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SPOTLIGHT

Maturing differentiated human pluripotent stem cells *in vitro*: methods and challenges

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ABSTRACT

Human pluripotent stem cells (hPSCs), derived from individuals or genetically modified with disease-related mutations and variants, have revolutionised studies of human disease. Researchers are beginning to exploit the extraordinary potential of stem cell technology to screen for new drugs to treat intractable diseases, ideally without side-effects. However, a major problem is that the differentiated cell types on which these models are based are immature; they resemble fetal and not adult cells. Here, we discuss the nature and hurdles of hPSC maturation, using cardiomyocytes as an example. We review methods used to induce cardiomyocyte maturation in culture and consider remaining challenges for their integration into research on human disease and drug development pipelines.

KEY WORDS: hiPSC-derived cardiomyocytes, Cardiac maturation, Cardiac organoids, Engineered heart tissue, Cardiac microtissues

Introduction

During development, cells divide rapidly and differentiate from early progenitor states to acquire cell- and tissue-specific identities that lead to fully functional organs at birth. In humans, many changes also occur in the first few postnatal months and years as organs mature and acquire the form and function of adult tissues. One of the first changes around birth is the switch from glucose to fatty acid metabolism as newborns derive nutrients from milk and begin breathing oxygen (Girard et al., 1992; Lopaschuk and Jaswal, 2010). Further hallmarks of maturation include anatomical and physiological changes (e.g. in cell shape and size), gene expression and cell cycle exit with terminal differentiation. Almost 25 years ago, the first human pluripotent stem cells (hPSCs) were derived from preimplantation embryos as human embryonic stem cells (hESCs) (Thomson et al., 1998). In the years that followed, many differentiated cell types were derived from hESCs, but although many had organ-specific identities, they all resembled fetal cells at 16-20 weeks of gestation, rather than their adult counterparts (DeLaughter et al., 2016). Studying maturation, and the methods to induce it, in hPSC derivatives has become an important area of research, whether for creating models of disease that manifest in adults [such as heart failure or brain disorders (Birket et al., 2015; Vera et al., 2016)] or using differentiated cells for therapy [such as pancreas cells for diabetes (Alvarez-Dominguez et al., 2020)] to restore function in adult organs.

The question of maturation became even more important with the advent of human induced pluripotent stem cell (hiPSC) technology by somatic cell reprogramming (Takahashi and Yamanaka, 2006). hiPSCs can be derived from individuals with a particular disease and differentiated into cell types in which the disease manifests. If the cells are mature, hiPSC derivatives from such individuals can be used not only to clarify disease mechanisms, but also determine the disease risk of a person (Terrenoire et al., 2013). Pharmaceutical or regulatory authorities are deploying hiPSC models to accelerate drug discovery, market approval and reduce costs caused by drug development failures (Galson et al., 2021). One way to study the mechanisms underlying cellular maturation is by comparing fetal and adult tissues, which can be exemplified using cardiomyocytes.

The differentiation of cardiomyocytes from hESCs (hESC-CMs) was first described over 20 years ago (Kehat et al., 2001; Mummery et al., 2003). Since then, simpler methods have been developed to generate hiPSC-CMs (Burridge et al., 2012) and there are now many *in vitro* models of the human heart (reviewed by Campostrini et al., 2021); these range from simple two-dimensional (2D) cultures on (rigid) tissue culture plastic or (flexible) polymers and hydrogels, to three-dimensional (3D) cultures, such as microtissues, engineered heart tissues, 'heart-on-chip' models (which have microfluidic channels mimicking blood flow) and self-organising cardioids (Hofbauer et al., 2021). These models all yield hPSC-CMs at various stages of maturation, but the fact that hPSC-CMs mature upon transplantation into the heart suggests that there is no intrinsic block in their capacity to form fully functional cardiomyocytes (Posern and Treisman, 2006). Maturation of hPSC-CMs must be addressed, as it can limit their utility in understanding normal physiology or late-onset cardiac diseases, in drug-discovery and even in regenerative therapies, where engraftment in host tissue can cause arrythmias unless treated (Nakamura et al., 2021).

In this Spotlight, we compare the salient features of mature cardiomyocytes compared with their immature counterparts in the fetal heart or those derived from hPSCs, and then consider methods currently being used to drive maturation *in vitro* in both 2D and 3D models. We discuss aspects of maturation that have yet to be investigated and provide a perspective on what we might expect in the future if fully mature or even aged cells and tissues can be created from hPSCs. Although we focus on cardiomyocytes, many of the principles we discuss, such as stromal cell co-culture, are likely relevant to other hPSC derivatives.

Recognising mature and immature cells

An important question is: how can we recognise a mature cell? Structural, functional, biochemical, molecular and genetic

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characteristics have all emerged as distinguishing features of fetal versus adult cells. For example, only mature β-cells in the pancreas are able to respond to glucose by insulin secretion, whereas fetal or immature β-cells show a poor insulin response to glucose (Cook and Hales, 1984; Hrvatin et al., 2014). In the liver, expression of the full complement of cytochrome P450 enzymes involved in drug metabolism marks mature, but not immature, liver cells (Sadler et al., 2016). In this Spotlight, we focus on mature versus immature cardiomyocytes, which can be recognised on the basis of morphological, transcriptional and functional features, as summarized below and in Fig. 1.

Morphology and ultrastructure of mature and immature cardiomyocytes

Cardiomyocytes proliferate during development but exit the cell cycle shortly after birth as terminal differentiation and maturation take place (Porrello et al., 2011). Cells also become hypertrophic, increasing their volume by ∼30-fold (Mollova et al., 2013). This is largely achieved by myofibril growth to accommodate the increased workload, and by polyploidisation, where cardiomyocyte nuclei become tetraploid (4n, where n is the haploid chromosome number) without nuclear division (Bergmann et al., 2009). This contrasts with mouse cardiomyocytes, which undergo cytokinesis and are binucleated (Hesse et al., 2018). Polyploidy increases with age to 64n, with corresponding increases in cell volume and finally, irreversible cell cycle exit. Adult cardiomyocytes are rod shaped, have a length-to-width (aspect) ratio of 7:1 and a sarcomere length (distance between sarcomeres) of ∼2.2 µm. By comparison, immature hPSC-CMs are round or triangular, with sarcomere lengths of less than 1.5µm (reviewed by Campostrini et al., 2021) (Fig. 1). Mature cardiomyocytes form a functional 'syncytium' in which spontaneous beating is suppressed until triggered by contraction of a neighbouring cell. This eventually becomes restricted to specialised pacemaker cells and the mature myocardium becomes electrically quiescent. hPSC-CMs, by contrast, retain spontaneous beating and, to some extent, continue to divide (Mills et al., 2017). Immature cardiomyocytes also have sparse, misaligned myofibrils and do not have transverse tubules (T-tubules), which are cell membrane invaginations derived from the sarcolemma that bring the cell membrane, sarcoplasmic reticulum and mitochondria in close proximity during sarcomere maturation (Brette and Orchard, 2003). T-tubules are therefore important in the correct formation of the electrochemical syncytium that mediates efficient excitation-contraction coupling (Brette and Orchard, 2003). Sarcomere development is key to promoting maturation, as sarcomeres act as hubs of signal transduction (Guo et al., 2018). Crucially, these morphological changes that are tightly linked to functional maturation are missing in immature hPSC-CMs.

Transcription networks and genetic switches underlying developmental maturation of the heart: isoforms and alternative splicing

Transcription networks in the human heart have evolved such that many genes essential for cardiac function have either multiple homologues or are produced in cardiac-specific isoforms. The latter is achieved by alternative splicing (AS) of the same precursor-mRNA (pre-mRNA) molecules (Wang et al., 2008). The precise temporal control of the appropriate homologues and cardiac-specific exons results in specific isoforms expressed in fetal and adult hearts. Although the functional relevance of isoform switching is not entirely clear for all genes, it has been linked to altered excitation-contraction coupling (Bedada et al., 2014; Link et al., 2009), increased myofibril stiffness (Opitz et al., 2004) and metabolic changes (Fig. 1). Importantly, because the expression of these isoforms changes throughout development, their relative expression has been used as a proxy for maturity in cultured cardiomyocytes (Table 1). For example, hPSC-CMs in 2D culture display isoform expression profiles similar to fetal cardiomyocytes (Bedada et al., 2014; Selewa et al., 2020), whereas cardiomyocytes from complex, multi-cell-type 3D structures express isoforms that correlate with *in vivo* maturation (Campostrini et al., 2022).

RNA-binding proteins in heart development and maturation

Different types of RNA-binding proteins (RBPs) regulate AS during heart development and maturation (Fig. 1). Quaking (QKI), for example, is active early during differentiation and is indispensable for cardiac sarcomerogenesis and contraction (Beqqali, 2018; Chen et al., 2021). Furthermore, myofibril development in the heart is controlled by RNA-binding Fox1 homologs 1 and 2 (RBFOX1 and RBFOX2), and cardiac morphogenesis is regulated by muscleblind-like splicing regulators 1 and 2 (MBNL1 and MBNL2) via modulation of gene expression and AS (e.g. *SCN5A* isoform switching) (Kalsotra et al., 2008). Ectopic expression of MBNL1 in hiPSC-CMs induces *SCN5A* isoform switching without other maturation stimuli, suggesting RBPs alone could promote maturation (Campostrini et al., 2022). To date, most expression profiling and RBP functional studies have used animal models (Blech-Hermoni and Ladd, 2013; DeLaughter et al., 2016; Kalsotra et al., 2008). However, recent high-resolution profiling of human tissues throughout development (Cardoso-Moreira et al., 2019; Mazin et al., 2021) will facilitate benchmarking of immature and mature cells. In turn, this may identify new ways to induce maturation of cells differentiated from hPSCs.

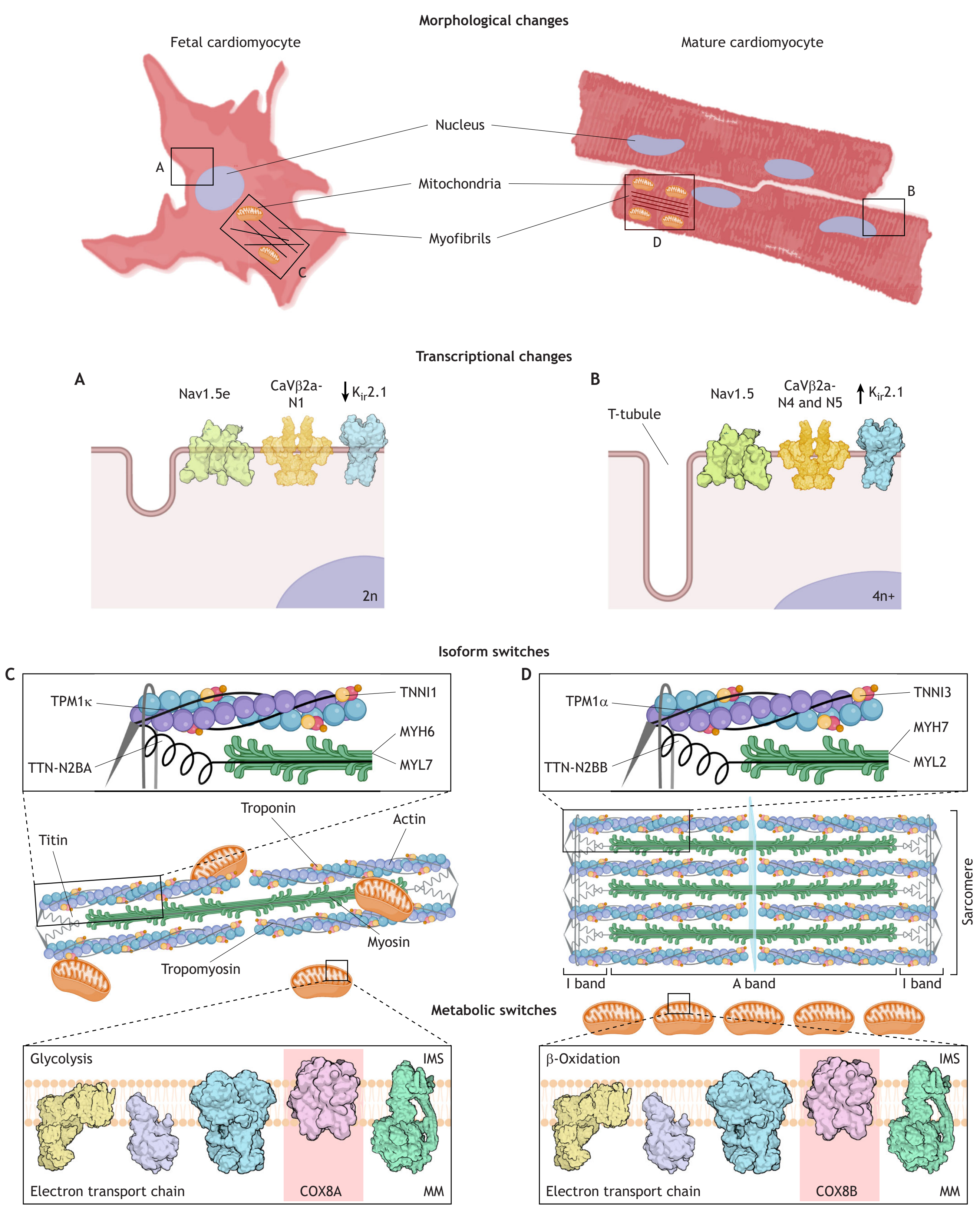
Promoting maturation *in vitro*

Although some hPSC-derived cells can mature effectively in extended culture periods, such as hPSC-derived neurons (Koch et al., 2012; Miller et al., 2013; Sandoe and Eggan, 2013), others may require the presence of tissue components, such as stromal cells or the extracellular matrix (ECM). For example, retinal organoids mature significantly in the presence of multiple cell types of the retina (Cowan et al., 2020) and heterotypic cell interactions in liver organoids enhance maturation beyond that in conventional 2D culture (Camp et al., 2017). Similarly, incorporation of cells of the brain vasculature into cerebral organoids promotes the formation of a structured and matured subventricular zone (Walsh et al., 2023 preprint).

Prolonged culture only enhances maturation in a subset of hPSC-CM features and T-tubules have not been observed (Snir et al., 2003). Even if effective, extended culture is costly, and has shown line-to-line variability for hiPSC-CMs (Veerman et al., 2017). Other approaches used to induce maturation *in vitro* are often based on recapitulating the biophysical, mechanical and biochemical cues present in the native heart, and building on the pathways underpinning cardiomyocyte maturation described above (Fig. 2). For a detailed review, see Campostrini et al. (2021).

Recapitulating the extracellular matrix

An important component of all organs is the ECM that naturally supports cells *in vivo*. Modulating the ECM was recently shown to mature the biochemical, functional and electrophysiological properties of hPSC-derived cortical and motor neurons in brain organoids (Álvarez et al., 2023). In addition, ECM has also been



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recognised as a key instructive stem cell regulatory element in organoid formation and maturation, especially in gastrointestinal organoids (Rezakhani et al., 2021). This is also true for cardiomyocytes; ECM proteins including laminins, collagens and fibronectin, promote functional maturation of hPSC-CMs via their cell surface receptors, such as integrins (Block et al., 2020; Ong et al., 2023; Yap et al., 2019). For example, the widely used ECM substrate Matrigel activates β-integrin signalling, resulting in

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Fig. 1. Features of immature and mature cardiomyocytes *in vivo*. Cardiomyocyte maturation from the fetal (top left) to the adult (top right) heart, including morphological, ultrastructural, transcriptional and metabolic changes. Fetal cardiomyocytes are round and mononucleated, while adult cardiomyocytes are mono/bi-nucleated and form a syncytium. The outlined areas are shown in more detail in A-D. (A-D) Fetal (A,C) and mature (B,D) cardiomyocytes. (A,B) Characteristic ion channels expressed at the plasma membrane of (A) immature versus (B) mature cardiomyocytes, including the embryonic sodium voltage-gated channel 1.5 (Nav1.5e/Nav1.5, green), the calcium voltage-gated channel subunit N1 β (CaVβ2a-N1, and CaVβ2a-N4 and N5, yellow) and the inward-rectifier potassium channel 2.1 (K_{ir}2.1, blue). The diploid (2n) and polyploid (4n+) nuclei of the immature and mature cardiomyocytes, respectively, are shown in purple. Mature cardiomyocytes also have fully developed T-tubules. Colour intensities in A and B reflect relative expression levels. (C,D) Characteristics and structure of muscle fibres in cardiomyocytes, including magnifications of the sarcomeres (top) and the mitochondrial electron transport chain (bottom). (C) In fetal cardiomyocytes, muscle fibres are misaligned and comprise multiple protein homologues or cardiac-specific isoforms expressed in the fetal heart. Sparse mitochondria (orange) mainly rely on glucose as their source of fuel and express cytochrome c oxidase subunit 8A in the in the electron transport chain. (D) In mature cardiomyocytes, muscle fibres are aligned in sarcomeres and characterised by 'I bands' made of thin filaments and 'A bands' where thin and thick filaments overlap. Mature cardiomyocytes express protein homologues or cardiac-specific isoforms that are expressed in only in the mature heart. Mitochondria (orange) are aligned along the muscle fibber. The metabolic switch from glycolysis to β-oxidation is also supported by expression of COX8B. See Table 1 for functional relevance of all genes and isoforms mentioned. COX8A, cytochrome c oxidase subunit 8A; IMS, intermembrane mitochondrial space; MM, mitochondrial matrix; MYH, myosin heavy chain; MYL, myosin light chain; TNN, cardiac troponin; TPM, tropomyosin; TTN, titin.

reduced cardiomyocyte proliferation and enhanced multinucleation (Jiang et al., 2022). Crucially, the cardiac ECM composition changes during development, in line with the functional requirements of the heart. ECM from the neonatal heart promotes cardiomyocyte proliferation, whereas adult ECM promotes maturation (Bassat et al., 2017). Even though Matrigel is widely used, its exact composition is not defined and varies from batch to batch. Therefore, synthetic culture matrices have been used to enhance maturation and reduce variability within and between experiments (Afzal et al., 2022).

As integrins are mechanosensitive receptors, the ECM composition also relays physical information on the tissue. Modulation of the biophysical properties of cell culture substrates can alter cardiomyocyte phenotypes (Körner et al., 2021). For example, there is a positive correlation between substrate stiffness and both myofibril alignment and contractile force (Querceto et al., 2022). Furthermore, cardiomyocyte maturation, including improved excitation-contraction coupling, has been observed in single cardiomyocytes cultured on optimised substrates (Kit-Anan et al., 2021).

Another physical element to consider is the structural organisation of the contractile apparatus, which relies on the rod shape of the cardiomyocyte. To this end, groove-patterned culture surfaces have been developed to drive alignment of cultured cardiomyocytes (Knight et al., 2021). Furthermore, alignment also supports the formation of specialized intercellular connections, e.g. gap junctions, that are important in cardiomyocyte maturation (Giacomelli et al., 2020). Micropatterned soft substrates coated with a broad range of matrix proteins showed synergistic maturation that exceeded prolonged culture (Afzal et al., 2022). These advances in 2D culture conditions have been paralleled by a shift towards 3D cell culture platforms, circumventing the need for ECM or patterned growth substrates (Hofbauer et al., 2021).

Supporting cardiomyocytes: the need for other cell types

As mentioned above, a major difference between the *in vivo* environment and *in vitro* culture conditions for differentiated cells is the variety of stromal cell types present in the organ, in addition to the 3D organisation (Goodyer et al., 2019; Litviňuková et al., 2020; Sim et al., 2021). Thus, co-culturing hPSC derivatives with these cell types may promote maturation. For example, the maturation of midbrain neurons is increased when in contact with astrocytes (Tang et al., 2022). In the heart, non-cardiomyocyte lineages not only provide physical support to shape the heart, but also promote cardiomyocyte maturation (Bassat et al., 2017; Ieda et al., 2009). During development, several fibroblast populations are crucial for normal cardiomyocyte maturation (Hortells et al., 2020). Subsequently, fibroblasts have been used to enhance the maturity of engineered 3D cardiac tissues (Giacomelli et al., 2020; Ieda et al., 2009; Tiburcy et al., 2017). Crucially, fibroblasts from different developmental stages have opposite effects on cardiomyocytes, with embryonic fibroblasts promoting cardiomyocyte proliferation and fibroblasts from aged hearts promoting hypertrophy (Ieda et al., 2009). Thus, the biological role of cardiac fibroblasts changes during development. Understanding this process may guide future cellular bioengineering of heart tissue.

Two other key cell types that affect cardiomyocyte function and maturation are epicardial and endothelial cells (ECs). When incorporated in 3D cardiac organoids, ECs and epicardial cells improve aspects of cardiomyocyte maturation, such as contractility, myofibril structure and alignment, excitation-contraction coupling and a switch from fetal to adult myosin heavy chain isoforms (Bargehr et al., 2019; Dunn et al., 2019) (Table 1). Finally, other cell types that might have supporting functions, such as macrophages, smooth muscle cells and cardiac neural cells, are being studied in cardiac organoids (Arslan et al., 2022).

Several studies highlight the elevated expression and intercellular localisation of the important gap junction protein connexin 43 (CX43) upon maturation (e.g. Giacomelli et al., 2020; Ieda et al., 2009; Tiburcy et al., 2017). Fibroblast-specific downregulation of CX43 inhibits the maturation of cardiomyocytes in 3D cardiac organoids or microtissues, suggesting that intercellular communication through these gap junctions plays a key role in cardiomyocyte maturation (Giacomelli et al., 2020). Upregulation of CX43 was observed in cardiomyocytes cultured on a pre-constructed vascular-like network developed to improve structural maturity of hiPSC-CMs (Koivisto et al., 2022). Furthermore, bioprinting of ECM, cardiomyocytes and ECs generates tissues with microvasculature, which may permit bioengineered muscle with integrated blood vessels (Lee et al., 2019; Noor et al., 2019). Taken together, these studies show that cardiomyocyte maturation is not solely an intrinsic cell process but relies on coordinated signalling from the ECM and non-cardiomyocytes. As the *in vivo* milieu is more closely mimicked, bioengineered cardiac constructs move closer to the physiological characteristics of the human heart. These advances permit dissection of the role of non-myocytes in cardiomyopathies and improve the predictive value of these platforms in drug testing (Giacomelli et al., 2020; Koivisto et al., 2022).

Developing self-organising cardiac organoids

Although the cellular composition of engineered cardiac tissues and organoids better supports cardiomyocyte maturation, their cellular complexity and spatial organisation does not reflect the native heart (Goodyer et al., 2019; Mehdiabadi et al., 2022). More complex, self-organising cardiac organoids (also called cardioids) recapitulate

Table 1. Isoform switches during cardiomyocyte maturation

Gene (family)	Dominant gene/isoform: Immature	Dominant gene/isoform: Mature	Function	Reference(s)
Cardiac troponin I (TNNI)	TNNI1	TNNI3	Ca^{2+}sensing and myofilament relaxation; the mature isoform is less sensitive to Ca^{2+}	Bedada et al. (2014)
Myosin heavy chain (MYH)	MYH6	MYH7	MYH7 has distinct enzymatic properties leading to decreased contractile velocity	Cui et al. (2019)
Myosin light chain (MYL)	MYL7	MYL2	Regulation of myosin motility and function. MYL2 is more energy efficient and results in lower contractile velocity.	Finck (2007)
Sodium voltage-gated channel alpha subunit 5 (SCN5A)	Nav1.5e (exon 6A)	Nav1.5 (exon 6B)	Fast upstroke of the action potential; the mature isoform shows faster (in)activation and faster recovery from inactivation.	Onkal et al (XXXX)
Calcium voltage-gated channel auxiliary subunit β2 (CACNB2)	CaVβ2a-N1	CaVβ2a-N4 and CaVβ2a-N5	A Cav1.2 chaperone. Regulation of activation and inactivation kinetics.	Link et al. (2009)
Titin (TTN)	N2BA	N2BB	The mature N2BB form increases fibril and myocardial passive stiffness	Opitz et al. (2004)
Tropomyosin (TPM1)	TPM1κ	TPM1α	Regulates the Ca^{2+}-dependent interaction between myosin and actin; increased Ca^{2+} sensitivity	Weeland et al. (2015)
Cytochrome c oxidase subunit 8 (COX8)	COX8A	COX8B	The isoform predominantly expressed upon maturation promotes oxidative phosphorylation	Calmettes et al. (2013); DeLaughter et al. (2016)

several aspects of heart development *in vivo*, including chamber formation with an EC inner lining (Lee et al., 2020). Cardioids are rather similar in their self-organising capacity to other self-organising organoids, such as brain (Lancaster et al., 2013) and kidney (Takasato et al., 2015) organoids, in that that they follow key steps of native organ developmental and are thus models for organ formation in the embryo. Activation of the STAT3 pathway in cerebral organoids, for example, promotes formation of an outer subventricular zone where cortical neurons become metabolically and functionally more mature (Walsh et al., 2023 preprint). Notably though, in cardioids, the non-cardiomyocyte cell types that also form have a mature gene expression profile, which could further enhance cardiomyocyte maturation (Hofbauer et al., 2021). Paracrine signalling from the foregut endoderm has been proposed as a key driver of cardiomyocyte maturation (Silva et al., 2021). Cardiomyocytes within cardiac organoids with foregut endoderm cells showed coordinated Ca^{2+} handling and higher maximum upstroke velocities, suggesting greater functional maturation compared with other organoids (Silva et al., 2021).

Electrical stimulation contributes to cardiomyocyte maturation, and is more efficient at increasing intensities across extended periods of time (Ribeiro et al., 2022; Ronaldson-bouchard et al., 2018; Ruan et al., 2016). The cardiomyocytes within electrically stimulated cardiac organoids are highly mature, showing T-tubules, metabolic switching and functional Ca^{2+} handling (Ronaldson-bouchard et al., 2018). These effects are observed only in early immature cardiomyocytes, suggesting that differentiation in the absence of these stimuli results in cells without the plasticity to undergo further maturation (Crestani et al., 2020; Ronaldson-bouchard et al., 2018).

Driving a metabolic switch *in vitro*

Over the past decade there has been increasing interest in metabolic maturation of cardiomyocytes. During post-natal development, the demand for energy increases, forcing cardiomyocytes to adapt their metabolism such that ATP is generated more efficiently, and cardiomyocytes transition from glycolysis to fatty acid oxidation (Lopaschuk and Jaswal, 2010). Primary cardiomyocytes mainly consume palmitoleic, stearic, oleic, arachidonic and linoleic acid (Feyen et al., 2020; Yoshida et al., 2022), and supplementing the culture medium with these fatty acids leads to higher intracellular ATP, increased oxygen consumption and reduced cell cycle activity in hiPSC-CMs (Correia et al., 2017; Mills et al., 2017; Zhang et al., 2022). Peroxisome proliferator-activated receptor (PPAR) is an important driver of the fatty acid-mediated metabolism and pharmacological modulation of this signalling cascade promotes cardiomyocyte maturation (Kim et al., 2022; Miao et al., 2020; Murphy et al., 2021; Zhang et al., 2022). PPAR signalling is needed for the efficient uptake of fatty acids through the upregulation of CD36 (Wickramasinghe et al., 2022). This suggests that fatty acid supplementation could be influenced by CD36 expression levels, which may vary between hiPSC-CMs. Furthermore, the transcriptional network regulated by co-activator PPAR gamma co-activator 1 (PGC1) includes estrogen-related receptor-γ (ERRγ); activation of ERRγ promotes maturation, including the formation of T-tubules (Miki et al., 2021). Thus, the concerted activation of PPAR signalling may more efficiently drive cardiac metabolic maturation.

Conclusions and perspectives

Differentiated cells from hPSCs do not reach full maturity *in vitro*. However, the continued improvement of 3D culture conditions, inclusion of the appropriate stromal cells, as well as microfluidic flow in 'organ-on-chip' platforms (Ingber, 2022) make this a realistic outcome of future work. Moreover, in the case of hPSC-CMs or other muscle cell types, forcing them to 'work' via electrical or mechanical stimulation may produce adult-like phenotypes. Furthermore, optimised media composition and small molecule activation of the required signalling cascades will likely improve the maturation of these systems. Ongoing transcriptional profiling of both *in vitro*-derived (cardiac or other) tissue and human organ samples provide robust benchmarks for cellular identity and maturation. In particular, these transcriptional benchmarks will be powerful tools for defining the 'transcriptional gap to maturity'; as outlined above, complex mRNA-based mechanisms driven by alternative splicing and RBPs may need to be manipulated to address this problem.

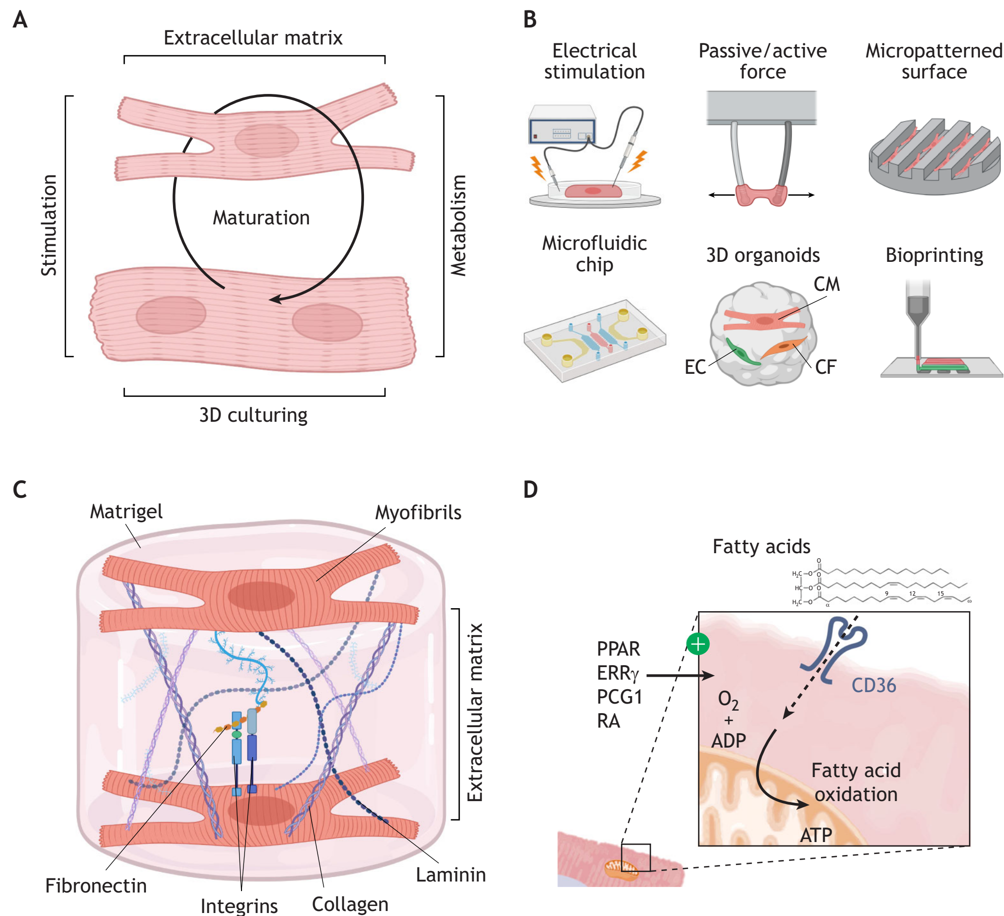

Fig. 2. Methods for maturing hPSC-CMs *in vitro*. Several aspects can be considered to tackle the intrinsic immaturity of cultured cardiomyocytes *in vitro* and induce their maturation. (A) The extracellular matrix (ECM) is a key component in cardiomyocyte maturation that can be manipulated, together with cell metabolism, electrical stimulation and 3D culturing. (B) Culturing techniques for maturing hPSC-derived cardiomyocytes. (C) hPSC-derived cardiomyocytes cultured in Matrigel, showing laminins, collagens and fibronectins together with their cell-surface mechanosensitive integrin receptors and the ECM substrate. (D) Cardiomyocyte maturation can also be enhanced by inducing metabolic switches *in vitro* from glycolysis to fatty acid oxidation. CD36, xxxxx; CF, cardiac fibroblast; CM, cardiomyocyte; EC, endothelial cell; ERRγ, estrogen-related receptor-γ; PCG1, PPAR gamma co-activator 1; PPAR, peroxisome proliferator-activated receptors; RA, retinoic acid.

The best model is one that is fit for purpose: as simple as possible, as complex as necessary (Campostrini et al., 2021). In this context, it is important to note that, in many cases, it may be sufficient to capture some (but not all) features of the mature cardiomyocyte. For example, less mature but more robust and reproducible hiPSC-CMs in 2D culture have proven to be highly predictive of cardiotoxicity (Saleem et al., 2020). Furthermore, many cardiac genetic diseases show a phenotype in 2D culture, permitting the use of these platforms in high-throughput drug screens for the next generation of therapeutics that target improving contractility (Bellin et al., 2012; Hnatiuk et al., 2021). Thus, existing monolayer and organoid cardiac cultures will continue to have crucial roles in disease modelling and drug discovery.

Finally, immature hPSC-based models do not capture either the dynamic traits of tissue healing and remodelling or diseases of aging. Attempts have been made to mimic aging with progeria, a syndrome caused by nuclear lamin gene mutations that lead to premature aging. However, this is pathological and not ‘physiological’ aging (Miller et al., 2013). Until we understand more about the molecular mechanisms underlying physiological aging, it will remain a challenge to recapitulate aging in a tissue culture dish. Nevertheless, refining existing 3D cardiac organoid platforms may offer scope to accurately model the cellular and molecular changes that come with aging.

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Summary: A Spotlight on the challenges of maturing cells that are differentiated from human pluripotent stem cells *in vitro*, discussing methods to drive cardiomyocyte maturation, as an example.

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