate maturation in response to hormone stimulation or intensity training. With regard to the regeneration of injured hearts, the finding that maturity-inducing treatments are more effective on early-stage iPSC-CMs than on late-stage iPSC-CMs suggests that cells at this stage, or perhaps even their committed progenitors, might be preferable for use in myocardial grafts. With regard to in vitro disease modelling, these studies provide a path to developing higher-fidelity, engineered cardiac tissue models to study human heart disease. The availability of iPSC-CMs with electrical and Ca^{2+}-handling properties similar to those of adult human cardiomyocytes will also enhance efforts to develop iPSC-CMs as a platform for pharmacological cardiotoxicity testing. However, work remains to be done: despite the success of Ronaldson-Bouchard and colleagues in improving some facets of cardiomyocyte maturation, room for improvement in other areas remains. For example, some properties of these engineered muscle bundles, such as specific contractile force, action potential upstroke velocity, and conduction velocity, were still low compared with native human myocardium[7] as well as other bioengineered heart tissues[8,9] (Supplementary Table 1). Furthermore, engineered cardiac tissue production methods will need to be optimized to yield tissues with a consistent and uniform level of maturity, in a format suitable for high-throughput assays. Achieving these goals will be facilitated by the development of standardized, quantitative measures of cardiac tissue maturation and function that can be compared across models and studies.

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Competing interests
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SUPPLEMENTARY INFORMATION

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Table 1 | **Characteristics and maturation parameters for several human 3D engineered heart tissue models**

Model		**Adult LV**	**Intensity-trained cardiac tissue**	**Cardiopatch**	**Cardiac muscle patch**	**Matrigel mattress**	**Engineered heart tissue**	**Engineered cardiac tissue**	**Biowire**
References		NA	1	2,3	4	5	6,7	8	9
Cells	**Type**	Native human LV	iPSC-CMs and human dermal fibroblasts (3:1)	PSC-CMs (~80% pure)	iPSC-CM, iPSC-SMC, iPSC-EC (2:1:1)	iPSC-CM	iPSC-CM (87% $cTnT^+$)	iPSC-CM (70% $cTnT^+$)	PSC-CM (48%) and nonmyocytes including 34% fibroblasts
	Time of differentiation prior to tissue seeding (days)	NA	12	16	19	16–30	10–14	14–21	20
	Time in 3D culture (days)	NA	28	7–21	7	5	10–29	14	14
Cardiac tissue	**Materials**	NA	6 mm x 2 mm fibrinogen gel	7 mm x 7 mm fibrinogen/ Matrigel	2 mm x 2 mm light-patterned gelatin methacrylate	Single cells on Matrigel mattress	100 µl fibrinogen/ Matrigel	20 x 3 mm collagen type I gel	~600 µm wide collagen type I gel on inelastic silk core
	Protocol	NA	Rocking culture, early ramped field stimulation 2–6 Hz, isotonic contraction	Rocking culture	Rocking culture	T3 + Dex	NA	2 weeks static stretch + electrical stimulation	EB differentiation; seeded 7 days then ramped field stimulation 1–6 Hz
Structure	***Cell morphology***								
	Size (μm^2)	2,565[10]	1,500	NA	NA	3,000, M 2,000, F	4–8 times lower membrane capacitance than adult CMs	795	917
	Shape	Rod	Rod-like	Rod-like	Rod-like	Rod-like	Rod-like	Elongated spindle	~50% rod-like
	Transverse tubules	Membrane, BIN1, $Ca_v1.2$, CAV3, and RYR2 staining aligned perpendicularly to CM long axis	Membrane, BIN1, $Ca_v1.2$, and RYR2 staining aligned perpendicularly to CM long axis	No	No	Present but sparse and poorly organized	Perinuclear, irregular CAV3 staining	No	No

	Intercalated discs	Cx43, $Na_v1.5$, N-cadherin localized at CM poles	Cx43 localized at CM poles	N-cadherin localized at CM poles. Cx43 less anisotropic	NA	NA	$Na_v1.5$ localized at CM poles. Cx43 not anisotropic.	Adherens junctions	NA
	Sarcomere length (μm)	2.2[11]	2.2	2.09	NA	NA	1.6	NA	NA
	Ultrastructure								
	Sarcomeres	Orderly register of sarcomeres; A-bands, I-bands, M-lines, and Z-lines present.	Orderly register of sarcomeres; A-bands, I-bands, M-lines, and Z-lines present.	Orderly register of sarcomeres; A-bands, I-bands, and Z-lines present. No M-lines.	NA	NA	Regular Z-lines, and inconsistent I-bands and A-bands. No M-lines.	NA	Regular Z-lines. I-bands and H-zones present. No M-lines.
	Mitochondria	Aligned to sarcomeres; ~40% cell area[12]	Aligned to sarcomeres; 30% cell area	NA	NA	NA	NA	NA	Closer to sarcomeres
	Other	Intercalated discs, desmosomes, T-tubules	Intercalated discs, desmosomes, T-tubules	Intercalated discs, desmosomes	NA	NA	Primitive intercalated discs; desmosomes	Junctions, desmosomes, myofibrils	Desmosomes, nascent intercalated discs
Physiology	**Membrane Potential (mV)**	Subepi, –79.5; subendo –78.8[13]	–70.0	–70.9	NA	NA	–73.5	NA	~ –80.0
	Maximum I_{Na} upstroke velocity (V/s)	278[14]	~23	38.1	NA	NA	219	NA	~125
	Action potential duration (ms)	APD_{80}: subepi 383; subendo 494[15]	APD_{90}: 500	APD_{80}: 450	APD_{80}: 270	NA	NA	NA	APD_{90}: ~120
	Conduction velocity (cm/s)	46[16]	25	28.5	18.8	NA	NA	2.76	~15
	Physiological responses								
	Force–frequency relationship	Positive	Positive	Flat to slightly negative	NA	NA	Flat	Flat	NA
	Post-pause potentiation	Present	Present	NA	NA	NA	Present	NA	NA
	Ca^{2+} release from intracellular stores	~70%	60% reduced Ca^{2+} amplitude by SR inhibition	NA	NA	50% reduced Ca^{2+} amplitude by SR inhibition	NA	NA	NA

	Contractility: maximum specific stress (mN/mm^2)	25–44[17,18]	~3	22.4	NA	NA	NA	1.3	NA

APD_{80} or APD_{90}, action potential duration at 80% or 90% recovery; BIN1, bridging integrator 1; $Ca_v1.2$, voltage-dependent L-type calcium channel; CAV3, caveolin 3; Cx43, connexin 43; Dex, dexamethasone; EB, embryoid body; iPSC-CM, induced pluripotent stem cell-derived cardiomyocyte; iPSC-SMC, induced pluripotent stem cell-derived smooth muscle cell; LV, left ventricle; NA, not available; $Na_v1.5$, cardiac sodium channel; PSC-EC, induced pluripotent stem cell-derived endothelial cell; PSC-CM, pluripotent stem cell-derived cardiomyocyte; RYR2, ryanodine receptor 2; SR, sarcoplasmic reticulum; Subendo, subendocardial; Subepi, subepicardial; T3, thyroid hormone.

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