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Learn from Your Elders: Developmental Biology Lessons to Guide Maturation of Stem Cell-Derived Cardiomyocytes

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Abstract

Human pluripotent stem cells (hPSCs) offer a multifaceted platform to study cardiac developmental biology, understand disease mechanisms, and develop novel therapies. Remarkable progress over the last two decades has led to methods to obtain highly pure hPSC-derived cardiomyocytes (hPSC-CMs) with reasonable ease and scalability. Nevertheless, a major bottleneck for the translational application of hPSC-CMs is their immature phenotype, resembling that of early fetal cardiomyocytes. Overall, bona fide maturation of hPSC-CMs represents one of the most significant goals facing the field today. Developmental biology studies have been pivotal in understanding the mechanisms to differentiate hPSC-CMs. Similarly, evaluation of developmental cues such as electrical and mechanical activities or neurohormonal and metabolic stimulations revealed the importance of these pathways in cardiomyocyte physiological maturation. Those signals cooperate and dictate the size and the performance of the developing heart. Likewise, this orchestra of stimuli is important in promoting hPSC-CM maturation, as demonstrated by current in vitro maturation approaches. Different shades of adult-like phenotype are achieved by prolonging the time in culture, electromechanical stimulation, patterned substrates, microRNA manipulation, neurohormonal or metabolic stimulation, and generation of human-engineered heart tissue (hEHT). However, mirroring this extremely dynamic environment is challenging, and reproducibility and scalability of these approaches represent the major obstacles for an efficient production of mature hPSC-CMs. For this reason, understanding the pattern behind the mechanisms elicited during the late gestational and early postnatal stages not only will provide new insights into postnatal development but also potentially offer new scalable and efficient approaches to mature hPSC-CMs.

Keywords Postnatal cardiac development · Human embryonic stem cells · Human induced pluripotent stem cells · Cardiomyocyte maturation

Introduction

The discovery of human pluripotent stem cells (hPSCs) has revolutionized cardiovascular biology [1–3]. Over the last two decades, methods to differentiate cardiomyocytes from human pluripotent stem cells (hPSCs, either embryonic stem cells—hESCs, or -induced pluripotent stem cells—hiPSCs) have been refined and streamlined [4–7]. State-of-the-art protocols allow the production of highly pure cells with the main features of either working (i.e., ventricular and atrial) or pacemaker myocytes [8–10]. Such bona fide hPSC-derived cardiomyocytes (hPSC-CMs) contract spontaneously and show action potentials and calcium transients similar to their in vivo counterparts. Accordingly, they express the main cardiac ion channels and sarcomeric proteins involved in excitation–contraction coupling. This provides a cost-effective

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and renewable source of cardiomyocytes for a plethora of applications such as developmental biology [11, 12], pharmacology [13, 14], disease modeling [15, 16], and regenerative medicine [17–19]. For instance, hPSC-CMs are becoming a prominent model to evaluate the potential cardiac toxicity of novel drugs, which represents one of the most significant bottlenecks in the drug development pipeline [14, 20, 21]. Moreover, hPSC-CMs are emerging as a promising therapeutic approach for cardiac regeneration [22, 23], as their transplantation into infarcted hearts of nonhuman primates leads to efficient remuscularization and electrical coupling, which can prevent adverse remodeling of the myocardium and restore its mechanical function [19, 22, 24].

Despite these exciting applications, a major obstacle that prevents harnessing the full potential of hPSC-CMs is represented by their immature phenotype. Among other features, hPSC-CMs are a fraction of the size of adult cells, are much weaker, exhibit persistent automaticity and lack their typical rod-shaped morphology. Moreover, hPSC-CMs are metabolically dependent on glucose rather than fatty acid oxidation and lack key structural features such as transverse tubules (T-tubules) [25, 26]. Indeed, from an epigenetic and transcriptional point of view, hPSC-CMs most closely resemble fetal cardiomyocytes [11, 27, 28]. Improving hPSC-CMs maturation is thereby one of the most ambitious challenges faced by the field today.

Lessons from embryonic development have been pivotal in informing the establishment of methods for the differentiation and maturation of hPSC-CMs [3, 7, 29]. For instance, the study of WNT signaling during early embryogenesis [7, 30] paved the way to methods which rely on biphasic modulation of the WNT pathway to first induce mesoderm (WNT activation) and then specify cardiac progenitors (WNT inhibition [7]). Similarly, the evaluation of hormone levels throughout fetal and postnatal development [31, 32] unveiled the role of triiodothyronine (T3) and glucocorticoids in promoting multiple aspects of hPSC-CM maturation [33, 34]. In a similar vein, we submit that the development of scalable and efficient strategies for hPSC-CM maturation will require careful examination of cardiac development during both the late gestational and postnatal periods. However, an updated and comprehensive collection of the available information appears, to the best of our knowledge, to be lacking.

This review addresses this limitation by summarizing our current understanding of the fundamental mechanisms in late gestation and postnatal life that drive the physiological maturation of embryonic cardiomyocytes toward an adult phenotype. We discuss how some of these paradigms have already been implemented to promote hPSC-CM maturation, and we highlight benefits and limitations of current technologies. We conclude by assessing the missing pieces in the cardiac maturation puzzle and by suggesting future areas of research both in vivo and in vitro. Due to its relevance to most current applications of hPSC-CM technology, this review is centered on the ventricular cardiomyocyte subtype.

Gestational Cardiac Development

Acquisition of the cardiac cell fate is tightly interwoven with the morphogenetic events that underpin the formation of the heart in utero. Throughout this process, a number of changing factors in the extracellular milieu contribute to the first key stages of cardiomyocyte maturation. Unfortunately, gestational maturation has been much less studied than the earlier processes of cell type specification and organ morphogenesis. Furthermore, animal models will not perfectly predict human cardiomyocyte maturation [35, 36], and the scarcity of human samples at this developmental stage limits our understanding human-specific elements of maturation. Here we summarize what we know about this "black box" of cardiac development.

Morphogenetic Events

The human heart is the first organ to form during embryogenesis. This is a complex, multistep process requiring fine orchestration of different cell populations [37, 38]. During gastrulation, the pluripotent epiblast gives rise to a multilayered structure composed by endoderm, mesoderm, and neuroectoderm [38]. A subset of cells localized in the lateral plate mesoderm gives rise to two main cardiogenic populations named first and second heart field (FHF and SHF). These are organized in a bilaterally symmetric structure detectable at human embryonic development day 18 (E18; E7.5 in mice) [37, 39]. Cells from the FHF migrate and fuse at the midline, generating the primitive heart tube by E20 (E8 in mice) [39]. This structure is composed by an outer layer of myocardial cells, which predominantly contribute to the working myocardium of the left ventricle, and an inner layer of endocardial cells, which develop into the heart valves and the deep coronary circulation [40]. These layers are separated by an acellular hydrophilic matrix named cardiac jelly [40]. This structure contains paracrine signals that drive the epithelial–mesenchymal transition (EMT) of endocardial cells into mesenchymal cells, which then proliferate and invade the cardiac jelly [41]. This process generates the endocardial cushion, which lines the atrioventricular canal and the outflow tract, and is the precursor of heart valves [42]. The slowly differentiating cells from the SHF integrate into the forming heart at the arterial and venous poles, and contribute to the right ventricle, to parts of the left and right atria, and to the outflow tract [43, 44]. Another population of

mesodermal cells in the so-called pro-epicardium, localized dorsally to the heart tube, gives rise to epicardial cells [45]. In addition to forming the mesothelial cells that line the heart's outer surface, epicardial cells are precursors of multiple cell lineages that contribute to the generation of the interstitium and the vasculature such as cardiac fibroblasts and coronary vascular smooth muscle cells [46, 47].

The heart's pacemaking centers, the sinoatrial node (SAN), and atrioventricular node (AVN), are another crucial component for the developing heart. Lineage tracing experiments in mouse and chick embryos demonstrated that pacemaker cells arise from a defined lateral plate mesoderm population separated from the FHF and the SHF, suggesting that it constitutes a "tertiary heart field" [48–50]. Cells of the SAN seems to be segregated early in the sinus venosus region of the heart tube under the regulation of T-box (Tbx) transcription factors Tbx18/Shox2/Tbx3 [51]. This cell population has the fastest beating rate and therefore dictates the pace of the entire heart [51]. At this stage, the ECG recording shows a sinusoidal wave, starting from the caudal region and slowly diffusing throughout the heart tube, resulting in peristaltic contraction [52, 53]. The AVN forms later than the SAN and rises from the crest of the interventricular septum under the control of Tbx3. This transcription factor, together with Tbx18 and Shox2, represses working myocardial genes (*Gja5*/Cx40*, Gja1*/Cx43*, Nppa*/ANF, *Scn5a*/Nav1.5) and promotes expression of Cx30.2 and Hcn channels [51]. The presence of the low-conductance Cx30.2 in these regions delays the propagation of the electrical impulse; indeed, in the mammalian heart, the electrical conductance of Cx30.2 is 9 pS compared to 162 pS of Cx40, expressed in the ventricular conduction system [54]. The different expression of gap junctions is crucial for establishing the unidirectional propulsion of the blood flow in the absence of the valves [55]. The His-Purkinje conduction system is derived from a population of working ventricular myocytes expressing Cx40, but negative for Tbx3, and is responsible for rapid propagation of the electrical impulse distal to the AVN [56, 57]. This population remains segregated within the trabecular myocytes during development, as demonstrated in lineage tracing experiments by labeling *Gja5*/Cx40-expressing cells [56].

Finally, cardiac neural crest cells migrating from the neural tube contribute to cardiac septation by mediating the symmetrical development of the endocardial cushion and the septation of the outflow tract into the pulmonary artery and aorta [58].

During early- and mid-gestational periods, the heart undergoes a number of complex morphological changes. The heart tube can be divided into the truncus arteriosus, bulbus cordis, ventricle, and sinus venosus, as shown in Fig. 1. Starting from E23 and until E35 (E8.5–E10.5 in mice), the straight heart tube gradually transforms into a helically wound structure in a process called cardiac looping [59]. This begins with the movement of the upper portion (truncus arteriosus and bulbus cordis) in a ventral and caudal direction, creating a C-loop shape pointing toward the right side of the embryo (Fig. 1, E24). In the consecutive S-looping phase, the ventricle moves dorsally and cranially, pulling along the atria. During this phase, the distance between the arterial pole and the venous pole shortens, and the ventricular portion shifts below the atrial segment [59]. This process establishes the left–right asymmetry of the future ventricle chambers (and indeed, the first lateral asymmetry in the embryo) and imparts the definitive topological relationship between the different parts of the heart, including the great vessels [59]. After cardiac looping, cardiomyocytes and endocardial cells in the ventricular wall proliferate and form sponge-like structures called trabecular ridges. Trabeculation both facilitates nutrient exchange and enhances contractile force generation in order to respond to the increasing blood and oxygen demands of the developing embryo, due to the lack of coronary vasculatures [60, 61]. The trabecular ridges are constituted by clusters of cardiomyocytes loosely attached to the compact myocardium and surrounded by endocardial cells [60, 62]. As development proceeds, cardiomyocytes adjacent to the epicardium are characterized by increased proliferation rate, whereas the cardiomyocytes closer to the endocardium are more differentiated [60, 62].

Heart partitioning in the four chambers is due to the formation of the interventricular septum, the interatrial septum, and the atrioventricular valves [63]. Between E35 and E40, the endocardial cushions move toward each other until they fuse to form the primitive interventricular septum, subdividing the right and left atrioventricular canals. The mesenchyme around each canal proliferates and forms the atrioventricular valves [42]. The two atria are subsequently separated by the septum primum and septum secundum, while the blood can flow from the right to the left atria through the foramen ovale, bypassing the pulmonary circulation (Fig. 1). Septation is completed by day E55 (E15.5 in mice), at which point the heart becomes a four-chambered, fully functional organ. During this time, epicardial cells undergo extensive proliferation and migration, to eventually cover the entire embryonic heart by E34 (E11.5 in mice) [45]. From gestational week 8 (E18.5 in mice) and throughout the postnatal period, most of the trabeculae collapse toward the myocardial wall forming a thicker, compact structure [61, 64]. Finally, the myocardial wall further matures in a multilayered spirally organized muscle during neonatal stage (Section II) [64–66].

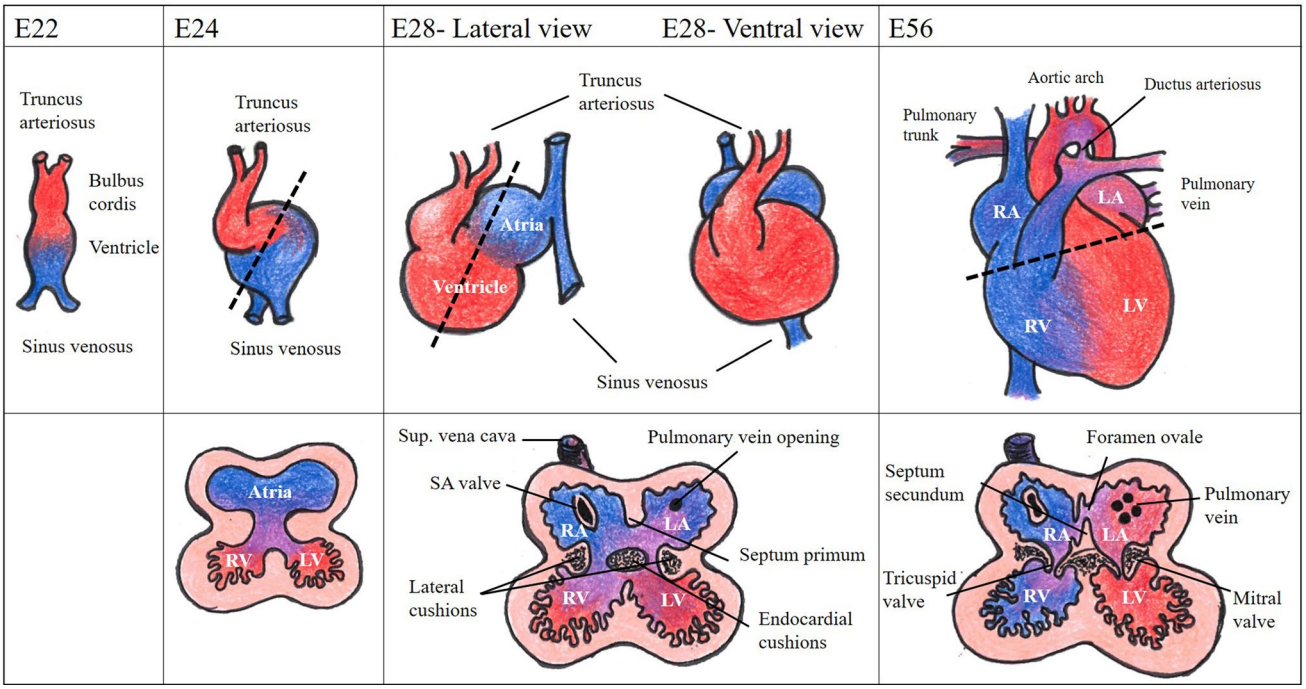

Fig. 1 Human embryonic heart development. The primitive heart tube forms by human embryonic day 22 (E22) and is divided in four different regions: from head to tail, the truncus arteriosus will give rise to the aorta and the pulmonary trunk; the bulbus cordis will develop into the right ventricle; the ventricle region will form the left ventricle and the anterior portion of the right and the left atria; and the sinus venosus develops into the posterior portion of the right atrium, the SA node, and the coronary sinus. By E24, the heart tube starts to bend in a primitive C-shape. Ventricular myocytes undergo trabeculation, whereas the endocardial cells start to migrate into the endocardial cushions. By E28, the heart tube creates a S-shape with the primordial division of the four chambers. By E56, the fetal heart is completely formed; the septa primum and secundum divide the two atria, which remain connected through the foramen ovale, while the endocardial cells from the cushions give rise to the tricuspid and the mitral valves. The dashed line indicates the coronal sections depicted below each stage

Environmental Dynamics of the Gestational Heart

The morphological changes happening during heart development are governed both by mechanical forces exerted on the myocardial wall by the blood flow and the availability of energy substrates like oxygen and carbon sources which ultimately affect cardiogenesis on both a cellular and a molecular level.

During the early stage of embryogenesis, the first heart beat in humans can be observed as soon as day E22 (E8 in mice), and this seems to drive the development of primordial hemodynamic forces that eventually will promote cardiac development [67]. In the straight heart tube phase, the blood flow is barely detectable but becomes substantial after cardiac looping, due to the formation of the chambers and the development of coronary vasculature [68]. This results in an increased blood pressure and mechanical strain to which fetal cardiomyocytes are exposed. The blood pressure measured in human fetuses showed a linear increase with advancing gestation, with the left and the right ventricular systolic pressures ranging between 28 and 45 mmHg from the 20th and 40th gestational weeks [67, 69]. Evaluation of mechanical strain across the myocardial wall also revealed an increasing strain gradient from the compact myocardium toward the inner layer of the heart [70]. These differences affect the phenotype of the fetal cardiomyocytes. Cells become elongated and have decreased mitotic activity in the region exposed to increased mechanical forces, like trabecular cardiomyocytes [60, 62, 70]. The formation of the different septa and valves, and the development of the coronary vasculature also modifies the hemodynamic flow within the heart, where laminar blood flow dominates the early fetal stage and turbulent flow becomes predominant during chamber formation. Indeed, wall shear stress increases from 80 to 250 dynes/cm^2 as evaluated in chick embryos between HH23–27 [67].

The role of hemodynamic forces in cardiac development has been demonstrated in a variety of animal models in which blood flow was impaired by either occlusion of the outflow tract [71] or ligation of the left atrium [72]. In these models, manipulation of blood flow resulted in impaired cardiac morphogenesis, with hypoplasia of the left ventricular wall and trabeculae noncompaction [67, 71, 72].

Although the precise molecular mechanism by which hemodynamic forces affect cardiomyocytes maturation is still not fully determined, endocardial–myocardial communication seems to control the architecture of the developing heart by expression of different signals, including Notch and neuregulin-1 (NRG1) [61, 73]. Indeed, apical expression of NRG1 promotes trabeculae expansion by increasing extracellular matrix (ECM) synthesis [61, 74]. Vice versa, the expression of the Notch signaling components delta-like canonical Notch ligand-4 and Notch1 intracellular domain (DLL4-N1ICD) at the base of the nascent trabeculae promotes cardiomyocytes proliferation by activating bone morphogenic protein 10 (BMP10) [75] and thus, suppressing the cell-cycle inhibitor $p57^{kip2}$ [75, 76].

The high proliferative rate of early fetal cardiomyocytes is also associated with their predominant metabolic substrate for energy production: glucose. Glycolysis is the catabolic pathway that most efficiently provides the main biosynthetic precursors for macromolecules such as nucleic acids, lipids and proteins, all of which are required at high levels in rapidly proliferating cells [77]. Accordingly, the ventricular wall of fetuses from diabetic mothers show hyperplasia and increased thickening, a feature that is often resolved within months after birth as the exposure to the hyperglycemic intrauterine milieu ends [78]. In the fetus, the abundance of glucose and lactate combined with the paucity of fatty acids (less than 0.1 mM) and the low oxygen levels favor anaerobic glycolysis over fatty acid β-oxidation [79, 80]. Indeed, plasma lactate levels in the fetus are ~ 5 mM, compared to approximately 0.5 mM in the adult [81]. Such high levels of lactate repress fatty acid β-oxidation via the so-called Randle Cycle, also known as the glucose/fatty acid cycle [79]. As a result, more than 80% of ATP produced by fetal cardiomyocytes comes from glycolysis [79]. The anaerobic metabolism of fetal cardiomyocytes is also favored by the expression of specific glycolytic enzymes [82]. In the late stages of pregnancy, the expression of transcripts related to fatty acid oxidation markedly increases [82]. Thus, during this late fetal stage the metabolism starts to switch to become less dependent on glucose and more favorable for fatty acid oxidation. The mechanism that regulates this metabolic switch is still largely unknown, but repression of HIF1 signaling represents a valid candidate for future investigation, due to its role in the regulation of both energy metabolism and cell proliferation [83–85]. Interestingly, there is a gradient of VHL/HIF1 expression from the compact myocardium (high expression) toward the trabecular myocytes (low expression) [85]. This could correlate with the increased proliferation, decreased differentiation, and reduced mitochondrial oxidative activity observed in the compact myocardium.

Postnatal Cardiac Development

Environment of the Postnatal Heart

The transition from the intrauterine to extrauterine life is arguably the most complex adaptation that occurs in human experience. At birth, the loss of the placenta deprives the fetus of an important source of energy substrates and hormones. The removal of the umbilical cord and the recruitment of the lungs, together with the exposure of the newborn to a relatively cold environment trigger both physical and endocrine signals in order to ensure the survival in the postnatal life [31, 32, 80, 86].

Cortisol represents one of the major regulatory hormones responsible for neonatal adaptation [32]. Indeed, during fetal life in human, plasma cortisol is very low but it gradually starts to increase around late gestational period (it ranges from 2 to 50 µg/dL in preterm infants [87]) and reaches a peak at labor, with levels four times higher compared to that of gestational stage [86]. This results in the maturation of the lungs associated with the clearance of fetal fluid, activation of thermogenesis as well as metabolic adaptation. Moreover, the expression of mitochondrial membrane protein UCP1 (uncoupling protein1) increases and, together with the boost in catecholamine secretion, thermogenesis is initiated in the brown adipose tissue [88]. Cardiomyocyte maturation is also promoted by glucocorticoids as demonstrated in vivo by administration of betamethasone to preterm piglets [89]. Indeed, treatment with glucocorticoids 24 and 48 h before delivery increases the number of binucleated cardiomyocytes in piglets, characterized also by reduced proliferation capability [89]. At the molecular levels, cortisol influences these processes potentially by increasing the expression of peroxisome proliferator-activated receptor PPAR-γ coactivator (PGC1α) [90]. PGC1α indeed acts as a coactivator for a variety of transcription factors involved in different biological responses such as thermogenesis, mitochondria biogenesis and metabolic control [91].

Thyroid hormones (TH) signaling in the fetus starts to be detectable only at 35–40 weeks of gestation. During fetal life, the thyroid receptor system is completely developed by the 12th gestational week, however the serum concentration of T3 and T4 are very low until the hypothalamus–pituitary–thyroid gland axis becomes fully functional [31]. Maternal thyroid hormones have limited ability to cross the placental barrier, indeed placental expression of type 3 iodothyronine monodeiodinase deactivates both T4 and T3 to their inactive counterparts [31, 92]. At birth, the amount of thyroid hormones dramatically increases to peak at high levels of about 300 ng/dL for T3 [31], 12 µg/dL for T4 as well as an eightfold increase of TSH levels [92]. This increase could be triggered by both the surge in

cortisol [86] and the acquisition of homeothermy with the initiation of thermogenesis [31, 92]. The effects of thyroid hormones are mainly due to the activation of thyroid hormone receptors (THRα and THRβ), expressed in the nucleus of the cells but also could be exerted by the αvβ3-mediated integrin signaling [31, 93], although this pathway is still not investigated in cardiomyocytes [94]. TH modulates cardiomyocytes inotropic maturation by modulating the expression of calcium handling and sarcomere proteins, and induces a hypertrophic response by increasing cell size [31, 94, 95]. The spike in TH at birth is also associated with cardiomyocytes cell-cycle withdrawal [31]. Indeed, cardiomyocytes with impaired TH signaling showed increased proliferation and decreased polyploidization [96]. Moreover, TH seems to control the expression of Glut1 and Glut4 during rat heart development. These two glucose transporters are differentially regulated during development, with GLUT1 highly expressed in the fetal stage, whereas GLUT4 becomes the main glucose transporter in the adult stage [80, 97]. Translocation of GLUT4 to the cytoplasmic membrane is regulated by insulin, whereas GLUT1 constitutively localizes to the plasma membrane once it is synthesized, independent of insulin. Administration of T3 to hypothyroid rats showed increased expression of *Glut4* combined with decreased levels of *Glut1*; thus, indicating a TH involvement in postnatal metabolic adaptation [97].

Circulating catecholamines, i.e., noradrenaline, adrenaline and dopamine increase up to tenfold at birth, due to both the maturation of adrenal medulla and the loss of placental clearance of catecholamines [86]. Cardiomyocytes' responsiveness to adrenergic stimuli is already present in fetal cardiomyocytes, although β-adrenergic stimulation results only in increased chronotropism without inotropic effects, potentially due to the low number of receptors and the absence of phospholamban, an adrenergic-responsive regulator of SERCA2a activity [98, 99]. This catecholamine surge participates in postnatal cardiac adaptation by increasing blood pressure and cardiac inotropic effects; it also is involved in metabolic switching by mediating glucagon secretion and decreasing insulin secretion as well as induction of thermogenesis [86, 92].

Insulin-like growth factor-1 (IGF-1) is an anabolic hormone that is involved in regulation of proliferation, differentiation and postnatal growth of the fetus. This hormone is supplied to the fetus from the placenta during early gestation, and it increases during the last trimester due to secretion from the fetal liver [100]. Indeed, IGF-1 increases from 40 µg/L at 18th gestational week to almost 160 µg/L at birth [101]. Circulating levels of IGF-1 are regulated by growth hormone (GH) and insulin at both transcriptional and posttranscriptional levels [101]. These three hormones are essential for metabolic homeostasis; indeed, they regulate nutrient availability in order to provide adequate substrates for growth [80, 102]. Insulin per se promotes fetal growth by enhancing the cellular uptake of glucose and potentially other energy substrates, like amino acids. Fetal pancreatectomy in sheep results in a significant decrease in glucose uptake and oxidation and 30% decrease in fetal growth; conversely, high fetal insulin levels (such as from maternal diabetes) are associated with increased body weight [78, 103]. Accordingly, rat cardiomyocytes treated with insulin increased plasma membrane expression of the fatty acid transporter Cd36 and Glut4, with a subsequent decrease in their localization in the microsomal fraction [104]. Growth hormone controls fetal development by stimulating hepatic release of IGF-1; excess production of GH (acromegalic cardiomyopathy) or decreased levels of GH (GH deficiency) both result in cardiac developmental defect [105]. IGF-1 stimulates protein synthesis by modulating the PI-3 kinase/mTOR pathway, and it also regulates free fatty acid utilization in skeletal muscles, potentially via modulation of Cd36 [106]. In vitro studies on neonatal rat cardiomyocytes demonstrated that IGF-1 treatment increases maturation of contractile apparatus as well as induces hypertrophy [107].

Postnatal adaptations are also mediated by air breathing, which induces the closure of the ductus arteriosus and the foramen ovale, along with marked vasodilation of the pulmonary vasculature, resulting in a low-pressure pulmonary circulation, a higher pressure systemic circulation and near-100% oxygenation of hemoglobin [108]. Indeed, the oxygen tension in the fetal circulation is only 25–28 mmHg, as evaluated in ascending aorta of fetal lamb [109]; this is due to the presence of the different shunts resulting in the mixing of arterial and venous blood [109].

On a tissue levels, the paracrine signals secreted by fibroblasts, endothelial cells and smooth muscle cells also modulate cardiomyocyte postnatal maturation [110, 111]. These stromal-vascular cells encounter almost 70% of the total cell population present in the heart and likely contribute to cardiac maturation [110], although we know much less about these interactions. Notably, fibroblasts from the fetal and the neonatal or adult heart secrete different signals [111]. Co-culture of embryonic cardiomyocytes and embryonic fibroblasts from E12.5 to E13.5 mouse embryos stimulate cardiac proliferation due to fibroblast-secreted fibronectin, collagen, and heparin-binding EGF-like growth factor. Conversely, when these same cardiomyocytes are co-cultured with adult fibroblast, the proliferative effect is replaced by a hypertrophic response [111]. This hypertrophic signaling seems to be mediated by increased expression of interleukin-6 (Il6) in the adult fibroblast (58-fold higher compared to the embryonic fibroblast), whereas ECM/β1-integrin signaling is required for cardiomyocytes proliferation in response to growth factors [111]. Accordingly, fibroblasts could be detected at E12.5 (mice) and this is associated with

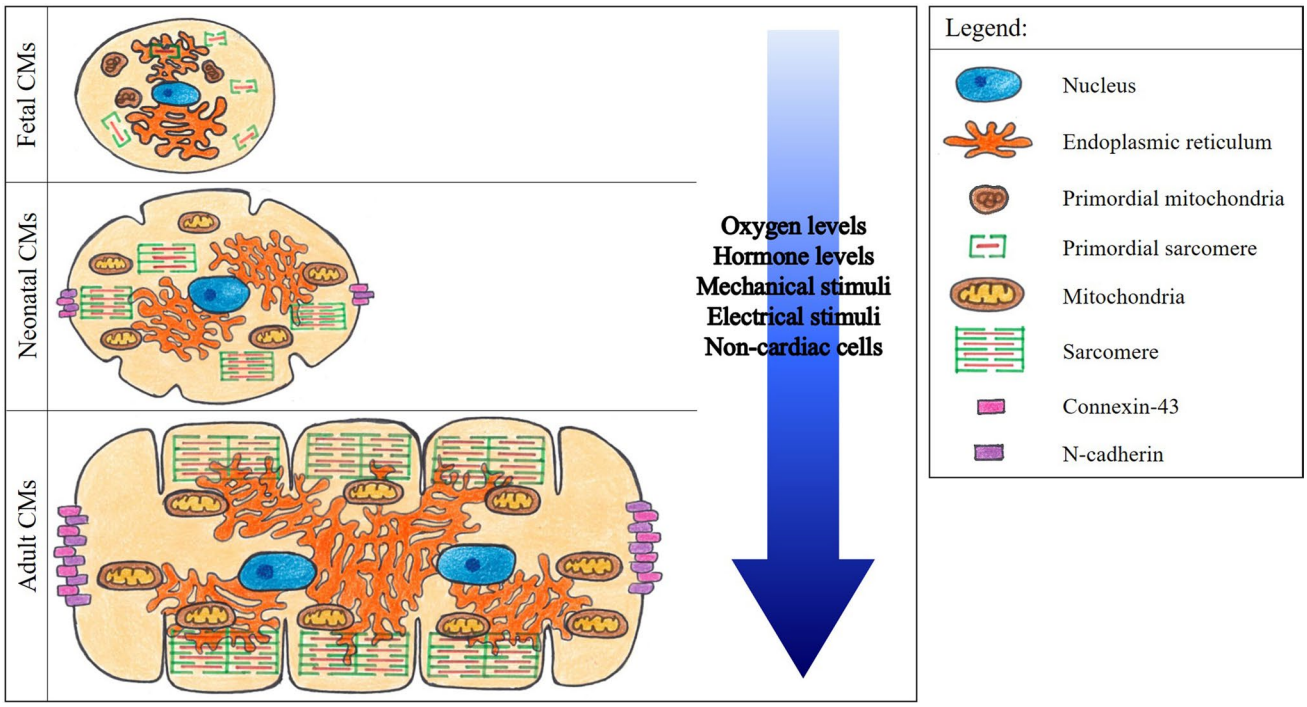

Fig. 2 Cardiomyocyte maturation. Fetal cardiomyocytes (CMs) are small and round-shaped, with undeveloped mitochondria and sarcomeres. Multiple environmental cues, such as mechanical and electrical stimuli, extracellular matrix interactions, and interactions with non-cardiomyocytes drive gradual maturation of CMs. Through neonatal and adult stages, CMs become elongated and display increased cytoskeletal organization. Expressions of Connexin-43 and N-cadherin increase, and mitochondria develop mature cristae. Adult CMs are also often binucleated and display robust T-tubules and intercalated disks

increased ECM synthesis, upregulation of β1 integrin and increased cardiomyocytes proliferation [111].

Postnatal cardiac adaptation results in a linear increase of cardiac output from 120 mL/min at 15–20 weeks of gestation to 1700 mL/min at birth, and finally to 5000 mL/min in the adult [112]. The surge in the afterload and preload, together with increasing levels of circulating vasoactive molecules, increase the left ventricular systolic pressure, which rises from approximately 30 mmHg during gestation, to 75 mmHg at birth, reaching ~ 90 mmHg at 1 year of age [67, 69, 86, 113]. This increased workload is accompanied by the physiological enlargement of the left ventricular chamber, with LV end-diastolic volume increasing from 1.4 mL at 36 weeks of gestation [114] to 2.6 mL in the early neonatal period (5 days after birth) and to 150 mL in the adult human [115].

Overall, changes in endocrine factors, oxygen tension, hemodynamic forces, and the phenotype of noncardiac cells are the key extracellular signals marking the end of the fetal stage and driving postnatal cardiomyocyte maturation (Fig. 2). In the next section, we discuss the main cellular modifications triggered by such signals.

Electrical Activity of Postnatal Cardiomyocytes

Early fetal cardiomyocytes differ substantially from their adult counterparts in terms of their electrophysiological properties, particularly with regards to automaticity (the ability to periodically and spontaneously depolarize until an action potential is triggered). As reviewed above, while automaticity is restricted to specialized cardiomyocytes in the adult heart, all early fetal cardiomyocytes show strong automaticity [49, 56].The precise molecular basis of cardiac automaticity is still being debated. Two main nonexclusive mechanisms have been described: (1) the "voltage clock," constituted by a slow, inward sodium current mediated by the hyperpolarization-activated cyclic nucleotide-gated channels encoded by the *HCN* gene family (the so-called "funny current," I_f); and (2) the "calcium clock," initiated by calcium leaks from the sarcoplasmic reticulum (SR) through the ryanodine receptor (RYR2), which lead to activation the sodium-calcium exchanger (NCX) and result in a net depolarizing current (as three Na^+ are imported for each exported Ca^{2+}). The progressive resting membrane potential depolarization resulting from these clocks may be further amplified by voltage-dependent T-type calcium channels (I_{CaT}, encoded by *CACNA1G*, *CACNA1H*, and *CACNA1I*) [116, 117]. Of note, while the role of these ion channels in

Table 1 Features of developing CMs versus hPSC-CMs

Parameters	Fetal CMs[a]	Neonatal CMs[a]	hPSC-CMs (< 30 days in culture)	Adult-CMs[a]
Morphology				
Surface area	1216 ± 44.6 μm^2 [193]	4395 ± 436 μm^2 [194]	1000–1300 μm^2 [193]	12,315 ± 2103 μm^2 [194]
Capacitance	20.3 ± 4.6 pF [231]	NA	24.6 ± 3.3 pF [231]	100–150 pF [195]
Length-to-width ratio	5:1 (mouse) [149]	5:1 (mouse) [149]	2:1 [27]	7–9.5:1 [197]
Contractility				
Sarcomere length	1.2 μm (piglet) [89]	1.95 μm (rat) [232]	1.6 μm [209]	2.2 μm [26]
Force production [201]	0.4 mN/mm^2	1.2 mN/mm^2	0.1 to 0.5 mN/mm^2	10–50 mN/mm^2
Isoform [26]				
Titin	N2BA	N2BA	N2BA	N2B
MHC	α	α	α	β
Troponin	ssTnI	ssTnI	ssTnI	cTnI
Electrophysiology				
RMP [202, 203]	− 40 mV	− 60 mV	− 40 mV	− 85 mV
I_f	− 7 pA/pF (mouse) [123]	− 3.5 pA/pF (mouse) [123]	− 4.1 pA/pF [203]	− 1.9 pA/pF [125]
I_{CaT} [202]	− 4 pA/pF (mouse)	− 2 pA/pF (mouse)	Present	Absent
I_{CaL} [202]	− 6 pA/pF (mouse)	− 8 pA/pF (mouse)	− 6 pA/pF	− 9 pA/pF
I_{K1}	6–8 pA/pF (mouse) [131]	6–8 pA/pF (mouse) [131]	Absent [26]	12 pA/pF [233]
Metabolism [26]	Glycolysis	Glycolysis	Glycolysis	FA OX

[a]Unless indicated otherwise, all parameters are reported for human cardiomyocytes

regulating the pacemaking activity of the postnatal heart is supported by studies in animal models and by clinical evidence [118, 119], whether these same mechanisms mediate automaticity in the early embryonic heart is still largely unknown [117, 120].

The expression of HCN4 in the working myocardium gradually decreases during development, and this channel is virtually absent at human postnatal day 37 (Table 1) [121, 122]. In ventricular mouse cardiomyocytes, the amount of inward current that flows through Hcn4 is ~ 7 pA/pF at day E10.5, and decreases by 50% at day E16.5 [123]. In humans, HCN4 and T-type calcium channels are still detectable in neonatal ventricular cardiomyocytes, which could explain their retained automaticity and the heart rate variability in the first week after birth [123, 124]. In contrast, in the human adult working myocardium, the amount of funny current is only ~ 1.9 pA/pF [125]. In agreement with its key function in calcium and sodium homeostasis during the cardiac cycle, expression of NCX increases linearly during embryogenesis and is highest at the fetal stage (19th week of gestation). Interestingly, during postnatal and adult stages, the levels of *NCX* mRNA are lower compared to that of fetal stage. However, the ratio of protein level to mRNA level is higher in the adult, indicating that NCX is subjected to substantial posttranscriptional regulation [116, 126, 127]. In E12.5 mouse embryos, calcium release from the SR is dependent on the inositol 1,4,5-triphosphate receptor (Itpr3) [128]. From mouse E16.5 onward, calcium release is mediated by the increasing levels of Ryr2, which mediates localized Ca^{2+}-induced-Ca^{2+}-release in the proximity of T-tubules due to its association with the L-type calcium channels [128, 129]. This more refined regulation of intracellular calcium levels leads to increased action potential amplitude and duration [130]. The expression of SERCA2a, which pumps calcium from the cytosol into the sarcoplasmic reticulum, linearly increases during development. Interestingly, levels of the *SERCA2a* mRNA do not substantially change between embryonic, fetal, neonatal, and adult stages, but SERCA2a protein levels largely increase during cardiac maturation, indicating the existence of posttranscriptional regulation also for this factor [116, 131].

The expression of ion channels involved in cardiac automaticity is thought to be regulated by regionally expressed transcription factors like Nkx2.5, Shox2 and T-box transcription factors [121]. Tbx3 is detectable only in the pacemaker progenitors cells and in the conduction system, and its ectopic expression in the working myocardium of mice leads to upregulation of *Hcn1*, *Hcn2*, *Hcn4*, and *Cacna1g* [56, 121]. On the contrary, Nkx2.5 represses the expression of these pacemaking ion channels, possibly via the inhibition of Shox2 [121, 132]. In addition, the presence of a conserved binding site for the transcriptional repressor NSRF (also known as Rest) in the first intron of *Hcn4* could also modulate its expression [133]. NSRF is a transcriptional repressor that acts through the recruitment of class I and class IIa histone deacetylases (HDACs). It is highly expressed in ventricular cardiomyocytes from 20-week-old mice compared to those from embryos at E13.5 [133].

NSRF is potentially involved also in the regional expression of T-type calcium channels (*Cacna1g* and *Cacna1h*), whose expression decreases over time and becomes undetectable in the adult working myocardium [131, 133, 134].

Another hallmark of cardiac maturation is the decrease in the resting membrane potential, which for ventricular myocytes shifts from − 40 to − 70 mV in the fetal stage (E12–E18 in mice) to − 85 mV in the adult [135]. The stabilization of the resting membrane potential at a hyperpolarized level further decreases the automaticity of the working myocardium. This process is predominantly controlled the inwardly rectifier potassium current I_{K1}, which is mediated by the Kir2.1 channel encoded by the *KCNJ2* gene. In the mouse, expression of Kir 2.1 is first detectable at E17.5, begins to increase at P1, and is strongly upregulated only 5 to 8 weeks after birth (Table 1) [127, 131]. This increase could be potentially related to the action of the Notch-responsive transcription factor Hey2, which is also involved in cardiac morphogenesis and ventricular cardiomyocyte terminal differentiation [136, 137].

In summary, the electrical activity of the postnatal heart is determined by the regional and temporal regulation of transcription factors that are expressed throughout the myocardial wall and gradually become restricted to specialized area starting from the 5th week of gestation (E12.5 in mice) and through the postnatal period [57].

Structural Organization of Postnatal Cardiomyocytes

Cardiomyocytes undergo extensive structural changes during the postnatal period. Many of the proteins required for sarcomere formation and cytoskeletal organization are already expressed when the heartbeat begins at E22 (E8 in mice), but at lower levels or in different isoforms compared to the adult (Fig. 2) [138]. A prominent example is titin, the largest human protein, which spans from the Z-disk to the M-line and is responsible for both sarcomere assembly and integrity. During development titin is expressed in multiple isoforms as a result of alternative splicing, a process controlled by the cardiac-specific splicing regulator RBM20 [139, 140]. The fetal isoform N2BA switches to the less compliant N2B isoform soon after birth, resulting in increased passive tension at a given sarcomere length [139]. This augments myocardial stiffness, thereby compensating for the large increase in the end-diastolic volume of the left ventricle, which increases up to 100-fold from late gestation stage [114] to the adult stages in humans [115], as discussed in the previous section. Myosin heavy chain (MHC) isoforms in human also switch from α-MHC to β-MHC, leading to slower ATPase activity and stronger actin binding, both of which are important for stronger contractions at slower heart rates [141]. Conversely, in rodents, myosin isoform switches from β to α postnatally, which accommodates the 3- to fourfold increase in mouse heart rate postnatally [142, 143]. Regulatory troponin I (TnI) is initially present as the slow skeletal TnI (ssTnI) isoform, and after birth it switches to the cardiac isoform (cTnI) [138, 144]. This is associated with an increase in myofibrillar sensitivity to Ca^{2+}, promoting stronger contractions soon after birth (Table 1) [145].

Besides mature sarcomeres, efficient excitation–contraction coupling (E–C coupling) and fast conduction rely on two structures that are detectable only in adult cardiomyocytes: the T-tubules and the intercalated disks. T-tubules are plasma membrane invaginations along the Z-line regions, which carry sarcolemmal ion channels deeply into the cell and allow juxtaposition with the sarcoplasmic reticulum [26]. Intercalated disks are cardiac-specific cell–cell junctional complexes containing desmosomes, intermediate junctions and gap junctions that together allow rapid transmission of contractile forces and electrical signals within cardiomyocytes [146]. Primordial T-tubules and intercalated disks are already detectable in the human fetus, but fully mature structures are not complete until age 7 [147–149]. T-tubulation requires the expression of developmentally regulated membrane proteins like caveolin-3 [150], whose expression strikingly increases after birth [95], as well as the expression of bridging integrator-1 [151] and junctophilin-2 [152]. The formation of intercalated disks dictates the structural anisotropy of the adult cardiomyocyte as it leads to the characteristic rod-shape and polarity. Indeed, the strong polarization of Connexin-43 and N-cadherin in adult cardiomyocytes determines a length-to-width ratio of 7 to 9.5. Conversely, the lower abundance and circumferential distribution of these structural proteins in neonates results in a length-to-width of ~ 5 [66, 149]. Maturation also leads to progressive myofibril polarization: in mice their orientation (evaluated as the angle difference made by the myofibril compared to the longitudinal axis) is of 6.14 at E18.5 compared to 5.00 at P4 [149], indicating increased alignment.

Collectively, changes in calcium handling proteins, formation of T-tubules and intercalated disks, improved sarcomere organization, and changes in myofibril protein isoforms largely improve the efficiency of E–C coupling and increase the myofilament sensitivity to calcium, leading to greatly increased contractility [153]. As a result, the contractile force of human ventricular myocardium goes from ~ 0.4 mN/mm^2 in the fetus to ~ 1.1 mN/mm^2 in the neonate, and finally to 10–50 mN/mm^2 in the adult [154, 155].

Metabolism of Postnatal Cardiomyocytes

The adult heart is one of the most energy-consuming organs of the body, followed by the kidney, the brain, and the liver [156]. Indeed, the ATP consumption of a healthy adult heart is around 30 $\mu mol/min^{-1}$ per gram of tissue

at rest [157]. Because of the relative shortage of energy reserves (ATP is found at ~ 5 µmol/g of heathy cardiac tissue [158]), the heart must continuously produce high amounts of energy. In the adult, more than 80% of ATP production is the result of fatty acid β-oxidation in the mitochondria [158, 159]. In contrast, as introduced in Section I, the preferential metabolic pathway in the embryo and fetus is glycolysis (contributing to more than 80% of all ATP) [77, 80, 160]. After birth, the increasing energy demand of the growing body combined with new availability of carbon substrate (feeding on lipid-rich milk) and arterial oxygen rapidly converts metabolism from anaerobic glycolysis to fatty acid oxidation [160, 161]. Whereas levels of fatty acids are less than 0.1 mM in the fetus, they increase immediately after birth to ~ 0.4 mM [80]. Moreover, the PaO_2 increases from ~ 28 to ~ 100 mmHg in the postnatal stage [108, 109]. Such an increase in oxygen levels is required for efficient fatty acid β-oxidation [162], as the oxidation of one gram of palmitate requires 2.4-fold more oxygen than a gram of glucose (with commensurately greater ATP yield). Fatty acid β-oxidation is also favored by the postnatal expression of different metabolic enzymes. As the newborn develops, the ATP consumption in the heart rises continuously, and this energy depletion activates the energy-sensing enzyme, AMP-kinase (AMPK), which contributes to the metabolic switch toward fatty acids' utilization by inhibiting acetyl-CoA carboxylase (ACC) [163, 164]. Indeed, ACC is highly expressed in the fetal and early postnatal periods but decreases dramatically within days after birth [165]. ACC produces malonyl-CoA, which in turn inhibits the activity of CPT1, a transporter required for lipid trafficking across the mitochondrial membrane. The activity of ACC is further inhibited by malonyl-CoA decarboxylase (MCD), which, like AMPK, is also more active postnatally [164]. Overall, the decreased expression and activity of ACC after birth improves CPT1 function, leading to increased mitochondrial lipid uptake.

The mitochondria themselves also become more functional postnatally due to both increased biogenesis and structural remodeling, which facilitates energy production and its transfer to the myofibrils and sarcoplasmic reticulum [166]. Neonatal mitochondria are small, lack fully developed cristae, and have a low transmembrane potential [135, 166]. The fusion and fission of mitochondria are the key processes that underlie mitochondria biogenesis and maturation [167], and are controlled by different factors that are highly dynamic after birth [168–170]. Among them, the transcriptional coactivators PGC-1α and PGC-1β regulate mitochondrial biogenesis in the postnatal heart. In particular, PGC-1 controls genes involved in mitochondrial fusion, such as the mitochondrial dynamin-like GTPase OPA1 and mitofusin 1 and 2 (MFN1 and MFN2), and genes involved in mitochondrial fission like dynamin-related protein 1 (DRP1) and mitochondrial fission protein 1 (FIS1) [168, 169]. The considerable amount of high-energy phosphates produced by the adult mitochondria is transferred to the myosin ATPase by the phosphocreatine shuttle [171]. This facilitated diffusion system is present only in the postnatal stage due to the increased cytoskeletal organization, and it is highly efficient compared to the simple diffusion mechanisms which dominates the fetal stage.

Overall, the increased energy demand of the postnatal heart is addressed by the metabolic switch from glycolysis to oxidative phosphorylation that, combined with mitochondrial development and a more refined energy transfer system, leads to a highly efficient coupling between energy production in the mitochondrion and utilization at the myofibril.

Cell-Cycle Regulation in Postnatal Cardiomyocytes

The final stage of cardiac growth, sometimes called terminal differentiation, is marked by a transition from hyperplasia to hypertrophy. DNA synthesis during murine heart development (as evaluated by tritiated thymidine incorporation) occurs in two temporally different phases: there is high DNA synthesis rates in the embryo, e.g., ~ 33% at E12, which drops to almost undetectable levels at birth [172]. In the early neonatal stage, a second wave of DNA synthesis occurs as the cells become binucleated, with a peak labeling of 10% at postnatal day P4–6. After postnatal day 10 (P10), DNA synthesis is again almost undetectable [172]. In humans, cardiomyocyte cell-cycle withdrawal is considerably slower: in the first decade of life, 0.01% of cardiomyocytes were in the mitotic phase; this percentage decreased significantly during adolescence (10–20 years) and it is barely detectable in the hearts of 40 years-old subjects [173–176]. The vast majority of both human and rodent cardiomyocytes undergo a final round of DNA synthesis without mitosis, resulting in polyploid cells [172, 175, 176]. Although the evolutionary basis for polyploid cardiomyocytes is unknown, it may be that the 20-fold myocyte enlargement that occurs during hypertrophic growth requires multiple genomes to achieve [177]. Interestingly, polyploidy is achieved in different ways by different species. In humans, cardiomyocytes undergo DNA synthesis without nuclear division, resulting in ~ 75% of cardiomyocytes having polyploid nuclei [175]. In rodents, cardiomyocytes undergo both DNA synthesis and nuclear division but do not undergo cytokinesis. This results in > 90% of adult rodent cardiomyocytes being binucleated, with each nucleus containing a diploid chromosomal content [172].

Cell-cycle progression is mediated by complexes of cyclins and cyclin-dependent kinases (CDK) like Cyclin E, D, and A, which mediate the progression through G1 to S,

and Cyclin A and B, which are responsible for the progression from M to G2. The expression and activity of such complexes is developmentally controlled, and reflects the distinct proliferative states of the developing heart [177]. The expression of these cell-cycle regulators is controlled by transcription factors such as the E2F family and Tbx10, which promote expression of cyclins and CDKs, whereas BTG2 and MEIS1, which are significantly upregulated in cardiomyocytes soon after birth, act as negative regulators [177–179]. Other transcription factors like SOX6, GATA4, and MEF2C affect cell proliferation by modulating the PI3K/AKT, WNT/β-catenin, and Hippo-YAP pathways [177, 180–182].

Amongst all of these regulators, the Hippo-YAP pathway has emerged as a major player in mediating cell-cycle withdrawal in cardiomyocytes [182, 183]. Evidence began to accumulate when multiple miRNAs were identified that enhance neonatal rat cardiomyocyte reentry in vitro and adult heart regeneration in vivo [184]. These subsequently were shown to target members of the Hippo pathway for degradation [185]. Genetic inhibition of the Hippo pathway induces cardiomyocyte hyperplasia in fetal mice, in part through unrestrained β-catenin signaling [186]. Genetic inhibition of Hippo in the adult heart induces cardiomyocyte cell-cycle re-entry [187], and when inhibited after myocardial infarction, this induces cardiac regeneration and improvement in cardiac function [188]. Thus, manipulation of the Hippo pathway has been the most successful means to re-induce division in cardiomyocytes. Conversely, activating this pathway seems to be an important step in postnatal maturity.

Oxygen has been proposed as another key regulator of cell-cycle withdrawal in postnatal cardiomyocytes. For instance, the elevated regenerative capacity of certain amphibians and fish correlates with their relatively hypoxic habitat [189, 190]. Conversely, the exposure to a hyperoxic environment after birth leads murine cardiomyocytes to exit the cell cycle, possibly through increasing concentration of reactive oxygen species (ROS) and subsequent oxidative DNA damage [190].

In summary, postnatal cues at the cellular, tissue, and organ levels drive cardiomyocyte maturation to the adult phenotype (Fig. 2). Taken together, this suggest that a similar multifaced approach will be required to increase the maturation of stem cell-derived cardiomyocytes.

The Peter Pan Syndrome of Pluripotent Stem Cell-Derived Cardiomyocytes

Despite substantial process, current approaches to increase the maturation of hPSC-CMs in vitro only manage to produce cardiomyocytes with an intermediate or late fetal phenotype (Table 1) [8, 25, 26]. This contrasts markedly with results from cell transplantation, when cardiomyocytes undergo almost complete structural and molecular maturation in rodent [191, 192], guinea pig [18] or nonhuman primate hearts [19, 22]. Thus, there is no intrinsic block to maturation in hPSC-CMs. The maturation impediment results from the inability of our in vitro systems to mimic faithfully their natural environment.

The surface area of hPSC-CMs generated by conventional protocols is in the range of 1000–1300 μm^2 [193], close to that of fetal CMs (1216 ± 45 μm^2 [193]). On the other hand, the surface area for neonatal CMs and adult CMs is 4395 ± 436 μm^2 and $12{,}315 \pm 2103$ μm^2, respectively [194]. In line with these size differences, the membrane capacitance of hPSC-CMs is 17.5 ± 7.6 pF [26], which is ten times lower compared to that of adult CMs (100–150 pF) [26, 195]. hPSC-CMs are also relatively flat, polygonal to round-shaped, and have an irregular morphology: The length-to-width ratio of these cells ranges from 2 to 4 [196], which is substantially different to that of adult CMs (7.7 – 9.5, as evaluated in isolated human adult CMs [197]). The contractile force generated by hPSC-CMs ranges from 0.1 to 4 mN/mm^2 [198–201], depending on the condition in which the force was measured, but it still significantly lower than that of adult CMs (10–50 mN/mm^2 [155, 201]).This difference may be explained by reduced myofibril content, myofibril disarray and sarcomeric immaturity: ultrastructural analysis revealed that hPSC-CMs have immature sarcomeres in which only the Z band is sometimes visible, whereas the I-band, A-band, and M-band are absent [27]. Moreover, the expression of fetal isoforms of titin (N2BA), troponin I (ssTnI), and myosin heavy chain (α-MHC), the absence of T-tubules and poor polarization of the intercalated disks, combined with the low expression of RYR2 and SERCA2a collectively lead to inefficient E–C coupling [26, 28, 202]. hPSC-CMs are also characterized by strong automaticity and significantly depolarized resting membrane potential (RMP), which ranges from − 50 to − 60 mV. This is substantially more positive than the adult RMP (− 85 mV), and even higher than the neonatal one (− 60 mV to − 70 mV) [202, 203]. Moreover, the electrical immaturity of hPSC-CMs is displayed by strong depolarizing currents (such as I_f and I_{CaT}), combined with low/absent I_{K1} hyperpolarizing current (Table 1).

With regards to hPSC-CM metabolism, mitochondria are small, fewer in number, and localized in the perinuclear region rather than being intermyofibrillar or subsarcolemmal as in the adult heart [204]. Further, the rudimentary cristae and the low expression of CPT1 on the mitochondrial membrane lead to a reduced uptake of fatty acids [205, 206]. Therefore, the preferred metabolic substrates of hPSC-CMs are glucose and lactate, which as described earlier are processed by glycolysis and inhibit fatty acid β-oxidation [77,

205–207]. The cell-cycle dynamics of hPSC-CMs are also indicative of immaturity, as the cells have a relatively high proliferative capacity [208] and low levels of polyploidy [209]. Finally, hPSC-CMs globally resemble fetal CMs from a transcriptional and epigenetic level, as confirmed by genome-wide assays, including RNA sequencing (RNA-seq), and the analysis of transcription factor-binding (ChIP-seq), chromatin accessibility (ATAC-seq), and 3D genome architecture (Hi-C) [28, 140, 210].

Strategies for hPSC-CM Maturation

Over the last decade, multiple approaches have been put forward to enhance hPSC-CM maturation, as discussed in a number of excellent reviews [26, 196, 211]. While the scope of this review is distinct, and thus we do not wish to present a complete overview of this extensive field, in this short section we summarize the state of the art and highlight current limitations.

Long-term culture of hPSC-CMs was the first and simplest approach to improve their maturation. Indeed, full physiological maturation of human cardiomyocytes occurs between 6 and 10 years of age [147]. This can be partially mimicked by culturing hPSC-CMs in vitro for up to one year [209, 210, 212, 213]. While this approach does not require specialized equipment, it is inefficient, expensive, and labor-intensive. Moreover, even after 200 days of culture, the phenotype of hPSC-CMs is nowhere near complete maturation (Table 2). Considering that this method also suffers from large batch-to-batch variability and limited yield, it is unsuitable for scalable production [196, 211].

Physical stimulation either by mechanical or electrical means can speed up hPSC-CM maturation by promoting expression of contractile proteins and structural organization (Table 2) [214], similarly to what is observed during natural development [66, 70]. This has been most successful in engineered heart tissues, where mechanical loading can be controlled more readily [198–200, 215]. Tissue engineering aims to closely mimic the environment of developing cardiomyocytes by embedding them into a 3D matrix in the presence of other noncardiac cells. For instance, progressive increase in afterload leads to increased twitch force, highly organized sarcomeres, and increased expression of β-MHC [214, 216]. Electrical stimulation alone or in combination with mechanical stress also enhances E–C coupling in engineered heart tissues by promoting gap junction formation, sarcomere organization, and SERCA2a and RYR2 expression [217, 218]. However, long-term exposure to excessive physical stimulation can induce markers of pathological hypertrophy and fibrosis [214, 217]. When 3D-EHTs are further stimulated by electrical and/or mechanical means, hPSC-CMs grow to develop adult-like features such as increased sarcomere length (~ 2.2 µm), higher mitochondrial density (up to 30% of the cell volume), presence of T-tubules, strong oxidative metabolism, and efficient E–C coupling (Table 2) [199, 200, 214, 215, 219]. Furthermore, despite substantial recent progress in automation and scalability [215, 220], generation of 3D-EHTs for high-throughput analyses and/or regeneration of large portions of the myocardium is still very challenging.

Multiple approaches have been identified that enhance cardiomyocyte maturation in 2D culture. For example, co-culturing hPSC-CMs with endothelial cells enhances maturity, in part through upregulation of miRNAs miR-125b-5p, miR-199a-5p, miR-221, and miR-222 [221]. Cardiomyocytes respond to substrate cues such as nanotopology, and culturing them on surfaces containing sub-micrometer grooves enhances alignment of the contractile cytoskeleton [222]. This has allowed isolation of single myofibrils from hPSC-CMs derived from patients with familial cardiomyopathy [223]. Further maturation of conduction velocities and calcium handling occurs when the conductivity of the nano-grooved substrate is enhanced with graphene oxide [224].

Maturation can also be promoted via stimulation with growth factors and/or hormones such IGF [208, 225], glucocorticoids [34], and T3 [33, 34, 226]. While these individual treatments improve to some degree sarcomere organization, electrical coupling, and contractility, hPSC-CMs are still largely immature. Overexpression of let-7 family miRNAs, which are highly upregulated during long-term hPSC-CM maturation in vitro, also leads to a more mature phenotype, including stimulating fatty acid metabolism, increased cell size, sarcomere length, force of contraction, and respiratory capacity [227].

We emphasize that, however, with the possible exception of electrically paced engineered heart tissues, in all of these cases hPSC-CMs are still only comparable to neonatal cardiomyocytes. Overall, while these approaches are in principle easily scalable and may be effective to improve cardiac maturation, the lack of a truly mature phenotype still hinders application of hPSC-CMs for certain sensitive applications such as pharmacology and toxicology studies.

Conclusion and Future Directions

Late gestational and postnatal cardiac development is characterized by multiple complex pathways that gradually promote the transition of fetal cardiomyocytes into neonatal and finally adult stages. Unfortunately, we still have incomplete mechanistic knowledge of this process, particularly with regard to late gestational development. Thus, we expect that major advances in our ability to mature hPSC-CMs will rely on much more extensive studies of the basic biology underlying physiological cardiac maturation. Nevertheless, as summarized above, there are a large number of pathways

Table 2 Features of hPSC-CMs matured with different approaches

Parameters	Long-term culture (> 30 days)	Biochemical stimulation (T3)	Physical stimulation	3D-EHT
Morphology				
Surface area	4067.9 ± 1814.6 μm^2 [213]	991 ± 58 μm^2 [33]	2000 μm^2 [216]	> 1000 μm^2
Shape	0.28 ± 0.02[a] [209]	0.54 ± 0.02[a] [33]	7:1 (length to width ratio) [216]	> 0.5 (length to width ratio) [200]
Contractility				
Sarcomere length	1.81 ± 0.01 μm [209]	1.73 ± 0.01 μm [33]	1.5–2.0 μm [214]	2.2 μm [200]
H-band; M-bands	Yes [213]	NA	NA	Yes [200]
Force production	NA	12.3 ± 0.7 nN/cell (twitch force) [33]	0.1–500 nN [214, 216]	4.4 nN/mm^2 [200]
Electrophysiology				
RMP	− 73.5 mV [203]	− 70 mV [226]	NA	− 57 mV [215]
Metabolism				
Mitochondria	Increased organization [209]	Increased OCR [33]	Increased organization [216]	Increased density [200, 215]
Nuclear organization				
% binucleated cells	35% [209]	No difference [33]	NA	25% [219]
Gene Expression				
Upregulated	*MYH6, MYH7, GJA1, HCN4* [209]	*ACTN2, MYBPC3, MYH6, TNNI3, SERCA2, RYR2, CACNA1C, SCN5A, KCNH2, KCND3, KCNJ12 KCNQ1, PGC1α* [33, 226]	*KCND2. KCNH2, ACTN2, GJA1* [216]	*NPPB, MAPK1, PRKACA, MYH7, GJA1, TNNI3, AKAP6, GJA5, JPH2, AKAP1, TFAM, PGC1α, CAV3, BIN1, ATP2A2, RYR2, ITPR3* [200]
Downregulated	NA	*HCN4, MYH7* [33]	NA	*HCN4* [200]

[a]Circularity index = 4π•area/perimeter^2

already known to control cardiac maturation, which have not yet been extensively tested in hPSC-CMs.

At least two important aspects must be considered while trying to achieve hPSC-CM maturation. First, the required level of maturation will heavily depend on the specific application. For instance, a truly adult phenotype would be highly advantageous for pharmacology, toxicology, and disease modeling studies. However, this might be unfavorable for regenerative medicine applications since adult cardiomyocytes do not survive transplantation into injured myocardium (while fetal and neonatal cells are more suitable [228]). Thus, cardiac maturation can represent a double-edged sword that must be carefully balanced: we submit that a "one size fits all" approach is neither the most efficient nor appropriate way to tackle this problem.

The second key issue is one of scalability of mature hPSC-CMs production. As discussed, while many efficient approaches already exist to increase hPSC-CM maturation, most of these cannot currently be applied in a high-throughput fashion and/or for the generation of the large numbers of hiPSC-CMs required for regenerative medicine applications (which is estimated to require billions of cells per patient). Indeed, current "natural engineering" strategies to drive hPSC-CM maturation achieve results that are roughly proportional to the number of nature's cues that are being replicated in vitro. However, increasing the complexity of the culture conditions inversely correlates with scalability, costs, and reproducibility. Thus, we propose that methods that take not just inspiration from developmental lessons but are also able to implement them in novel ways will be highly valuable in the future. For instance, the ability to engineer cellular phenotypes with high efficiency by means of gene editing with CRISPR/Cas9 technology [229, 230] opens the opportunity to specifically alter transcriptional gene networks and/or specific metabolic pathways, ion channels, or sarcomeric proteins.

Overall, we hope that the knowledge gathered in this review may catalyze further explorations of the fundamental link between "natural" and "engineered" development to unlock the full potential of hPSC-CMs in the biomedical sciences.

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Compliance with Ethical Standards

Conflict of interest CEM is a scientific founder and equity holder in Cytocardia.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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