Cardiac Regeneration
Lessons From Development

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Abstract: **Palliative surgery for congenital heart disease has allowed patients with previously lethal heart malformations to survive and, in most cases, to thrive. However, these procedures often place pressure and volume loads on the heart, and over time, these chronic loads can cause heart failure. Current therapeutic options for initial surgery and chronic heart failure that results from failed palliation are limited, in part, by the mammalian heart's low inherent capacity to form new cardiomyocytes. Surmounting the heart regeneration barrier would transform the treatment of congenital, as well as acquired, heart disease and likewise would enable development of personalized, in vitro cardiac disease models. Although these remain distant goals, studies of heart development are illuminating the path forward and suggest unique opportunities for heart regeneration, particularly in fetal and neonatal periods. Here, we review major lessons from heart development that inform current and future studies directed at enhancing cardiac regeneration. (*Circ Res*. 2017;120:941-959. DOI: 10.1161/CIRCRESAHA.116.309040.)**

Key Words: cardiac development ■ cardiac regeneration ■ cardiomyocyte maturation ■ signaling pathways ■ transcriptional regulation

Since Robert Gross inaugurated pediatric cardiac surgery in 1938 and since the advent of open-heart surgery on cardiopulmonary bypass in the 1950s, mortality from congenital heart disease has plummeted. However, many of the more complex forms of congenital heart disease still cannot be physiologically corrected; rather, patients are surgically palliated, permitting survival but leaving significant residual cardiac pressure and volume loads. Over time, these patients are at risk for heart failure, and some ultimately require heart transplantation.

A major biological factor that constrains treatment options for congenital heart disease patients, as well as adults with acquired heart disease, is the limited ability of mature mammalian cardiomyocytes to proliferate. The low innate regenerative

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Nonstandard Abbreviations and Acronyms	
BMP	bone morphogenic protein
E	embryonic day (days of gestation)
FGF	fibroblast growth factor
FHF	first heart field
iCM	induced cardiomyocyte
MESP1	mesoderm posterior-1
miRNA	microRNA
P	postnatal day (days after birth)
PS	primitive streak
PSC-CM	pluripotent stem cell-derived cardiomyocyte
SHF	second heart field
WNT	wingless-type MMTV integration site family member

capacity of the heart narrows initial treatment strategies and the therapeutic options for maximizing the longevity of pressure- or volume-loaded hearts. Overcoming this barrier by developing methods to engineer new myocardium or to regenerate injured myocardium would transform the management of congenital heart disease patients who are confronted primarily by declining pump function.

Not long ago, this vision of cardiac regeneration was confined to science fiction; however, expanding mechanistic understanding of heart formation and development achieved over the past decades has enabled cardiac biologists to make significant inroads into the challenge of therapeutic heart regeneration. In this Compendium article, we review the key signaling pathways and transcriptional regulators that govern cardiogenesis from gastrulation through the formation of cardiac mesoderm to the specification of cardiomyocytes. We go on to review mechanisms that control cardiomyocyte proliferation and maturation. We highlight recent studies that exemplify redeployment of developmental signaling pathways to further the goals of cardiac regeneration and discuss further challenges that must be overcome for achieving success in myocardial regeneration. This review is focused on myocardial regeneration. Because of space limitations, we do not address valve and pulmonary vascular disease, additional important problems faced by some palliated congenital heart disease patients, which will need to be corrected to make regenerative approaches viable for the full spectrum of congenital heart disease.

Development of the Cardiac Mesoderm

Mesodermal Commitment From Epiblast

During gastrulation, the developing embryo reorganizes itself into 3 primary germ layers: the ectoderm, mesoderm, and endoderm. Cardiomyocytes are derived from the mesoderm that forms during early development. Besides the heart, the mesoderm also gives rise to skeletal muscle, bones, blood, and other tissues.[1] It has been recognized that molecular signaling and cell-to-cell interactions between germ layers play significant roles in mesoderm induction and cardiac differentiation.

At embryonic day 5 (E5) in the mouse, the embryo exhibits an elongated cylinder shape that is divided into 2 portions—the proximal and distal regions. A combination of signaling events leads to initial patterning and separation of the embryo into embryonic and extraembryonic layers. Along the distal region of the extraembryonic visceral endoderm, NODAL (nodal growth differentiation factor) is expressed and forms a concentration gradient along the proximal–distal axis.[1,2] Through a positive feedback loop, NODAL activates its expression throughout the epiblast and induces an organizing center in the distal extraembryonic visceral endoderm that later becomes known as the anterior visceral endoderm.[3] As the embryo further develops, the anterior visceral endoderm migrates toward the anterior of the embryo and expresses NODAL antagonists such as CER1 (cerberus-like) and LEFTY1 proteins.[4] These antagonists prevent NODAL signaling from taking place in the anterior but not in the posterior of the epiblast (Figure 1). Along with NODAL,[5] fibroblast growth factor (FGF),[6] BMP (bone morphogenic protein),[7–9] and wingless-type MMTV integration site family member 3 (WNT3A)[10] are expressed in the posterior of the embryo.[11,12] Together, NODAL, FGF, BMP, and WNT signaling induce early mesoderm that is marked by the primitive streak (PS)/pan-mesoderm marker, Brachyury.

The PS marks the site where epiblast cells begin to ingress into the embryo and differentiate into the 3 embryonic germ layers. Several NODAL signaling gradients pattern epiblast cells along the streak. High concentrations of NODAL at the anterior PS specify definitive endoderm precursors, whereas lower levels of NODAL in concert with BMP and WNT signaling specify mesodermal precursors in the intermediate and posterior PS.[1,13] Cardiac mesoderm precursors form anterior and lateral to the PS as a result of this morphogen patterning.[2] The T-box transcription factor eomesodermin, expressed at the anterior PS, is important for both definitive endoderm and cardiac mesoderm development.[14] Eomesodermin-positive cells activate the basic-helix-loop-helix transcription factor, mesoderm posterior-1 (MESP1),[14] which subsequently regulates the activation of several cardiac and mesodermal genes and plays a vital role in allowing mesodermal precursor cells to migrate through the PS.[15] Although MESP1 is broadly expressed throughout mesoderm precursors,[16] a subpopulation of MESP1$^+$ cells anterior to the PS become specified to cardiac lineages as they ingress through the PS[17] (Figure 1).

Migration From Primitive Streak to Cardiogenic Mesoderm

With the patterning of the primitive streak and the activation of MESP1, mesoderm cells begin to migrate away from the PS. A subpopulation of these MESP1$^+$ cells marches anteriorly and laterally away from the mid and posterior aspect of the mesoderm to become the anterior lateral plate mesoderm.[17] The migration of these early cardiac precursors away from the WNT/β-catenin–rich PS, mediated by WNT chemorepulsion,[18] marks a significant step in the commitment of early mesoderm cells toward a cardiac fate. At E7.5 in the mouse/week 3 of human development, these migratory cells make 2 pools of cardiac progenitors known as the first and second heart fields (FHF and SHF).[19]

Canonical WNT signaling, mediated by β-catenin, plays a crucial role in the differentiation of these cardiac progenitors. First, as noted above, WNT3A from the posterior of the embryo induces early mesoderm. Subsequently, WNT signaling from

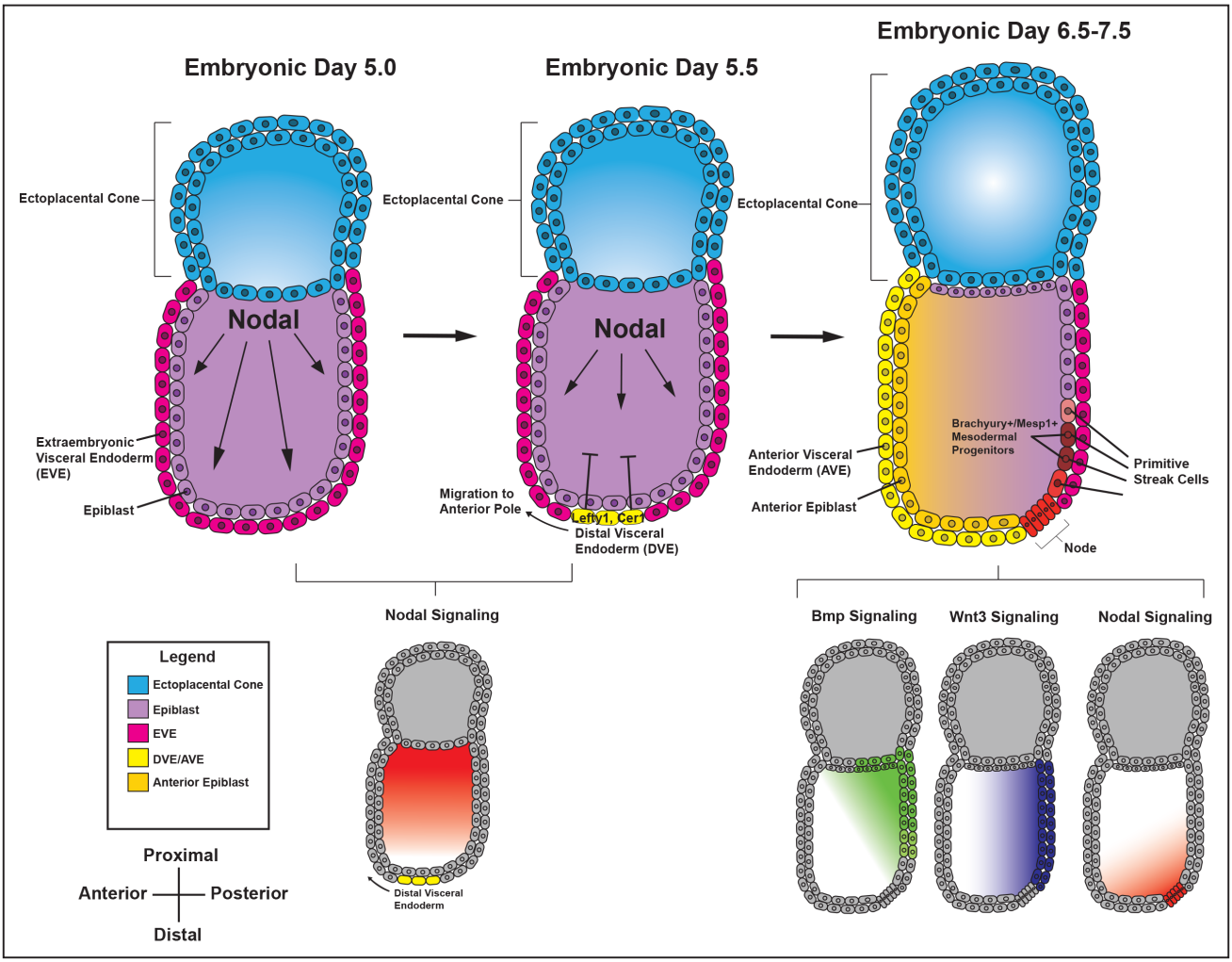

Figure 1. Specification of mesodermal precursors. Schematic representing the signaling events leading to mesodermal specification during early development. NODAL (nodal growth differentiation factor) is first expressed proximally at embryonic day (E) 5.0. Through an autoregulatory loop, NODAL activates its own expression throughout the epiblast (shown in light purple) and goes on to induce the expression of NODAL antagonists, LEFTY1 and CER1, in the distal visceral endoderm at E5.5 (DVE). The DVE migrates anteriorly where it specifies the anterior portion of the embryo as shown in the yellow hues at E6.5 to 7.5. The anterior visceral endoderm (AVE, yellow) limits NODAL signaling to the posterior of the embryo. Along with wingless-type MMTV integration site family member 3 (WNT3) and bone morphogenic protein (BMP) signaling, NODAL specifies early primitive streak progenitors to the mesoderm fate.

the overlying ectoderm inhibits cardiac induction in the region posterior to the cardiac crescent, limiting the cardiogenic region to the anterior lateral plate mesoderm.[20,21] Canonical WNT signaling, thus, has a biphasic effect on cardiac differentiation by enhancing mesoderm formation followed by repression of cardiac fates in mesoderm-specified cell populations.[21–24] This biphasic role of WNT is evolutionarily conserved. In chick embryos, canonical WNT inhibition by Dickkopf WNT signaling pathway inhibitor 1 and crescent secreted from the anterior endoderm are necessary for cardiac differentiation, whereas ectopic expression of canonical WNT ligands WNT3A and WNT8C induces erythroid cell formation in cardiac precursor regions.[21] Other chemotactic and inductive signals are likely involved in mesoderm migration and differentiation into the cardiac fate; however, their identities remain to be determined.

This understanding of the major signaling pathways that govern mesoderm formation has permitted the development of robust protocols to differentiate pluripotent stem cells into cardiomyocytes.[25,26] These protocols rely on activation of canonical WNT signaling to induce mesoderm formation, followed by WNT inhibition to stimulate cardiomyocyte differentiation. These protocols are remarkably efficient, often yielding 100 cardiomyocytes per input pluripotent stem cells and at purities of >90%.[25,26] Efficient pluripotent stem cell to cardiomyocyte differentiation protocols are fundamental to using pluripotent cell differentiation systems to study cardiac differentiation,[27] model human heart disease,[28] and produce cardiomyocytes as replacement therapy for heart failure.[29]

Cardiomyogenesis in the FHF and SHF

Signals Regulating Commitment to Cardiac Progenitor Cells From Mesodermal Precursors

Although the early signals regulating mesoderm induction have been well characterized, the molecular signaling and genetic regulation driving MESP1$^+$ mesoderm precursors to FHF and SHF cardiac progenitors have only been uncovered in recent years. The FHF gives rise to the left ventricular

free wall, part of the septum, and a portion of the atria, whereas the SHF gives rise to the right ventricle, a portion of the septum, the outflow tracts, and a portion of the atria.[30] Identifying the signals and molecular markers that regulate the segregation of early mesodermal progenitors to either of these fields is an important step toward understanding cardiac development in vivo and developing novel differentiation protocols that can give rise to specific subpopulations within the heart.

During mesodermal cell migration, the earliest cells to reach the anterolateral plate form the FHF, whereas later cells form the SHF.[17] Recent work from Birket et al[31] has shown that modulating FGF and BMP signaling in vitro can direct pluripotent stem cell–derived cardiac progenitors into either FHF- or SHF-like cells, suggesting that differential signaling underlies the differentiation of precursor cells into SHF or FHF lineages. At E7.5 in mouse development, FHF cells are spatially organized in a crescent-shaped structure, the cardiac crescent. SHF cells lie more medial and dorsal to these FHF cells (Figure 2A). These FHF progenitors receive BMP2,[32] FGF8,[33] and noncanonical WNT[34] signals from the underlying endoderm to promote their differentiation. Meanwhile, the SHF progenitors receive FGFs,[35] sonic hedgehog,[36] and canonical WNT/β-catenin[37] signals to promote their proliferation and multilineage differentiation (Figure 2B). Although the signaling pathways described above are important for the multipotency of their respective heart fields, the question remains as to how the MESP1$^+$ mesodermal progenitors are appropriately allocated into FHF or SHF populations and what factors regulate the size of each heart field.

Despite the incomplete understanding of FHF and SHF progenitor cell regulation from their mesodermal precursors,

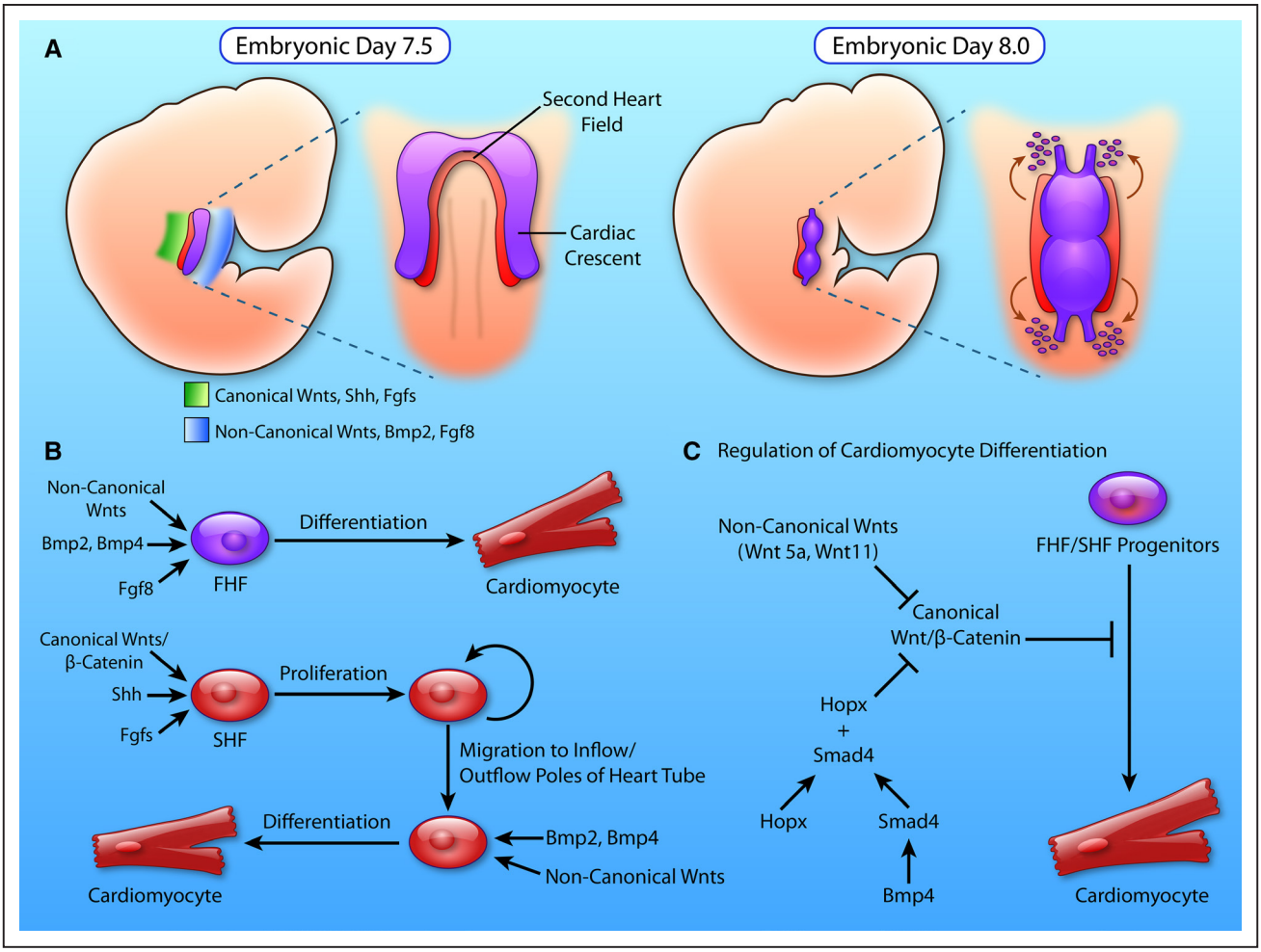

Figure 2. Regulation of cardiac progenitor proliferation and differentiation. **A**, Schematic showing the anterolateral position of first heart field (FHF) progenitors and dorsomedial position of second heart field (SHF) progenitors at embryonic day (E) 7.5. Canonical wingless-type MMTV integration site family member (WNTs), Sonic Hedgehog (SHH), and fibroblast growth factors (FGFs) are expressed dorsally in the region encompassed by the SHF, whereas noncanonical WNTs, BMP2, and FGF8 are expressed ventrally, where the FHF is present. FHF progenitors make up the cardiac crescent and differentiate before the SHF to form the developing heart tube at E8.0. SHF maintains their proliferative state and elongate the heart tube by migrating and differentiating at the inflow and outflow poles of the heart. **B**, Noncanonical WNTs, BMP2/4, and FGF8 signaling drives FHF progenitors to differentiates toward the myocyte lineage. Meanwhile, canonical WNT/β-catenin, SHH, and FGFs maintain SHF progenitor proliferation. SHF progenitor migration to the outflow and inflow poles of the heart tube exposes them to BMP2/4 and noncanonical WNTs, which drives SHF progenitors to exit their proliferative state and differentiate. **C**, Canonical WNT/ β-catenin signaling inhibits the differentiation of cardiac progenitors to the myocytes. BMP signaling activates SMAD4 that binds to the transcription factor HOPX to directly inhibit canonical WNT/β-catenin. Moreover, noncanonical WNTs such as WNT5a and WNT11 also inhibit canonical WNT/β-catenin to drive cardiac progenitor differentiation (illustration credit: Ben Smith).

there has been enthusiasm for the generation of renewable cardiac progenitor cells from the application of these signaling pathways for cardiac regeneration. Aside from deriving FHF/SHF cardiac progenitor cells from pluripotent stem cells,[31] investigators have recently generated cardiac progenitor cells by direct reprogramming of fibroblasts using cardiac transcription factors and small molecules that activate WNT3A, JAK/STAT (Janus kinsase/signal transducer and activator of transcription), BMP, and TGF-β (transforming growth factor-beta) signaling to regulate the formation and renewal of induced, expandable cardiac progenitor cells.[38,39] Remarkably, when transplanted in vivo into an infarcted heart, these cells differentiated into cardiomyocytes and improved cardiac function. Further studies will be necessary to reproduce and validate the findings from these studies. Nevertheless, these studies illustrate the potential importance of harnessing early developmental signaling to generate cardiac progenitor cells for therapeutic benefit.

Signaling Pathways Regulating Cardiomyogenesis From FHF and SHF Progenitors

The regulation of cardiomyogenesis from multipotent cardiovascular progenitors remains a major focus of ongoing research because of a combination of interest in understanding the mechanisms of cardiac disease and an ever-growing interest in generating cardiomyocyte subtypes (eg, atrial, ventricular, and nodal) for therapeutic purposes. BMP2 and BMP4, secreted by underlying endoderm, induce cardiomyogenesis of the overlying lateral plate mesoderm, and exogenous BMP2 and BMP4 induced ectopic cardiac differentiation in chick embryos.[32] SHF progenitors switch from a proliferative state toward cardiac differentiation in the setting of increased BMP expression as they migrate to the outflow tract.[40,41] Notably, inhibition of BMP signaling via Noggin resulted in complete lack of cardiac differentiation in chick embryos, highlighting the critical importance of this pathway in heart development.[32] The central role of BMP signaling in promoting cardiomyogenesis is in part mediated by the induction of *Gata4, Mef2c, Srf*, and *Nkx2-5* expression.[42,43] Within SHF, BMP is also required for upregulation of T-Box2 (*Tbx2)* and T-Box3 (*Tbx3)*, which are required for maintaining the slow conduction velocity and reduced proliferation of myocardium within the outflow tract, atrioventricular canal, and sinus horns.[44,45] Compared with FHF cells, SHF cells have delayed commitment to the cardiomyocyte lineage. Canonical WNT/β-catenin signaling is critical for maintaining SHF progenitors in a proliferative state while inhibiting differentiation toward terminal lineages.[46–48] In developing mouse hearts, as SHF progenitors migrate into the developing outflow tract, canonical Wnt signaling is significantly downregulated, coinciding with activation of cardiomyocyte-specific gene expression.[41,49]

Noncanonical WNT signaling, through calcium-dependent pathways involving protein kinase C and calmodulin-dependent kinase or through so-called planar cell polarity pathways involving Rho-associated kinase 2 and Jun amino-terminal kinase, is crucial for normal cardiomyocyte specification.[50] Two noncanonical WNT ligands, WNT5A and WNT11, are essential for heart development across multiple species. WNT11 was necessary for expression of cardiac genes and induced ectopic heart formation in both chick and frog embryos.[34,51] Developing mouse embryos lacking both *Wnt5a* and *Wnt11* showed a dramatic reduction in SHF progenitors. These roles of *Wnt5a* and *Wnt11* are thought to be due in part to the suppressive effect of noncanonical WNT signaling on the canonical WNT/β-catenin pathway.[52]

Recent work has identified HOPX, a homeodomain-containing transcriptional repressor, as a key link between canonical WNT/β-catenin and BMP signaling pathways during heart development.[41] HOPX expression initiates in FHF and SHF derivatives that are exclusively committed to the cardiomyocyte lineage. Notably, *Hopx* expression in FHF occurs earlier than its expression in the SHF, consistent FHF preceding SHF differentiation. Using an embryoid body–directed differentiation system, it was further shown that *Hopx* promotes cardiomyocyte differentiation via inhibition of WNT signaling (Figure 2C). This inhibition is mediated by direct interaction between HOPX and SMAD4, a transcription factor that is essential for transducing BMP signals. These data suggest that as SHF cells migrate into the outflow tract, they are exposed to increased BMP4 signaling, which cooperates with newly expressed HOPX to diminish canonical WNT/β-catenin signaling and thereby promote cardiomyocyte differentiation.[41]

Transcriptional Events During Cardiomyogenic Commitment

The signaling pathways described above act in a coordinated fashion with a complex network of cardiac transcription factors to regulate cardiomyogenesis. The cardiac genetic program is initiated during the MESP1$^+$ precardiac mesoderm stage.[15] As MESP1$^+$ precardiac progenitors march toward the anterolateral plate mesoderm, a subunit of the SWI/SNF (switch/sucrose non-fermentable) chromatin remodeling complex, SMARCD3, is expressed after MESP1 is downregulated and before upregulation of cardiac progenitor markers NKX2-5 and ISL homeobox 1 (ISL1).[53] SMARCD3 allows the zinc-finger transcription factor GATA4 to bind the enhancer regions of several transcription factors that initiate the cardiac gene program,[54] including GATA4, NKX2-5, ISL1, T-box 5 (TBX5), and myocyte enhancer factor 2c (MEF2C).[53,55] Along with GATA4, yin yang 1 (YY1) has been shown to bind directly to a cardiac enhancer region of NKX2-5 and plays a vital role in its early cardiac transcriptional activation.[56] The central roles of GATA4 and NKX2-5 in early cardiac development are highlighted by the observations that GATA4/6 double mutants lack hearts entirely, and NKX2-5 is the most frequently mutated gene in congenital heart disease patients.[57,58]

FHF progenitors are the earliest cells to express NKX2-5 and constitute the earliest wave of developing cardiac progenitors. Early FHF progenitors quickly activate TBX5, which interacts with GATA4 and NKX2-5 to drive cardiac muscle development and specification of the left ventricle through induction of many cardiac genes, including *Nppa* (natriuretic peptide A) and the *Gja5* (gap junction protein connexin 40).[59,60] TBX5 misexpression can lead to the improper positioning of the interventricular septum or the complete absence of the septum.[60] GATA4 and NKX2-5 repress the hemangiogenic gene program, through suppression of the hematopoietic transcription factor *GATA1*, and upregulate cardiac-specific

genes including *Hand1*, *Mef2c*, myosin light chain-2v (*Myl2*, also known as *Mlc2v*), and other genes necessary for cardiomyocyte structure and function.[61–63] Study of FHF progenitors has been complicated by their transience and by a paucity of well-established markers. One of the few markers for FHF progenitors is hyperpolarization-activated cyclic nucleotide gated potassium channel 4 (HCN4),[64,65] which is initially expressed in the cardiac crescent and gradually becomes confined to the cardiac conduction system.

SHF progenitors persist after the formation of the heart tube.[66] These cells, located at the tube's arterial and venous poles, migrate and differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells,[67,68] thereby contributing to the growth of the heart tube and formation of the inflow and outflow tracts. SHF progenitor cells are marked by sustained expression of ISL1,[66,67] although the FHF also transiently expresses this transcription factor.[69] *ISL1* knockout mice exhibit severe defects in the looping of the heart and the development of the right ventricle and outflow tract. ISL1 activates FGF and BMP genes, which play important roles in cardiac progenitor proliferation and differentiation as discussed above.[66] Moreover, ISL1 cooperates with GATA4 to activate *Mef2c*, which activates the expression of the basic-helix-loop-helix transcription factor HAND2.[70–72] HAND2 is an essential transcription factor that regulates right ventricular development, as seen by *Hand2* knockouts that display varying degrees of right ventricular hypoplasia.[73] Moreover, *Hand2* is required for the survival and proliferation of SHF progenitors.[73] As SHF progenitors differentiate, *Nkx2-5* is activated and directly represses *ISL1* to limit progenitor proliferation and promote progenitor differentiation.[74] Interestingly, a recent study has shown that the direct repression of *Isl1* by NKX2-5 is required for the proper development of ventricular cardiomyocytes.[75] In contrast, this same study showed that overexpression of ISL1 in mouse ESCs enhanced specification of cardiac progenitors and led to increased nodal-type cardiomyocytes relative to ventricular subtypes, thus demonstrating complex regulatory feedback between *Isl1* and *Nkx2-5* in cardiac progenitor specification and differentiation. The role of these transcription factors in regulating cardiac progenitor proliferation and differentiation continues to be a point of investigation. Understanding the complex interactions that regulate cardiac progenitor proliferation and differentiation is vital for understanding the cause of congenital heart defects and for applications of cardiac regeneration to congenital and acquired heart disease.

Role of MicroRNAs in Cardiac Differentiation

MicroRNAs (miRNAs) are single-stranded, noncoding RNA molecules that negatively affect gene expression at the post-transcriptional level, either by guiding mRNA degradation or by preventing protein translation.[76,77] DICER is an RNase that is critical for cytoplasmic processing of premiRNAs into mature miRNAs, which will subsequently be incorporated into the RNA-induced silencing complex. Selective knockout of *Dicer* in murine cardiac precursors using Nkx2-5^{Cre} resulted in embryonic lethality because of dilated cardiomyopathy, ventricular hypoplasia, and heart failure by E12.5.[78] However, when *Dicer* was knocked out in cardiomyocytes via myosin heavy chain 6 (Myh6)-Cre, which initiates recombination later in heart development, the phenotype was milder, as mice survived to birth but died soon after from dilated cardiomyopathy and heart failure.[79] These studies demonstrate the critical importance of miRNAs in cardiac differentiation and morphogenesis.

Several specific miRNAs have been shown to play key roles in cardiac development. The miR-1 and miR-133 families are cotranscribed as miR-1-1/miR-133a-2 and miR-1 to 2/miR-133a-1 and are expressed in the developing heart and skeletal muscle.[76,80] In the developing heart, miR-1 and miR-133 family members are regulated by serum response factor and MEF2 transcription factors, which are intricately involved in myocyte differentiation.[81,82] Homozygous deletion of miR-1 to 2 resulted in a wide range of severe cardiac defects that lead to embryonic or perinatal lethality, including ventricular septal defect, heart failure, and dysrhythmias.[78] Notably, driving overexpression of miR-1 from the myosin heavy chain 7 (*Myh7*) promoter, active in fetal cardiomyocytes, resulted in thinning of the ventricular wall and heart failure.[78] Intriguingly, double miR-1-1 and miR-1 to 2 knockout caused predominantly postnatal lethality before weaning as a result of severe cardiac dysfunction.[83] Although the milder phenotype in these double knockout mice compared with miR-1 to 2 single knockout requires further clarification, it seems that miR-1 plays a key role in modulating the relative proliferation and differentiation of cardiac precursors. Similar to miR-1 to 2 deletion, mice lacking both miR-133a-1 and miR-133a-2 developed severe heart failure because of ventricular septal defects and dilated cardiomyopathy.[84] Interestingly, these mutants also showed ectopic expression of smooth muscle genes, pointing to a role for miR-133a in mediating the lineage decision between cardiac and smooth muscle. Deletion of individual miR-1-1/miR-133a-2 or miR-1 to 2/miR-133a-1 gene clusters did not affect survival to birth, cardiac morphogenesis, or function, suggesting functional redundancy.[85] However, double knockout of both miR-1-1/miR-133a-2 and miR-1 to 2/miR-133a-1 caused embryonic lethality at E11.5 with marked thinning of the compact ventricular myocardium and impaired cardiomyocyte maturation and proliferation. This effect is, in part, mediated through loss of suppression of the transcription factor *Myocardin*, leading to persistent expression of smooth muscle genes and incomplete cardiomyocyte maturation.

Fibroblast to Cardiomyocyte Reprogramming

One exciting application of our knowledge of the factors that govern cardiomyocyte specification and differentiation is direct reprogramming of nonmyocytes such as fibroblasts to cardiomyocyte-like cells (induced cardiomyocytes or iCMs). This can be achieved by transduction of nonmyocytes with a cocktail of transcriptional regulators that promote myocardial transdifferentiation. Although this strategy is still in its infancy, results have been promising. Multiple groups have reported direct reprogramming via transduction of fibroblasts with retrovirus-expressing transcription factor cocktails.[86–89] The most commonly used cocktails have used GATA4, MEF2C, and TBX5,[87] with some reports indicating that HAND2 enhances reprogramming.[89] The combination of MESP1 and ETS2 has also been reported to successfully reprogram human

fibroblasts to cardiac progenitors.[90] An additional recent study using a cocktail of 4 miRNAs also reported direct reprogramming of cardiac fibroblasts to cardiomyocytes.[91] A range of phenotypes were observed in iCMs, with some cells demonstrating contractile and calcium-handling characteristics of cardiomyocytes. However, the robustness of reprogramming in vitro and the extent to which in vitro iCMs express complex physiological phenotypes characteristic of bona fide cardiomyocytes require further improvement.[92] The efficiency of nonmyocyte to iCM reprogramming and the extent to which iCMs can be induced to attain functional properties of mature bona fide cardiomyocytes (see sections on Cardiomyocyte Maturation, below) will determine whether this exciting concept ultimately will bear fruit.

Regulation of Cardiomyocyte Proliferation

Cardiomyocyte Proliferation During Embryonic Heart Development

After cardiac progenitor cells differentiate into cardiomyocytes, new cardiomyocytes are generated through division of existing cardiomyocytes, and this expansion of cardiomyocyte number primarily accounts for embryonic heart growth. Early events of cardiomyocyte proliferation have been studied through lineage tracing experiments and clonal analyses. One of the earliest cardiomyocyte clonal analyses was performed in chicken embryos,[93] using a low dose of retrovirus to genetically label a small fraction of cardiomyocytes and cardiac progenitor cells. Later on, these labeled cells were observed to form clusters, and the number of cells per cluster increased during heart development. This result suggested a coherent expansion of cardiomyocytes through their local proliferation. More precise labeling and tracing experiments done in mouse embryos showed that coherent cardiomyocyte colonies derived from single cardiomyocytes could be found at E8.5, when heart tube looping initiates.[94] This clonal expansion of cardiomyocytes during heart growth was further demonstrated in zebrafish. In elegant studies that took advantage of the ease with which the developing zebrafish heart can be visualized, stochastic cardiomyocyte labeling by combinations of multiple fluorescent proteins permitted detailed clonal analysis of heart growth.[95] Proliferation and expansion of as little as ≈55 cardiomyocytes during early development was sufficient to generate all of the cardiomyocytes of the ventricular myocardium.[95]

Several signaling pathways precisely control cardiomyocyte proliferation to regulate heart growth and morphogenesis. Among the best characterized is the Hippo/YAP signaling pathway, which controls the size of several organs during development.[96,97] Elevated cell density signals through incompletely understood upstream pathways to activate Hippo pathway kinases (MST1/2 and LATS1/2), which put the brakes on cardiomyocyte proliferation by phosphorylating and inactivating the transcriptional coactivators YAP and TAZ (formally known as WWTR1), partially redundant drivers of cell proliferation.[98,99] Hippo/YAP regulation of cardiomyocyte proliferation and heart growth have been well characterized using genetic mouse models to manipulate pathway components. Inactivation of Salvador (*Sav1*), encoding a scaffold protein required for Hippo kinase activity, in cardiomyocytes increased cardiomyocyte proliferation, resulting in overgrowth of the fetal ventricular walls and trabeculae.[100] Forced expression of constitutively active YAP in cardiomyocytes also strongly stimulated their proliferation, whereas YAP/TAZ loss of function in cardiomyocytes reduced their proliferation, causing embryonic lethal cardiac hypoplasia and hypotrabeculation.[101–103] YAP functions by interacting with transcription factor TEAD1,[102,104] which activates downstream mitogenic pathways, including the PI3K-AKT pathway.[105,106] YAP could also interact with β-catenin and directly modify WNT signaling to upregulate cardiomyocyte proliferation.[100] Thus, the Hippo/YAP pathway is an essential regulator of cardiomyocyte proliferation that matches cardiac growth to physiological needs of the fetus.

Proper spatiotemporal control of cardiomyocyte proliferation is required for normal cardiac morphogenesis. Cardiomyocytes in the compact myocardium proliferate more rapidly than trabecular cardiomyocytes. Differences in Hippo/YAP signaling may contribute to this regional difference in cardiomyocyte proliferation because relieving Hippo inhibition or increasing YAP activity abrogates the difference and causes dramatic trabecular myocardial hyperplasia.[100,102] Gradients of mitogenic factors may also contribute to more rapid proliferation of cardiomyocytes in the compact myocardium. The epicardium, an epithelial sheet that covers the outer surface of the heart, is essential for growth of the compact myocardium, as disrupting epicardium perturbs the proliferation of underlying myocardium.[107] Recent studies showed that epicardium secretes IGF2, which activates IGF1R and subsequently ERK in cardiomyocytes to induce proliferation.[108,109] Follistatin-like 1 (FSTL1) is another epicardially secreted cardiac mitogen,[110] although the importance of FSTL1 for cardiac development remains to be determined. Gradients of environmental stimuli, such as oxygen or blood flow, also regulate regional cardiomyocyte proliferation rates. Nuclear localization of hypoxia-inducible factor 1 α, which is governed by oxygen tension, was enriched in cardiomyocytes within the outer compact myocardium and interventricular septum, where it was required for robust cardiomyocyte proliferation.[111]

Another heart morphogenic process that is tightly linked to cardiomyocyte proliferation is trabeculation (Figure 3). Trabeculae are ridge-shaped myocardial protrusions that are derived from the subendocardial myocardium. Although the key mechanistic events are still being elucidated, in mouse, the process of trabeculation is thought to be initiated when a subset of subendocardial cardiomyocytes alter their polarity from parallel to perpendicular relative to the chamber wall.[112] These cardiomyocytes then proliferate and expand to form the trabeculae.[113–115] Rodent models show that cardiomyocyte proliferation during trabeculation is tightly controlled by communication between myocardium and trabecular endocardium, which involves a complex signaling pathway composed of NOTCH1, BMP10, ephrin B2 (EFNB2), HAND2, and neuregulin-1 (NRG1).[113–116] Zebrafish-based studies of trabecular development support crucial roles for Notch and neuregulin signaling; however, the expression profiles and presumed functions of individual pathway components seems to be markedly different from rodent models.[95,117–119] Furthermore, trabeculation in zebrafish initiates through depolarization and delamination of a

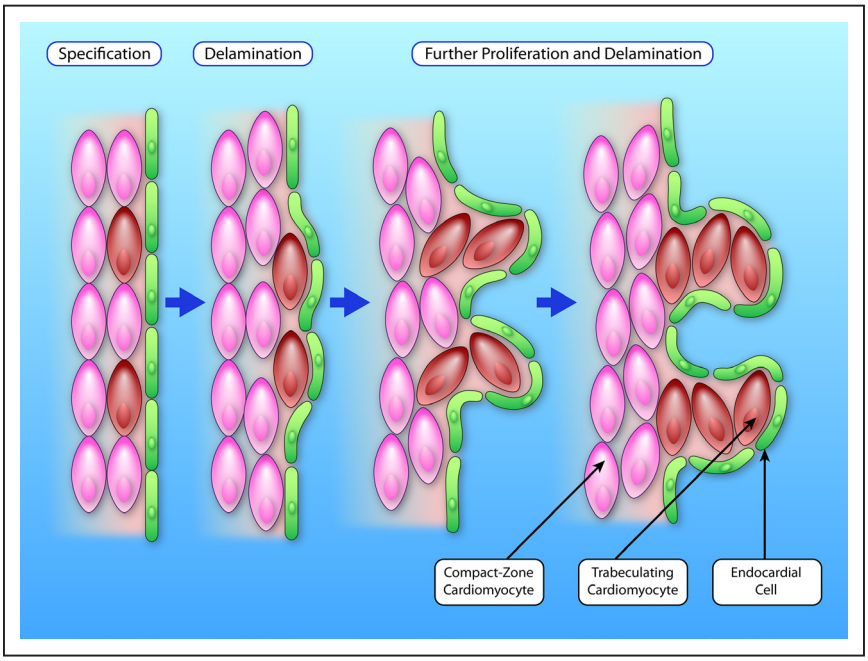

Figure 3. Model of the trabeculation process. During trabeulation, a small fraction of cardiomyocytes (CMs) in the compact myocardium (pink) are first specified as trabeculating CMs (brown). These cells delaminate from the compact myocardium and migrate inward to form the first trabecular CMs. CMs in both compacted and trabecular myocardium further proliferate. This proliferation, together with CM migration and rearrangement, results in protrusion and expansion of the trabecular myocardium (illustration credit: Ben Smith).

subset of cardiomyocytes rather than altered polar orientation as is thought to occur in mouse.[120]

Cardiomyocyte Cell Cycle Exit During Postnatal Development

Although cardiomyocyte proliferation is responsible for fetal heart growth, shortly after birth, mammalian cardiomyocytes largely exit the cell cycle. In adult hearts, there is measurable albeit limited cardiomyocyte turnover.[121–124] By taking advantage of the spike in atmospheric C_{14} that accompanied aboveground nuclear bomb tests, Bergmann et al[121,124] showed that human cardiomyocyte turnover rate is ≈1% per year at 20 years of age and gradually decreases with advancing age. This estimate is similar to the 0.76% annual turnover rate measured in adult mouse heart by stable isotope labeling coupled with imaging mass spectrometry and genetic fate mapping.[122] Cardiomyocyte proliferation extends into the first postnatal week in mice.[125,126] In humans, this postnatal proliferative window may last for several years,[127] suggesting a potential window in infancy for therapeutic regenerative approaches in congenital heart disease. Several factors that trigger and enforce adult cardiomyocyte cell cycle exit have been identified. Mitogenic signaling pathways that drive fetal cardiomyocyte proliferation are attenuated postnatally. For example, expression of ERBB2 quickly decreases in cardiomyocytes after birth, which reduces the mitogenic potency of NRG1.[128] The mitogenic activity of YAP is likewise restrained by multiple mechanisms, including activity of Hippo pathway kinases,[100] downregulation of YAP and TEAD1, and TEAD1 sequestration by the protein VGLL4.[104] In addition to loss of promitotic signals, several mechanisms actively inhibit expression and function of the cell cycle machinery in adult cardiomyocytes. Transcriptional regulators such as MEIS (myeloid ecotropic viral integration site) homeobox 1 (MEIS1)[129] and retinoblastoma protein (RB),[130,131] epigenetic modifiers such as polycomb repressive complex 2 (PRC2),[132] and microRNAs such as the miR-15 family[133] actively repress core cell-cycle activators and/or activate cell-cycle inhibitors. P38 MAP kinase signaling also inhibits cardiomyocyte proliferation.[134,135] Increased oxidative stress in postmitotic cardiomyocytes has been implicated in causing DNA damage response-mediated cell-cycle arrest.[136] Downregulation of telomerase activity has been shown to block proliferation in a p21-dependent manner.[137,138] Disassembly of centrosomes, a hub of mitogenic signaling[139] as well as the organizing center for mitotic spindle assembly,[140,141] in postnatal cardiomyocytes may add another layer of negative regulation of cardiomyocyte proliferation.[142]

Heart Regeneration Through Cardiomyocyte Proliferation

Heart injury such as myocardial infarction causes massive cardiomyocyte death. In response, the rate of cardiomyocyte proliferation increases in the peri-infarct region by ≈5-fold above its low basal rate.[122] However, this level of innate cardiac

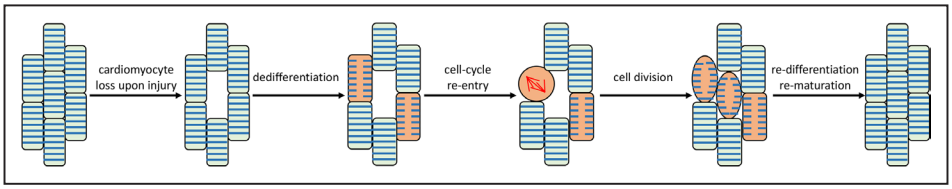

Figure 4. Model of cardiomyocyte regeneration. During heart regeneration, myocardial injury and cardiomyocyte (CM) loss induce the dedifferentiation of a fraction of CMs (pink). These CMs reenter the cell cycle, produce new CMs, and replenish the lost CMs. CMs that are generated from cell division redifferentiate to a fully mature state to improve heart contraction.

regeneration is far too limited to effectively replace the lost cardiomyocytes. As a result, the remaining cardiomyocytes become overburdened and experience chronically elevated biomechanical stress that induces further cardiomyocyte loss, precipitating a vicious cycle that ultimately leads to heart failure and pathological heart remodeling.[143,144] Interrupting or reversing this process requires replacing or regenerating the lost cardiomyocytes.

To gain insights into how heart regeneration could be therapeutically enhanced, cardiac biologists have studied models competent in effective heart regeneration. Several species including fish and amphibians retain heart regenerative capacity throughout life.[143,145–147] A key finding from the zebrafish system is that cardiomyocytes lost in heart injury models are replaced by proliferation of pre-existing cardiomyocytes, rather than by generation of cardiomyocytes from nonmyocytes.[148,149] Enhanced cardiomyocyte proliferation rate is observed in zebrafish hearts after several different types of injuries such as resection and genetic ablation,[148–150] indicating that a robust injury response mechanism is present in zebrafish that promotes cardiomyocyte proliferation. Regenerating cardiomyocytes had unique morphological and molecular signatures consistent with sarcomere disassembly, which has been described as cardiomyocyte dedifferentiation (Figure 4). Sarcomere disassembly has been inferred to be a prerequisite for productive mitosis and cytokinesis.[148,149] Thus, cardiomyocyte proliferation provides the basis of heart regeneration in zebrafish.

Cardiomyocyte proliferation also compensates for the damage or loss of cardiomyocytes in fetal and neonatal mice. At fetal stages, cardiomyocyte-specific mosaic knockout of Hccs, an X-linked gene that is essential for mitochondrial function, damages ≈50% cardiomyocytes in E10.5 heterozygous females because of random X chromosome inactivation. However, this perturbation does not cause embryonic lethality because the healthy cardiomyocytes proliferate to compensate for the injury. Consequently, only ≈10% cardiomyocytes are Hccs deficient at birth, and >85% of these animals develop and survive normally.[151] Similarly, we used diphtheria toxin A to ablate various fractions of cardiomyocytes in embryonic hearts. We found that embryos tolerated loss of ≤60% of cardiomyocytes and maintain normal heart development by upregulating the proliferation of remaining unablated cardiomyocytes.[152]

Murine cardiac regeneration capacity remains robust into the first postnatal week of life, as hearts injured by apical resection on postnatal day 1 efficiently replenish the lost cardiomyocytes, resulting in hearts with minimal residual scar and with normal heart morphology and function by 1 month of life.[126,133] Although multiple groups have reproduced this result,[153] confirmation has not been uniform,[154] possibly because the extent of scarring and regeneration depends on surgical technique and the extent of myocardial resection.[155] Interestingly, macrophages are essential for robust neonatal cardiac regeneration.[156] Lineage tracing showed that the proliferation of pre-existing cardiomyocytes also underlies murine heart regeneration.[122,126] Cardiomyocyte dedifferentiation, characterized by sarcomere disassembly, has also been implicated in mammalian cardiomyocyte proliferation[128,133,157–159] (Figure 4), although it has not yet been observed directly during in vivo mammalian cardiomyocyte proliferation. Thus, induction of cardiomyocyte proliferation is a conserved mechanism of heart regeneration from zebrafish to mammals.

Enhancing Cardiac Regeneration by Driving Cardiomyocyte Proliferation

The induction of cardiomyocyte proliferation as the major mechanism for heart regeneration in lower vertebrates and fetal and neonatal mice provides a rational foundation for current efforts to augment cardiomyocyte proliferation in mature mammalian hearts to achieve therapeutic cardiac regeneration after heart injury. Developmental cardiomyocyte cell cycle exit is accompanied by the silencing of fetal pathways that drive proliferation; thus, reactivating and augmenting these fetal pathways is an attractive strategy to stimulate the adult cardiomyocyte proliferation. Although many strategies are currently being pursued, because of space constraints, here we will focus on NRG1 and Hippo/YAP. We refer readers to other excellent reviews that cover other efforts to stimulate cardiomyocyte proliferation.[160–163]

Several studies have shown that activating NRG1/ERBB signaling boosts proliferation of adult cardiomyocytes and improves heart repair on injury. In zebrafish, overexpressing NRG1 in cardiomyocytes was sufficient to stimulate cardiomyocyte proliferation, resulting in accelerated myocardial expansion after injury.[164,165] NRG1 treatment stimulated proliferation of in vitro cultured neonatal and adult murine cardiomyocytes,[158,166] as well as cardiomyocytes from myocardium removed from pediatric patients at the time of heart surgery.[166] NRG1 delivery to mice, or overexpression of a constitutive active form of ErbB2, improved heart structure and function after myocardium infarction.[128,158] In patients with stable chronic heart failure, NRG1 infusion has been shown to be safe and to have beneficial hemodynamic effects.[167] Although several studies have suggested that NRG1's therapeutic effect is due in part to stimulation of adult cardiomyocyte proliferation, other studies have demonstrated that NRG1's cardiomyocyte mitogenic activity quickly diminishes after birth.[128,166] Indeed, one group could not detect a mitogenic effect of NRG1 on adult cardiomyocytes and assigned its salutary effects to other mechanisms.[168]

Because YAP has robust cardiomyocyte mitogenic activity during fetal heart development, elevating YAP activity in cardiomyocytes is another potential approach to stimulate cardiomyocyte proliferation and heart regeneration. Forced expression of constitutively active YAP in cardiomyocytes stimulated cardiomyocyte proliferation in both neonatal and adult hearts.[101,103] In the neonatal murine heart regeneration model, abolishing YAP activity disrupted the regenerative capacity of the neonatal murine heart, whereas YAP overexpression enhanced it.[162] A study that used adeno-associated virus, a promising gene therapy vector, to selectively overexpress activated YAP in adult cardiomyocytes, provided proof of concept that this treatment can improve myocardial function and survival after myocardial infarction.[101] Importantly, YAP activation did not have deleterious effects on the heart. Thus,

YAP activation has therapeutic potential to stimulate heart repair and regeneration.

It is worth noting that NRG1 and YAP both play additional biological functions other than regulating cardiomyocyte proliferation, including cardiomyocyte survival,[169] migration,[117,119] and calcium handling.[170] NRG1 also acts on nonmyocytes to promote neovascularization after heart injury.[164] YAP regulates cardiomyocyte survival[105,171] and transcriptional responses to mechanical stress.[172,173] In cardiomyocytes, YAP promotes actin cytoskeleton remodeling and the formation of cardiomyocyte protrusions at heart injury sites.[174] Recently, YAP was also implicated in controlling cardiomyocyte oxidative stress.[175] Thus, the beneficial effects of NRG1 and YAP activation in injured hearts are likely to be multifactorial.

Although stimulating cardiomyocyte proliferation is a promising strategy to boost myocardial regeneration in adult hearts, multiple obstacles need to be surmounted before this strategy can be used in clinical applications. First, induction of cardiomyocyte proliferation remains inefficient in adult hearts, likely because of multiple layers of negative regulation that block mature cardiomyocyte cell cycle activity. Thus, a critical task in future research is to precisely define the signals that trigger and maintain cell-cycle withdrawal in adult cardiomyocytes and design methods to remove or circumvent these obstacles. A second challenge is the potential for increased cancer risk because of cell cycle activation in noncardiomyocytes. For example, both NRG1 and Yap signals have oncogenic potentials and are known to be involved in cancers.[176,177] Precise targeting of mitogenic stimuli to cardiomyocytes is necessary to minimize oncogenic risk.

The dedifferentiation of regenerating cardiomyocytes has been observed in both zebrafish and mouse. This process, marked by sarcomere disassembly, occurs during regeneration in response to heart injury[126,133,148] and during NRG1-stimulated myocardial regeneration.[128,164] These data imply the reversing cardiomyocyte differentiation state might stimulate cardiomyocyte proliferation, a strategy that could potentially be more efficient and less risky than direct manipulation of cell cycle regulators. However, the merits of this strategy are unknown and depend on better understanding the dedifferentiation process. Although sarcomere disassembly has been suggested as a prerequisite for completion of cardiomyocyte mitosis,[157,178] it is unclear whether the dedifferentiation observed during regeneration is a true reversal of differentiation state or simply a change of cytoskeletal architecture of cardiomyocytes undergoing mitosis. Second, whether dedifferentiation will necessarily enhance proliferation is also unclear. Although correlation between dedifferentiation and the increase of cardiomyocyte proliferation has been well documented,[126,128,133,148,164] the causal relationship between these 2 processes has not been established.

How newly regenerated cardiomyocytes re-establish the morphology of myocardium damaged by injury is also an open question. During heart regeneration in adult zebrafish and fetal and neonatal mice, regenerated heart walls largely retain the morphology of the original structures.[95,126,152] Given that cardiomyocyte proliferation plays an essential role in morphogenesis during development, it is hoped that the proliferation of regenerative cardiomyocytes follows similar rules to rebuild the adult myocardium. Understanding these rules and their underlying molecular mechanisms will be important to properly enhance heart regeneration by cardiomyocyte proliferation or to properly sculpt myocardium regenerated by other means (eg, by delivery of stem cell–derived cardiomyocytes).

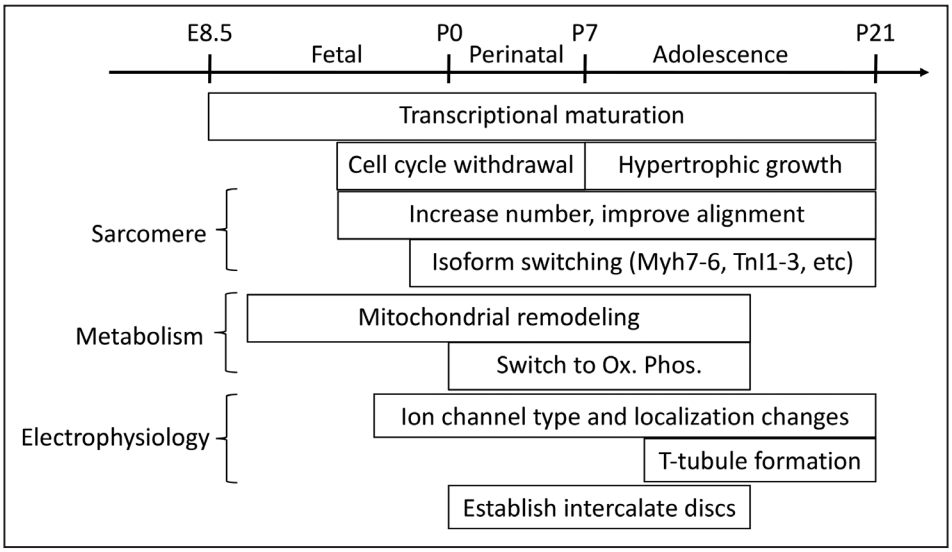

Figure 5. Representative cellular events during cardiomyocyte (CM) maturation. During CM maturation, transcriptional changes occur throughout both embryonic and postnatal development. CMs stop hyperplastic growth at the perinatal stage and transition to hypertrophic growth, in which sarcomere number increases and sarcomere alignment improves. Altered expression of critical sarcomere isoforms modifies the contractile properties of CMs. Mitochondria increase in number and size and become well organized with respect to sarcomeres. After birth, CMs switch from deriving most of their energy from glycolysis to depend on oxidative phosphorylation. The maturation of electrophysiological properties is characterized by proper expression and localization of ion channels characteristic of adult CMs. T-tubules form at a late stage of postnatal development, perhaps to permit rapid AP penetration with rapidly enlarging CMs. Maturation is also characterized by the postnatal establishment of intercalated discs and proper formation of cell–cell contacts.

Table. Hallmarks of Cardiomyocyte Maturation

Phenotype	Immature (Late Fetal)	Mature (Adult)	PSC-CMs	Regulators	References
Cell size	Small	Large	Small	GATA4/6	183,208
Cell shape	In situ: rod-shaped (4:1::L:W); cultured: stellate	In situ and dissociated: rod-shaped (7:1::L:W)	Irregular/round	ECM composition and patterning, mechanical load, ERK1/2 signaling	182,184,188,222
Sarcomere alignment	Organized	Superbly organized	Disorganized	Cell shape, mechanical load, substrate stiffness	188,214,215
Sarcomere components	TNNI1, MYH7, TTN-N2BA, EH-MYOM1,TNNT2 exon 5 inclusion	TNNI3, Myh6, TTN-N2B, TNNT2 exon 5 exclusion	Variable	Thyroid hormone; maturation-related transcriptional programs are currently not well understood	189–193,219
Metabolism	Glycolysis	Oxidative phosphorylation	Glycolysis	PPAR signaling, greater oxygen availability after birth	198,203,206
Mitochondria	Low number, small and round	High number, large and ovoid, close proximity to myofibers	Ovoid, clustered around nucleus and cell periphery	AMPK, PPAR signaling, mitofusins 1 and 2	199–201
Proliferative capacity	High	Very low	Can proliferate for ≈1 mo after contraction begins	NRG1/ERBB2/4, YAP/TEAD, IGF2/IGF1R/PI3K/ERK, BMP10, WNT/β-catenin, NOTCH	100,102,108,113,114,158
Nucleus	Mononucleated	Multinucleated or polyploid	Mononucleated	Proliferation regulators above	100,102,108,113,114,158
T-tubules	None	Extensive network	None	JPH2, BIN1	196,197

PPAR indicates peroxisome proliferator-activated receptor; and PSC-CM, pluripotent stem cell–derived cardiomyocyte.

Cardiomyocyte Maturation

Hallmarks of Cardiomyocyte Maturation

The dynamic process of heart development includes dramatic alterations in cardiomyocyte metabolism, form, and function, particularly in the neonatal period (Figure 5; Table). Fetal cardiomyocytes actively proliferate, and the resulting increase in cardiomyocyte number largely accounts for fetal heart growth. These cells are adapted to the hypoxic intrauterine environment, as they rely primarily on glycolytic metabolism. At birth, cardiomyocytes undergo dramatic changes linked to greater demand for left ventricular pump function and greater availability of oxygen. Postnatal cardiomyocytes become highly specialized for efficient contraction: they shift to oxidative phosphorylation as their primary energy source increase in volume by 30- to 40-fold between birth and adulthood and develop accompanying ultrastructural specializations and changes in gene expression that enable efficient and coordinated cardiomyocyte contraction. Perhaps intimately linked with specialization for efficient contraction, postnatal cardiomyocytes largely exit the cell cycle and become predominantly polyploid (a single polyploid nucleus in humans or two diploid nuclei in rodents).

Although much has been learned about the fetal specification of cardiomyocytes, much less is known about how cardiomyocyte maturation is coordinately regulated. Understanding cardiomyocyte maturation is critical to advance the field of cardiac regeneration, because any new source of cardiomyocytes must be sufficiently mature to effectively contribute to cardiac function and to seamlessly electrically couple with pre-existing myocardium. Indeed, a recent landmark study demonstrated that although significant numbers of human pluripotent stem cell–derived cardiomyocytes (PSC-CMs) could be engrafted in infarcted primate hearts, all treated hearts exhibited ventricular arrhythmias that were attributed to insufficient maturity of donor cardiomyocytes.[29] Immaturity of stem cell–derived cardiomyocytes is also a major barrier to their use for in vitro modeling of human heart disease.[179–181] Thus, discovering ways to induce cardiomyocyte maturation has become a major goal in the field. Progress will require developing a detailed understanding of the normal processes that drive developmental cardiomyocyte maturation. Here, we review key aspects of postnatal cardiomyocyte maturation, survey current strategies to mature PSC-CMs, and recommend directions for future study.

Structural remodeling during postnatal physiological maturation is extensive and affects virtually all aspects of cardiomyocyte cytoarchitecture. However, 3 prominent hallmarks stand out: an increase in cardiomyocyte size leading to formation of large rod-shaped cells with high length:width aspect ratio, a higher myofibrillar density with increased sarcomere prominence, and formation of T-tubules. The molecular mechanisms that control these morphological and structural changes are incompletely understood. The length and diameter of cardiomyocytes seem to be regulated by distinct types of mechanical loads, with cardiomyocyte elongation occurring through addition of new sarcomere units at the cell poles in response to diastolic strain, and cardiomyocyte girth increasing through parallel addition of filaments in response to systolic strain.[182,183] In the disease state, this process is mediated by ERK1/2 signaling, which likely also plays important roles in maturation-related remodeling.[184] These mechanisms are influenced by substrate stiffness, which together with additional

cues make the ECM an important determinant of cardiomyocyte shape.[185–187]

Myofibrillar density and organization directly correlate with cardiomyocyte maturation status, with fully mature cardiomyocytes having superbly organized sarcomeres. Sarcomere organization is intimately linked to cell shape, as patterning cardiomyocytes with microcontact printing to induce the formation of different cell geometries directly impacts sarcomere alignment, with rectangular shape and higher length to width ratios driving increased organization.[188] This increase in sarcomeric organization and prominence corresponds to alterations in the expression ratio of many crucial sarcomere protein isoforms. In mouse, this transition includes shifts in gene transcription from fetal to adult isoforms (eg, troponin I1 (TNNI1) to troponin I3 (TNNI3); Myh7 to Myh6) and altered gene splicing (eg, titin [TTN]-N2BA to TTN-N2B; myomesin 1 embryonic isoform [EH-MYOM1] to mature isoform; troponin T2 [TNNT2] exon 5 inclusion to exclusion).[189–193] T-tubules, invaginations of the plasma membrane that allow membrane depolarization to quickly penetrate to the cardiomyocyte interior, are a hallmark of mature cardiomyocytes. Development of the murine T-tubule network initiates at ≈2 weeks after birth and similar to sarcomere development gradually becomes better defined and organized as the cardiomyocytes mature to adulthood.[194,195] Currently, besides junctophilin 2 and Bin 1 (Table), few proteins involved in T-tubule formation have been identified, and the process is poorly understood.[196,197]

In addition to structural remodeling during maturation, neonatal cardiomyocytes undergo dramatic alterations in metabolism, shifting from predominantly nonoxidative to oxidative metabolism in the neonatal period.[198] This metabolic transition coincides with an increase in mitochondrial density, and a mitochondrial shift in shape from small and round to large and ovoid.[199,200] Positional differences are also observed, with mitochondria of mature cardiomyocytes being located in closer proximity with myofibrils and the sarcoplasmic reticulum.[201] Although distinguishing cause and effect relationships from associations has been difficult, there is mounting evidence that metabolic remodeling is a key driver of cytoskeletal and sarcomeric maturation.[202–205]

Many aspects of the structural and metabolic remodeling that takes place during cardiomyocyte maturation have been appreciated for some time. However, understanding the regulatory networks driving these changes has been more challenging. External signals influencing maturation include neurohormonal factors and mechanical stimuli related to hemodynamic load, force generation, and ECM composition. These external signals activate internal cell-signaling pathways, which in turn activate transcriptional regulators to orchestrate global changes in gene expression. One recent study that analyzed hundreds of microarray data sets identified stage-specific gene regulatory networks,[203] and future studies will determine whether major nodes within these networks act as regulators of maturation. One strong theme of the analysis was the presence of multiple networks related to metabolism, with PPAR (peroxisome proliferator-activated receptor)-signaling pathways constituting central components. Indeed, PPAR signaling acts as an activator of fatty acid oxidation, and pathway activation increases as maturation proceeds.[206] Although this study succeeded in describing how the cardiomyocyte gene expression profile changes during maturation, proposed transcriptional regulators have not been functionally validated in vivo. This is a difficult task, as global cardiomyocyte-specific overexpression or ablation of important transcriptional regulators inevitably results in cardiac stress or failure, which confounds efforts to gauge the effect on maturation. Indeed, because of this complication, many maturation investigations have been limited to the study of neonatal rat cardiomyocytes or PSC-CMs in an artificial culture environment. Although this line of study has yielded some notable advances, such as the identification of Let-7 miRNA family members as positive regulators of maturation,[207] the field continues to be hampered by insufficient knowledge of maturational regulation in vivo. Our recent study of the postnatal roles of transcription factors GATA4 and GATA6 offers clues as to how regulation of cardiomyocyte maturation can be dissected in vivo.[208] Mosaic gene knockout, achieved by administering a low dose of Cre-expressing adeno-associated virus serotype 9 that only transduced a fraction of cardiomyocytes, enabled the study of mutant cardiomyocytes in functionally healthy hearts. In this context, GATA4/6 double knockout cardiomyocytes were dramatically smaller and less mature than control cardiomyocytes, indicating that GATA4 and GATA6 are crucial cell autonomous regulators of postnatal cardiomyocyte growth and maturation. Combining this mosaic gene manipulation strategy with single cell RNA-seq and emerging technologies such as somatic CRISPR/Cas9 mutagenesis[209,210] offers an experimental strategy to dissect the regulatory networks that govern cardiomyocyte maturation.

Enhancing Maturation of Cardiomyocytes Differentiated From Nonmyocytes or Pluripotent Stem Cells

Despite limitations in our understanding of the mechanisms driving cardiomyocyte maturation, many approaches to improving PSC-CM maturity have capitalized on knowledge of developmental paradigms. Approaches such as 3-dimensional tissue engineering, mechanical loading, modulation of substrate stiffness, and electric stimulation have all had varying degrees of success by more closely mimicking the in vivo environment.[211–215] Indeed, just as tension-sensing mechanisms are currently generating considerable interest as determinants of how the heart remodels in response to disease,[216] similar mechanosensing signaling pathways have been shown to play important roles in maturation-related remodeling.[217,218] In addition, treatment with hormonal factors has also been shown to modulate cardiomyocyte maturation, with thyroid hormone being a major stimulant of fetal cardiomyocyte maturation.[219] Finally, long-term culture of PSC-CMs has been shown to induce more complete maturation.[220] Although expression profile analyses of cardiomyocytes cultured for ≤1month show arrest at or before a stage equivalent to the late fetal period,[203] reportedly culture for ≈1 year results in PSC-CMs with expression profiles similar to adult cardiomyocytes.[207] Although these results are impressive, the inconvenience and cost of the long-term culture approach makes it unsuitable as an experimental model system

or as a regenerative therapeutic approach. However, combinations of the above strategies are currently being examined, and better understanding of the mechanisms that govern normal cardiomyocyte maturation promises to enable further progress.

Interestingly, cardiomyocytes differentiated from nonmyocytes seem to undergo enhanced maturation within the native milieu of the beating heart. For example, when human PSC-CMs were injected into a nonhuman primate model of myocardial infarction.[29] The injected PSC-CMs engrafted, and over 3 months, they were observed to have increased myofibril alignment and sarcomere registration. The engrafted PSC-CMs attained a diameter comparable to the size of normal adult monkey cardiomyocytes. On the contrary, transient ventricular arrhythmias were common early in the engraftment process and spontaneously resolved. The proarrhythmic effect may have resulted from the immaturity and heterogeneous electric coupling of the engrafting PSC-CMs. Generalizing from this experience, proarrhythmia may be a consequence of many regenerative strategies that yield immature cardiomyocytes, and cardiac regeneration experiments need to be designed to detect these adverse events. A second example is the maturation of iCMs reprogrammed from fibroblasts by viral delivery of cardiac transcription factors (see non-cardiomyocyte to cardiomyocyte reprogramming, above). iCMs created by reprogramming in vitro often are highly immature in size, morphology, and functional parameters such as calcium handling.[92] When reprogramming was performed in an in vivo context, a significant fraction iCMs properly localized structural maturation markers and had mature electrophysiological and calcium-handling phenotypes.[87,89] Indeed, although the overall number of in vivo reprogrammed cardiomyocytes is still limited, recent reprogramming studies showed a surprisingly potent salutary effect on heart function and myocardial outcome in an experimental murine MI model.[87,89] It is possible that in vivo reprogramming has additional benefits beyond cell autonomous gains in contractile ability. Translating this exciting work toward clinical applications will require further improvements in reprogramming efficiency and iCM maturation, development of reprogramming platforms that do not require viral integration, evaluation of this strategy's efficacy in large animal models, and improving efficiency of reprogramming human fibroblasts, which have been more refractory to reprogramming compared with murine cells.[88,221] The enhanced maturity achieved by in vivo cardiomyocyte differentiation likely reflects limitations of current in vitro culture conditions and the overall importance of the cell's environment on cardiomyocyte differentiation and maturation.

Conclusions

By following the lessons of normal development, cardiac biologists have made rapid inroads toward generating cardiomyocytes to make human disease models and to enhance myocardial outcomes in heart injury models. The conceptual focus of most work in therapeutic cardiac regeneration has been ischemic heart disease. However, myocardial failure in congenital heart disease typically results from chronic volume and pressure loads produced by palliated circulations. Greater appreciation of the need for therapeutic cardiac regeneration in congenital heart disease and its specific biology will allow advances to be applied to this rapidly growing patient population. The spectrum of therapeutic options for congenital heart disease could expand through creative uses of regenerative and stem cell biology to engineer heart tissue. Although this review has focused on myocardial regeneration, parallel efforts in engineering valves and vessels are also needed to address the panoply of difficulties faced by patients with palliated congenital heart disease. It is our hope that the ongoing efforts of investigators in developmental and regenerative biology will one day lead to groundbreaking advances in the diagnosis, prevention, and treatment of congenital and acquired heart disease in children and adults.

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Disclosures

None.

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