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Cardiomyocyte Maturation: New Phase in Development

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Abstract

Maturation is the last phase of heart development that prepares the organ for strong, efficient, and persistent pumping throughout the mammal's lifespan. This process is characterized by structural, gene expression, metabolic, and functional specializations in cardiomyocytes (CMs) as the heart transits from fetal to adult states. CM maturation gained increased attention recently due to the maturation defects in pluripotent stem cell-derived CMs (PSC-CMs), its antagonistic effect on myocardial regeneration, and its potential contribution to cardiac disease. Here we review the major hallmarks of ventricular CM maturation and summarize key regulatory mechanisms that promote and coordinate these cellular events. With advances in the technical platforms used for CM maturation research, we expect significant progress in the future that will deepen our understanding of this process and lead to better maturation of PSC-CMs and novel therapeutic strategies for heart disease.

Keywords

cardiomyocyte maturation; cardiac development; regulatory mechanism; regenerative medicin; stem cell; heart regeneration

Subject Terms:

Stem Cells

1. Background and Significance

Mammalian heart development is a highly dynamic process that can be conceptually divided into specification, morphogenesis, and maturation (Fig. 1A). Specification refers to the differentiation of the major cardiac lineages from uncommitted mesodermal progenitors. Morphogenesis includes the events that spatially organize cardiac cells, create the structural components of the heart, and properly connect them together. Maturation encompasses the cell- and tissue-level changes that optimize the heart for strong and efficient pumping

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Disclosures

None.

throughout the animal's lifespan. While the first two phases have been focal points for developmental cardiology, heart maturation has been less studied until recently.

Cardiomyocytes (CMs) drive heart contraction. In maturation, CMs undergo changes that permit the cells to sustain billions of cycles of forceful contraction and relaxation. The term "CM maturation" refers to the constellation of changes to cell structure, metabolism, function, and gene expression that convert fetal CMs to adult CMs. This term also refers to the overarching developmental program that drives and coordinates the wide spectrum of phenotypic changes.

The recent attention to CM maturation has been driven by a surging interest in cardiac regenerative medicine (Fig. 1B). Although current technology allows for efficient differentiation of human pluripotent stem cells (PSCs) into CMs, these PSC-CMs exhibit immature phenotypes that resemble fetal CMs[1, 2]. Despite tremendous progress in promoting PSC-CM maturation by tissue engineering-based methods[3, 4], recently reviewed in references[5] and[6], complete maturation of PSC-CMs has yet to be achieved. This maturation bottleneck severely impairs the use of PSC-CMs in in vitro modeling for pathological, pharmacological, or therapeutic purposes. Electrophysiological maturation defects of PSC-CMs also result in arrhythmogenic risk from cell replacement therapy[7]. New knowledge in the developmental biology of maturation is essential for tissue engineers to rationally design better approaches to promote the maturation of PSC-CMs.

CM maturation research is also significant due to its connection to CM regeneration. Natural CM regeneration occurs through proliferation of existing CMs[8–10]. While CMs exhibit proliferative capacity in the fetus, they quickly lose this potential after birth[11], concurring with changes characteristic of CM maturation. Factors that promote CM maturation, such as thyroid hormone[12, 13] and oxygen[14], are antagonistic to CM proliferation. On the other hand, proliferative CMs undergo "dedifferentiation" that includes sarcomere disassembly and upregulation of genes characteristic of fetal CMs[15–17]. Forced proliferation of adult CMs by over-expression of activated Yap[18] or miR199a[19] adversely impacts heart function and causes lethality. Therefore, understanding the Yin and Yang between maturation and proliferation is essential to design strategies to stimulate CM regeneration while minimizing its side effects.

Defective CM maturation could also contribute to heart diseases. For example, sarcomere gene mutations that cause cardiomyopathy have largely been studied for their impact on sarcomere function and Ca^{2+} sensitivity[20]. However, sarcomere assembly is a key driver of CM maturation that not only organizes intracellular structures[21], but also modulates signal transduction[22]. Thus, sarcomere mutations could cause cardiomyopathy by impairing the programs that coordinate CM maturation. As another example, a subset of congenital heart disease patients develops late heart failure. Although this has been attributed to complications of cardiac surgery or the longstanding impact of aberrant hemodynamic loads, some congenital heart disease mutations could affect genes that regulate CM maturation[22–25] and thereby predispose to late myocardial dysfunction.

In this review, we first describe the phenotypic hallmarks of CM maturation and next summarize regulatory mechanisms that trigger and coordinate CM maturation. Ventricular, atrial, and nodal CMs undergo distinct changes during maturation. Most research to date has focused on ventricular CMs, and accordingly we restrict the scope of this review to ventricular CMs.

2. Major Hallmarks of CM Maturation

Major biological processes in CM maturation are described below. Experientially measurable parameters are summarized in Table 1. Selected recent efforts to mature PSC-derived CMs using a combination of three dimensional culture and physical and biological stimuli are summarized in Table 2.

2.a. Myofibril Maturation

Myofibrils are specialized cytoskeletal structures that serve as the contractile apparatuses of CMs[35, 36]. Sarcomeres are longitudinally repeated subunits of myofibrils. A mature sarcomere comprises thin filaments (sarcomeric actin, troponins, tropomyosin), thick filaments (myosin heavy and light chains and their associated proteins, such as myosin binding protein C), titin filaments, Z-lines (actinin and its interacting proteins), and M-lines (myomesin, and its interacting proteins) (Fig. 2A). In a process powered by ATP hydrolysis, myosin complexes exert “power strokes” on thin filaments that slide thick filaments toward the barbed end of sarcomeric actin filaments, which are anchored at Z-lines. This action shortens the distance between Z-lines and results in muscle contraction. Z-lines and M-lines cross-link thin and thick filaments respectively and ensure their alignment. Titin is a gigantic protein with N- and C-termini anchored to Z- and M- lines, respectively. Z-lines are also attached with other cytoskeletal components such as desmin (a type of intermediate filament), microtubules, and the non-sarcomeric actomyosin system, which mechanically integrates these cytoskeletal structures.

Sarcomere assembly initiates at cardiac specification, and continuously occurs in both immature and mature CMs. Thus, the emergence of sarcomeres should be treated as a marker of CM identity, but not maturation. However, CM maturation is characterized by massive expansion of myofibrils (Fig. 2B), as new sarcomeres are continuously added in alignment with pre-existing myofibrils both longitudinally and laterally. Very little is known about the molecular mechanisms that drive sarcomere expansion.

Sarcomere maturation also features changes in ultrastructural organization. When observed by transmission electron microscopy (TEM), mature sarcomeres exhibit more clear banding as compared to immature sarcomeres, suggesting improved alignment of sarcomere filaments. Z-lines increase in width and alignment, and the distance between Z-lines (often called sarcomere length) also increases to ~2.2 μm in diastole in mature, loaded CMs. Although the M-line protein myomesin is present in fetal sarcomeres, the M-line is difficult to visualize by TEM in fetal heart. With maturation, the M-line becomes distinct, likely due to increased thick filament alignment[37].

An integral element of myofibril maturation is sarcomeric isoform switching, in which several sarcomere components switch from a fetal to an adult isoform due to transcriptional changes or alternative splicing. In rodents, among the most well-known is the myosin heavy chain switch from fetal *Myh7 to* adult *Myh6*. By contrast, *MYH7* is the predominant isoform in adult heart of humans, and this isoform preference is already established by 5 weeks of gestation[38, 39]. Whether a *MYH6*-to-*MYH7* switch occurs at an earlier stage of human cardiogenesis remains undetermined, but this event is suggested by predominant expression of *MYH6* in newly differentiated human induced PSC-CMs (hiPSC-CMs)[40].

Isoform switching also affects other sarcomere components. For example, the regulatory light chain of myosin was predominantly expressed by the gene *MYL7* (often known as *MLC-2a*) in all early fetal CMs. However, this isoform switches to *MYL2* (also known as *MLC-2v*) as ventricular CMs mature, and *MYL7* expression becomes restricted to atrial CMs[41, 42]. Fetal CMs primarily express slow skeletal troponin I (*TNNI1*), and this is replaced by cardiac troponin I (*TNNI3*) in mature CMs[43].The more compliant splicing isoform of titin (N2BA isoform) is preferentially expressed in fetal hearts, and after birth the stiffer N2B isoform predominates[44]. Likewise, the fetal isoform of myomesin (EH-myomesin) is expressed in fetal CMs, and this transits to myomesin isoforms lacking the EH domain in mature CMs. This isoform transition has been associated with the appearance of the M-line[37]. Cardiac troponin T and tropomyosin also undergo maturationally regulated alternative splicing[45].

2.b. Maturation of Electrophysiology and Ca^{2+} Handling

The strength, speed, and rhythm of CM contraction and relaxation are tightly controlled by electrical impulses and oscillations of cytoplasmic Ca^{2+} concentration. The electrical signals take the form of the action potential, which is determined by cardiac ion channels. In mature CMs, the resting membrane potential is maintained at ~−85 mV by the inward rectifying current I_{K1}[46]. Potassium channels Kir2.1 and Kir2.2, encoded by genes *KCNJ2* and *KCNJ12*, respectively, are the major channels that establish and maintain the resting membrane potential. The action potential is initiated by rapid opening of voltage-gated sodium channels (mainly Nav1.5; encoded by *SCN5A*), which permits Na^+ influx (I_{Na}) and membrane depolarization. Depolarization is followed by the activity of transient outward potassium current (I_{to}) that results in a unique “notch” shape in the action potential of maturation CMs. Membrane depolarization opens the L-type Ca^{2+} channels (Cav1.2), which generate the Ca^{2+} current ($I_{Ca,L}$) responsible for the “plateau” phase of the action potential in human CMs. Action potential of murine CMs do not exhibit a clear plateau phase. The depolarizing effect of $I_{Ca,L}$ is counteracted by an array of temporally controlled repolarizing potassium currents, including I_{Ks}, I_{Kr}, and I_{K1}. Upon Cav1.2 inactivation, the repolarizing potassium currents re-establish the resting membrane potential.

Immature CMs differ in important ways from mature CMs in electrophysiology. First, the resting membrane potential of immature CMs is less negative (~−50 to −60 mV) as a result of insufficient expression of Kir2.1 and Kir2.2[47]. Second, the upstroke velocity of immature CMs (~15–30 V/s) is slower due to lower activity and expression of *SCN5A* and other sodium channels[48, 49]. Third, the plateau phase of the action potential is longer in mature

CMs, partly due to higher expression of Cav1.2 core component *CACNA1C*[50] and alternative splicing of its auxiliary subunit *CACNB2*[51].

Membrane depolarization is coupled to sarcomere contraction through Ca^{2+}-induced Ca^{2+} release (CICR). In systole, Cav1.2 activation allows a small amount of extracellular Ca^{2+} to enter cells, where it activates the ryanodine receptor 2 (RYR2) to release Ca^{2+} from the sarcoplasmic reticulum (SR, specialized endoplasmic reticulum in CMs). In diastole, Ca^{2+} is cleared from the cytosol to the SR via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2), and to the extracellular space via the Na^{+}-Ca^{2+} exchanger (NCX).

CICR occurs in proximity to plasma membrane. In small, immature CMs where sarcomeres are relatively proximal to the cell surface, Ca^{2+} that is released at the cell periphery is sufficient to trigger sarcomere contraction. However, as CMs enlarge and sarcomeres expand toward the cell interior, Ca^{2+} that is released at the cell periphery cannot rapidly activate interior sarcomeres. To solve this problem, CMs evolved transverse-tubules (T-tubules; Fig. 2), which are invaginations of plasma membrane that penetrate transversely into the center of mature CMs. This structural specialization juxtaposes the plasma membrane with subdomains of SR to form dyads, where Cav1.2 and RYR2 cluster in proximity to form Ca^{2+} release units. These structural specializations allow the action potential to travel rapidly along T-tubules to the interior of cells, where they trigger dyads to release Ca^{2+} in close proximity to sarcomeres.

The structural basis of T-tubule maturation is poorly understood. Caveolin-3 (CAV3) is thought to regulate plasma membrane invagination[52], but T-tubules still form in *Cav3* knockout mice[53]. BIN1 increases membrane curvature of T-tubules in mice[54], and BIN1 overexpression induces T-tubule-like structures in PSC-CMs[55]. However, the transverse alignment of T-tubules is preserved in *Bin1* knockout CMs in mice[54]. JPH2 is required to juxtapose T-tubule and SR membranes[56], but JPH2 disruption only results in mild cell-autonomous loss of T-tubule organization in murine CMs[57]. Although ACTN2 is essential for T-tubule organization[22], how T-tubules are anchored to Z-lines remains unclear. A recent study identified a Z-line component nexilin (NEXN) as a new regulator of T-tubules[58]. Whether NEXN mediates Z-line-T-tubule association remains to be determined.

Whereas mature ventricular CMs exhibit low automaticity, immature CMs and PSC-CMs spontaneously beat, a phenotype that likely contributes to arrhythmia when PSC-CMs are transplanted in myocardial infarction models[7]. Multiple factors contribute to the automaticity of PSC-CMs, including the expression of pacemaker channels such as hyperpolarization activated cyclic nucleotide gated potassium channel 4 (HCN4), the resting membrane potential that is closer to the action potential activation threshold, and spontaneous Ca^{2+} release, which drives membrane depolarization through the Ca^{2+}-Na^{+} exchanger[59].

2.c. Metabolic Maturation

An adult human heart is estimated to use ~6 kg ATP per day[60], with the primary consumers being myosin ATPases, which are needed for sarcomere contraction, and SERCA, which

drives Ca^{2+} clearance and sarcomere relaxation. This ATP is primarily produced through oxidative phosphorylation using lipid substrates[61].

In maturation, CMs undergo multiple adaptations to enable a high and sustained rate of ATP production. Chief among them is increased number and size of mitochondria, which occupy up to 40% of cell volume[62]. The morphology and size of mitochondria is controlled by their fusion and fission. Perturbation of pro-fusion proteins such as mitofusin 1/2 (MFN1/2[63, 64]), or overexpression of pro-fission proteins such as DRP1[65], resulted in decreased mitochondrial size in maturing CMs. Mitochondria also become associated with sarcomeres during maturation (Fig. 2). Sarcomere disassembly caused decreased mitochondrial size[21], suggesting a functional link between sarcomeres and mitochondrial morphology. Mitochondria are also attached to SR, potentially through ER-mitochondria contact sites. This close organization leads to efficient ATP transport from mitochondria to ATPases in sarcomeres and SR[66].

Mature mitochondria contain densely organized cristae, the foldings of the inner mitochondrial membranes that house the electron transport chain and ATP synthase. By contrast, in immature CMs, which primarily produce ATP through glycolysis, mitochondria exhibit few and poorly-aligned cristae[67]. Cristae maturation requires an array of molecules such as OPA1[68, 69],the MICOS complex[70] and cardiolipin-based lipid-protein microdomains[71]. ATP synthase[72] may also drive cristae curvature formation.

The metabolic transition from immature CMs to mature CMs is driven by activation of metabolic transcriptional regulators including *Ppargc1a/b*, *Ppara*, *Nrf1/2*, and *Esrra/b/g*[73], upregulation of genes involved in fatty acid metabolism, oxidative phosphorylation, and mitochondrial biogenesis, and downregulation of glycolytic genes[74, 75]. Isoform switching also contributes to metabolic maturation. Hexokinase, which executes the first committed step of glycolysis, is predominantly hexokinase 1 (HK1) in fetal and neonatal CMs[76]. In adult CMs, the predominant isoform is hexokinase 2 (HK2)[77], which exhibits less glycolytic activity. Cytochrome c oxidase (COX) subunit 8, a component of complex IV of the electron transport chain, also switches between COX8A and COX8B isoforms in CM maturation[78],although the contribution of this switch to CM maturation remains to be determined.

Less is known about anabolic metabolism changes in CM maturation. Immature, proliferative CMs create a high demand for nucleotide biosynthesis, which is suppressed after CMs mature. Conversely, high glucose promotes nucleotide biosynthesis through the pentose phosphate pathway and inhibits CM maturation[79]. Because CM maturation involves a remarkable increase of protein-built components such as myofibrils, and extensive expansion of lipid bilayers in T-tubules, SR and mitochondria, protein and lipid biosynthesis are also expected to be highly active. However, little work has been done to characterize these two anabolic processes during CM maturation.

2.d. Proliferation-to-hypertrophy Transition

In mice, CM cell cycle exit occurs within the first postnatal week[11]. In humans, CM proliferation rate declines rapidly postnatally, but does not reach the steady-state rate of <

1% per year until the second decade of life[80, 81]. Central cell cycle regulators, such as the cyclin-dependent kinase (CDK) complexes, are tightly repressed during CM maturation[82]. Recently, it was reported that co-overexpression of CDK1:CCNB and CDK4:CCND complexes, which activate M phase and G1-S phase respectively, was sufficient to reactivate CM proliferation[82]. This exciting finding awaits confirmation by independent groups. The mechanisms that enforce CM cell cycle exit include the downregulation of mitogenic signals, such as the neuregulin-ErbB axis[83], and the inhibition of YAP, a potent activator of CM proliferation[84, 85]. During postnatal CM maturation, YAP activity is restrained by Hippo kinases[84, 85], interactions with cell adhesion complexes[86, 87], and nuclear antagonists[88].

Despite cell cycle withdrawal, the postnatal heart increases in size by ~30-fold through proportional increase of CM volume, a process called maturational hypertrophy. The liquid-phase cytoplasm is unlikely the major contributor to increased cell volume, as mature CMs are tightly packed and myofibrils and mitochondria occupy most intracellular space. Myofibril expansion is critical for maturational hypertrophy, as the ablation of sarcomeres by *Myh6* depletion or *Actn2* mutation dramatically decreased CM size during murine CM maturation[21, 22]. However, whether mitochondria biogenesis and enlargement cell-autonomously contributes to maturational hypertrophy is unclear[21, 89].

Another hallmark of CM maturation during the proliferation-to-hypertrophy transition is polyploidization. In murine CMs, the final round of the cell cycle involves karyokinesis without cytokinesis, leading most mature CMs (~90%) to contain two diploid nuclei ("binucleation")[90, 91] (Fig. 2). By contrast, in adult humans, ~75% CMs are mononuclear, but the majority of these nuclei are polyploid due to DNA endoreplication without karyokinesis[92, 93]. This polyploidization largely develops in the second decade of life[80].

CM polyploidization negatively correlates with cell cycle withdrawal[94]. Residual CM cell cycle activity in adult hearts resides in the mononuclear diploid subset of CMs[80, 81, 94]. The introduction of a genetic modifier associated with higher mononuclear diploid fraction increased CM cell cycle activity after adult heart injury[94]. Forced CM polyploidization by ECT2 inhibition, which blocks cytokinesis, is sufficient to suppress the proliferative capacity of CMs in regeneration[95, 96]. For many cell types, the ploidy of a cell is positively correlated with cell size[97], thus CM polyploidization likely promotes maturational hypertrophy. Consistent with this hypothesis, the induction of CM polyploidization was sufficient to increase CM size[95, 96]. Together, CM polyploidization is partially causative for both CM cell cycle withdrawal and maturational hypertrophy in CM maturation.

2.e. CM Integration into a Mature Tissue

Maturational integration of CMs into cardiac tissues require the formation of specialized CM-CM junctions called intercalated discs (ICDs), which occurs 2–3 weeks after birth in mice. ICDs are hybrid junctions comprising three major types of cell adhesions: fascia adherens, desmosomes, and gap junctions[98]. Fascia adherens comprise N-cadherin and its associated proteins. Desmosomes comprise desmoglein-2, desmocollin-2, and their anxilary proteins such as plakoglobin, plakophilin-2, and demoplakin. Gap junctions are composed of connexin 43. While fascia adherens and desmosomes mechanically couple the actin

cytoskeleton and intermediate filaments of neighboring CMs, gap junctions mediate propagation of electrical and small molecule signals between CMs.

Immature CMs lack ICDs, and ICD components are either not expressed, localized to the interior of cells, or throughout the cell surface. During CM maturation, these molecules redistribute to cell termini to form ICDs. The mechanisms that regulate the targeted localization of ICD components to CM termini are incompletely elucidated, but likely involve protein trafficking along “microtubule highways” extending from the trans-golgi network to cell termini[99].

CM integration into tissues also requires attachment to the extracellular matrix (ECM) through specialized focal adhesion-like structures called costameres[100]. The transmembrane adaptors of costameres include both the integrin complexes and the dystrophin-associated glycoprotein complexes, which anchor to sarcomere Z-lines and non-sarcomere cytoskeleton at the lateral CM membrane.

Beyond tissue integration, ICDs and costameres are likely to play additional roles in CM maturation. For example, both ICDs and costameres harbor vinculin-based actomyosin organizers that are essential for sarcomere assembly[101], and potentially mediate longitudinal and lateral sarcomere expansion respectively. ICDs and costameres are also critical sensors of biophysical signals[98, 100]. Thus, further investigation of ICD and costamere is essential to understand how biophysical signals promote CM maturation (see next section).

3. The Regulation of CM Maturation

CM maturation involves a spectrum of diverse cellular events that occur concurrently. The mechanisms that activate these events and integrate them into a coordinated program is an overarching question for CM maturation research.

3.a. Microenvironmental Instruction

The microenvironment of the maturing myocardium provides necessary and sufficient information to instruct CM maturation. This notion is supported by two lines of evidence. First, in vitro culture of primary mature CMs leads to loss of hallmarks of maturity[102]. Second, immature CMs developed toward an adult-like state after being transplanted into maturing myocardium[103]. These studies provide the logical basis to search for CM maturation cues by dissecting the physicochemical properties of maturing myocardium.

3.a.i. Biophysical Cues (Fig. 3A)—Adult CMs exhibit a rod shape with an average length-to-width ratio of 7:1[104]. This unique shape cannot be solely explained by the cell-autonomous effect of sarcomere elongation, as CMs with sarcomere ablation due to *Myh6* knockout retained an elongated morphology in a genetic mosaic model in mice, although the cell width was drastically decreased[21]. Both neonatal and adult CMs are elongated in vivo but cannot maintain this shape after cell culture. PSC-CMs on regular cell culture dishes are round- or triangular- shaped and require physical cues to adopt a rod shape. Therefore, the microenvironment of myocardium establishes geometric cues that induce uniaxial CM elongation (Fig. 3A).

Patterning CMs to adopt a rod-shaped morphology promotes CM maturation. For example, PSC-CM growth on rectangular micropatterns[105] or uniaxially aligned ridges and grooves[106, 107] were sufficient to improve sarcomere organization and contractile and electrophysiological function of CMs in a two dimensional (2D) system. CM maturation was further improved by assembling CMs into three dimensional (3D) tissue with anisotropically directed strain, such as engineered heart tissue (EHT)[108, 109] or cardiac microtissue (CMT)[108, 109].

The viscoelastic properties of ECM also modulate CM maturation (Fig. 3A). The elastic modulus of ECM progressively increases from neonatal (<10 kPa) to adult (~25 kPa) heart[110]. Culturing CMs on matrix with tunable elastic moduli showed that physiological matrix stiffness is optimal for CM maturation parameters such as sarcomere organization, Ca^{2+} handling, and contractility[111–113].

Maturing CMs experience escalating mechanical force during development[114]. Cyclic mechanical stress during systole and passive stretch during diastole both induced CM maturation in cell culture[115–117] (Fig. 3A). Mechanical force not only improved structural maturation but also induced gene expression changes[115–117]. A recent study showed that cardiac contractile force regulated the distribution of vinculin and activated slingshot protein phosphatase 1 and the actin depolymerizing factor cofilin to promote myofilament maturation[118]. How mechanotransduction pathways convert mechanical force into transcriptional changes remains to be clarified.

Electrical pacing also enhances the ultrastructure and gene expression of cultured CMs (Fig. 3A), as well as their contractile, electrophysiological, and metabolic activity[119–121]. A recent study reported the production of adult-like CMs after 3D engineered heart tissue was paced at supraphysiological rates from an early point in their differentiation[4, 27]. The striking degree of maturation achieved in this study requires further validation and replication by other groups. The mechanisms by which electrical stimulation enhances CM maturation remain poorly explored. A key unanswered question is whether electrical pacing directly impacts CM maturation or acts indirectly through induction of mechanical stress.

3.a.ii. Biochemical Cues (Fig. 3B)—Among the best characterized biochemical cues that promote CM maturation is the thyroid hormone T3 (triiodothyronine). The serum level of T3 rises dramatically in the perinatal period. T3 exerted a broad impact on CM maturation, including isoform switching of myosin heavy chain and titin[122, 123], induction of SERCA expression, hypertrophy and cell polyploidization[12, 13]. T3 treatment was sufficient to enhance CM contractility, Ca^{2+} handling, and mitochondrial respiration in vitro[124, 125]. One study linked a proliferative burst of mouse cardiomyocyte proliferation on postnatal day 15 to a transient surge of thyroid hormone[126]; however, others groups have not replicated the proposed surge of proliferating cardiomyocytes[127, 128]. The major thyroid hormone receptors in the heart are nuclear receptors (NRs) that are encoded by *Thra* and *Thrb* (Fig. 3B). Inactivation of *Thra* cell-autonomously suppressed CM maturation[25].

Similar to T3, glucocorticoids also modulate CM maturation[129]. Glucocorticoids are ligands for the glucocorticoid receptor, another NR encoded by *Nr3c1*. Mutation of *Nr3c1* impaired

myocyte alignment, disruption of sarcomere organization and the expression of genes regulating sarcomere assembly and Ca^{2+} handling[130].

Insulin-like growth factors (IGFs) regulate CM maturation through the insulin-like growth factor 1 receptor (IGF1R) and the insulin receptor (INSR), which are receptor tyrosine kinases that signal through the PI3K-AKT and RAF-MEK-ERK pathways. IGF1 is predominantly produced in the liver, and also locally produced in the heart[131]. Circulating IGF1 quickly increases after birth in response to growth hormone[132, 133]; changes to local production of cardiac IGF1 were not well-described. Overexpression of IGF1R in CMs caused physiological hypertrophy[134]. Double knockout of INSR and IGF1R in murine CMs resulted in early-onset dilated cardiomyopathy within a month after birth, with disrupted sarcomere and mitochondrial morphology and reduced heart function[135]. However, deletion of either INSR or IGF1R alone did not cause phenotypic abnormalities, consistent with functional redundancy.

Circulating fatty acids also increase at birth, and this could serve as a biochemical signal for CM maturation. Culture of engineered cardiac tissues with palmitate, the most abundant long-chain free fatty acid in the neonatal circulation[136], matured multiple parameters, including gene expression, contractile force, action potential, Ca^{2+} transient and oxidative respiration[29]. In another study, treatment of PSC-CMs with palmitate-albumin complexes along with carnitine, which facilitate mitochondrial fatty acid transport, promoted structural and functional maturation, suggesting that in vitro promotion of oxidative phosphorylation stimulates overall CM maturation[137]. However, perturbation of metabolic maturation did not impair structural maturation in a cell-autonomous manner in vivo, since neonatal, mosaic ablation of genes essential for mitochondrial function (*Tfam*) or dynamics (*Mfn1/2*) did not impair structural maturation of the mutant CMs[21, 89].

Oxygen tension is another environmental cue that modulates CM maturation. Increased oxygen tension inhibits HIF1α (hypoxia-inducible factor 1α) activity and promotes the metabolic switch to oxidative phosphorylation during murine heart development[138], whereas hypoxia impaired PSC-CMs differentiation and maturation in vitro[139]. Inhibition of HIF1α and its downstream target lactate dehydrogenase A promoted hiPSC-CM maturation, enhancing not only metabolism but also gene expression, sarcomere organization and contractility[140].

Biochemical signals function synergistically to promote CM maturation. For example, T3 and dexamethasone, a synthetic glucocorticoid, in combination with culture on “matrigel mattresses” cooperatively triggered CM maturation by inducing T-tubule formation[141]. A cocktail of T3, dexamethasone, and IGF1 induced several adult features in iPSC-CMs cultured in 3D cardiac tissues[3]. Cross-talk between T3 and AKT-PI3K, a downstream branch of IGF1 signaling, stimulated TTN isoform switching in cultured, late gestation rat CMs[122]. Thus, a sophisticated signaling network is present that integrates diverse extracellular signals into a robust and coordinated program of CM maturation.

3.a.iii. Non-CMs—Although CMs occupy ~70–85% of myocardial volume, they constitute only ~20–30% of the total cell number[80, 142, 143]. Numerically, non-CMs,

including endothelial cells (64%), cardiac fibroblasts (27%), and leukocytes (9%), are the major cell types in the heart[143]. In the fetal heart, CMs constitute a higher fraction of cells, with the proportion declining during maturation due to the greater proliferation of non-CMs.

Non-CMs regulate CM maturation, as co-culture of CMs with non-CMs promotes CM maturation in vitro[144–146]. The impact of non-CMs on CM maturation could occur through direct physical adhesion, and through paracrine molecules that are secreted from non-CMs and act on CMs[146]. In addition, non-CMs build the microenvironment that delivers biophysical and biochemical cues to CMs. For example, cardiac fibroblasts create the appropriate ECM to support CM maturation, and endothelial cells construct coronary vasculature that transport circulating signals to instruct CM maturation.

3.b. Intracellular regulation

3.b.i. Transcriptional regulation of gene expression—The coordination of diverse phenotypic changes during CM maturation and the association of those changes with altered gene expression suggest an overarching transcriptional program that orchestrates CM maturation.

Several transcriptional regulators of CM maturation have been identified. One of these is serum response factor (SRF)[21]. In murine CMs undergoing maturation, SRF depletion resulted in a wide spectrum of transcriptional dysregulation, including defective sarcomere isoform switching, global downregulation of the transcriptional programs of lipid metabolism, mitochondria biogenesis and oxidative respiration, and the reversal of maturational changes of key electrophysiological and Ca^{2+} handling genes, such as upregulation of *Hcn4* and downregulation of *Kcnj2*, *Serca2a* and *Ryr2*[21]. Structurally, SRF depletion impaired sarcomere expansion, T-tubule formation, and mitochondrial organization.

The broad impact of SRF on nearly every aspect of CM maturation is partly due to its key role in regulating sarcomere genes. Sarcomere disassembly by mosaic inactivation of the major Z-line protein ACTN2 not only recapitulated structural CM maturation defects, but also the transcriptomic signature of mosaic SRF depletion[22]. This relationship demonstrates that sarcomere-based signaling impacts gene transcription and highlights a hierarchical organization of the subprograms of CM maturation: Sarcomere maturation is upstream of most other aspects of CM maturation[21], whereas metabolic maturation was dispensable for structural maturation in vivo[21, 89].

Three myocardin-family transcriptional regulators, MYOCD, MRTFA and MRTFB, are major coactivators of SRF in CMs[147]. MRTFA and MRTFB are functionally redundant. *Mrtfa*$^{-/-}$*; Mrtfb*$^{fl/fl}$*; Myh6Cre* mice caused lethality of most mutants within a month after birth[148]. *Myocd*$^{fl/fl}$*; Myh6Cre* mice developed later onset, lethal cardiomyopathy, with a median survival of about 10 months[149]. Although *Mrtfa/b* double knockout mice exhibit a more severe cardiac phenotype than *Myocd* mutant mice, both mice exhibit cardiac phenotypes that are less severe than *Srf* knockout mice, suggesting a synergistic role of all three factors in SRF activation and CM maturation. The MRTF-SRF axis could convert mechanical stress into transcriptional changes[150], thus MRTF-SRF signaling potentially

mediates regulation of CM maturation in response to biomechanical cues, including mechanical stretch and ECM matrix stiffness.

A recent transcriptomic analysis revealed another SRF-binding transcription cofactor, HOPX, as a novel activator of CM maturation, especially in the process of myofibrillar isoform switching and CM hypertrophy[151]. In vivo, overexpression of HOPX in CMs resulted in progressive concentric cardiac hypertrophy with preserved systolic function[152], whereas *Hopx* knockout caused partial embryonic lethality[153, 154], with postnatal survivors exhibiting normal cardiac contractility and cardiomyocyte hyperplasia due to delayed cell cycle exit[154]. Paradoxically, HOPX was classically thought to be a transcriptional corepressor that reduces SRF-DNA binding[153, 154]. Further studies are necessary to determine how SRF-HOPX interaction impacts CM maturation.

SRF functions in synergy with other transcription factors. For instance, SRF ChIP-Seq in maturing hearts revealed co-enrichment of GATA and MEF2 motifs[21]. GATA4 and GATA6 are the major GATA family transcription factors expressed in CMs, and these factors are redundantly essential for neonatal CM maturation[25, 155]. Four MEF2 family transcription factors, MEF2A~D, are expressed in hearts[156] and their functions can be factor-specific, overlapping, or, in some cases, antagonistic[157, 158]. A systematic comparison has yet to be performed to determine the overlapping and unique roles of MEF2 factors in CM maturation.

In addition to SRF-related factors, NRs are another major group of transcription regulators that control CM maturation. Among these factors, thyroid hormone receptors and glucocorticoid receptors mediate the role of T3 and glucocorticoids in CM maturation as described in the previous section. Additional NRs play key roles in metabolic maturation. One family of such factors are peroxisome proliferator-activated receptors (PPARs), which form heterodimers with retinoid X NRs to activate and balance the transcription of genes involved in fatty acid and carbohydrate metabolism[159, 160]. The ligands of PPARs are fatty acid metabolites[161], thus PPARs probably mediate the impact of circulating fatty acids on CM maturation. The estrogen-related receptors (ERRα, β, and γ) are another group of NRs essential for the maturational switch to oxidative respiration, by activating genes involved in fatty acid oxidation, citric acid cycle, electron transport chain, ATP synthase and mitochondrial dynamics[162, 163]. These factors belong to the orphan NR family and do not bind to estrogen. Interestingly, myofibril and Ca^{2+} handling genes are also direct downstream targets of ERRs[162, 163]. Both PPARs and ERRs directly interact with PGC1α/β, encoded by *Ppargc1a* and *Ppargc1b*, which are master regulators of both oxidative respiration and its associated mitochondrial biogenesis[73]. Interestingly, a recent study showed additional functions of PGC1/PPARα in the maturation of calcium handling and hypertrophy, implicating broader roles of these factors beyond metabolism[164].

Epigenetic mechanisms, such as DNA methylation and covalent histone modifications, exert a profound impact on transcriptional regulation. DNA hypermethylation is associated with gene silencing in CM maturation, while DNA demethylation results in gene activation[75, 165, 166]. Activating histone modifications H3K27ac, H3K4me1, H3K4me3 and H3K9ac are associated with actively expressed genes in maturation[165, 167], while repressive

histone marks H3K27me3 and H3K9me2 are maintained or acquired by inactivated genes[165, 167–170]. Treatment of cultured human cardiac progenitor cells with polyinosinic-polycytidylic acid yielded PSC-CMs with enhanced maturity, which was attributed to "epigenetic priming" that enhanced Notch signaling and expression of cardiac myofilament genes[171]. Recently, a clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-based forward genetic screen in vivo identified RNF20/40 as a novel epigenetic regulator of CM maturation. This enzyme deposits histone H2B lysine 120 monoubiquitination marks at genes that are active in CM maturation[25]. Mutations that disrupt this pathway cause congenital heart disease[24], suggesting that the same mutations that cause congenital heart disease could also impact CM maturation and late cardiac outcomes.

Chromatin organization changes are also correlated with transcriptional changes in CM maturation. ATAC-Seq revealed decreased chromatin accessibility of silenced genes such as cell cycle genes between neonatal and adult hearts, while metabolic and muscle contraction genes acquired a more open chromatin state in mature hearts[172]. Histone remodeling factor BRG1 modulates myosin heavy chain isoform switching[173]. Mutation of CTCF, a crucial regulator of chromatin-architecture, was recently reported to cause premature activation of the CM maturation program in embryonic cardiomyocytes[174].

3.b.ii. Post-transcriptional regulation of gene expression—RNA splicing is a critical regulatory component of CM maturation, as isoform switching often occurs through alternative splicing. One representative splicing regulator is RBM20, mutation of which causes dilated cardiomyopathy[175–177]. RBM20 is essential for proper splicing of *Ttn* (Titin) transcripts and other maturationally regulated genes[175, 178].

Additional splicing regulators could potentially impact CM maturation: CELF proteins are down regulated in heart development while MBNL proteins are upregulated. The antagonistic regulation of these two splicing regulators[179] has been proposed to trigger a large fraction of developmental splicing changes and to be essential for T-tubule organization and Ca^{2+} handling[180, 181]. Serine/arginine-rich family of splicing factors, including SRSF1[182], SRSF2[183] and SRSF10[184], were each shown to regulate postnatal heart development by modulating Ca^{2+} handling genes. CM-specific *Hnrnpu* knockout resulted in splicing defects in *Ttn* and Ca^{2+} handling genes and triggered perinatal dilated cardiomyopathy[185]. The RNA splicing regulator RBFOX1 markedly increases in expression during CM maturation[180, 186] and is another potential activator of CM maturation[187].

MicroRNA (miRNA)-based mRNA silencing is another mechanism that modulates gene expression in CM maturation. For example, miR-1, a miRNA enriched in mature CMs, facilitated electrophysiological maturation in stem-cell derived CMs in vitro[188]. Let-7 family miRNAs were highly enriched in CMs matured for 1 year in vitro, and they were necessary and sufficient to promote hypertrophy, sarcomere organization, contractile force, and respiratory capacity of cultured PSC-CMs[189]. Co-culture of CMs with endothelial cells promoted CM maturation in association with upregulation of multiple miRNAs[145]. Overexpression of four such miRNAs (miR-125b-5p, miR-199a-5p, miR-221, and miR-222) in PSC-CMs resulted in improvement of several maturation hallmarks such as *Myh6/7*

switching, sarcomere alignment, mitochondrial cristae formation, and improved Ca^{2+} handling[145]. Recently, a new miRNA maturation cocktail that overexpressed Let-7i and miR-452 and repressed miR-122 and miR-200a was shown to promote transcriptomic maturation as well as contractility, cell size, and fatty acid oxidation without sharing predicted target genes with previous microRNA cocktails[190].

Cardiac protein synthesis is very active at fetal and neonatal stages, but regulation of protein translation, modification, and stability in CM maturation has been poorly studied. Recent advances in proteomics have started to characterize protein changes in CM maturation[191–193]. Integration of these data with RNA-Seq and Ribo-Seq analyses will provide an improved understanding of regulation at the protein level.

3.b.iii. Ultrastructural regulation—Major ultrastructural maturation hallmarks -- myofibrils, mitochondria, and T-tubules -- are not independent of each other. As the major cytoskeletal structures of CMs, myofibrils are essential for the organization of other intracellular structures. Mutagenesis of key myofibril genes, such as *Myh6* and *Actn2*, impaired mitochondrial enlargement as well as the organization of T-tubules[21, 22]. By contrast, perturbation of T-tubule (by mutagenesis of *Jph2*[57]*)* or mitochondria (by mutagenesis of *Mfn1/2*[21] or *Tfam*[89], or by overexpression of *Drp1*[21]*)* did not impair myofibril organization. Thus, proper sarcomere organization and expansion is central to overall structural maturation.

4. Model systems to study CM maturation

Innovations in the model systems and techniques used to study CM maturation will fuel future discoveries. Here we review some of the recent advances in model systems used to study CM maturation.

4.a. Mouse genetic mosaic and Cas9-mediated somatic knockout models

Genetically modified mice have been gold standards to understand mammalian heart development. This approach is particularly important in CM maturation research because so far no in vitro system can induce, or even maintain, full maturity of CMs. However, traditional genetic manipulation of the murine heart has several caveats. First, it is slow and expensive to generate or obtain alleles to knockout each gene of interest. Achieving spatiotemporal control of the knockout in perinatal CMs requires further complexity. Second, organ-wide mutagenesis of a gene essential for CM maturation often triggers lethality or secondary effects that can confound identification of the direct functions of the gene. This is particularly problematic in CM maturation research as the secondary effects of heart dysfunction, such as fetal gene reactivation and mitochondria/T-tubule remodeling, are similar to CM maturation defects[57, 194].

These problems can be circumvented using adeno-associated virus (AAV), which efficiently and stably manipulates genes in CMs following subcutaneous or intraperitoneal injection to newborn mice. Gain-of-function via AAV-directed overexpression is straightforward. Loss-of-function can be achieved by using AAV to delivery CRISPR/Cas9 components (CRISPR/Cas9 and AAV-mediated somatic mutagenesis, CASAAV, Fig. 4A)[57, 195]. The CRISPR/Cas9

system further reduces the need to obtain conditional alleles. This technology allows mutagenesis of many genes at once[21, 57] and even high-throughput genetic screening in vivo[25].

To pinpoint the direct, cell-autonomous effects of gene manipulation, the dose of AAV is titrated so that a minority (e.g. <15%) of CMs are transduced, leaving most CMs, and the overall cardiac function, unaffected. Single-cell readouts on the transduced cells are used to deduce cell-autonomous gene function[21, 22, 25, 57, 89]. In genetic mosaics, mutant and control CMs are mixed in the same heart, thus analysis is limited to single cell readouts, or readouts compatible with a cell purification method such as flow cytometry. These analyses rely heavily on the ability to distinguish individual mutant and control cells, usually through immunostaining of the targeted proteins or introduction of fluorescent proteins as surrogate markers. Genetic mosaic approaches are most well suited to cell autonomous phenotypes and would difficult to apply to genes that produces secreted products.

4.b. Engineered tissue model

CM maturation demonstrates substantial interspecies differences. For instance, adult zebrafish CMs lack T-tubules[196] and exhibit much lower mitochondrial content[14] than mammalian CMs. Mouse and human CMs also exhibit several distinct maturation features, such as Myh6/7 isoform switching, contraction rates, and action potential profiles. Therefore, a human model is necessary to validate knowledge that was learnt in other model organisms.

In addition, a major practical goal of studying CM maturation is to improve the maturation of hPSC-CMs in vitro for translational medicine. The current consensus is that 3D engineered cardiac tissues that are assembled by hPSC-CMs, non-myocytes and ECMs provide the necessary platforms to best mature CMs in vitro. Additional biochemical (T3, Dex, IGF1, palmitate) and biophysical treatments (electrical pacing; mechanical stress) on these engineered tissues are essential to produce adult-like CMs (Fig. 4B, Table 2)[3, 4]. These technologies are useful to validate knowledge that is generated in animal models and to allow de novo discovery of CM maturation regulators. In vivo validation is still necessary to determine the physiological relevance of novel CM maturation factors that are identified in these tissue models. Importantly, factors that drive CM maturation in vitro may incompletely overlap with those that promote maturation in vivo during normal heart development.

Disease modeling is another application of these hPSC-CMs and engineered tissues. The immaturity of these cells is an important hurdle to disease modeling. Nevertheless these model systems have yielded important insights into disease mechanisms and led to new potential therapeutic strategies[197]. The properties of the model system, such as its electrical or metabolic maturity, should be considered with respect to the disease being studied. Key findings may require validation in alternative model systems that exhibit greater physiological maturity.

4.c. Neonatal xenotransplantation model

Human PSC-CMs could be matured toward a near-adult state by transplantation into rat myocardium (Fig. 4C)[103, 198], which is a promising solution to the partial maturation defects

observed in in vitro engineered tissue models. However, human PSC-CMs matured by this method exhibit more binucleation than normal human adult CMs[103], raising the question of whether the transplanted human PSC-CMs become rat-like CMs or remain human-like. Although some comparisons between donor and host CMs were documented[198], a more comprehensive analysis is necessary to determine if xenotransplants are viable models to study human-specific features of CM maturation.

5. Concluding Remarks

Here we reviewed major hallmarks of CM maturation and known regulators of this process. Although differences between immature and mature CMs have been well documented, the molecular mechanisms that mediate the change from immature to mature states remain incompletely understood. Accumulated evidence demonstrates interdependence between individual maturation events. Thus, research in this area should not only study individual hallmarks, but also how the maturation events are coordinated. With technical advances in model systems and increased collaboration between basic scientists with tissue engineers, a more comprehensive picture of CM maturation is warranted in the near future. This effort is critical to design better strategies to mature PSC-CM, stimulate CM regeneration, and treat diseases that involve CM maturation defects.

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Non-standard Abbreviations and Acronyms:

CM	cardiomyocyte
PSC	pluripotent stem cell
hiPSC-CM	human induced PSC-derived CM
TEM	transmission electron microscopy
CICR	calcium induced calcium release
SR	sarcoplasmic reticulum
T-tubule	transverse tubule
ECM	extracellular matrix
ICD	intercalated disc
EHT	engineered heart tissue

CMT	cardiac microtissue
CDK	cyclin-dependent kinase
T3	triiodothyronine
NR	nuclear receptor
IGF	Insulin-like growth factor
PPAR	peroxisome proliferator-activated receptor
ERR	estrogen-related receptor
CRISPR	clustered regularly interspaced short palindromic repeats
miRNA	microRNA
AAV	adeno-associated virus
CASAAV	CRISPR/Cas9/AAV9-mediated somatic mutagenesis

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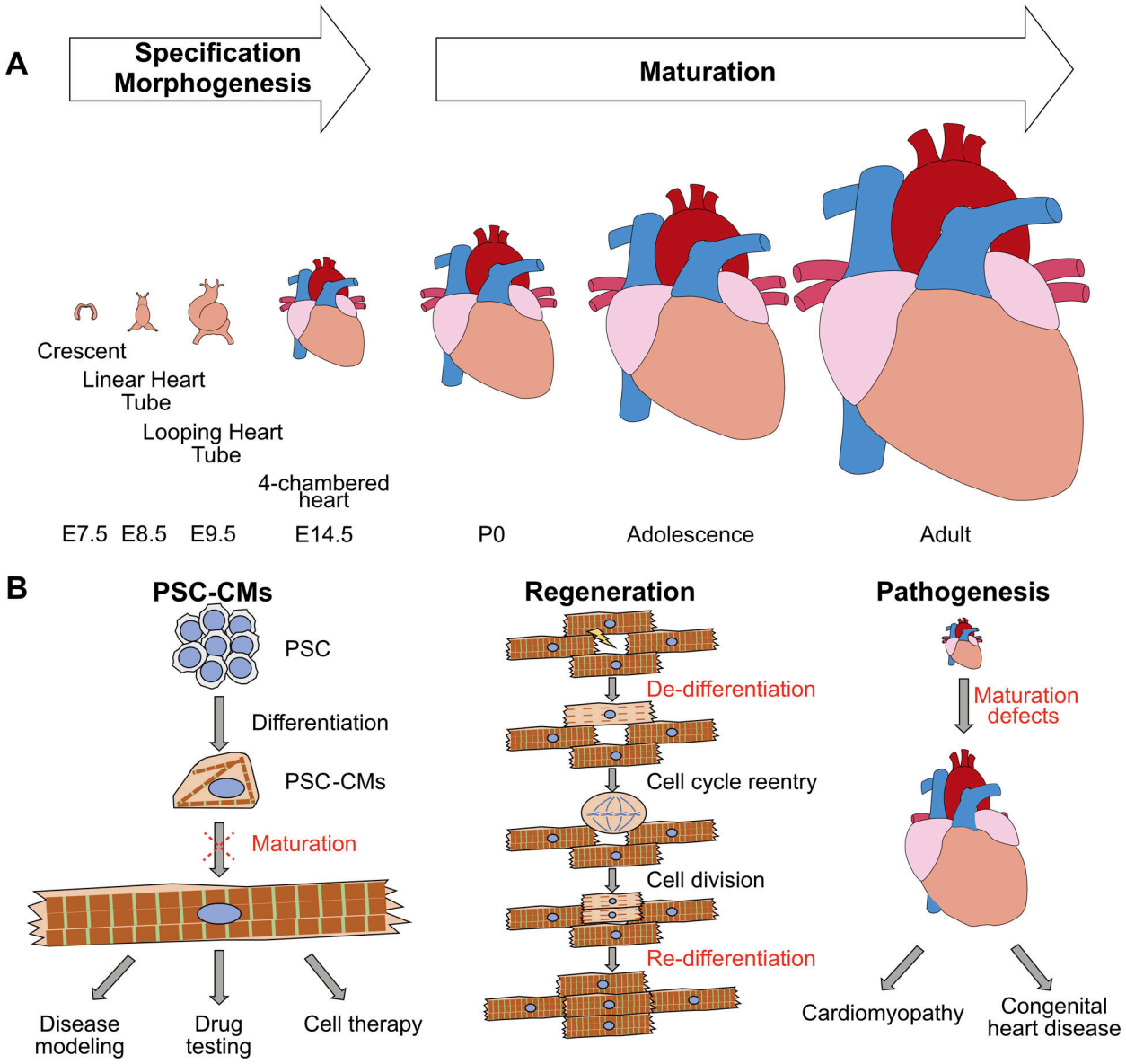

Figure 1. Heart maturation and its implication in translational medicine.

(A) Conceptual scheme of the maturation phase of heart development. Mouse stages are labeled at bottom. **(B)** Major applications of CM maturation studies. Left: to promote the maturation of PSC-CMs. Mid: to optimize CM regeneration conditions. Right: to better understand cardiac pathogenesis.

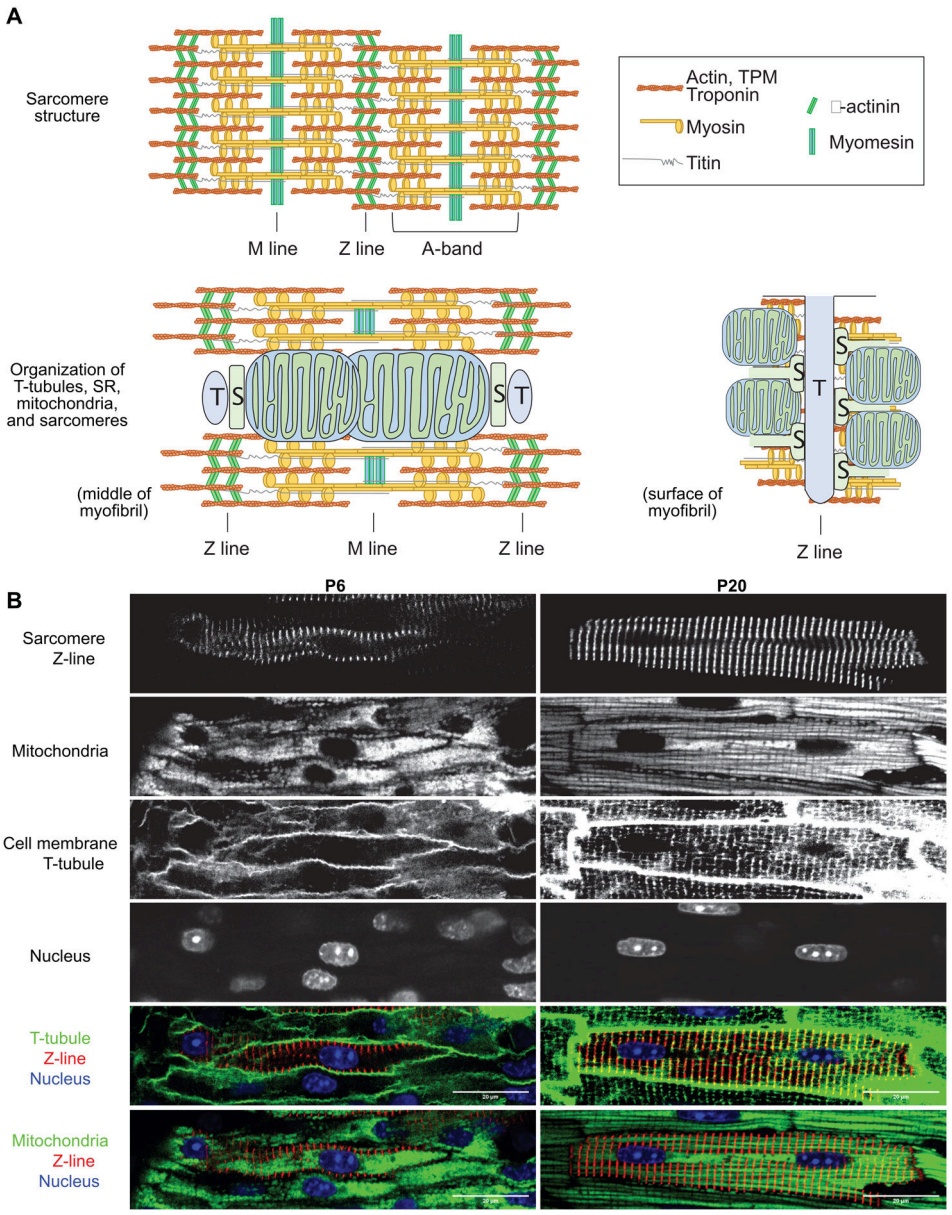

Figure 2. Structural maturation of CMs.

(A) A schematic view of sarcomere components in mature CMs (top) and spatial relationship between sarcomeres and T-tubule (T), SR (S) and mitochondria in mature CMs (bottom). Bottom left: a view across the middle of a myofibril. Bottom right: a view on the cytoplasmic surface of a myofibril. **(B)** In situ confocal images of murine myocardium at postnatal day 6 (P6) and P20. Sarcomere Z-lines were labeled by AAV-Actn2-GFP infection. Mitochondria, T-tubules and nuclei were stained by TMRM (polarized mitochondria), FM 4–64 (plasma membrane), and Hoechst (DNA), respectively, through Langendoff perfusion. Merged images highlight T-tubule-sarcomere and mitochondria-sarcomere associations that are established during postnatal maturation.

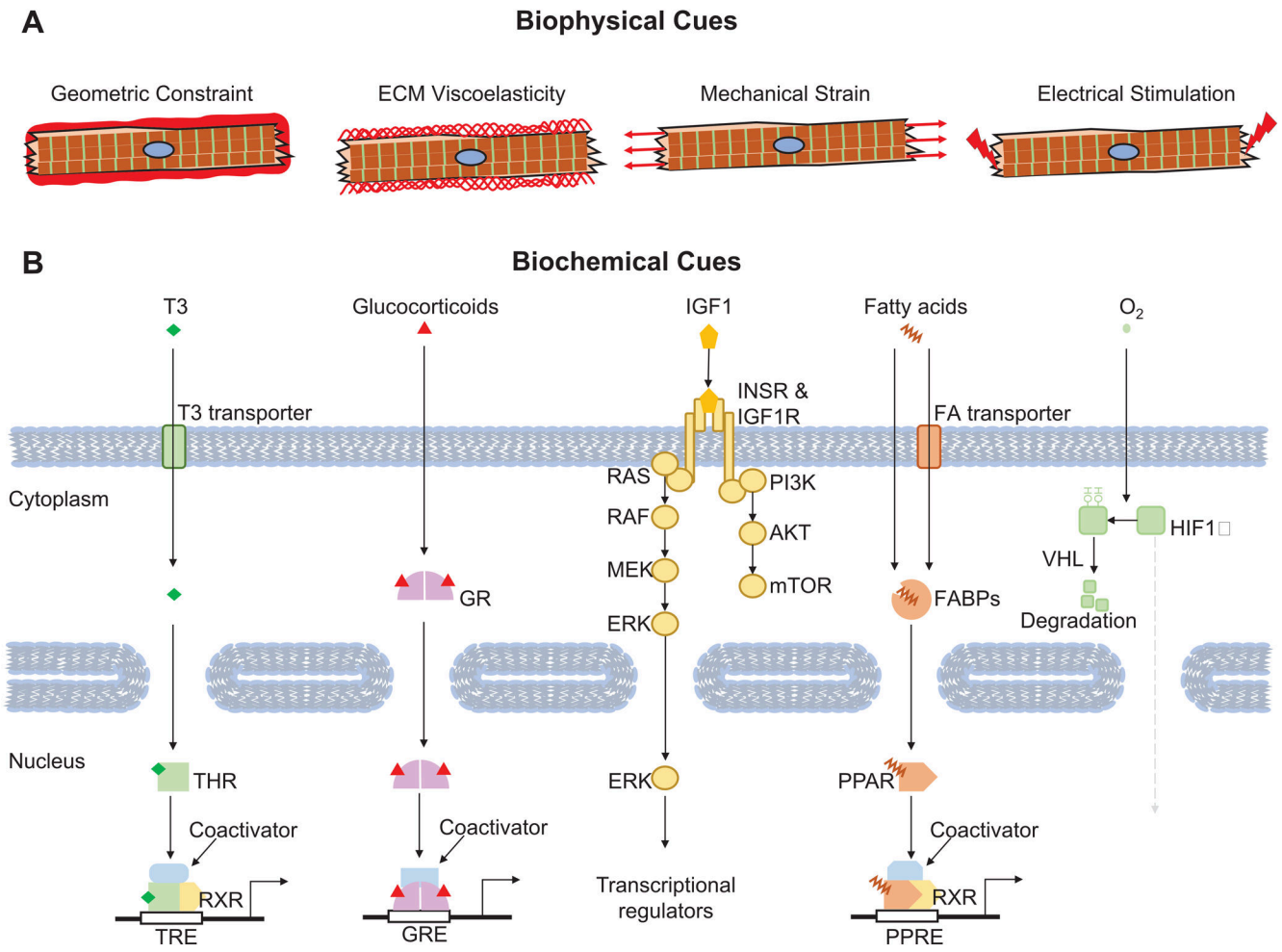

Figure 3. Representative environmental cues that regulate CM maturation.

(A) Key biophysical factors that affect CM maturation. **(B)** Critical biochemical cues that regulate CM maturation. Representative signal receptors, messengers and transcriptional regulators are also depicted.

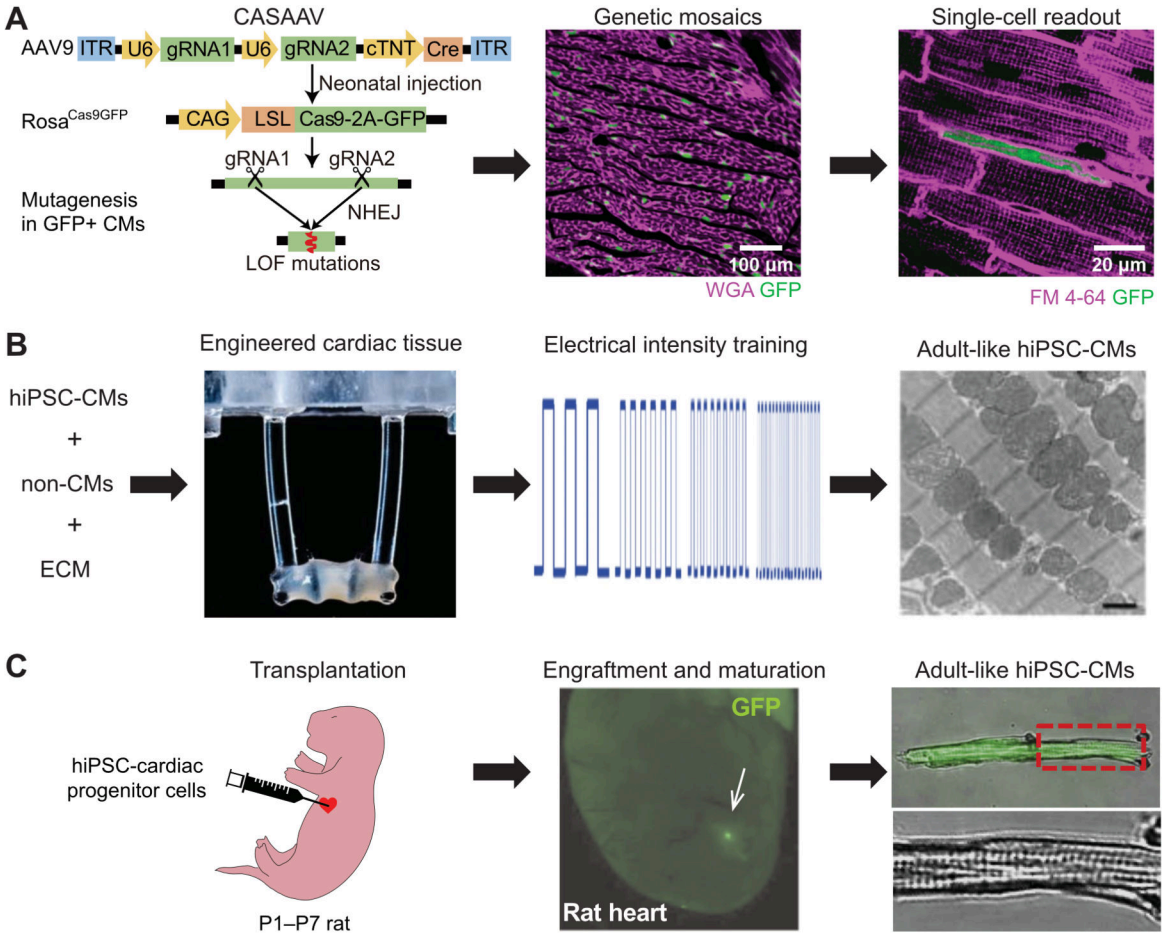

Figure 4. Model systems to study CM maturation.

(A) CASAAV-based genetic mosaic analysis of murine CM maturation in vivo[57]. Expression of genome-encoded Cas9-P2A-GFP was activated by AAV-delivery of single or dual gRNAs and Cre, expressed from the cardiomyocyte-specific cTNT promoter (left). When the AAV is given at a low dose, mosaic transduction and Cas9-mediated somatic mutagenesis at genes targeted by gRNA(s) occurs (GFP^+ cells, middle). The phenotype of single GFP+ cells is then analyzed (right, illustrating T-tubule and maturational growth defects caused by *Srf* depletion[21]). WGA, wheat germ agglutinin. FM 4–64, membrane dye. Left panel reprinted ref. 21. with permission. **(B)** In vitro maturation of PSC-CMs by tissue engineering and electrical pacing. 3D cultured engineered heart tissue was assembled from PSC-CMs (left). Elastomeric posts apply anisotropic stress on muscle bundle. Rapid electrical pacing protocol was applied from early in the PSC-CM differentiation process (middle), resulting in well organized, mature PSC-CMs, as evaluated by transmission electron microscopy (right). Reprinted from ref. 4 with permission. **(C)** In vivo maturation of PSC-CMs. Human PSC-CMs expressing GFP were injected into the hearts of immunodeficient neonatal rats (left). After several weeks, engrafted PSC-CMs (GFP^+, middle) have mature morphology (right). Reprinted from ref.[103] with permission.

Table 1.

Major Parameters of CM maturation

	Gene Expression	Morphology	Functional Readouts
Myofibril	Overall increase of mature sarcomere components Isoform switching: MYH6 to MYH7 (hs) MYH7 to MYH6 (mm) TNNI1 to TNNI3 TTN-N2BA to TTN-N2B MYL7 to MYL2	Sarcomere assembly and expansion Improved sarcomere alignment Increased sarcomere length (~2.2 μm) M-line formation	Sarcomere contraction: Diastolic sarcomere length Fractional shortening Shortening velocity Contractile Force
Electrophysiology & Ca^{2+} handling	Increase of ventricular ion channels, e.g. KCNJ2 Decrease of automaticity ion channels, e.g. HCN4 Increase of Ca^{2+} handling molecules, e.g. LTCC, RYR2 and SERCA2	T-tubule formation and organization SR expansion and organization Dyad formation and distribution	Action potential: Resting Vm (~−85 mV) Max dVm/dt (~200 V/s) Duration and shape Ca^{2+} transient: Peak amplitude Time to peak Decay time Diastolic Ca^{2+}
Metabolism	Glycolysis decrease Mitochondria biogenesis increase FAO increase Oxidative phosphorylation increase Energy transfer system increase	Mitochondria # and size incr. (up to 40% cell volume) Cristae formation and organization Inter-myofibrillar localization	Oxygen consumption rate Electron transport chain activity IMM electrochemical gradient Extracellular acidification rate
Other	Cell cycle gene silencing Hypertrophy gene upregulation Changes of cell adhesion genes, e.g. ICD and costamere components	Polyploidization Binucleation in >80% rodent CMs but only ~25% human CMs Maturational hypertrophy (~30 fold) ICD formation	

Abbreviations: FAO, fatty acid oxidation; Vm, membrane potential; ICD, intercalated disk; hs, Homo sapiens; mm, Mus musculus; IMM, inner mitochondrial membrane

Table 2.

Efforts to promote hiPSC-CM maturation by 3D tissue engineering

Reference		Huang et al.[26]	Ronaldson-Bouchard et al.[4, 27]	Shadrin et al.[28]	Mills et al.[29]	Ruan et al.[30]	Hirt et al., Mannhardt et al., Lemoine et al.[31–33]	Nunes et al.[34]
Engineered tissue size and treatments		0.5 mm × 0.2 mm, T3 + Dex + IGF1 for 1 wk	6 mm × 2 mm, early ramped field stim. 2–6 Hz	7 mm × 7 mm, RPMI + B27 + insulin for 1wk, 5% FBS for 2 wks	1 mm × 0.5 mm, low glucose, high palmitate, no insulin	20 mm × 0.3 mm, static stress for 2 wks + electrical stim. for 1 wk	8 mm × 0.2–1.3 mm, ± pacing	~600 μm wide gel on inelastic silk core; ramped field stim. 1–6 Hz
Myofibril assembly	**isoform switching**	↑MYH6, ↑MLC2v; ↓MYH7, ↓MLC2a, ↓ TNNI1	↑MYH7, ↑TNNI3	↑MLC2V, ↑TNNI3, ↑MYH7; ↓MLC2a	↑MLC2v; ↑TTN N2B, ↑MYH7/6, ↑TNNI3/1	not described	MLC2v detectable	↓MYH6
	Sarcomere organization	Orderly register of A-bands, I-bands, H-zone and Z-lines; no M-lines.	Orderly register of A-bands, I-bands, Z-lines and M-lines.	Orderly register of A-bands, I-bands, H-zone and Z-lines; no M-lines.	Clear Z-lines, I-bands and A-bands; no M-line	Improved; lack detailed analysis of TEM	Regular Z-lines; inconsistent I- and A-bands; no M-line	Regular Z-lines; I-band and H-zone detectable; no M-line
	sarcomere length	2 μm	2.2 μm	2.1 μm	2.3 μm	not described	1.6 μm	not described
Electrophysiology and Ca^{2+} handling	**expression of channels & regulators**	↑KCNJ2, ↑RYR2, ↑SERCA, ↑NCX1	↑RYR2, ↑SERCA; ↓HCN4	↑CASQ2, ↑S100A1	not described	↑SERCA,↑RYR2	no detectable changes	↑KCNJ2
	T-tubule	adjacent to sarcomeres; unclear alignment	well developed and aligned	not detectable	adjacent to sarcomeres; unclear alignment	not detectable	not detectable	not detectable
	resting Vm	not quantified	–70 mV	–71 mV	–60 mV	nd	–73.5 mV	–80 mV
	Max dV/dt	not quantified	23 V/s	38 V/s	148 V/s	nd	219 V/s	125 V/s
	APD	APD80 1000 ms at 0.5 Hz pacing	APD90 500 ms	ADP80 450 ms	APD90 110 ms, APD50 60 ms	nd	nd	APD90 120 ms
	AP “notch”	not detectable	Yes	not described	Yes	nd	Yes	nd
	Ca^{2+} transient	enhanced	enhanced	visible	enhanced	nd	nd	nd
	Ca^{2+} storage & SR release	nd	enhanced	nd	nd	nd	nd	enhanced
metabolism	**metabolic gene expr.**	↑PPARA, ↑PGC1a	↑TFAM, ↑PGC1a	↑COX6A2, ↑CKMT2, ↑CKM	↑redox and FAO genes	nd	nd	nd
	mitochondria amount	increase by TEM	increase by TEM	increase by TEM	mtDNA increase	nd	nd	nd
	mitochondria alignment	close to sarcomeres	close to sarcomeres	nd	close to sarcomeres	nd	nd	close to sarcomeres

Reference		Huang et al.[26]	Ronaldson-Bouchard et al.[4, 27]	Shadrin et al.[28]	Mills et al.[29]	Ruan et al.[30]	Hirt et al., Mannhardt et al., Lemoine et al.[31–33]	Nunes et al.[34]
Proliferation & hypertrophy	mitochondria cristae	nd	well developed	well developed	nd	nd	immature	nd
	mitochondria functions	nd	OCR and ECAR increased	nd	Incr. maximal OCR and OCR reserve	nd	nd	nd
	cell-cycle gene expr.	nd	nd	nd	cell cycle gene downregulation	nd	nd	nd
	proliferation rate	nd	nd	decrease	decrease	nd	nd	decrease
	CM size	Incr. to 735 μm^2	Incr. to 1500 μm^2	nd	nd	Incr. to 795 μm^2	nd	Incr. to 917 μm^2
	ICD	ICD on TEM, Cx43 at cell poles	ICD on TEM, Cx43 at cell poles	NCad at cell poles; Cx43 mislocalized	ICD on TEM; Cx43 and NCad mislocalized	Primitive ICD on TEM	Cx43 mislocalized,	nascent ICD
	contractility	2.1–4.4 mN/mm^2	3 mN/mm^2	23 mN/mm^2	0.3 mN	1.3 mN/mm^2	up to 0.15 mN	nd
	Frank-Starling relationship	nd	nd	detectable	nd	detectable	detectable	nd
	Force-freq. relationship	flat	positive	flat or slightly negative	nd	positive	flat	nd
Tissue integration and physiology	Response to β-agonists	Incr. contraction rate & amplitude	Incr. contraction rate & amplitude	nd	Incr. contraction rate & amplitude	Incr. contraction rate but not force amplitude	Incr. force amplitude; rate not described	Incr. rate, force not described
	Post-pause potentiation	nd	present	nd	nd	nd	present	nd
	Conduction vel. (cm/s)	up to 40	25	25.1	nd	2.76	nd	15
	inotropic response to extracellular Ca^{2+} (EC_{50})	nd	~0.4 mM	nd	1 mM	nd	0.6 mM	nd

Abbreviations: Dex, dexamethasone; stim, stimulation; Vm, membrane voltage, APD, action potential duration; AP, action potential; nd, not described; ICD, intercalated disc; TEM, transmission electron microscopy; NCad, N-cadherin