


REVIEW

Cardiomyocyte Maturation

New Phase in Development

Yuxuan Guo, William T. Pu 

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 as the heart transits from fetal to adult states. Cardiomyocyte maturation gained increased attention recently due to the maturation defects in pluripotent stem cell–derived cardiomyocyte, its antagonistic effect on myocardial regeneration, and its potential contribution to cardiac disease. Here, we review the major hallmarks of ventricular cardiomyocyte maturation and summarize key regulatory mechanisms that promote and coordinate these cellular events. With advances in the technical platforms used for cardiomyocyte maturation research, we expect significant progress in the future that will deepen our understanding of this process and lead to better maturation of pluripotent stem cell–derived cardiomyocyte and novel therapeutic strategies for heart disease.

Key Words: development ■ heart disease ■ mammals ■ pluripotent stem cell ■ regeneration ■ stem cell

Mammalian heart development is a highly dynamic process that can be conceptually divided into specification, morphogenesis, and maturation (Figure 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 throughout the animal's lifespan. Although the first 2 phases have been focal points for developmental cardiology, heart maturation has been less studied until recently.

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

The recent attention to cardiomyocyte maturation has been driven by surging interest in cardiac regenerative medicine (Figure 1B). Although current technology allows for efficient differentiation of human pluripotent stem cells (PSCs) into cardiomyocytes, these PSC-cardiomyocytes exhibit immature phenotypes that resemble fetal cardiomyocytes.^{1,2} Despite tremendous progress in promoting PSC-derived cardiomyocyte (PSC-CM) maturation by tissue engineering-based methods,^{3,4} recently reviewed in Karbassi et al⁵ and Scuderi et al,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.

Cardiomyocyte maturation research is also significant due to its connection to cardiomyocyte regeneration. Natural cardiomyocyte regeneration occurs through proliferation of existing cardiomyocytes.^{8–10} Although cardiomyocytes exhibit proliferative capacity in the

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Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus
ECM	extracellular matrix
hiPSC-CM	human induced PSC-derived cardiomyocyte
ICD	intercalated disc
IGF	insulin-like growth factor
miRNA	microRNA
MRTF	myocardin-related transcription factor
NR	nuclear receptor
PPAR	peroxisome proliferator-activated receptor
PSC	pluripotent stem cell
SR	sarcoplasmic reticulum
T3	triiodothyronine
T-tubule	transverse-tubule

fetus, they quickly lose this potential after birth,¹¹ concurring with changes characteristic of cardiomyocyte maturation. Factors that promote cardiomyocyte maturation, such as thyroid hormone^{12,13} and oxygen,¹⁴ are antagonistic to cardiomyocyte proliferation. However, proliferative cardiomyocytes undergo dedifferentiation that includes sarcomere disassembly and upregulation of genes characteristic of fetal cardiomyocytes.^{15–17} Forced proliferation of adult cardiomyocytes by overexpression of activated Yap (Yes-associated protein)¹⁸ or miR199a (microRNA-199a)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 cardiomyocyte regeneration while minimizing its side effects.

Defective cardiomyocyte 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 Ca²⁺ sensitivity.²⁰ However, sarcomere assembly is a key driver of cardiomyocyte 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 cardiomyocyte 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 cardiomyocyte maturation^{22–25} and thereby predispose to late myocardial dysfunction.

In this review, we first describe the phenotypic hallmarks of cardiomyocyte maturation and next summarize regulatory mechanisms that trigger and coordinate

cardiomyocyte maturation. Ventricular, atrial, and nodal cardiomyocytes undergo distinct changes during maturation. Most research to date has focused on ventricular cardiomyocytes, and accordingly, we restrict the scope of this review to ventricular cardiomyocytes.

MAJOR HALLMARKS OF CARDIOMYOCYTE MATURATION

Major biological processes in cardiomyocyte maturation are described below. Experimentally measurable parameters are summarized in Table 1. Selected recent efforts to mature PSC-derived cardiomyocytes using a combination of 3-dimensional culture and physical and biological stimuli are summarized in Table 2.

Myofibril Maturation

Myofibrils are specialized cytoskeletal structures that serve as the contractile apparatuses of cardiomyocytes.^{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), TTN (titin) filaments, Z-lines (actinin and its interacting proteins), and M-lines (myomesin, and its interacting proteins; Figure 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. TTN 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 nonsarcomeric actomyosin system, which mechanically integrates these cytoskeletal structures.

Sarcomere assembly initiates at cardiac specification and continuously occurs in both immature and mature cardiomyocytes. Thus, the emergence of sarcomeres should be treated as a marker of cardiomyocyte identity but not maturation. However, cardiomyocyte maturation is characterized by massive expansion of myofibrils (Figure 2B), as new sarcomeres are continuously added in alignment with preexisting 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, mature sarcomeres exhibit more clear banding as compared to immature sarcomeres, suggesting improved alignment of sarcomere filaments.

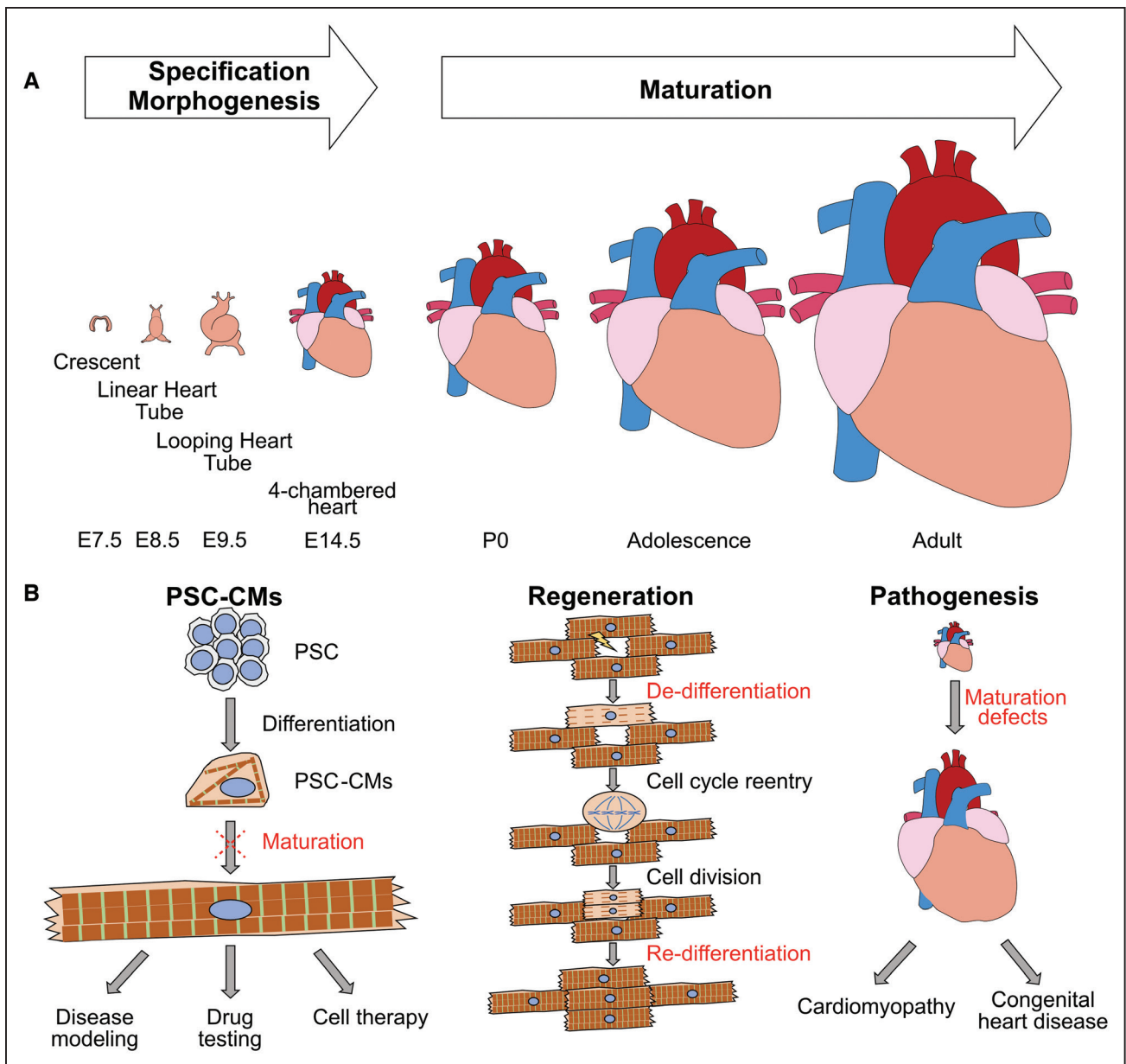


Figure 1. Heart maturation and its implication in translational medicine.

A, Conceptual scheme of the maturation phase of heart development. Mouse stages are labeled at **bottom**. **B**, Major applications of cardiomyocyte (CM) maturation studies. **Left**, To promote the maturation of pluripotent stem cell-derived CM (PSC-CMs). **Mid**, To optimize CM regeneration conditions. **Right**, To better understand cardiac pathogenesis. E indicates embryonic day.

Z-lines increase in width and alignment, and the distance between Z-lines (often called sarcomere length) also increases to $\approx 2.2 \mu\text{m}$ in diastole in mature, loaded cardiomyocytes. Although the M-line protein myomesin is present in fetal sarcomeres, the M-line is difficult to visualize by transmission electron microscopy 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 (Myh) 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 an *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-cardiomyocytes.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 *MLC2a*) in all early fetal cardiomyocytes. However, this isoform switches to *MYL2* (also known as *MLC2v*)

Table 1. Major Parameters of CM maturation

	Gene Expression	Morphology	Functional Readouts
Myofibril	Overall increase of mature sarcomere components <u>Isoform switching:</u> 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 ($\approx 2.2 \mu\text{m}$) M-line formation	<u>Sarcomere contraction:</u> Fractional shortening Shortening velocity Contractile Force
Electrophysiology and Ca^{2+} Handling	Increase of ventricular ion channels, for example, KCNJ2 Decrease of automaticity ion channels, for example, HCN4 Increase of Ca^{2+} handling molecules, such as Cav1.2, RYR2, and SERCA2	T-tubule formation and organization SR expansion and organization Dyad formation and distribution	<u>Action potential:</u> Resting Vm ($\approx -85 \text{ mV}$) Max dVm/dt ($\approx 200 \text{ V/s}$) Duration and shape <u>Ca^{2+} transient:</u> Peak amplitude Time to peak Decay time Diastolic Ca^{2+}
Metabolism	Glycolysis decrease, eg, HK1, PKM Activation of mitochondrial biogenesis, fatty acid oxidation, and oxidative phosphorylation, eg, PPARC1A, PPARA, ESRRA	Mitochondria number and size incr. (up to 40% cell volume) Cristae formation and organization Intermyofibrillar localization	<u>Metabolism:</u> Oxygen consumption rate Electron transport chain activity IMM electrochemical gradient Extracellular acidification rate
Other	Cell cycle gene silencing, eg, CDK1, CCNB1, AURKB Changes of cell adhesion genes, such as ICD and costamere components, eg, GJA1	Polyplodization Binucleation in $>80\%$ rodent CMs but only $\approx 25\%$ human CMs Maturation hypertrophy ($\approx 30\text{-fold}$) ICD formation	<u>Other readouts:</u> Force-frequency relationship Post-pause potentiation Conduction velocity Response to beta-agonists

CM indicates cardiomyocyte; FAO, fatty acid oxidation; HCN4, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; hs, *Homo sapiens*; ICD, intercalated disk; IMM, inner mitochondrial membrane; incr., increase; KCNJ2, potassium inwardly rectifying channel subfamily J member 2; LTCC, L-type calcium channel; mm, *Mus musculus*; Myh, myosin heavy chain; N2B, contains only the N2B element; N2BA, contains both N2A and N2B elements; RYR2, ryanodine receptor 2; SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; TNN, troponin; T-tubules, transverse tubules; TTN, titin; and Vm, membrane potential.

as ventricular cardiomyocytes mature, and *MYL7* expression becomes restricted to atrial cardiomyocytes.^{41,42} Fetal cardiomyocytes primarily express slow skeletal troponin I (*TNNI1*), and this is replaced by cardiac troponin I (*TNNI3*) in mature cardiomyocytes.⁴³ The more compliant splicing isoform of TTN (N2BA isoform, which includes both N2B and N2A elements of titin) is preferentially expressed in fetal hearts, and after birth, the stiffer N2B isoform, which exclude the N2A element, predominates.⁴⁴ Likewise, the fetal isoform of myomesin (EH-myomesin) is expressed in fetal cardiomyocytes, and this transits to myomesin isoforms lacking the EH domain in mature cardiomyocytes. 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

Maturation of Electrophysiology and Ca^{2+} Handling

The strength, speed, and rhythm of cardiomyocyte 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 cardiomyocytes, the resting membrane potential is maintained at $\approx -85 \text{ mV}$ by the inward rectifying current I_{K1} .⁴⁶ Inwardly rectifying potassium channels (Kir) 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 cardiomyocytes. Membrane depolarization opens the L-type Ca^{2+} channels (Cav1.2), which generate the Ca^{2+} current (I_{CaL}) responsible for the plateau phase of the action potential in human cardiomyocytes. Action potential of murine cardiomyocytes does not exhibit a clear plateau phase. The depolarizing effect of I_{CaL} 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 reestablish the resting membrane potential.

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 wk	1 mm×0.5 mm, low glucose, high palmitate, no insulin	20 mm×0.3 mm, static stress for 2 wk+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 V _m	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 and 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
	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
Proliferation and hypertrophy	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
Tissue integration and physiology	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
	Response to β-agonists	Incr. contraction rate & amplitude	Incr. contraction rate and amplitude	nd	Incr. contraction rate & amplitude	Incr. contraction rate but not force amplitude	Incr. force amplitude; rate not described	Incr. rate, force not described

(Continued)

Table 2. Continued

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
	Postpause 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 mmol/L	nd	1 mmol/L	nd	0.6 mmol/L	nd

3D, 3-dimensional; AP, action potential; APD, action potential duration; CASQ2, calsequestrin 2; CKMT, creatine kinase S-type, mitochondrial; CM, cardiomyocyte; COX6, cytochrome c oxidase subunit 6, mitochondrial; Cx43, connexin 43; Dex, dexamethasone; ECAR, extracellular acidification rate; expr., expression; HCN4, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; hiPSC-CM, human induced PSC-derived cardiomyocyte; ICD, intercalated disc; IGF1, insulin-like growth factor 1; incr., increase; MLC, myosin light chain; mtDNA, mitochondrial DNA; Myh, myosin heavy chain; NCad, N-cadherin; NCX, Na⁺-Ca²⁺ exchanger; nd, not described; OCR, oxygen consumption rate; PGC1, peroxisome proliferator-activated receptor gamma coactivator 1; PPAR, peroxisome proliferator-activated receptor α ; RYR2, ryanodine receptor 2; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase; SR, sarcoplasmic reticulum; stim, stimulation; T3, triiodothyronine; TEM, transmission electron microscopy; TFAM, transcription factor A, mitochondrial; TNN, troponin; TTN, titin; T-tubules, transverse tubules; vel, velocity; and Vm, membrane voltage.

Immature cardiomyocytes differ in important ways from mature cardiomyocytes in electrophysiology. First, the resting membrane potential of immature cardiomyocytes 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 cardiomyocytes (≈ 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 cardiomyocytes, 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 Ca²⁺-induced Ca²⁺ release. In systole, Cav1.2 activation allows a small amount of extracellular Ca²⁺ to enter cells, where it activates the RYR2 (ryanodine receptor 2) to release Ca²⁺ from the sarcoplasmic reticulum (SR, specialized endoplasmic reticulum in cardiomyocytes). In diastole, Ca²⁺ is cleared from the cytosol to the SR via the SERCA2 (sarco/endoplasmic reticulum Ca²⁺-ATPase) and to the extracellular space via the NCX (Na⁺-Ca²⁺ exchanger).

Ca²⁺-induced Ca²⁺ release occurs in proximity to plasma membrane. In small, immature cardiomyocytes where sarcomeres are relatively proximal to the cell surface, Ca²⁺ that is released at the cell periphery is sufficient to trigger sarcomere contraction. However, as cardiomyocytes enlarge and sarcomeres expand toward the cell interior, Ca²⁺ that is released at the cell periphery cannot rapidly activate interior sarcomeres. To solve this problem, cardiomyocytes evolved transverse-tubules (T-tubules; Figure 2), which are invaginations of plasma membrane that penetrate transversely into the center of mature cardiomyocytes. This structural specialization juxtaposes the plasma membrane with subdomains of SR to form dyads, where Cav1.2 and RYR2 cluster in proximity to form Ca²⁺ 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 Ca²⁺ in close proximity to sarcomeres.

The structural basis of T-tubule maturation is poorly understood. CAV3 (caveolin-3) is thought to regulate plasma membrane invagination,⁵² but T-tubules still form in *Cav3* knockout mice.⁵³ BIN1 (bridging integrator 1) 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 cardiomyocytes in mice.⁵⁴ JPH2 (Junctophilin 2) 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 cardiomyocytes.⁵⁷ Although ACTN2 (α -actinin-2) 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 NEXN (nexilin) as a new regulator of T-tubules.⁵⁸ Whether NEXN mediates Z-line-T-tubule association remains to be determined.

Whereas mature ventricular cardiomyocytes exhibit low automaticity, immature cardiomyocytes, 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 HCN4 (hyperpolarization-activated cyclic nucleotide-gated potassium channel 4), the resting membrane potential that is closer to the action potential activation threshold, and spontaneous Ca²⁺ release, which drives membrane depolarization through the Ca²⁺-Na⁺ exchanger.⁵⁹

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

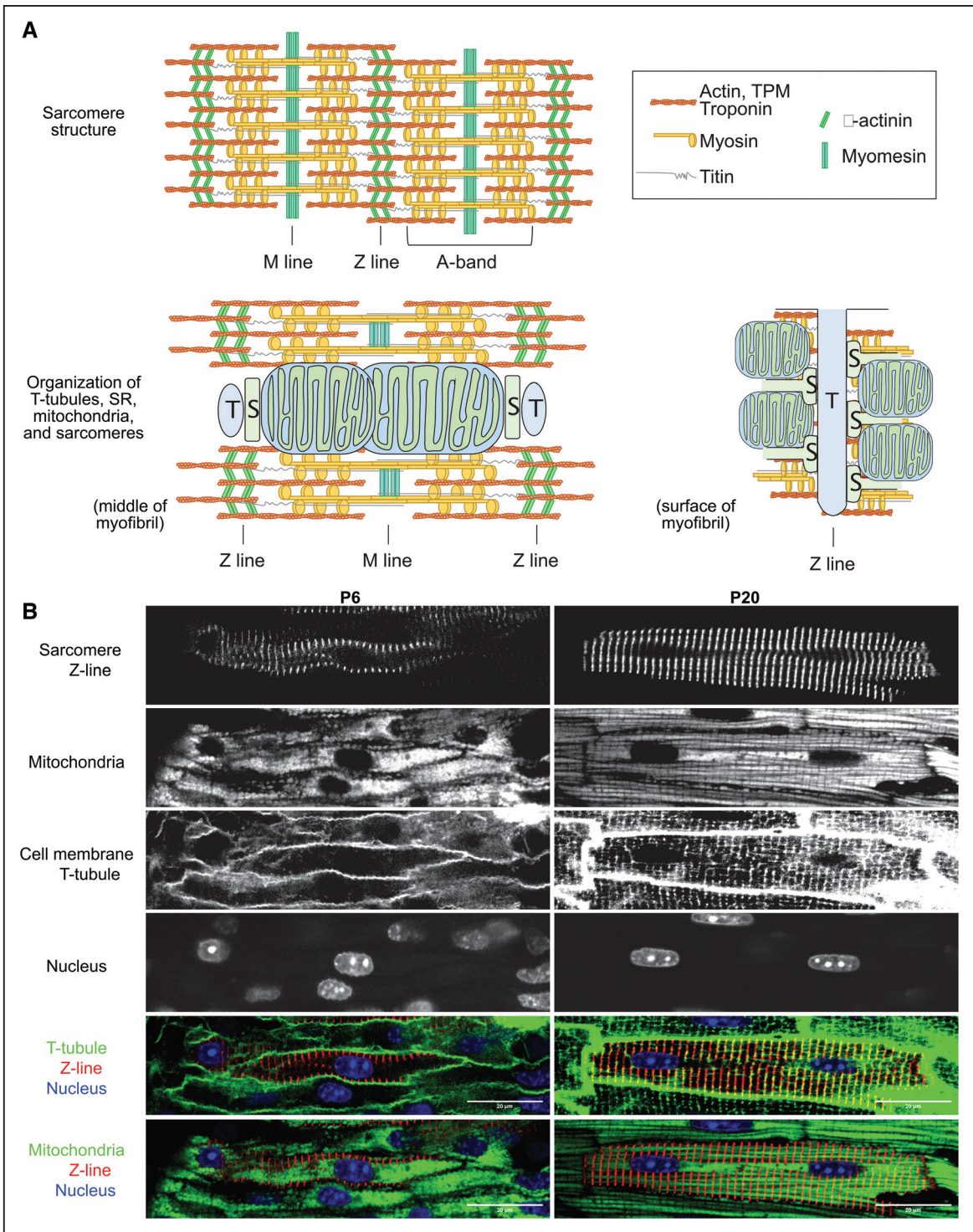


Figure 2. Structural maturation of cardiomyocytes (CMs).

A, A schematic view of sarcomere components in mature CMs (**top**) and spatial relationship between sarcomeres and transverse-tubule (T-tubule; T), sarcoplasmic reticulum (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 adeno-associated virus-Actn2-GFP (green fluorescent protein) infection. Mitochondria, T-tubules, and nuclei were stained by TMRM (polarized mitochondria), FM 4-64 (plasma membrane), and Hoechst (DNA), respectively, through Langendorff perfusion. Merged images highlight T-tubule-sarcomere and mitochondria-sarcomere associations that are established during postnatal maturation. Actn2 indicates α -actinin-2; FM 4-64, N-(3-(triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide; TMRM, tetramethylrhodamine, methyl ester; and TPM, tropomyosin.

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, cardiomyocytes 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 are controlled by their fusion and fission. Perturbation of profusion proteins such as MFN1/2 (mitofusin 1/2^{63,64}) or overexpression of profission proteins, such as DRP1 (dynamin-related protein 1),⁶⁵ resulted in decreased mitochondrial size in maturing cardiomyocytes. Mitochondria also become associated with sarcomeres during maturation (Figure 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 cardiomyocytes, which primarily produce ATP through glycolysis, mitochondria exhibit few and poorly aligned cristae.⁶⁷ Cristae maturation requires an array of molecules such as OPA1 (optic atrophy 1),^{68,69} the MICOS (mitochondrial contact site and cristae organizing system) complex,⁷⁰ and cardiolipin-based lipid-protein microdomains.⁷¹ ATP synthase⁷² may also drive cristae curvature formation.

The metabolic transition from immature cardiomyocytes to mature cardiomyocytes 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 HK1 (hexokinase 1) in fetal and neonatal cardiomyocytes.⁷⁶ In adult cardiomyocytes, the predominant isoform is HK2 (hexokinase 2),⁷⁷ which exhibits less glycolytic activity. COX (cytochrome c oxidase) subunit 8, a component of complex IV of the electron transport chain, also switches between COX8A and COX8B isoforms in cardiomyocyte maturation,⁷⁸ although the contribution of this switch to cardiomyocyte maturation remains to be determined.

Less is known about anabolic metabolism changes in cardiomyocyte maturation. Immature, proliferative cardiomyocytes create a high demand for nucleotide biosynthesis, which is suppressed after cardiomyocytes mature. Conversely, high-glucose promotes nucleotide

biosynthesis through the pentose phosphate pathway and inhibits cardiomyocyte maturation.⁷⁹ Because cardiomyocyte 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 cardiomyocyte maturation.

Proliferation-to-Hypertrophy Transition

In mice, cardiomyocyte cell cycle exit occurs within the first postnatal week.¹¹ In humans, cardiomyocyte 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 CDK (cyclin-dependent kinase) complexes, are tightly repressed during cardiomyocyte maturation.⁸² Recently, it was reported that cooverexpression of CDK1:CCNB1 (cyclin B1) and CDK4:CCND complexes, which activate M phase and G1 to S phase respectively, was sufficient to reactivate cardiomyocyte proliferation.⁸² This exciting finding awaits confirmation by independent groups. The mechanisms that enforce cardiomyocyte cell cycle exit include the downregulation of mitogenic signals, such as the neuregulin-ErbB (Erb-B2 receptor tyrosine kinase) axis,⁸³ and the inhibition of YAP, a potent activator of cardiomyocyte proliferation.^{84,85} During postnatal cardiomyocyte 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 cardiomyocyte volume, a process called maturational hypertrophy. The liquid-phase cytoplasm is unlikely the major contributor to increased cell volume, as mature cardiomyocytes 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 cardiomyocyte size during murine cardiomyocyte maturation.^{21,22} However, whether mitochondria biogenesis and enlargement cell-autonomously contributes to maturational hypertrophy is unclear.^{21,89}

Another hallmark of cardiomyocyte maturation during the proliferation-to-hypertrophy transition is polyploidization. In murine cardiomyocytes, the final round of the cell cycle involves karyokinesis without cytokinesis, leading most mature cardiomyocytes ($\approx 90\%$) to contain 2 diploid nuclei (binucleation^{90,91}; Figure 2). By contrast, in adult humans, $\approx 75\%$ of cardiomyocytes 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

Cardiomyocyte polyploidization negatively correlates with cell cycle withdrawal.⁹⁴ Residual cardiomyocyte cell cycle activity in adult hearts resides in the mononuclear diploid subset of cardiomyocytes.^{80,81,94} The introduction of a genetic modifier associated with higher mononuclear diploid fraction increased cardiomyocyte cell cycle activity after adult heart injury.⁹⁴ Forced cardiomyocyte polyploidization by ECT2 (epithelial cell transforming 2) inhibition, which blocks cytokinesis, is sufficient to suppress the proliferative capacity of cardiomyocytes in regeneration.^{95,96} For many cell types, the ploidy of a cell is positively correlated with cell size⁹⁷; thus, cardiomyocyte polyploidization likely promotes maturational hypertrophy. Consistent with this hypothesis, the induction of cardiomyocyte polyploidization was sufficient to increase cardiomyocyte size.^{95,96} Together, cardiomyocyte polyploidization is partially causative for both cardiomyocyte cell cycle withdrawal and maturational hypertrophy in cardiomyocyte maturation.

Cardiomyocyte Integration into a Mature Tissue

Maturational integration of cardiomyocytes into cardiac tissues require the formation of specialized cardiomyocyte-cardiomyocyte junctions called intercalated discs (ICDs), which occurs 2 to 3 weeks after birth in mice. ICDs are hybrid junctions comprising 3 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 ancillary proteins, such as plakoglobin, plakophilin-2, and desmoplakin. Gap junctions are composed of connexin 43. Although fascia adherens and desmosomes mechanically couple the actin cytoskeleton and intermediate filaments of neighboring cardiomyocytes, gap junctions mediate propagation of electrical and small molecule signals between cardiomyocytes.

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

Cardiomyocyte integration into tissues also requires attachment to the ECM (extracellular matrix) 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 nonsarcomere cytoskeleton at the lateral cardiomyocyte membrane.

Beyond tissue integration, ICDs and costameres are likely to play additional roles in cardiomyocyte 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 cardiomyocyte maturation (see next section).

REGULATION OF CARDIOMYOCYTE MATURATION

Cardiomyocyte 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 cardiomyocyte maturation research.

Microenvironmental Instruction

The microenvironment of the maturing myocardium provides necessary and sufficient information to instruct cardiomyocyte maturation. This notion is supported by 2 lines of evidence. First, in vitro culture of primary mature cardiomyocytes leads to loss of hallmarks of maturity.¹⁰² Second, immature cardiomyocytes developed toward an adult-like state after being transplanted into maturing myocardium.¹⁰³ These studies provide the logical basis to search for cardiomyocyte maturation cues by dissecting the physicochemical properties of maturing myocardium.

Biophysical Cues

Adult cardiomyocytes 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 cardiomyocytes 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 cardiomyocytes 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 cardiomyocyte elongation (Figure 3A).

Patterning cardiomyocytes to adopt a rod-shaped morphology promotes cardiomyocyte maturation. For example, PSC-CM growth on rectangular micropatterns¹⁰⁵ or uniaxially aligned ridges and grooves^{106,107} were sufficient to improve sarcomere organization and contractile and electrophysiological function of cardiomyocytes in a 2-dimensional system. Cardiomyocyte maturation was further improved by assembling cardiomyocytes

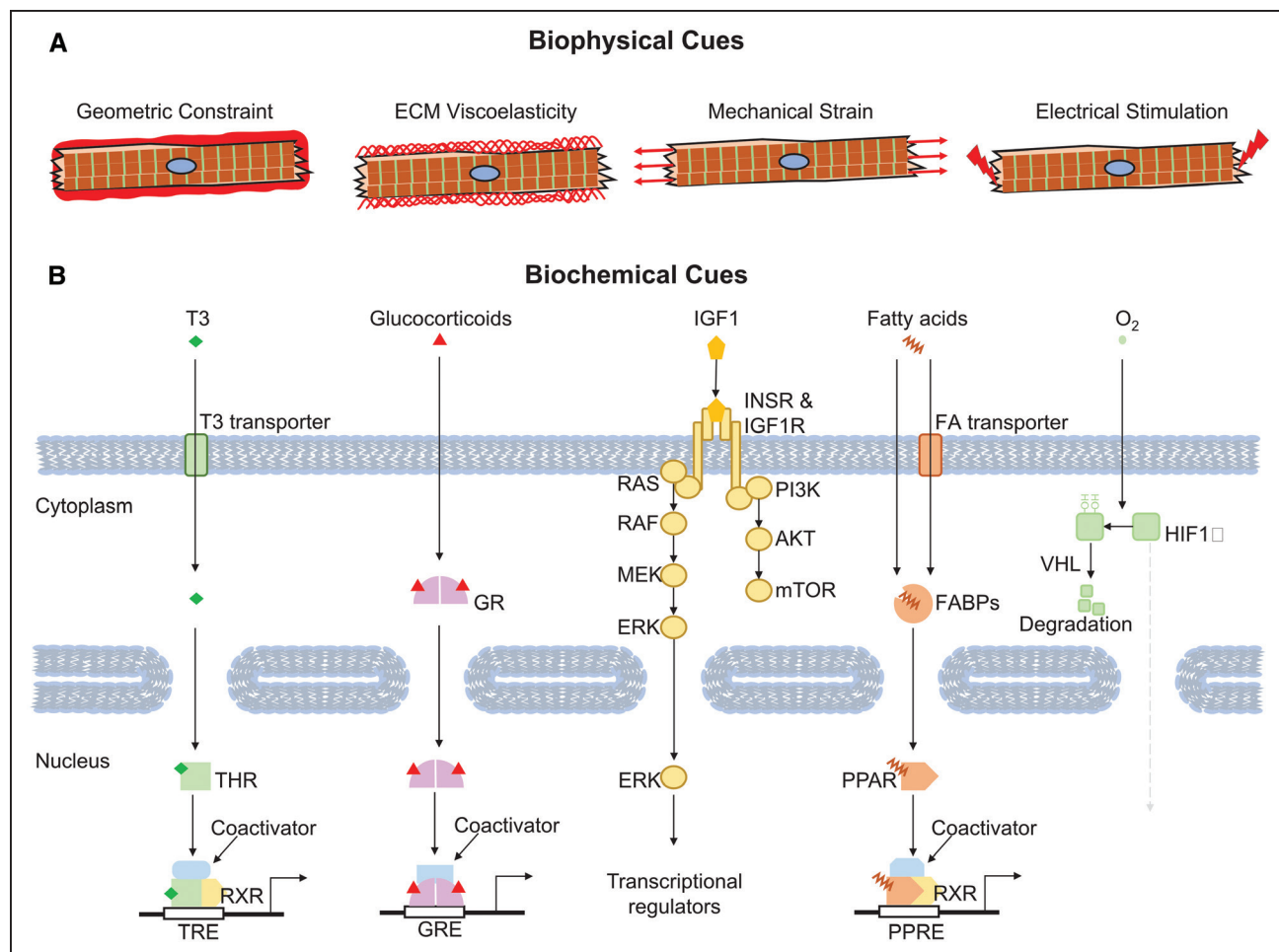


Figure 3. Representative environmental cues that regulate cardiomyocyte (CM) maturation.

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. AKT indicates AKT serine/threonine kinase; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FA, fatty acid; FABP, fatty acid-binding protein; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HIF, hypoxia-inducible factor; IGF, insulin-like growth factor; IGF1R, insulin-like growth factor 1 receptor; INSR, insulin receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mechanistic target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; PPPE, peroxisome proliferator response element; RAF, Raf proto-oncogene serine/threonine protein kinase; RAS, Ras proto-oncogene, GTPase; RXR, retinoid X receptor; THR, thyroid hormone receptor; TRE, thyroid hormone response element; and VHL, Von Hippel-Lindau tumor suppressor.

into 3-dimensional tissue with anisotropically directed strain, such as engineered heart tissue^{108,109} or cardiac microtissue.^{108,109}

The viscoelastic properties of ECM also modulate cardiomyocyte maturation (Figure 3A). The elastic modulus of ECM progressively increases from neonatal (<10 kPa) to adult (≈25 kPa) heart.¹¹⁰ Culturing cardiomyocytes on matrix with tunable elastic moduli showed that physiological matrix stiffness is optimal for cardiomyocyte maturation parameters such as sarcomere organization, Ca²⁺ handling, and contractility.^{111–113}

Maturing cardiomyocytes experience escalating mechanical force during development.¹¹⁴ Cyclic mechanical stress during systole and passive stretch during diastole both induced cardiomyocyte maturation in cell culture^{115–117} (Figure 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 cardiomyocytes (Figure 3A), as well as their contractile, electrophysiological, and metabolic activity.^{119–121} A recent study reported the production of adult-like cardiomyocytes after 3-dimensional 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 cardiomyocyte maturation remain poorly explored. A key unanswered question is whether electrical pacing directly impacts cardiomyocyte maturation or acts indirectly through induction of mechanical stress.

Biochemical Cues

Among the best characterized biochemical cues that promote cardiomyocyte maturation is the thyroid hormone T3 (triiodothyronine). The serum level of T3 rises dramatically in the perinatal period. T3 exerted a broad impact on cardiomyocyte maturation, including isoform switching of myosin heavy chain and TTN,^{122,123} induction of SERCA expression, hypertrophy, and cell polyploidization.^{12,13} T3 treatment was sufficient to enhance cardiomyocyte contractility, Ca²⁺ 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 hormone¹²⁶; however, other groups have not replicated the proposed surge of proliferating cardiomyocytes.^{127,128} The major thyroid hormone receptors in the heart are NRs (nuclear receptors) that are encoded by *Thra* and *Thrb* (Figure 3B). Inactivation of *Thra* cell-autonomously suppressed cardiomyocyte maturation.²⁵

Similar to T3, glucocorticoids also modulate cardiomyocyte 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 Ca²⁺ handling.¹³⁰

IGFs (insulin-like growth factors) regulate cardiomyocyte maturation through the IGF1R (insulin-like growth factor 1 receptor) and the INSR (insulin receptor), 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 cardiomyocytes caused physiological hypertrophy.¹³⁴ Double knockout of INSR and IGF1R in murine cardiomyocytes 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 cardiomyocyte 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, Ca²⁺ transient, and oxidative respiration.²⁹ In another study, treatment of PSC-CMs with palmitate-albumin complexes along with carnitine, which facilitates mitochondrial fatty acid transport, promoted structural and functional maturation, suggesting that in vitro promotion of oxidative phosphorylation stimulates overall cardiomyocyte 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 cardiomyocytes.^{21,89}

Oxygen tension is another environmental cue that modulates CM maturation. Increased oxygen tension inhibits HIF1 α (hypoxia-inducible factor 1 α) 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 HIF1 α and its downstream target lactate dehydrogenase A promoted human-induced PSC-cardiomyocyte maturation, enhancing not only metabolism but also gene expression, sarcomere organization, and contractility.¹⁴⁰

Biochemical signals function synergistically to promote cardiomyocyte maturation. For example, T3 and dexamethasone, a synthetic glucocorticoid, in combination with culture on matrigel mattresses cooperatively triggered cardiomyocyte maturation by inducing T-tubule formation.¹⁴¹ A cocktail of T3, dexamethasone, and IGF1 induced several adult features in induced PSC cardiomyocyte cultured in 3-dimensional 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 cardiomyocytes.¹²² Thus, a sophisticated signaling network is present that integrates diverse extracellular signals into a robust and coordinated program of cardiomyocyte maturation.

Noncardiomyocytes

Although cardiomyocytes occupy \approx 70% to 85% of myocardial volume, they constitute only \approx 20-30% of the total cell number.^{80,142,143} Numerically, noncardiomyocytes, including endothelial cells (64%), cardiac fibroblasts (27%), and leukocytes (9%), are the major cell types in the heart.¹⁴³ In the fetal heart, cardiomyocytes constitute a higher fraction of cells, with the proportion declining during maturation due to the greater proliferation of noncardiomyocytes.

Noncardiomyocytes regulate cardiomyocyte maturation, as coculture of cardiomyocytes with noncardiomyocytes promotes cardiomyocyte maturation in vitro.¹⁴⁴⁻¹⁴⁶ The impact of noncardiomyocytes on cardiomyocyte maturation could occur through direct physical adhesion and through paracrine molecules that are secreted from noncardiomyocytes and act on cardiomyocytes.¹⁴⁶ In addition, noncardiomyocytes build the

microenvironment that delivers biophysical and biochemical cues to cardiomyocytes. For example, cardiac fibroblasts create the appropriate ECM to support cardiomyocyte maturation, and endothelial cells construct coronary vasculature that transport circulating signals to instruct cardiomyocyte maturation.

Intracellular Regulation

Transcriptional Regulation of Gene Expression

The coordination of diverse phenotypic changes during cardiomyocyte maturation and the association of those changes with altered gene expression suggest an overarching transcriptional program that orchestrates cardiomyocyte maturation.

Several transcriptional regulators of cardiomyocyte maturation have been identified. One of these is SRF (serum response factor).²¹ In murine cardiomyocytes 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 Ca²⁺ 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 cardiomyocyte 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 cardiomyocyte 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 cardiomyocyte maturation: sarcomere maturation is upstream of most other aspects of cardiomyocyte maturation,²¹ whereas metabolic maturation was dispensable for structural maturation *in vivo*.^{21,89}

Three myocardin-family transcriptional regulators, MYOCD (myocardin), MRTFA (myocardin-related transcription factor A), and MRTFB, are major coactivators of SRF in cardiomyocytes.¹⁴⁷ MRTFA and MRTFB are functionally redundant. *Mrtf^{f/f}; 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 3 factors in SRF activation

and cardiomyocyte maturation. The MRTF (myocardin-related transcription factor)-SRF axis could convert mechanical stress into transcriptional changes¹⁵⁰; thus, MRTF-SRF signaling potentially mediates regulation of cardiomyocyte maturation in response to biomechanical cues, including mechanical stretch and ECM matrix stiffness.

A recent transcriptomic analysis revealed another SRF-binding transcription cofactor, HOPX (homeodomain-only protein), as a novel activator of cardiomyocyte maturation, especially in the process of myofibrillar isoform switching and cardiomyocyte hypertrophy.¹⁵¹ *In vivo*, overexpression of HOPX in cardiomyocytes 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 cardiomyocyte maturation.

SRF functions in synergy with other transcription factors. For instance, SRF ChIP-Seq (chromatin immunoprecipitation followed by sequencing) in maturing hearts revealed coenrichment of GATA and MEF2 motifs.²¹ GATA4 (GATA-binding protein 4) and GATA6 are the major GATA family transcription factors expressed in cardiomyocytes, and these factors are redundantly essential for neonatal cardiomyocyte maturation.^{25,155} Four MEF2 (myocyte enhancer factor 2) 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 cardiomyocyte maturation.

In addition to SRF-related factors, NRs are another major group of transcription regulators that control cardiomyocyte maturation. Among these factors, thyroid hormone receptors and glucocorticoid receptors mediate the role of T3 and glucocorticoids in cardiomyocyte maturation, as described in the previous section. Additional NRs play key roles in metabolic maturation. One family of such factors is PPARs (peroxisome proliferator-activated receptors), 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 metabolites¹⁶¹; thus, PPARs probably mediate the impact of circulating fatty acids on cardiomyocyte maturation. The ERRs (estrogen-related receptors α , 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 Ca²⁺ handling genes are also direct downstream targets of ERRs.^{162,163} Both PPARs and ERRs directly interact with PGC1 α/β (PPAR γ coactivator α/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/PPAR α (peroxisome proliferator-activated receptor α) 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 cardiomyocyte maturation, whereas 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} whereas 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 (ring finger protein 20/40) as a novel epigenetic regulator of cardiomyocyte maturation. This enzyme deposits histone H2B lysine 120 monoubiquitination marks at genes that are active in cardiomyocyte 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 cardiomyocyte maturation and late cardiac outcomes.

Chromatin organization changes are also correlated with transcriptional changes in cardiomyocyte maturation. ATAC-Seq (assay for transposase-accessible chromatin using sequencing) revealed decreased chromatin accessibility of silenced genes such as cell cycle genes between neonatal and adult hearts, whereas metabolic and muscle contraction genes acquired a more open chromatin state in mature hearts.¹⁷² Histone remodeling factor BRG1 (BRM/SWI2-related gene 1) modulates myosin heavy chain isoform switching.¹⁷³ Mutation of CTCF (CCCTC-binding factor), a crucial regulator of chromatin-architecture, was recently reported to cause premature activation of the cardiomyocyte maturation program in embryonic cardiomyocytes.¹⁷⁴

Posttranscriptional Regulation of Gene Expression

RNA splicing is a critical regulatory component of cardiomyocyte maturation, as isoform switching often occurs through alternative splicing. One representative splicing regulator is RBM20 (RNA-binding motif protein 20), mutation of which causes dilated cardiomyopathy.^{175–177} RBM20 is essential for proper splicing of *Ttn* transcripts and other maturationally regulated genes.^{175,178}

Additional splicing regulators could potentially impact cardiomyocyte maturation: CELF (CUGBP Elav-like family member) proteins are downregulated in heart development, whereas MBNL (muscleblind-like splicing regulator) proteins are upregulated. The antagonistic regulation of these 2 splicing regulators¹⁷⁹ has been proposed to trigger a large fraction of developmental splicing changes and to be essential for T-tubule organization and Ca²⁺ 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 Ca²⁺ handling genes. Cardiomyocyte-specific *Hnrnpu* knockout resulted in splicing defects in *Ttn* and Ca²⁺ handling genes and triggered perinatal dilated cardiomyopathy.¹⁸⁵ The RNA splicing regulator RBFOX1 (RNA-binding Fox-1 homolog 1) markedly increases in expression during cardiomyocyte maturation^{180,186} and is another potential activator of cardiomyocyte maturation.¹⁸⁷

MicroRNA (miRNA)-based mRNA silencing is another mechanism that modulates gene expression in cardiomyocyte maturation. For example, miR-1, a miRNA enriched in mature cardiomyocytes, facilitated electrophysiological maturation in stem cell-derived cardiomyocytes in vitro.¹⁸⁸ Let-7 (Lethal-7) family miRNAs were highly enriched in cardiomyocytes 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 Coculture of cardiomyocytes with endothelial cells promoted cardiomyocyte maturation in association with upregulation of multiple miRNAs.¹⁴⁵ Overexpression of 4 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 Ca²⁺ 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 cardiomyocyte maturation have been poorly studied. Recent advances in proteomics have started to characterize protein changes

in cardiomyocyte maturation.^{191–193} Integration of these data with RNA-Seq (RNA sequencing) and Ribo-Seq (ribosome profiling) analyses will provide an improved understanding of regulation at the protein level.

Ultrastructural Regulation

Major ultrastructural maturation hallmarks—myofibrils, mitochondria, and T-tubules—are not independent of each other. As the major cytoskeletal structures of cardiomyocytes, 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*¹ 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.

MODEL SYSTEMS TO STUDY CARDIOMYOCYTE MATURATION

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

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 cardiomyocyte maturation research because, to date, no *in vitro* system can induce, or even maintain, full maturity of cardiomyocytes. 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 cardiomyocytes requires further complexity. Second, organ-wide mutagenesis of a gene essential for cardiomyocyte maturation often triggers lethality or secondary effects that can confound identification of the direct functions of the gene. This is particularly problematic in cardiomyocyte maturation research as the secondary effects of heart dysfunction, such as fetal gene reactivation and mitochondria/T-tubule remodeling, are similar to cardiomyocyte maturation defects.^{57,194}

These problems can be circumvented using adeno-associated virus (AAV), which efficiently and stably manipulates genes in cardiomyocytes 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 deliver CRISPR/Cas9 components (CRISPR/Cas9 and AAV-mediated somatic mutagenesis, CASA, Figure 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 (eg, <15%) of cardiomyocytes are transduced, leaving most cardiomyocytes, 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 cardiomyocytes 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 be difficult to apply to genes that produce secreted products.

Engineered Tissue Model

Cardiomyocyte maturation demonstrates substantial interspecies differences. For instance, adult zebrafish cardiomyocytes lack T-tubules¹⁹⁶ and exhibit much lower mitochondrial content¹⁴ than mammalian cardiomyocytes. Mouse and human cardiomyocytes 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 learned in other model organisms.

In addition, a major practical goal of studying cardiomyocyte maturation is to improve the maturation of hPSC-CMs *in vitro* for translational medicine. The current consensus is that 3-dimensional engineered cardiac tissues that are assembled by hPSC-CMs, nonmyocytes, and ECMs provide the necessary platforms to best mature cardiomyocytes *in vitro*. Additional biochemical (T3, dexamethasone, IGF1, palmitate) and biophysical treatments (electrical pacing; mechanical stress) on these engineered tissues are essential to produce adult-like cardiomyocytes (Figure 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 cardiomyocyte maturation regulators. *In vivo* validation is still necessary to determine the physiological relevance of novel cardiomyocyte maturation factors that are identified in these tissue models. Importantly, factors that drive cardiomyocyte maturation *in vitro* may

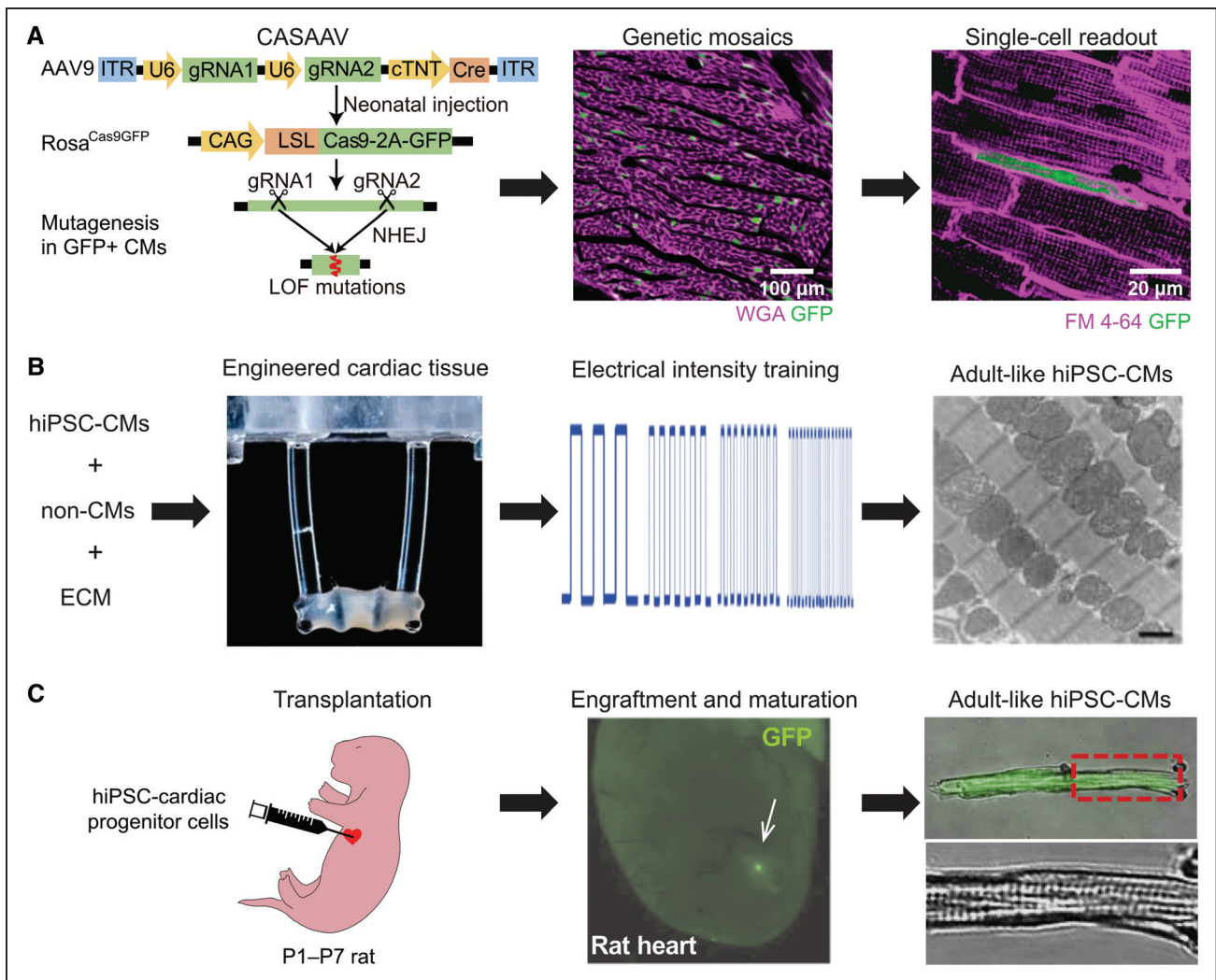


Figure 4. Model systems to study cardiomyocyte (CM) maturation.

A, CRISPR/Cas9 and adeno-associated virus (AAV)-mediated somatic mutagenesis (CASAAB)-based genetic mosaic analysis of murine CM maturation in vivo.⁵⁷ Expression of genome-encoded Cas9-P2A-GFP (green fluorescent protein) 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 transverse-tubule and maturational growth defects caused by *Srf* depletion²¹). FM 4-64, membrane dye. Left, Reprinted from Guo et al.²¹ Copyright ©2018 (see: <http://creativecommons.org/licenses/by/4.0/>). **B**, In vitro maturation of pluripotent stem cell-derived CMs (PSC-CMs) by tissue engineering and electrical pacing. Three-dimensional 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 Ronaldson-Bouchard et al⁴ with permission, Copyright ©2018, Springer Nature. **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 Cho et al.¹⁰³ Copyright ©2017 (see: <http://creativecommons.org/licenses/by-nc-nd/4.0/>). ECM indicates extracellular matrix; hiPSC, human induced PSC-derived cardiomyocyte; P, postnatal day; and WGA, wheat germ agglutinin.

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.

Neonatal Xenotransplantation Model

Human PSC-CMs could be matured toward a near-adult state by transplantation into rat myocardium (Figure 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 cardiomyocytes,¹⁰³ raising the question of whether the transplanted human PSC-CMs become rat-like cardiomyocytes or remain human-like. Although some comparisons between donor and host cardiomyocytes were documented,¹⁹⁸ a more comprehensive analysis is necessary to determine if xenotransplants are viable models to study human-specific features of cardiomyocyte maturation.

CONCLUDING REMARKS

Here, we reviewed major hallmarks of cardiomyocyte maturation and known regulators of this process. Although differences between immature and mature cardiomyocytes 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 cardiomyocyte maturation is warranted in the near future. This effort is critical to design better strategies to mature PSC-CM, stimulate cardiomyocyte regeneration, and treat diseases that involve cardiomyocyte maturation defects.

ARTICLE INFORMATION

Affiliations

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Disclosures

None.

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