



Published in final edited form as:

Circ Res. 2017 March 17; 120(6): 941–959. doi:10.1161/CIRCRESAHA.116.309040.

Circulation Research Compendium – 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 (CMs). 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.

Subject Terms

Congenital heart Disease; Treatment

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Author Contributions

Co-first authors are listed in alphabetical order. FXG and SLP were primarily responsible for the cardiomyocyte specification and differentiation sections, respectively. YG and NVD were primarily responsible for the cardiomyocyte proliferation and maturation sections, respectively. SMW and WTP contributed equally to organize and edit the manuscript.

Conflict of Interest

None

Keywords

Cardiac development; regeneration; proliferation; cardiac progenitor cell; transcription factor

Introduction

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 (CMs) to proliferate. The low innate regenerative capacity of the heart narrows initial treatment strategies as well as 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 CMs. We go on to review mechanisms that control CM 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. Due to space limitations, we do not address valve and pulmonary vascular disease, additional important problems faced by some palliated congenital heart disease patients that 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 three primary germ layers: the ectoderm, mesoderm, and endoderm. CMs 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 tissues¹. 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 two 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 (EVE), NODAL 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 EVE that later becomes known as the anterior visceral endoderm³. As the embryo further develops, the anterior visceral endoderm migrates towards the anterior of the embryo and expresses NODAL antagonists such as Cerberus-like (CER1) and LEFTY1 proteins⁴. These antagonists prevent NODAL signaling from taking place in the anterior but not the posterior of the epiblast (Figure 1). Along with NODAL⁵, fibroblast growth factor (FGF)⁶, bone morphogenic protein (BMP)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 three 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, while 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 patterning². The T-box transcription factor eomesodermin (EOMES), expressed at the anterior PS, is important for both definitive endoderm and cardiac mesoderm development¹⁴. EOMES⁺ 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 PS¹⁵. While MESP1 is broadly expressed throughout mesoderm precursors¹⁶, a subpopulation of MESP1⁺ cells anterior to the PS become specified to cardiac lineages as they ingress through the PS¹⁷ (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 mesoderm¹⁷. The migration of these early cardiac precursors away from the WNT/ β -catenin rich PS, mediated by WNT chemorepulsion¹⁸, marks a significant step in the commitment of early mesoderm cells towards a cardiac fate. At E7.5 in the mouse/week 3 of human development, these migratory cells make two 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 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 (DKK1) and Crescent secreted from the anterior endoderm is necessary for cardiac differentiation, while ectopic expression of canonical WNT ligands WNT3A and WNT8C induces erythroid cell formation in cardiac precursor regions²¹. 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 (CMs)^{25, 26}. These protocols rely on activation of canonical WNT signaling to induce mesoderm formation, followed by WNT inhibition to stimulate CM differentiation. These protocols are remarkably efficient, often yielding 100 CMs per input pluripotent stem cells, and at purities of over 90%^{25, 26}. Efficient pluripotent stem cell to CM differentiation protocols are fundamental to using pluripotent cell differentiation systems to study cardiac differentiation²⁷, model human heart disease²⁸, and produce CMs as replacement therapy for heart failure²⁹.

Cardiomyogenesis In The First and Second Heart Fields

Signals regulating commitment to cardiac progenitor cells from mesodermal precursors

While 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, while the second heart field gives rise to the right ventricle, a portion of the septum, the outflow tracts, and a portion of the atria³⁰. Identifying the signals and molecular markers that regulate the segregation of early mesodermal progenitors to either of these fields is an important step towards understanding cardiac development *in vivo* as well as 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 while later cells form the SHF¹⁷. Recent work from the Mummery lab 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 lineages³¹. 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 BMP2³², FGF8³³, and non-canonical WNT³⁴ signals from the

underlying endoderm to promote their differentiation. Meanwhile, the SHF progenitors receive FGFs³⁵, sonic hedgehog³⁶, and canonical WNT/ β -catenin³⁷ signals to promote their proliferation and multilineage differentiation (Figure 2B). While 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, 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, BMP, and TGF- β 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 CMs 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 First and Second Heart Field Progenitors

The regulation of cardiomyogenesis from multipotent cardiovascular progenitors remains a major focus of ongoing research due to a combination of interest in understanding the mechanisms of cardiac disease as well as an ever-growing interest in generating CM subtypes (e.g. atrial, ventricular, 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 up-regulation 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 to FHF cells, SHF cells have delayed commitment to the CM lineage. Canonical WNT/ β -catenin signaling is critical for maintaining SHF progenitors in a proliferative state while inhibiting differentiation towards 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 CM specific gene expression.^{41, 49}

Non-canonical 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 (ROCK2) and Jun amino-terminal kinase, is crucial for normal CM specification.⁵⁰ Two non-canonical 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 non-canonical 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 CM 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 CM 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 CM 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⁺ pre-cardiac mesoderm stage¹⁵. As MESP1⁺ pre-cardiac progenitors march toward the anterolateral plate mesoderm, a subunit of the SWI/SNF 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 program⁵⁴ 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 activation⁵⁶. 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 natriuretic peptide A (*Nppa*) and the gap junction protein connexin 40 (*Gja5*)^{59, 60}. TBX5 misexpression can lead to the improper positioning of the interventricular septum or the complete absence of the septum⁶⁰. 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 tube⁶⁶. These cells, located at the tube's arterial and venous poles, migrate and differentiate into CMs, 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 factor⁶⁹. *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 above⁶⁶. 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 hypoplasia⁷³. Moreover, *Hand2* is required for the survival and proliferation of SHF progenitors⁷³. As SHF progenitors differentiate, *Nkx2-5* is activated and directly represses *ISL1* to limit progenitor proliferation and promote progenitor differentiation⁷⁴. Interestingly, a recent study has shown that the direct repression of *Isl1* by *NKX2-5* is required for the proper development of ventricular cardiomyocytes⁷⁵. 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 etiology of congenital heart defects and for applications of cardiac regeneration to congenital and acquired heart disease.

Role of Micro RNAs in Cardiac Differentiation

Micro-RNAs (miRNAs) are single-stranded, non-coding 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 pre-miRNAs into mature miRNAs, which will subsequently be incorporated into the RNA-induced silencing complex. Selective knock-out of *Dicer* in murine cardiac precursors using *Nkx2-5*^{Cre} resulted in embryonic lethality due to dilated cardiomyopathy, ventricular hypoplasia, and heart failure by E12.5⁷⁸. However, when *Dicer* was knocked out in CMs 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 co-transcribed as miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1, and are expressed in the developing heart as well as skeletal muscle.^{76, 80} In the developing heart, miR-1 and miR-133 family members are regulated by serum response factor (SRF) and MEF2 transcription factors, which are intricately involved in myocyte differentiation.^{81, 82} Homozygous deletion of miR-1-2 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 over-expression of miR-1 from the myosin heavy chain 7 (*Myh7*) promoter, active in fetal CMs, resulted in thinning of the ventricular wall and heart failure.⁷⁸ Intriguingly, double miR-1-1 and miR-1-2 knockout caused predominantly postnatal lethality prior to weaning as a result of severe cardiac dysfunction.⁸³ While the milder phenotype in these double knockout mice compare with miR-1-2 single knockout requires further clarification, it appears that miR-1 plays a key role in modulating the relative proliferation and differentiation of cardiac precursors. Similar to miR-1-2 deletion, mice lacking both miR-133a-1 and miR-133a-2 developed severe heart failure due to 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-1-2/miR-133a-1 gene clusters did not affect survival to birth, cardiac morphogenesis, or function, suggesting functional redundancy⁸⁵. However, double knock-out of both miR-1-1/miR-133a-2 and miR-1-2/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 CM reprogramming

One exciting application of our knowledge of the factors that govern CM specification and differentiation is direct reprogramming of non-myocytes such as fibroblasts to CM-like cells (induced CMs or iCMs). This can be achieved by transduction of non-myocytes with a cocktail of transcriptional regulators that promote myocardial transdifferentiation. While 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 TBX5⁸⁷, with some reports indicating that HAND2 enhances reprogramming⁹⁰. The combination of MESP1 and ETS2 has also been reported to successfully reprogram human fibroblasts to cardiac progenitors⁹¹. An additional recent study utilizing a cocktail of four miRNAs also reported direct reprogramming of cardiac fibroblasts to CMs⁹². A range of phenotypes were observed in iCMs, with some cells demonstrating contractile and calcium handling characteristics of CMs. However, the robustness of reprogramming in vitro, and the extent to which in vitro iCMs express complex physiological phenotypes characteristic of bona fide CMs requires further improvement⁹³. The efficiency of non-myocyte to iCM

reprogramming, and the extent to which iCMs can be induced to attain functional properties of mature bona fide CMs (see sections on CM maturation, below) will determine whether this exciting concept ultimately will bear fruit.

Regulation of CM proliferation

CM proliferation during embryonic heart development

After cardiac progenitor cells differentiate into CMs, new CMs are generated through division of existing CMs, and this expansion of CM number primarily accounts for embryonic heart growth. Early events of CM proliferation have been studied through lineage tracing experiments and clonal analyses. One of the earliest CM clonal analyses was performed in chicken embryos⁹⁴, using a low dose of retrovirus to genetically label a small fraction of CMs 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 CMs through their local proliferation. More precise labeling and tracing experiments done in mouse embryos showed that coherent CM colonies derived from single CMs could be found at E8.5, when heart tube looping initiates⁹⁵. This clonal expansion of CMs 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 CM labeling by combinations of multiple fluorescent proteins permitted detailed clonal analysis of heart growth⁹⁶. Proliferation and expansion of as little as ~55 CMs during early development was sufficient to generate all of the CMs of the ventricular myocardium⁹⁶.

A number of signaling pathways precisely control CM 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^{97, 98}. Elevated cell density signals through incompletely understood upstream pathways to activate Hippo pathway kinases (MST1/2 and LATS1/2), which put the brakes on CM proliferation by phosphorylating and inactivating the transcriptional co-activators YAP and TAZ (formally known as WWTR1), partially redundant drivers of cell proliferation^{99, 100}. Hippo/YAP regulation of CM proliferation and heart growth has 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 CMs increased CM proliferation, resulting in overgrowth of the fetal ventricular walls and trabeculae¹⁰¹. Forced expression of constitutively active YAP in CMs also strongly stimulated their proliferation, while YAP/TAZ loss-of-function in CMs reduced their proliferation, causing embryonic lethal cardiac hypoplasia and hypotrabeulation^{102–104}. YAP functions by interacting with transcription factor TEAD1^{105, 106}, which activates downstream mitogenic pathways, including the PI3K-AKT pathway^{107, 108}. YAP could also interact with β -catenin and directly modify WNT signaling to upregulate CM proliferation¹⁰¹. Thus the Hippo/YAP pathway is an essential regulator of CM proliferation that matches cardiac growth to physiological needs of the fetus.

Proper spatiotemporal control of CM proliferation is required for normal cardiac morphogenesis. CMs in the compact myocardium proliferate more rapidly than trabecular

CMs. Differences in Hippo/YAP signaling may contribute to this regional difference in CM proliferation, since relieving Hippo inhibition or increasing YAP activity abrogates the difference and causes dramatic trabecular myocardial hyperplasia^{101, 103}. Gradients of mitogenic factors may also contribute to more rapid proliferation of CMs 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 myocardium¹⁰⁹. Recent studies showed that epicardium secretes IGF2, which activates IGF1R and subsequently ERK in CMs to induce proliferation^{110, 111}. Follistatin-like 1 (FSTL1) is another epicardially-secreted cardiac mitogen¹¹², 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 CM proliferation rates. Nuclear localization of hypoxia inducible factor 1 alpha (HIF1A), which is governed by oxygen tension, was enriched in CMs within the outer compact myocardium and interventricular septum, where it was required for robust CM proliferation¹¹³.

Another heart morphogenic process that is tightly linked to CM proliferation is trabeculation (Figure 3). Trabeculae are ridge-shaped myocardial protrusions that are derived from the sub-endocardial 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 sub-endocardial CMs alter their polarity from parallel to perpendicular relative to the chamber wall¹¹⁴. These CMs then proliferate and expand to form the trabeculae^{115, 116, 117}. Rodent models show that CM 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)¹¹⁵⁻¹¹⁸. 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 appears to be markedly different from rodent models^{96, 119, 120; Han, 2016 #139}. Furthermore, trabeculation in zebrafish initiates through depolarization and delamination of a subset of CMs rather than altered polar orientation as is thought to occur in mouse¹²¹.

CM cell cycle exit during postnatal development

While CM proliferation is responsible for fetal heart growth, shortly after birth mammalian CMs largely exit the cell cycle. In adult hearts, there is measurable albeit very limited CM turnover¹²²⁻¹²⁵. By taking advantage of the spike in atmospheric C₁₄ that accompanied above ground nuclear bomb tests, Bergmann et al. showed that human CM turnover rate is about 1% per year at 20 years-of-age and gradually decreases with advancing age^{122, 125}. 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 mapping¹²³. CM proliferation extends into the first postnatal week in mice^{126, 127}. In humans, this postnatal proliferative window may last for several years¹²⁸, suggesting a potential window in infancy for therapeutic regenerative approaches in congenital heart disease. Several factors that trigger and enforce adult CM cell cycle exit have been identified. Mitogenic signaling pathways that drive fetal CM proliferation are attenuated postnatally. For example, expression of ERBB2 quickly decreases in CMs after birth, which

reduces the mitogenic potency of NRG1¹²⁹. The mitogenic activity of YAP is likewise restrained by multiple mechanisms, including activity of Hippo pathway kinases¹⁰¹, downregulation of YAP and TEAD1, and TEAD1 sequestration by the protein VGLL4¹⁰⁵. In addition to loss of pro-mitotic signals, several mechanisms actively inhibit expression and function of the cell cycle machinery in adult CMs. Transcriptional regulators such as MEIS homeobox 1 (MEIS1)¹³⁰ and retinoblastoma protein (RB)^{131, 132}, epigenetic modifiers such as polycomb repressive complex 2 (PRC2)¹³³, and microRNAs such as the miR-15 family¹³⁴ actively repress core cell-cycle activators and/or activate cell-cycle inhibitors. P38 MAP kinase signaling also inhibits CM proliferation^{135, 136}. Increased oxidative stress in post-mitotic CMs has been implicated in causing DNA damage response-mediated cell-cycle arrest¹³⁷. Downregulation of telomerase activity has been shown to block proliferation in a p21-dependent manner^{138, 139}. Disassembly of centrosomes, a hub of mitogenic signaling¹⁴⁰ as well as the organizing center for mitotic spindle assembly^{141, 142}, in postnatal CMs may add another layer of negative regulation of CM proliferation¹⁴³.

Heart regeneration through CM proliferation

Heart injury such as myocardial infarction causes massive CM death. In response, the rate of CM proliferation increases in the peri-infarct region by about 5-fold above its very low basal rate¹²³. However, this level of innate cardiac regeneration is far too limited to effectively replace the lost CMs. As a result, the remaining CMs become overburdened and experience chronically elevated biomechanical stress that induces further CM loss, precipitating a vicious cycle that ultimately leads to heart failure and pathological heart remodeling^{144, 145}. Interrupting or reversing this process requires replacing or regenerating the lost CMs.

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^{144, 146–148}. A key finding from the zebrafish system is that CMs lost in heart injury models are replaced by proliferation of pre-existing CMs, rather than by generation of CMs from non-myocytes^{149, 150}. Enhanced CM proliferation rate is observed in zebrafish hearts following several different types of injuries such as resection and genetic ablation^{149–151}, indicating a robust injury response mechanism is present in zebrafish that promotes CM proliferation. Regenerating CMs had unique morphological and molecular signatures consistent with sarcomere disassembly, which has been described as CM “dedifferentiation” (Figure 4). Sarcomere disassembly has been inferred to be a pre-requisite for productive mitosis and cytokinesis^{149, 150}. Thus CM proliferation provides the basis of heart regeneration in zebrafish.

CM proliferation also compensates for the damage or loss of CMs in fetal and neonatal mice. At fetal stages, CM-specific mosaic knockout of Hccs, an X-linked gene that is essential for mitochondrial function, damages ~50% CMs in E10.5 heterozygous females due to random X chromosome inactivation. However, this perturbation does not cause embryonic lethality because the healthy CMs proliferate to compensate for the injury. Consequently, only ~10% CMs are Hccs deficient at birth and over 85% of these animals develop and survive normally¹⁵². Similarly, we used diphtheria toxin A to ablate various

fractions of CMs in embryonic hearts. We found that embryos tolerated loss of up to 60% of CMs and maintain normal heart development by upregulating the proliferation of remaining unablated CMs¹⁵³.

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 CMs, resulting in hearts with minimal residual scar and with normal heart morphology and function by 1 month of life^{127, 134}. Although multiple groups have reproduced this result¹⁵⁴, confirmation has not been uniform¹⁵⁵, possibly because the extent of scarring and regeneration depends on surgical technique and the extent of myocardial resection.¹⁵⁶ Interestingly, macrophages are essential for robust neonatal cardiac regeneration¹⁵⁷. Lineage tracing showed that the proliferation of preexisting CMs also underlies murine heart regeneration^{123, 127}. CM “dedifferentiation”, characterized by sarcomere disassembly, has also been implicated in mammalian CM proliferation^{129, 134, 158–160} (Figure 4), although it has not yet been observed directly during in vivo mammalian CM proliferation. Thus induction of CM proliferation is a conserved mechanism of heart regeneration from zebrafish to mammals.

Enhancing cardiac regeneration by driving CM proliferation

The induction of CM 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 CM proliferation in mature mammalian hearts to achieve therapeutic cardiac regeneration after heart injury. Developmental CM 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 CM proliferation. Although many strategies are currently being pursued, due to space constraints here we will focus on NRG1 and Hippo/YAP. We refer readers to other excellent reviews that cover other efforts to stimulate CM proliferation^{161–164}.

Several studies have shown that activating NRG1/ERBB signaling boosts proliferation of adult CMs and improves heart repair upon injury. In zebrafish, overexpressing NRG1 in CMs was sufficient to stimulate CM proliferation, resulting in accelerated myocardial expansion after injury^{165, 166}. NRG1 treatment stimulated proliferation of *in vitro* cultured neonatal and adult murine CMs^{159, 167}, as well as CMs from myocardium removed from pediatric patients at the time of heart surgery¹⁶⁷. NRG1 delivery to mice, or over-expression of a constitutive active form of ErbB2, improved heart structure and function after myocardium infarction^{129, 159}. In patients with stable chronic heart failure, NRG1 infusion has been shown to be safe and to have beneficial haemodynamic effects¹⁶⁸. Although several studies have suggested that NRG1’s therapeutic effect is due in part to stimulation of adult CM proliferation, other studies have demonstrated that NRG1’s CM mitogenic activity quickly diminishes after birth^{129, 167}. Indeed, one group could not detect a mitogenic effect of NRG1 on adult CMs and assigned its salutary effects to other mechanisms¹⁶⁹.

Because YAP has robust CM mitogenic activity during fetal heart development, elevating YAP activity in CMs is another potential approach to stimulate CM proliferation and heart regeneration. Forced expression of constitutively active YAP in CMs stimulated CM

proliferation in both neonatal and adult hearts^{102, 104}. In the neonatal murine heart regeneration model, abolishing YAP activity disrupted the regenerative capacity of the neonatal murine heart, whereas YAP overexpression enhanced it¹⁶³. A study that used adeno-associated virus, a promising gene therapy vector, to selectively overexpress activated YAP in adult CMs, provided proof-of-concept that this treatment can improve myocardial function and survival after myocardial infarction¹⁰². 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 CM proliferation, including CM survival¹⁷⁰, migration^{119, 171} and calcium handling¹⁷². NRG1 also acts on non-myocytes to promote neo-vascularization after heart injury¹⁶⁵. YAP regulates CM survival^{107, 173} and transcriptional responses to mechanical stress^{174, 175}. In CMs, YAP promotes actin cytoskeleton remodeling and the formation of CM protrusions at heart injury sites¹⁷⁶. Recently, YAP was also implicated in controlling CM oxidative stress¹⁷⁷. Thus the beneficial effects of NRG1 and YAP activation in injured hearts are likely to be multifactorial.

Although stimulating CM 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 CM proliferation remains inefficient in adult hearts, likely due to multiple layers of negative regulation that block mature CM 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 CMs and design methods to remove or circumvent these obstacles. A second challenge is the potential for increased cancer risk due to cell cycle activation in non-CMs. For example, both NRG1 and Yap signals have oncogenic potentials and are known to be involved in cancers^{178, 179}. Precise targeting of mitogenic stimuli to CMs is necessary to minimize oncogenic risk.

The “dedifferentiation” of regenerating CMs has been observed in both zebrafish and mouse. This process, marked by sarcomere disassembly, occurs during regeneration in response to heart injury^{127, 134, 149} as well as during NRG1-stimulated myocardial regeneration^{129, 165}. These data imply the reversing CM differentiation state might stimulate CM proliferation, a strategy which 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 upon better understanding the “dedifferentiation” process. Although sarcomere disassembly has been suggested as a prerequisite for completion of CM mitosis^{158, 180}, it is unclear whether the “dedifferentiation” observed during regeneration is a true reversal of differentiation state or simply a change of cytoskeletal architecture of CMs undergoing mitosis. Second, whether dedifferentiation will necessarily enhance proliferation is also unclear. Although correlation between dedifferentiation and the increase of CM proliferation has been well documented^{127, 129, 134, 149, 165}, the causal relationship between these two processes has not been established.

How newly regenerated CMs 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^{96, 127, 153}. Given that CM proliferation plays an essential role in morphogenesis during development, it is hoped that the proliferation of regenerative CMs 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 CM proliferation, or to properly sculpt myocardium regenerated by other means (e.g. by delivery of stem cell-derived CMs).

CM maturation

Hallmarks of CM maturation

The dynamic process of heart development includes dramatic alterations in CM metabolism, form, and function, particularly in the neonatal period (Figure 5; Table 1). Fetal CMs actively proliferate, and the resulting increase in CM 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, CMs undergo dramatic changes linked to greater demand for left ventricular pump function and greater availability of oxygen. Postnatal CMs become highly specialized for efficient contraction: they shift to oxidative phosphorylation as their primary energy source, increase in volume by 30-40-fold between birth and adulthood, and develop accompanying ultrastructural specializations and changes in gene expression that enable efficient and coordinated CM contraction. Perhaps intimately linked with specialization for efficient contraction, postnatal CMs largely exit the cell cycle and become predominantly polyploid (a single polyploid nucleus in humans, or two diploid nuclei in rodents).

While much has been learned about the fetal specification of CMs, much less is known about how CM maturation is coordinately regulated. Understanding CM maturation is critical to advance the field of cardiac regeneration, since any new source of CMs 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 while significant numbers of human pluripotent stem cell derived CMs (PSC-CMs) could be engrafted in infarcted primate hearts, all treated hearts exhibited ventricular arrhythmias that were attributed to insufficient maturity of donor CMs²⁹. Immaturity of stem-cell derived CMs is also a major barrier to their use for in vitro modeling of human heart disease.¹⁸¹⁻¹⁸³ Thus discovering ways to induce CM maturation has become a major goal in the field. Progress will require developing a detailed understanding of the normal processes that drive developmental CM maturation. Here we review key aspects of postnatal CM 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 CM cytoarchitecture. However, three prominent hallmarks stand out: an increase in CM 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 CMs appear to be regulated by distinct types of mechanical loads, with CM elongation occurring through

addition of new sarcomere units at the cell poles in response to diastolic strain, and CM girth increasing through parallel addition of filaments in response to systolic strain.^{184, 185} In the disease state this process is mediated by ERK1/2 signaling, which likely also plays important roles in maturation-related remodeling.¹⁸⁶ These mechanisms are influenced by substrate stiffness, which together with additional cues make the ECM an important determinant of CM shape.^{187–189}

Myofibrillar density and organization directly correlate with CM maturation status, with fully mature CMs having superbly organized sarcomeres. Sarcomere organization is intimately linked to cell shape, as patterning CMs with micro-contact 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.¹⁹⁰ 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 (e.g. troponin I1 (TNNI1) to troponin I3 (TNNI3); Myh7 to Myh6) and altered gene splicing (e.g. titin (TTN)-N2BA to TTN-N2B; myomesin 1 embryonic isoform (EH-MYOM1) to mature isoform; troponin T2 (TNNT2) exon 5 inclusion to exclusion).^{191–195} T-tubules, invaginations of the plasma membrane that allow membrane depolarization to quickly penetrate to the CM interior, are a hallmark of mature CMs. Development of the murine T-tubule network initiates at approximately two weeks after birth, and similar to sarcomere development gradually becomes better defined and organized as the CMs mature to adulthood.^{196, 197} Currently, besides junctophilin 2 and Bin 1 (Table 1), few proteins involved in T-tubule formation have been identified, and the process is poorly understood.^{198, 199}

In addition to structural remodeling during maturation, neonatal CMs undergo dramatic alterations in metabolism, shifting from predominantly non-oxidative to oxidative metabolism in the neonatal period.²⁰⁰ This metabolic transition coincides with an increase in mitochondrial density, as well as a mitochondrial shift in shape from small and round to large and ovoid.^{201, 202} Positional differences are also observed, with mitochondria of mature CMs being located in closer proximity with myofibrils and the sarcoplasmic reticulum.²⁰³ While 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.^{204–207}

Many aspects of the structural and metabolic remodeling that takes place during CM 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 micro-array datasets identified stage specific gene regulatory networks,²⁰⁵ and future studies will determine if 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 signaling pathways constituting central components. Indeed, PPAR signaling acts as an activator of

fatty acid oxidation, and pathway activation increases as maturation proceeds.²⁰⁸ While this study succeeded in describing how the CM gene expression profile changes during maturation, proposed transcriptional regulators have not been functionally validated in vivo. This is a very difficult task, as global CM 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, due to this complication many maturation investigations have been limited to the study of neonatal rat CMs or PSC-CMs in an artificial culture environment. While this line of study has yielded some notable advances, such as the identification of Let-7 miRNA family members as positive regulators of maturation,²⁰⁹ 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 CM maturation can be dissected in vivo.²¹⁰ Mosaic gene knockout, achieved by administering a low dose of Cre-expressing adeno-associated virus serotype 9 that only transduced a fraction of CMs, enabled the study of mutant CMs in functionally healthy hearts. In this context, GATA4/6 double knockout CMs were dramatically smaller and less mature than control CMs, 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^{211, 212} offers an experimental strategy to dissect the regulatory networks that govern CM maturation.

Enhancing maturation of CMs differentiated from non-myocytes or pluripotent stem cells

Despite limitations in our understanding of the mechanisms driving CM maturation, many approaches to improving PSC-CM maturity have capitalized on knowledge of developmental paradigms. Approaches such as three 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.^{213–217} Indeed, just as tension-sensing mechanisms are currently generating considerable interest as determinants of how the heart remodels in response to disease,²¹⁸ similar mechano-sensing signaling pathways have been shown to play important roles in maturation related remodeling.^{219, 220} In addition, treatment with hormonal factors has also been shown to modulate CM maturation, with thyroid hormone being a major stimulant of fetal CM maturation.²²¹ Finally, long term culture of PSC-CMs has been shown to induce more complete maturation.²²² While expression profile analyses of CMs cultured for up to one month show arrest at or before a stage equivalent to the late fetal period,²⁰⁵ reportedly culture for approximately one year results in PSC-CMs with expression profiles similar to adult CMs.²⁰⁹ 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 CM maturation promises to enable further progress.

Interestingly, CMs differentiated from non-myocytes appear to undergo enhanced maturation within the native milieu of the beating heart. For example, when human PSC-CMs were injected into a non-human primate model of myocardial infarction²⁹. The injected

PSC-CMs engrafted and over three 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 mouse cardiomyocytes. On the other hand, transient ventricular arrhythmias were common early in the engraftment process and spontaneously resolved. The pro-arrhythmic effect may have resulted from the immaturity and heterogeneous electrical coupling of the engrafting PSC-CMs. Generalizing from this experience, pro-arrhythmia may be a consequence of many regenerative strategies that yield immature CMs, 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-CM to CM reprogramming, above). iCMs created by reprogramming in vitro often are highly immature in size, morphology, and functional parameters such as calcium handling⁹³. 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, 90}. Indeed, while the overall number of in vivo reprogrammed CMs 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, 90}. It is possible that in vivo reprogramming has additional benefits beyond cell autonomous gains in contractile ability. Translating this exciting work towards 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 to murine cells^{88, 223}. The enhanced maturity achieved by in vivo CM differentiation likely reflects limitations of current in vitro culture conditions and the overall importance of the cell's environment on CM differentiation and maturation.

Conclusions

By following the lessons of normal development, cardiac biologists have made rapid inroads towards 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

WTP was supported by grants from NIH (HL098166 and HL116461) and by a charitable contribution from Dr. and Mrs. Edwin A. Boger. SMW was supported by the NIH Office of Director's Pioneer Award LM012179, NIH/NHLBI U01 0099776, the Cardiovascular Institute, the Division of Cardiology, Department of Medicine, the Institute for Stem Cell Biology and Regenerative Medicine, and an endowed faculty scholar award from the Child Health Research Institute/Lucile Packard Foundation for Children at Stanford (S.M.W). SLP was supported by the Department of Pediatrics at Stanford, and FXG was supported by the Stanford Medical Scientist Training Program NIH T32 GM007365. NVD was supported by F32HL13423501.

Non-standard abbreviations and acronyms

CM	Cardiomyocyte
E	Embryonic day (days of gestation)
P	Postnatal day (days after birth)
FHF	First heart field
SHF	Second heart field
BMP	Bone morphogenic protein
WNT	wingless-type MMTV integration site family member
FGF	Fibroblast growth factor
miRNA	Micro-RNA
PSC-CM	pluripotent stem cell-derived cardiomyocyte

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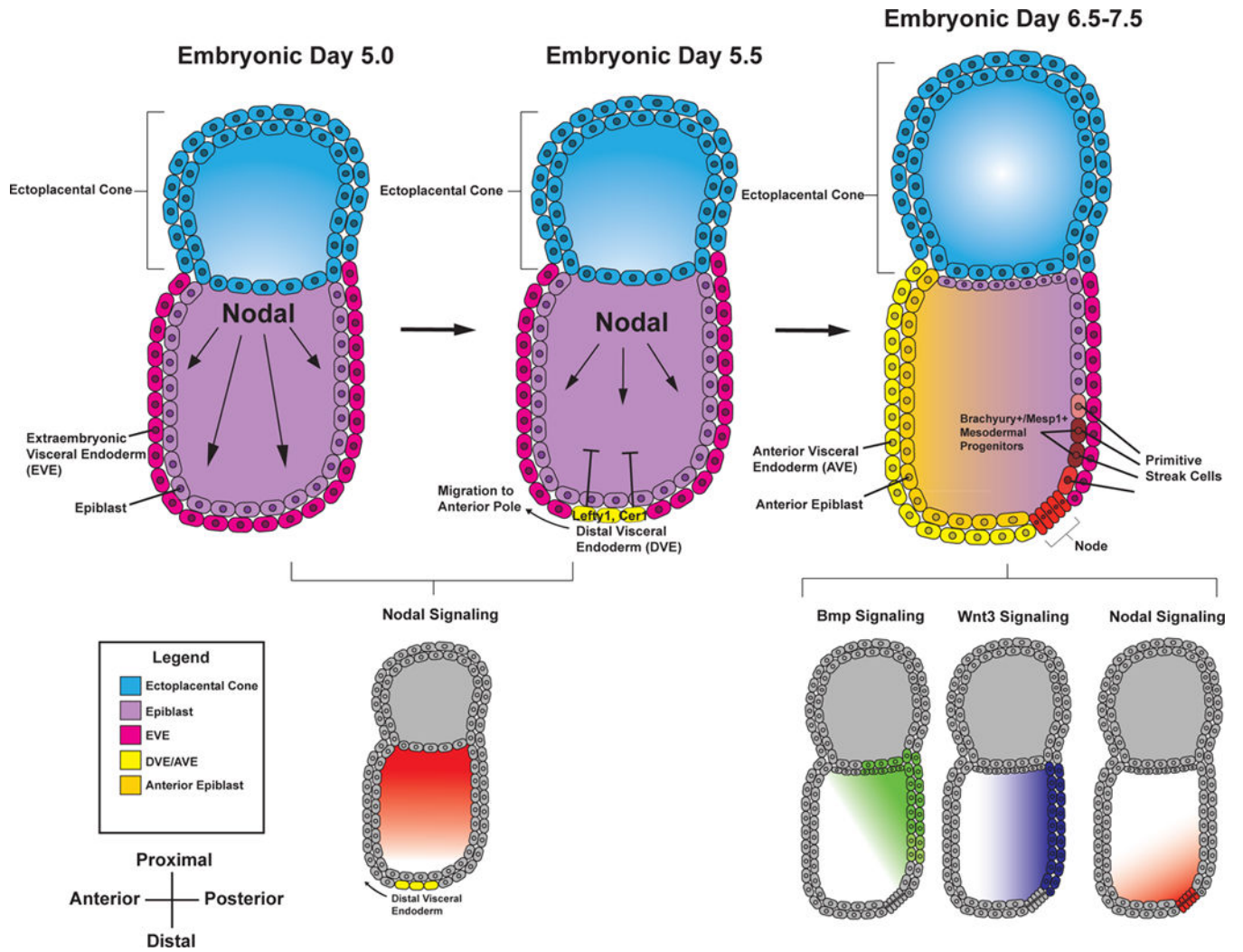


Figure 1. Specification of mesodermal precursors

Schematic representing the signaling events leading to mesodermal specification during early development. NODAL is first expressed proximally at E5.0. 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 E6.5-7.5. The anterior visceral endoderm (AVE, yellow) limits NODAL signaling to the posterior of the embryo. Along with WNT3 and BMP signaling, NODAL specifies early primitive streak progenitors to the mesoderm fate.

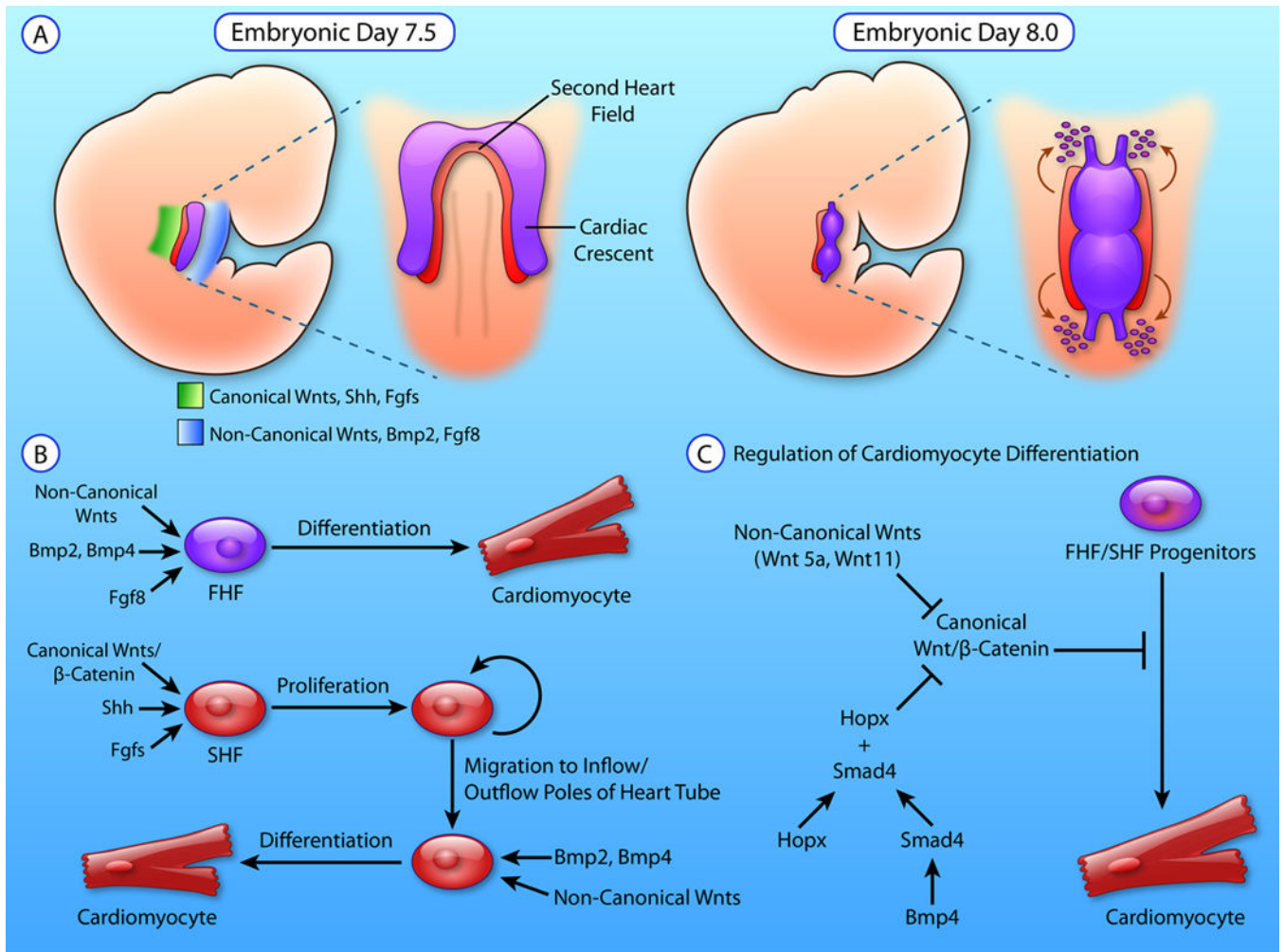


Figure 2. Regulation of cardiac progenitor proliferation and differentiation

A. Schematic showing the anterolateral position of first heart field (FHF) progenitors and dorsomedial position of second heart field (SHF) progenitors at E7.5. Canonical WNTs, Sonic Hedgehog (SHH), and fibroblast growth factors (FGFs) are expressed dorsally in the region encompassed by the SHF, while non-canonical WNTs, BMP2, and FGF8 are expressed ventrally, where the FHF is present. FHF progenitors make up the cardiac crescent and differentiate prior to the SHF in order to form the developing heart tube at E8.0. SHF maintain their proliferative state and elongate the heart tube by migrating and differentiating at the inflow and outflow poles of the heart. B. Non-canonical WNTs, BMP2/4, and FGF8 signaling drives FHF progenitors to differentiate towards 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 non-canonical 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 which binds to the transcription factor HOPX to directly inhibit Canonical WNT/ β -catenin. Moreover, non-

canonical WNTs such as WNT5a and WNT11 also inhibit Canonical WNT/ β -catenin to drive cardiac progenitor differentiation. (Illustration credit: Ben Smith)

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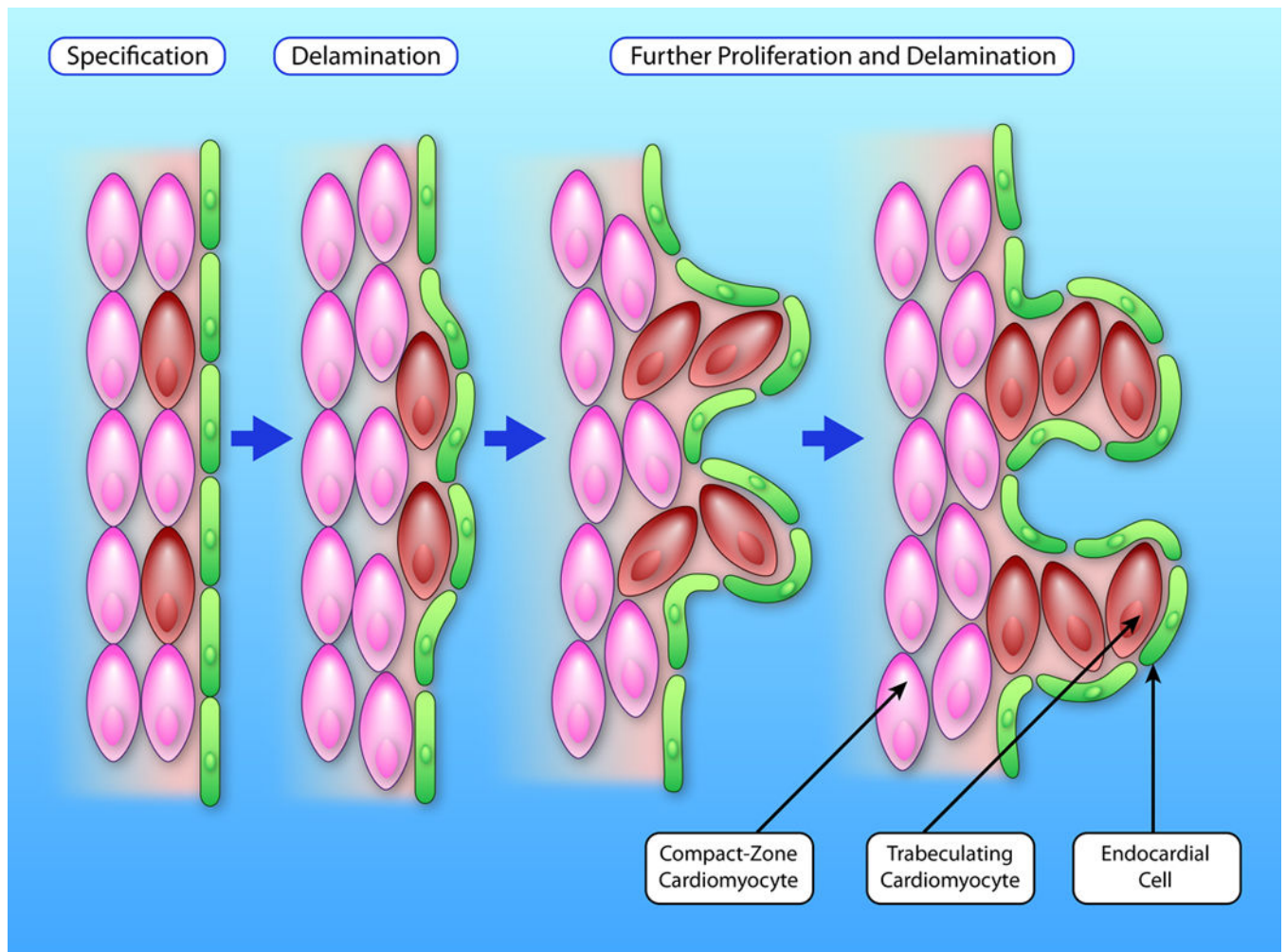


Figure 3. Model of the trabeculation process

During trabeculation, a small fraction of 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)

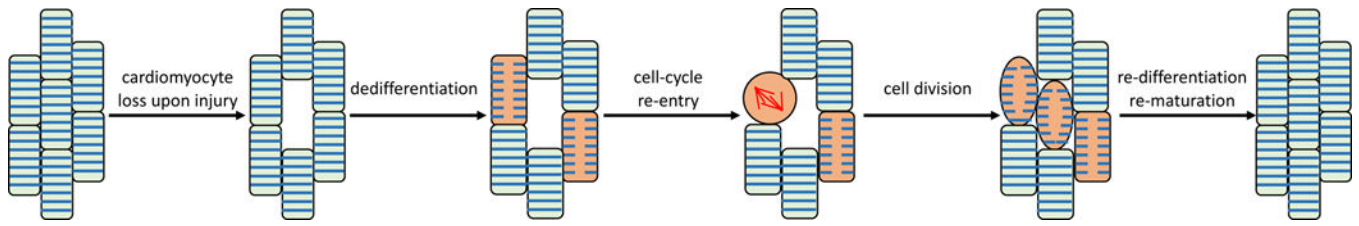


Figure 4. Model of cardiomyocyte regeneration

During heart regeneration, myocardial injury and 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 re-differentiate to a fully mature state to improve heart contraction.

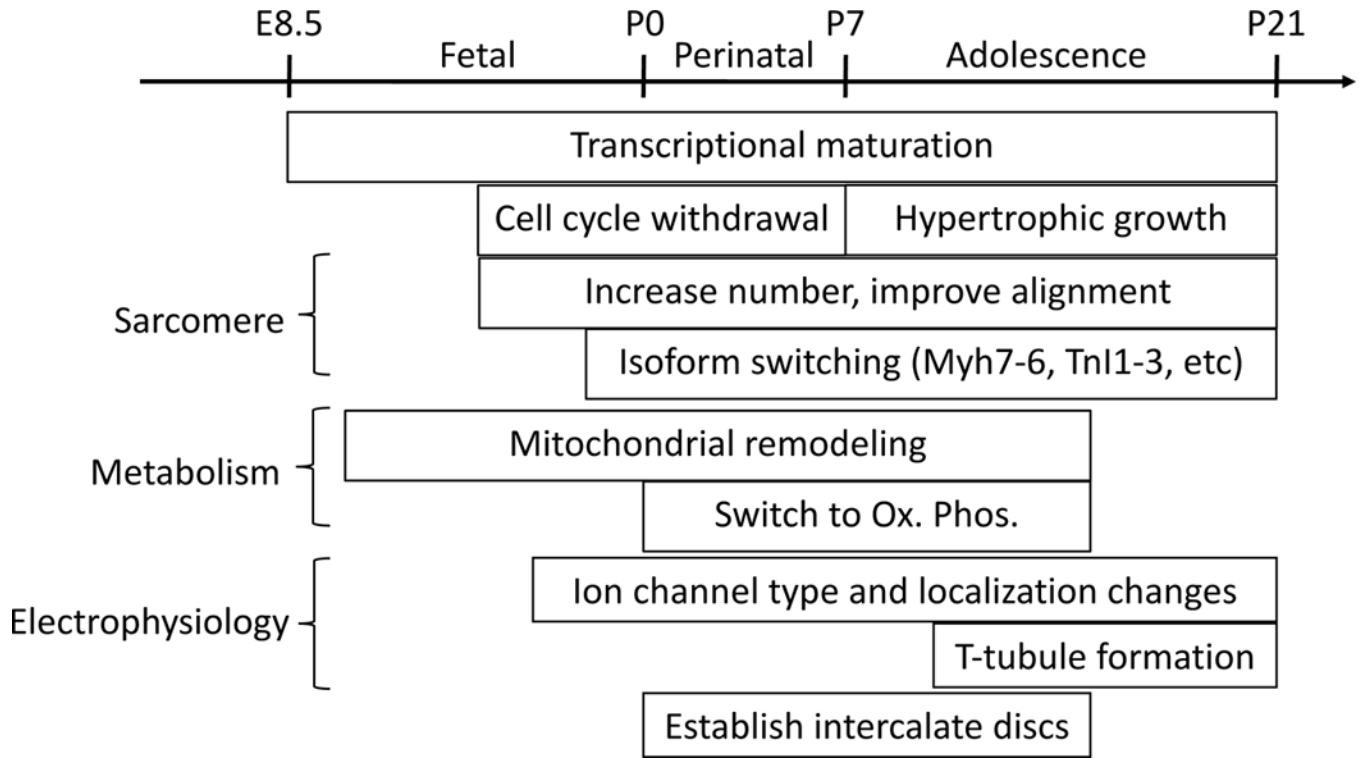


Figure 5. Representative cellular events during CM maturation

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 depending upon oxidative phosphorylation. The maturation of electrophysiologic 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 1

Hallmarks of CM Maturation.

Phenotype	Immature (late fetal)	Mature (Adult)	PSC-CMs	Regulators	References
Cell Size	small	large	small	GATA4/6	185, 210
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	184, 186, 190, 224
Sarcomere Alignment	organized	superbly organized	disorganized	cell shape, mechanical load, substrate stiffness	190, 216, 217
Sarcomere Components	TNNI1, MYH7, TTN-N2BA, EH-MYOM1, TNNI2 exon 5 inclusion	TNNI3, MYH6, TTN-N2B, TNNI2 exon 5 exclusion	variable	Thyroid hormone; maturation related transcriptional programs are currently not well understood	191-195, 221
Metabolism	glycolysis	oxidative phosphorylation	Glycolysis	PPAR signaling, greater oxygen availability after birth	200, 205, 208
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	201-203
Proliferative Capacity	high	very low	can proliferate for ~1 month after contraction begins	NRG1/ERBB2/4, YAP/TEAD, IGF2/IGFIR/PI3K/ERK, BMP10, WNT/β-catenin, NOTCH	101, 103, 110, 115, 116, 159
Nucleus	mononucleated	multinucleated or polyploid	mononucleated	Proliferation regulators above	101, 103, 110, 115, 116, 159
T-Tubules	none	extensive network	none	JPH2, BIN1	198, 199