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New Myocyte Formation in the Adult Heart: Endogenous Sources and Therapeutic Implications

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Abstract

Death of adult cardiac myocytes and supportive tissues resulting from cardiovascular diseases such as myocardial infarction (MI) is the proximal driver of pathological ventricular remodeling that often culminates in heart failure. After MI the heart may lose up to a billion myocytes[1], resulting in impaired pump function, persistent sympathetic neurohormonal activation, pathological hypertrophy of surviving myocytes, and replacement fibrosis[2]. Despite advances in the diagnosis and early treatment of acute MI, the lifetime risk and overall health burden from heart failure remain profoundly high[3]. This is partially because no currently available therapeutic, barring heart transplantation, can directly replenish myocytes lost from the injured heart. For decades, the field has struggled to define the intrinsic capacity and cellular sources for endogenous myocyte turnover in pursuing more innovative therapeutic strategies aimed at regenerating the injured heart. While controversy persists to this day as to the best therapeutic regenerative strategy to employ, a growing consensus has been reached that the very limited capacity for new myocyte formation in the adult mammalian heart is due to proliferation of existing cardiac myocytes, but not due to the activity of an endogenous progenitor cell source of some sort. Hence, future therapeutic approaches should take into consideration the fundamental biology of myocyte renewal in designing strategies to potentially replenish these cells from the injured heart.

Keywords

cardiac regeneration; myocardial infarction; stem cell; proliferation

Subject Terms

Myocardial Biology; Myocardial Regeneration; Stem Cells

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New Myocyte Formation Across Development, Homeostasis, and Injury

Myocyte formation in the adult non-mammalian heart

Studies in amphibians and teleost fish such as the zebrafish were among the first to demonstrate experimentally that the postnatal heart could generate new myocytes[4, 5]. In adult zebrafish, surgical resection of up to 20% of the ventricle resulted in the formation of a transient fibrin-rich clot, followed by a robust increase in cycling myocytes as indicated by BrdU incorporation[6]. A similar response has been documented in amphibians such as the newt[7] and the axolotl[8]. The expansion of new myocytes following partial resection of adult zebrafish or amphibian hearts can result in virtually complete structural and functional recovery of the ventricle, although some studies have demonstrated that this regenerative response is inadequate in the face of a more severe injury[9]. Genetic fate mapping studies in the zebrafish have concluded that pre-existing myocytes are the predominant cellular source for new myocyte formation in non-mammalian hearts[10, 11]. However, the precise mechanisms governing the generation of new myocytes in these systems have not been fully delineated and may involve direct cell cycle re-entry of differentiated myocytes[10], dedifferentiation to a more plastic state which precedes proliferation[12, 13], or even cellular fusion[14]. Determining the biological distinctions underlying the profound cardiac regenerative capacity in select non-mammalian species, as opposed to the limited capacity observed in mammalian hearts, is an area of active investigation[15, 16]. Perhaps most striking, fundamental differences in cardiovascular physiology, tissue oxygen content, or growth kinetics, which are considerable across amphibians, fish, and mammals, may not fully explain disparities in regeneration. For example, recent studies comparing zebrafish to a related freshwater teleost fish, the medaka, have revealed a near-complete loss of cardiac regenerative capacity in the latter[17, 18], suggesting that an even more subtle mechanism may be involved.

Myocyte formation in the fetal mammalian heart

Cardiomyogenesis during mammalian heart development is initiated with the commitment of cardiogenic precursor cells derived from the embryonic mesoderm[19, 20], which themselves are specified through a series of paracrine and autocrine gene regulatory signals culminating in activation of the transcription factor Mesp1[21]. During the early stages of heart development, Mesp1+ mesodermal progenitors are further specified into cardiac progenitor cells of the first and second heart fields[19, 22–24]. These committed cardiac progenitors undergo rapid proliferation and differentiation to generate and expand the early heart tube[25, 26]. Coordination of this process is tightly regulated by several key cardiogenic transcription factors including Nkx2.5, Tbx5, and Gata4[20, 27]. In terms of proliferative capacity, fate mapping and BrdU pulse-label studies suggest that cardiac progenitors are more proliferative than the early cardiac myocytes they give rise to[28, 29]; however, development of the early linear heart tube into the four-chambered heart is driven by substantial proliferation of differentiated myocytes[30, 31]. This second wave of proliferation from differentiated myocytes is essential for expansion of the ventricular chambers and establishment of the cardiac trabeculae[32], consistent with the increase in myocyte proliferation that drives chamber formation during development of the zebrafish heart[15].

Genetic manipulations in mice have revealed the extensive capacity of the fetal mammalian heart for new myocyte generation. In one study, mice with partial myocyte loss during development were generated via *Nkx2.5*-Cre-driven excision of holocytochrome c synthase (Hccs)[33]. Hcss is an X-linked gene required for mitochondrial function and cell viability. By taking advantage of random X chromosome inactivation, this model resulted in a roughly 50% loss of cardiac myocytes in a cell-autonomous fashion in females carrying *Nkx2.5*-Cre and one loxP targeted Hccs allele. Male mice of this same genotype were not viable beyond embryonic day 14.5. By coupling this genetic system with a dual reporter strategy that allowed for differential fate mapping of X-inactivated cells with either LacZ or GFP, this study demonstrated a compensatory 27% increase in proliferation in the remaining myocytes that were spared via X-inactivation of the Hccs floxed allele, which allowed the majority of these animals to develop normally into adulthood[33]. A more recent approach used *Nkx2.5*-Cre-driven diphtheria toxin coupled with blastocyst complementation to generate a series of mouse lines with quantifiable embryonic myocyte ablation ranging from 10% to roughly 95%[34]. This study demonstrated that mice depleted for up to 60% of myocytes during embryogenesis could survive into adulthood with no overt cardiac phenotype, by rapidly upregulating proliferation of the remaining myocytes. Thus, the embryonic mammalian heart retains a remarkable degree of plasticity, driven by myocyte cell cycle activity[35].

Myocyte formation in the neonatal mammalian heart

During the first days of postnatal life, the mammalian heart undergoes substantial growth and physiological remodeling to accommodate an increased demand for cardiac output. Hypertrophic growth of cardiac myocytes is the predominant effector in this process[36] but additional, temporally restricted myocyte hyperplasia also plays a role. In rodents, myocyte cell volume increases more than two-fold within the first week of postnatal life[37] with concomitant increases in myocyte DNA synthesis rates and myocyte binucleation that peak by postnatal day 7[30]. Estimates of the amount of myocyte proliferation and the absolute increase in myocyte number that occurs during this period can vary, largely due to technical issues related to identifying proliferation events in vivo from histological sections. Using thymidine analog (BrdU or EdU)-based detection methods, several investigators reported rates of 5–10% of myocytes undergoing active DNA synthesis within postnatal days 3–4 in rodent hearts, which rapidly declined to ~1% after this point[38]. More recent studies have examined isolated myocyte nuclei by flow cytometry[39, 40] and have documented largely comparable rates. These rates are also in agreement with data obtained using quantitative non-radioactive multi-isotope mass spectrometry to track cellular turnover of myocytes using ^{15}N-thymidine[41] which demonstrated a 1% myocyte turnover rate in the postnatal day 4 mouse heart. Collectively these results suggest that postnatally, new myocyte formation is highest within the first four days after birth but then rapidly declines as cells enter mitotic arrest and terminal differentiation around postnatal day 7[42]. Early postnatal cardiomyogenesis also differs between the left and right ventricle, as chamber-specific rapid proliferation is responsible for the marked expansion of left ventricular myocardium in mammals soon after birth[43–45]. Differences in regional signaling cues likely underlie the preferential induction of proliferation in the left versus right ventricles; for example, loss of Fgf10 results in myocyte hypoplasia only in the right ventricle[46]. This chamber-specific distinction in myocyte proliferative capacity or responsiveness to proliferative signals may

also play a role in the ability of the left ventricle to regenerate following injury in neonatal mammals, which we discuss next.

Some of the strongest evidence for myocyte proliferation in the postnatal heart comes from models of cardiac injury in mice within the first week after birth. Surgical resection of the left ventricular apex in 1 day-old neonates resulted in a robust upregulation of myocyte proliferation and near complete regeneration of the injury site by day 21 [47]. This enhanced regenerative capacity of p1 neonatal mouse hearts was also observed by multiple different investigators using coronary artery ligation-induced MI[48, 49] or cryoinjury[50, 51]. However, other groups have reported a limited or completely absent capacity for neonatal heart regeneration in mice[52, 53]. Technical variations in surgical technique or mode of injury have been suggested as reasons for the negative results[54–56], thus ongoing efforts to standardize experimental models of neonatal heart injury will be needed, although the positive results reported would appear to be undeniable and indicate that regeneration is indeed observed in the postnatal mouse heart with the greatest capacity immediately after birth [57–59].

A second round of increased myocyte cell cycle activity and proliferation has been described in the postnatal mouse heart, occurring between postnatal days 10–14 through a burst of thyroid hormone signaling, and accounting for almost a doubling in myocyte number[60, 61]. However, attempts to replicate this finding using either nuclear LacZ-targeted transgenic mice, or radiolabeled thymidine uptake failed[62, 63]. In contrast, a marked increase in myocyte DNA content was observed during this time[62], but this was instead associated with an increase in ploidy. A recently developed lineage tracing system to fate map cycling myocytes in the mouse also did not demonstrate an increase in proliferative cells at postnatal day 15 [64]. Thus, reactivation of myocyte DNA synthesis pathways may be occurring during the second week of postnatal growth in the mouse, but this appears to be unlikely to reflect myocyte cell division leading to new myocyte formation.

Myocyte formation in the adult mammalian heart

As previously discussed, significant myocyte cell cycle withdrawal occurs by the first week of postnatal life in the mammalian heart, concurrent with an increase in the number of binucleated myocytes due to a final round of karyokinesis without cytokinesis, at least in rodent models[42]. As a result, new myocyte formation during adulthood is highly limited. Multi-isotope mass spectrometry showed a frequency of 0.015% ^{15}N-thymidine labeled cardiac myocyte nuclei in young adult mice over an eight-week period[41], consistent with previous reports that demonstrated a sharp decline in myocyte DNA synthesis during postnatal life[30, 38]. Myocardial infarction (MI) injury resulted in a significant increase in labeled myocytes. However, only a rare fraction of these showed evidence for completion of cytokinesis, demonstrating that cardiac myocyte DNA synthesis and true cellular division are not directly correlative, and that endogenous cardiomyogenesis in the adult heart post-MI remains extremely modest. Advances in genetic lineage tracing systems have also allowed for more precise quantitation of myocyte turnover and dynamics in the mouse heart under homeostatic conditions and with aging. A 2014 report used a Cre-dependent dual GFP or RFP labeling system to distinguish myocytes resulting from complete cytokinesis versus binucleation[65]. Combining this approach with an inducible *Myh6*-driven Cre recombinase

demonstrated that approximately 0.01%–0.02% adult myocyte renewal could be observed in adult mice. Of note, this study did not identify an appreciable increase in new myocytes following MI, in contrast to other reports with alternative fate mapping approaches that have observed an increase in new myocytes derived from pre-existing myocytes in the peri-infarct zone after MI[41]. Collectively, these studies demonstrate a detectable but considerably reduced capacity for new myocyte formation in adult mammalian hearts soon after birth. Strategies to enhance the limited capacity of myocyte derived new myocyte formation should be pursued as a therapeutic strategy aimed at improving the function of the injured heart.

Myocyte formation in the healthy and diseased human heart

Evidence for myocyte turnover in human hearts during aging or after disease stimuli comes primarily from retrospective analyses. However recent novel approaches have provided considerable insight. Specifically, a landmark series of studies took advantage of pre-existing ^{14}C incorporation found in postmortem heart samples from subjects living during the sharp rise in atmospheric ^{14}C associated with nuclear bomb testing from the early 1950s until 1963[66, 67]. Mathematical modeling using levels of ^{14}C incorporation in isolated human myocyte nuclei demonstrated a maximal turnover rate of about 1% myocyte DNA per year in non-diseased adult human hearts, which declines by more than half with advancing age[66]. Moreover, juvenile and early adolescent hearts showed the largest annual rates of new myocyte DNA labeling, reaching a peak of 5% per year, which sharply declined after 10 years of age[67]. However, this approach only indirectly measures cellular turnover and does not detect or report actual cytokinesis, so it remains likely that the true rates of new myocyte formation in the human heart are lower. Moreover, these rates of cardiac myocyte formation via ^{14}C incorporation are highly dependent on assumptions and parameters used in the mathematical models. Independent analysis of these data has suggested that variations in parameters such as atmospheric ^{14}C levels or myocyte attrition rates can confound the interpretation of human cardiomyogenesis via ^{14}C incorporation[68]. The modeling approaches using ^{14}C incorporation also concluded that new myocyte formation ceases in the early postnatal human heart, whereas other studies have documented indices of myocyte proliferation in young human heart tissues[69], albeit only at the post-mortem histological level. Despite these caveats, Cold War-era ^{14}C incorporation does provide compelling evidence that the adult human heart retains a finite but detectable degree of cardiac myocyte DNA turnover throughout postnatal life, and that this rate is highest early after birth and during early adolescence, with a sharp decline thereafter and a continual decrease with advanced age. These findings are in accordance with experimental data from mammalian model systems in which actual cytokinesis can also be evaluated.

Evidence also supports the potential for an increase in endogenous cardiac myocyte turnover in certain human disease contexts. A small cohort of patients with advanced heart failure who received left ventricular assist devices showed significant increases in myocytes positive for histological markers of cell cycle activation and cytokinesis[70]. Clinical presentations in newborns with severe cardiac damage, perinatal MI, or genetic cardiomyopathies have also demonstrated evidence for myocyte cell cycle re-entry[71] and in some cases complete structural and functional recovery[72, 73], suggesting that the neonatal

human heart may retain some inherent cardiac plasticity, as observed in small animal models.

Endogenous Sources of New Myocytes in the Adult Mammalian Heart

The longstanding historical view of the adult heart as a terminally differentiated organ lacking considerable myocyte turnover[74] was challenged over the last 15 years with reports of profound intrinsic regenerative capacity via resident cardiac stem cells. However, based on the sum of available data as of 2018 the original historical model appears to have been correct all along; that new myocyte formation in the adult heart is very limited. After many studies in animal models and more recent analyses of human tissue samples, the consensus view in the field is that new myocyte formation in healthy adult hearts occurs at a rate around 0.5%–1% per year[75]. Here we review the available evidence that addresses the potential cell sources for cardiac myocyte turnover; either proliferation of pre-existing myocytes, or differentiation from a non-myocyte cell type.

In vivo evidence for endogenous myocyte proliferation in mammalian hearts

Much of the foundational work for assessing cardiac myocyte proliferation was based on measures of mitotic figures and DNA synthesis in rodent hearts[30, 38, 39, 76, 77]. These studies suggested that myocytes were themselves the source of identified cycling cells, but only the adoption of genetic lineage tracing models allowed the field to definitively address this question. Pulse-labeling of pre-existing myocytes with a GFP reporter using the αMHC-driven MerCreMer demonstrated that the proportion of GFP^+ labeled myocytes did not change over physiological aging out to one year[78], suggesting that a non-myocyte (GFP^-) cell source did not replenish myocytes. An extensive follow-up study from the same group combined this αMHC-driven lineage tracing model with mass cytometry of labeled thymidine to track cycling cells in a highly sensitive way, which showed an annual myocyte turnover rate in the mouse of 0.76% under physiological conditions[41], a rate consistent with total myocyte DNA turnover in humans[66, 67]. Of note, these thymidine-labeled cycling myocytes were GFP^+, having been previously labeled by αMHC-MerCreMer lineage tracing, indicating they were pre-existing myocytes. A more recent report has also used this thymidine incorporation and mass cytometry protocol to track new myocyte formation after prolonged voluntary exercise in mice[79]. Voluntary exercise was associated with a striking 4-fold increase in thymidine incorporation and an increase in mononuclear diploid myocytes. Of note, the determination of myocyte ploidy in this study was limited to in situ hybridization labeling of serial tissue sections, so future confirmatory experiments will be needed to directly assess the extent that increased DNA synthesis after exercise reflects new myocyte formation. Additionally, a lineage tracing study that coupled inducible Cre recombinase to a hypoxia-regulated element of Hif-1α found that myocytes under local tissue hypoxia preferentially re-enter the cell cycle and undergo proliferation[80], while a newly developed cell-cycle-specific reporter system in the mouse heart also demonstrated early postnatal myocyte proliferative capacity in vivo[64]. Taken together these findings are consistent with numerous studies demonstrating that the hearts of zebrafish[6, 10] and other lower vertebrates[8] as well as neonatal rodent hearts[34, 47] all are repopulated with new myocytes via proliferation of pre-existing myocytes after various injuries.

Isolation of human myocytes from transplant or cadaveric hearts demonstrated the presence of mitotic markers, as well as markers of cytokinesis, which correlated with patient age[69]. Specifically, myocytes showing phosphorylation of histone H3 and assembly of the contractile ring required for cytokinesis were most frequently present in patients less than one year of age (roughly 0.01–0.02%), while myocytes from patients after age 20 showed rates roughly half that (0.005%), which was estimated in this same study to reflect an approximate yearly myocyte turnover of 1.9% in human hearts ranging from ages 19 to 23[69]. An age-dependent decrease in myocyte proliferation is also in agreement with retrospective carbon dating studies, which have demonstrated that postnatal myocyte turnover in humans is highest soon after birth and precipitously diminishes with age[66, 67]. In line with this, individual case studies in human neonates have also documented histological indices suggestive of myocytes going through the cell cycle[71, 73].

In vivo evidence for myocyte differentiation from an endogenous non-myocyte cell type in mammalian hearts

In the early 2000s, studies reporting the presence of endogenous cardiac stem cells in adult hearts that could generate de novo myocytes[81–83] transformed the field and quickly led to clinical application. Follow-up studies would report that cardiac stem cells, identified predominantly by surface marker expression of the receptor tyrosine kinase c-Kit[84] or stem cell antigen-1 (Sca-1[85]), or side population (SP) phenotype cells[85, 86], could robustly generate new myocardium when isolated, expanded in culture, and delivered into infarcted hearts in rodents. However, if the adult heart contained biologically active endogenous stem cells with cardiomyogenic capacity, it stands to reason that these cells could act in vivo without culture manipulation to replenish the cardiac myocyte pool after injury, as is the case for other known adult stem cells and their respective tissues of residence[87]. We have previously discussed at length the limitations of using cells in culture as a surrogate for the in vivo biological activity of those cells in their native environment[88, 89]. Thus, here we focus on the in vivo evidence that the adult mammalian heart does (or does not) contain an endogenous stem cell that contributes to new myocyte formation.

One of the initial observations that led to the hypothesis of myocyte formation from non-myocytes was the presence of sex-mismatched myocyte nuclei in human hearts following heart transplant. A study published in 2002 analyzed post-mortem heart tissue from male patients who had received a female donor heart and found that up to 10% of the cardiac myocytes and vascular cells in these patients carried the Y chromosome, suggesting that they were newly formed cells derived from the patient after transplant[90]. Similar analyses were carried out by other investigators, but these studies showed rates of myocyte chimerism of less than 0.05%[91, 92] or none at all[93]. Nonetheless, the conclusion made from these studies was that non-myocyte cell types derived from outside the heart had migrated into the tissue and differentiated into new myocytes. The presumption that these were stem cells was based largely on the immunohistochemical detection of surface markers such as c-Kit and Sca-1, also present on hematopoietic stem cells[90], although Sca-1 is a murine gene with no known human homologue[94], making it uncertain which cell types in the human heart were detected using a Sca-1 antibody in that previous study. However, a more fundamental limitation of these human studies was the inability to rule out heterotypic cell fusion as the

source of these chimeric myocytes. Studies in mice that received myocardial infarction demonstrated that circulating immune cells as well as endothelial cells or skeletal muscle myoblasts could fuse with pre-existing cardiac myocytes in vivo[95, 96]. A genetic approach was also used to label mouse or hamster donor hearts with nuclear LacZ in a xenograft model of heterotopic heart transplant into GFP^+ transgenic rats[97]. demonstrating that donor cell myocyte nuclei were due to fusion of circulating cells with a pre-existing recipient myocyte. Thus, the presence of sex-mismatched myocyte nuclei following heart transplantation, which is exceptionally rare, is not an indicator of new myocyte formation from a non-myocyte stem cell.

Following these initial descriptions, several studies performed in the setting of myocardial injury documented myocyte proliferation events that may have arisen from a non-myocyte source[78, 84, 98]. Based on this and a series of papers demonstrating cardiomyogenic potential of cultured c-Kit cells using cellular transplantation, a conclusion was drawn that endogenous c-Kit cells underlie the turnover of myocytes observed under physiological conditions[99]. However, there was no direct evidence documenting the origin of these new myocytes as derived from c-Kit stem cells until the development of genetic fate mapping tools to track the contribution of endogenous c-Kit cells in the heart. One of the first approaches, using transgenic mice containing a bacterial artificial chromosome (BAC) system to drive eGFP in *Kit* expressing cells, demonstrated a limited degree of cardiomyogenesis from c-Kit cells only in the neonatal heart[50, 100], but this system was limited by the inability to permanently mark *Kit* expressing cells and the artificial nature of the transgenic approach in faithfully recapitulating endogenous *Kit* allele gene expression. Thus, several groups independently generated knock-in mice with Cre recombinase under the control of the endogenous *Kit* promoter, which were used with Cre-dependent reporter alleles to irreversibly mark c-Kit expressing cells and their progeny with genetically encoded fluorescent indicator proteins[101–103]. In agreement with prior reports using the BAC approach, these studies demonstrated a modest but detectable contribution of c-Kit cells to cardiomyogenesis during heart development and in the neonatal heart. However, in the adult heart these studies all demonstrated that the cardiac myocyte contribution from endogenous c-Kit cells remained less than 0.03% during physiological growth, aging, or after injury by myocardial infarction or isoproterenol infusion[101–104]. Moreover, up to 80% of this already low myocyte contribution was not from c-Kit cell differentiation but the result of cell fusion between existing myocytes and circulating hematopoietic cells from bone marrow-derived c-Kit cells[105, 106]. As a result, the field at large has concluded that c-Kit cells are not a physiological source of new myocyte formation in the adult heart[75].

Although cells marked by c-Kit are not cardiac myocyte forming stem cells[107], other purported stem cell classes remain less defined at the present day. One recent study performed lineage tracing of Sca-1 cells in mice using a partial Sca-1 (gene name *Ly6a*) promoter fragment[108]. This study observed that roughly 2–4% of all Sca-1 lineage-traced cells in the adult heart were myocytes. The conclusion drawn was that at least a few percent of myocytes were replenished from a Sca-1-derived source throughout aging, although this would be above the consensus myocyte turnover rate of maximally 1% per year documented by numerous approaches as discussed above. An alternate explanation of these findings is that the *Ly6a* promoter fragment that was employed does not accurately recapitulate

endogenous *Ly6a* gene expression. Indeed, the use of a transgenic line that employed a partial promoter construct to track c-Kit cells also precluded definitive analysis of endogenous c-Kit cell fates because such constructs do not contain the full sequence of gene regulatory elements and are often ectopically expressed in other cell types[109] including differentiated myocytes[110]. Even the more rigorous “knock-in” lineage tracing strategy introduces heterozygosity of the targeted allele, which might produce confounding effects[110]. For example, a recent report argued that *Kit* allele heterozygosity due to introduction of Cre impairs ex vivo functionality and/or in vivo labeling efficiency of c-Kit resident cardiac stem cells[111]. Genetic lineage tracing also depends on the chosen marker gene not being expressed in other differentiated cell types, which is not always the case. For example, ABCG2, the ATP-binding cassette transporter that confers the side population (SP) phenotype to another population of cells with purported cardiomyogenic capacity[83, 85, 86], is also expressed in fully differentiated adult cardiac myocytes[112].

In light of these concerns, a new series of genetic tools was developed to allow for fate mapping and quantitation of cardiomyogenesis from any non-myocyte cell sources, without the need for marker-specific lineage tracing[113, 114]. This model used four distinct mouse models and combined Cre-lox and Dre-rox dual recombinases with interleaved cell fate reporters to irreversibly and differentially label all pre-existing cardiac myocytes, as well as all cardiac non-myocytes with a different fluorescent protein. This approach demonstrated that non-myocytes of any source do not contribute to the new myocyte formation in the adult heart at baseline or after infarction injury[114]. Importantly, none of the four models used in this paper relied on targeted “knock-ins” for any of the stem cell marker genes, eliminating concerns of *Kit* allele heterozygosity raised previously. This study also demonstrated that differentiated non-myocyte cell types, such as epicardial cells, fibroblasts or endothelial cells, do not transdifferentiate into cardiac myocytes in vivo, which had been previously proposed[115, 116]. The results of Zhou and colleagues [113, 114] are in support of previous lineage tracing studies that did not observe cardiomyogenesis or transdifferentiation from differentiated non-myocyte cell sources in vivo[117–119].

Thus, more than 15 years after the initial reports of endogenous cardiac stem cells, rigorous genetic lineage tracing efforts from numerous investigators have demonstrated that non-myocyte cell types have essentially no contribution to endogenous myocyte turnover in the adult mammalian heart[107]. Proliferation from pre-existing myocytes is the mechanism by which the adult heart appears to replace new myocytes under homeostatic conditions and diseased conditions, albeit at very low levels[75]. While we are certain that the controversy will continue for years to come and more papers will be published suggesting the existence of some “flavor” of adult stem cell that can repair the heart, we believe that the rigorous lineage tracing results discussed above, especially the definitive approach of Zhou and colleagues,[113, 114], are collectively indisputable and definitely show that the heart is not repaired by any sort of progenitor cell, and that the adult mammalian heart simply lacks a myocyte producing stem cell.

Therapeutically Augmenting New Myocyte Formation in Adult Mammalian Hearts

The consensus rate of 1% myocyte DNA synthesis in the adult mammalian heart each year is several orders of magnitude too small to produce sufficient numbers of new myocytes and replace those lost by injury and thereby impart functional benefit [2, 120]. Cell therapy was originally proposed as a solution to this limitation, as transplanted adult stem cells were at first proposed to engraft, differentiate into myocytes and thus repopulate the infarcted heart with new myocardium[99]. However, the functional benefits observed following transplant of most adult cell types has now been attributed to indirect mechanisms and not de novo cardiomyogenesis[121]. Several of these mechanisms, such as revascularization, amelioration of fibrosis, or release of cardioprotective paracrine factors have been extensively reviewed previously[121–123]. Thus, here we will discuss strategies that have been proposed specifically with the goal of increasing total myocyte numbers in the injured heart through one or more cellular mechanisms. We also assess the potential advantages for each strategy in terms of therapeutic potential, as well as the inherent limitations and risks of each (Figure).

Reactivation or enhancement of endogenous myocyte cell cycle activity

The sharp decline in proliferative capacity in the postnatal heart has been directly correlated to downregulation and repression of cell cycle regulators in myocytes[42, 124]. However, adult cardiac myocytes do retain some capacity for DNA synthesis and to a lesser extent, cytokinesis. Therefore, one major avenue that has been pursued for potential therapeutic application is the reactivation of myocyte cell cycle activity in the injured adult heart (Figure). In terms of in vivo feasibility, mouse models that employed over-expression of known proliferative genes, or direct cell cycle regulators, laid much of the groundwork. An early study using transgenic over-expression of the oncogenic transcription factor c-myc in the heart demonstrated approximately a 40% increase in myocardial mass and a doubling of both myocyte DNA content and absolute myocyte numbers[125]. Likewise, transgenic expression of the SV40 large T-antigen in mice also produced a profound hyperplasia of atrial[126] or ventricular[127] myocytes in vivo. While such results established that adult myocytes could be coaxed back into cell cycle, use of cell cycle genes to mediate such events also produces the potential for tumorigeicity[128]. Moreover, induction of cell cycle activity in this way was not purely a proliferative signal, as additional studies of c-myc over-expression in adult hearts demonstrated myocyte hypertrophy and multinucleation instead of overt proliferation[129], suggesting that the hyperplasia seen in earlier models was predominantly via enhanced cell cycle activity during development or early postnatal growth. As a result, efforts shifted towards a more systematic induction of cell cycle checkpoint pathways to achieve adult myocyte proliferation. Using viral-mediated gene delivery, over-expression of the transcription factor E2F1, which governs the G1/S cell cycle transition, was shown to drive adult myocytes into S-phase and promote myocyte DNA synthesis in vivo[130]. However, E2F1 induction also caused rapid myocyte apoptosis and early-onset lethality. In contrast, this was not observed with overexpression of E2F2 by viral-mediated gene delivery[131], suggesting that the cell cycle regulator chosen, and the mode of overexpression, are important points of control. In line with this, another study using transgenic over-expression of cyclin D1 demonstrated a 41% increase in cardiac mass

and a sustained increase in DNA synthesis as measured by thymidine incorporation assays, without lethality[132]. Similarly, over-expression of CDK2 enhanced total cardiac mass in neonatal mouse hearts but resulted in a greater percentage of small, mononucleated myocytes[133]. Numerous additional studies over the last decade have provided confirmatory evidence that adult myocytes can re-enter cell cycle upon introduction of cell cycle activators using a transgenic approach in the mouse[134, 135] (also summarized in [42, 136]). More recently, combinatorial approaches by introducing multiple regulators simultaneously have demonstrated that synergistic increases in myocyte cell cycle activity are achievable, which may provide further benefit[137]. However, a key observation drawn from these studies is that enhancing cell cycle activity in adult myocytes is not a direct surrogate for new myocyte formation. In many cases, enhanced DNA synthesis results in myocyte hypertrophy or multinucleation without complete cell division. An ongoing challenge in the field is to decipher the molecular roadblocks that prevent cellular division even in the context of enhanced cell cycle activity. For this, multi-step manipulation of several distinct but convergent pathways may be required, to both enhance myocyte cycling and facilitate cytokinesis.

Modulation of endogenous signaling pathways governing myocyte growth and division

During embryonic development and postnatal growth, cardiac myocyte proliferation, differentiation, and cell cycle exit are all controlled by coordinated signal transduction, the details of which have been thoroughly dissected both in vitro and in vivo[20]. In the context of new myocyte formation in adult hearts, many of these pathways have been implicated as a means to promote endogenous proliferation, thereby possibly recapitulating the signals present during development and early heart growth (Figure). Here we will highlight some of the recent developments and experimental findings that suggest reactivation of endogenous signaling cascades in the adult heart as a means to promote therapeutic new myocyte formation. We would also direct the reader to comprehensive past reviews on some of these key pathways[138–141].

The Hippo-YAP/TAZ kinase cascade

The Hippo-YAP signaling pathway is an evolutionarily conserved central regulator of organ size and organ growth[142]. In mammals, the core Hippo signaling pathway acts via the kinases Mst1 and Mst2, homologues of Drosophila Hippo, which phosphorylate Lats1/2 in order to induce Lats1/2-dependent phosphorylation of the transcriptional co-activators yes-associated protein (YAP) and TAZ. Phosphorylation of YAP or TAZ triggers export of these proteins from the nucleus and therefore loss of YAP/TAZ-mediated TEAD transcription factor binding[143]. TEAD transcription factors govern expression of core proliferation gene programs, so the overall result of active Hippo signaling is repression of proliferation.

Coordinated Hippo-YAP signaling is required for proper fetal heart growth, as conditional deletion of YAP1 during early heart development resulted in significantly impaired myocyte proliferation and lethal hypoplasia[144]. Moreover, cardiac-specific deletion of salvador (Salv), a scaffold protein that facilitates Lats1/2 phosphorylation by Mst1 and Mst2, resulted in severe cardiomegaly and hyperproliferation of myocytes, due to loss of YAP/TAZ repression[145]. Because active Hippo-YAP/TAZ signaling suppresses proliferation and is

regulated at several nodal points, the notion that removal of key components of the pathway could diminish Hippo signaling and thus reactivate proliferation in the adult heart has received considerable attention. Myocyte deletion of either Salv or Lats1/2 specifically in adult mice promoted cell cycle re-entry and increased indices of proliferation in vivo[146], and this was associated with enhanced reparative ability of hearts after MI, as well as after apical resection at postnatal day 8, a time-point at which the mouse heart normally is unable to mount a regenerative response. Induction of YAP-mediated proliferation via over-expression of a constitutively active mutant YAP was also sufficient to enhance myocyte proliferation and improve heart function after MI in adult mice[147]. Similar benefits were observed with adeno- associated virus (AAV)-mediated delivery of human YAP in mice post-MI[148].

These studies discussed above have encouraged the use of therapeutics that modulate YAP/TAZ signaling. However, there are still key steps before this approach can be realized. Like all strategies that attempt to enhance cell cycle activity in the heart, the potential for tumorigenesis or other off-target effects will necessitate an approach that can be rigorously controlled temporally and spatially, ideally enhancing YAP/TAZ-mediated proliferation in areas of injury and for a limited period of time. However, a more fundamental question relates to the endogenous signals that serve to repress myocyte proliferation via YAP/TAZ, either by retaining Hippo pathway activity, or directly regulating YAP/TAZ. Several recent studies have implicated a structural segregation of YAP in the myocyte as an additional layer of control. Combined deletion of the myocyte intercalated disk components αE and αT catenin resulted in enhanced myocyte proliferation, and this was shown to be driven by release of sequestered YAP from the intercalated disk[149], suggesting that cytoskeletal remodeling might be an important downstream step toward myocyte proliferative potential. Indeed, YAP-meditated target gene profiling in the heart is enriched in regulators of the actin cytoskeleton[150], and inhibition of RhoA-mediated cytoskeletal remodeling prevented the nuclear accumulation of YAP in isolated myocytes[151]. As a final point of future consideration, beneficial effects of YAP activity might not be limited to proliferation, as YAP also has been implicated as an anti-apoptotic factor in the heart[152], in part via modulation of the oxidative stress response[153, 154]. Thus Hippo-YAP/TAZ signaling remains an intriguing candidate pathway for enhancing myocyte proliferation in the adult heart.

The neuregulin-ErbB signaling pathway

The ErbB family of receptor tyrosine kinases encompasses four family members, the prototypical member being the epidermal growth factor receptor (EGFR), or ErbB1. Activating ligands for ErbB family members include EGF itself, as well as a subfamily of four ligands called neuregulins (NRGs), which predominantly activate ErbB3 and ErbB4[155, 156]. NRG-ErbB signaling has diverse biological functions but is intricately linked to enhanced cell proliferation, perhaps best highlighted by the discovery of activating mutations in ErbB2, also called HER2/neu in a large subset of breast cancers[156]. During cardiac development NRG-ErbB signaling acts primarily in the formation of the valves, as well as the ventricular trabeculae[157]. However, during heart development NRG-ErbB signaling also acts to promote differentiation[158, 159] as opposed to proliferation.

Much of the focus on NRG-ErbB signaling in the adult heart stemmed from observations that breast cancer patients receiving the HER2-targeted therapeutic trastuzumab showed elevated incidence of cardiotoxicity, which was exacerbated in patients also receiving anthracyclines[160]. Studies in gene-targeted mice lacking myocyte ErbB2 or ErbB4, which are the highest-expressed family members[161] also revealed developmental phenotypes consistent with cardiomyopathy[162, 163], collectively signifying that NRG-ErbB signaling might also be involved in postnatal cardiac homeostasis. Subsequently, a study that employed cardiac myocyte-specific over-expression of ErbB4, or administration of recombinant NRG1, suggested that activation of NRG-ErbB signaling could improve cardiac function and lessen injury post-MI[164]. In line with this finding, increasing NRG1 levels also promoted myocyte proliferation and tissue repair in the injured zebrafish heart[165]. Enhanced mammalian myocyte proliferation and subsequent reparative effects were also observed with over-expression of a constitutively active form of ErbB2[166] or delivery of recombinant NRG1[167]. However, a second study failed to demonstrate enhancement of myocyte proliferation in mice treated with recombinant NRG1[168]. NRG-ErbB in the heart is remarkably pleiotropic, having effects on vascular tone, cellular metabolism, and signaling at neuromuscular junctions[157]. Thus, the benefits reported in injured hearts treated with NRG may in fact be due to multiple mechanisms of action in addition to myocyte proliferation. Currently, recombinant NRG peptides are in clinical trial for chronic heart failure patients[169] with some initial encouraging results. Future studies will need to address the proper duration and magnitude at which elevated NRG-ErbB signaling might benefit the injured heart. However, persistent/constitutive NRG activation can ultimately lead to cardiac hyperplasia and heart failure in animal models[170], suggesting that targeting the NRG-ErbB pathway will not be straightforward[171].

Exogenous Cellular Therapeutics for New Myocyte Formation

As discussed earlier in this review, a number of cell types initially proposed for direct myocyte formation and regeneration in the adult heart, such as freshly isolated bone marrow mononuclear cells[172, 173] or cardiac c-Kit cells[174], are now known to lack the ability to generate myocytes. Other classes of adult-derived cells, such as bone marrow mesenchymal stem cells, were reported to possess a modest degree of cardiomyogenic potential in animal models[175, 176], albeit at levels insufficient to directly repopulate an infarcted heart. Hence, there are currently no reproducible data between laboratories to demonstrate the existence of any sort of adult progenitor cell that can be isolated or grown in culture and then injected into the adult heart to create physiologically meaningful de novo myocytes. Thus, to generate therapeutically relevant numbers of new myocytes directly, the field has largely turned to embryonic stem (ES) cells or more recently, to a variety of strategies intended to coax adult cell types back to a pluripotent-like state first (Figure), then differentiated into myocytes in culture before injecting into the heart.

Differentiation of embryonic stem cells into myocytes

Embryonic stem (ES) cells have been an attractive cell source for studying cardiac myocyte differentiation and potentially generating large numbers of myocytes for therapeutic application, as ES cell technology is relatively mature and well-established[177] compared to more recent work with reprogrammed cell types. Human ES cells will form embryoid bodies

that contain beating myocytes when cultured in suspension[178] or when co-cultured with endodermal cells[179]. ES cell-derived myocytes express characteristic transcription factors such as Nkx2.5, GATA4, and MEF2, as well as sarcomeric genes, and can be propagated in extended culture[180]. Moreover, ES cell-derived myocytes are heterogeneous, consisting of cells with atrial, ventricular, and nodal-like properties[181]. However, this plasticity is a double-edged sword, as one of the most pressing concerns with use of ES cell-derived myocytes has been the potential for arrhythmias due to immature or dysregulated electrical coupling[182]. As a result, studies have explored the use of modified protocols to preferentially generate more highly differentiated atrial or ventricular ES cell-derived myocytes by modulation of defined signaling pathways or with modified culture conditions[183, 184].

Delivery of embryoid body-derived ES cells selected for cardiomyogenic potential into infarcted rodent hearts was shown to produce sustained improvements in cardiac function with evidence for cell engraftment and differentiation in vivo[185]. Human ES cells were also able to engraft into the hearts of pigs with ablation-induced complete heart block and restore normal cardiac rhythm[186], suggesting that ES-derived myocytes have the potential to electrically couple to host myocardium. Currently, studies in non-human primates have provided the most relevant evidence to-date that generation of new myocardium from transplanted ES cells may be clinically feasible[187–189]. For example, it has been shown that human ES cell-derived myocytes could be delivered into the infarcted macaque heart, and that these cells formed cardiac muscle grafts with mature characteristics in vivo[189]. These data provide evidence that cardiac remuscularization from human ES cells may be possible. However, much more work remains to optimize the therapeutic potential of ES cell-derived myocytes, particularly in the areas of graft survival and immunogenicity, electrical coupling, and maturation of the ES cell derived myocytes that remain poorly differentiated[190]. Indeed, transplant of ES cell-derived myocytes is particularly challenging in chronically infarcted hearts, as the benefits can be transient and limited to acute functional improvement[191, 192]. Arrhythmogenesis from poorly or incompletely coupled ES cell-derived myocardium to host muscle is also a major concern, and this was particularly evident in the macaque model with human ES cell transplant, as all transplanted animals showed arrhythmia[189]. Another known long-term concern with an ES cell-derived myocyte approach is the potential that some cells escape through the rigorous myocyte differentiation procedure and retain pluripotency, potentially resulting in teratoma formation. As a result, better-defined and more progressively differentiated ES cell-derived myocytes will be needed before this approach could be attempted in humans[193, 194].

Reprogramming of adult cells into myocytes

Technical and ethical hurdles associated with ES cells have driven an exhaustive search for alternate sources of newly generated myocytes. To this end, the development of induced pluripotent stem cell (iPS) technology, allowing for the directed conversion of adult somatic cells into an embryonic-like pluripotent state, transformed cardiac regenerative medicine approaches and opened up a wealth of diagnostic and potential therapeutic options[195, 196]. Currently, strategies to generate new myocytes via cellular reprogramming are being explored through multiple approaches – including the differentiation of converted iPS cells

directly into myocytes or the direct conversion of adult fibroblasts into myocytes, bypassing an iPS or progenitor-like intermediate (Figure). These strategies all possess both advantages and limitations and at present there is no clear frontrunner, although we will attempt to highlight the progress and future questions that must be addressed in each instance.

Differentiation of iPS cells into myocytes—Introduction of the transcription factors c-Myc, Oct3/4, Sox2, and Klf4 into mouse or human fibroblasts converts them into a pluripotent cell type (iPS cell) with the potential to generate all three germ layers in vitro, teratogenicity in vivo, and the ability to generate fully penetrant mouse chimeras when injected into blastocysts[197, 198]. iPS cells can be differentiated into beating myocytes in culture via addition of BMP4 and activin A, mirroring observations in ES cells[198]. Similar results could be obtained when culturing iPS cells as embryoid bodies[199, 200] or on a collagen IV matrix, although myocyte differentiation in this latter case was suggested to occur through an Flk-1+ mesodermal precursor intermediate cell stage[201]. As is the case for all proposed therapies that begin with undifferentiated pluripotent cell types, the potential for tumorigenicity remains a major concern. Determining approaches to mitigate this risk without removing the differentiation potential of iPS cells is ongoing but still requires extensive development. Also, like ES cells, myocytes from iPS cells are structurally and functionally immature compared to adult myocytes, particularly in terms of sarcomeric protein organization and calcium handling properties[202]. Hence, novel strategies that enhance iPS-derived myocyte maturation[203], bioengineering modifications[204] and/or addition of defined chemical factors[205, 206] have shown promise and will likely be required to achieve fully functional myocytes that integrate more effectively into a host myocardium.

Comprehensive characterization and reliable approaches for differentiation of myocytes from ES cells or iPS cells[207] should be part of future experimentation. When these issues are resolved the field can turn to development of approaches that include vascularizing areas seeded with new myocytes and improving electromechanical synchrony of transplanted and host myocytes. It is important to keep in mind that even if a robust and uniform ability to generate new cardiac muscle from previously undifferentiated pluripotent cells in vivo was developed, the ability to provide functional vascularization to this graft would still be required to allow for full tissue restoration. In addition, the proper alignment of myocytes within the graft would be beneficial in better coordinating contractility activity of bands of cells within the ventricle.

Another key possibility to consider is that ES or iPS cells may impart functional benefits to the injured heart that are not due to direct remuscularization. Indeed, delivery of human ES or iPS-derived myocytes into MI-injured immunodeficient mice has been shown to significantly improve contractile performance and lessen remodeling even with negligible retention of the transplanted cells[208, 209]. Cell delivery in these studies was associated with an upregulation of circulating angiogenic and cardioprotective cytokines, suggesting that paracrine-mediated repair pathways may have been activated with ES or iPS cell therapy. A recent large study of non-human primates treated with human ES cells that had been differentiated into cardiovascular progenitors also showed improvement in cardiac function, despite very limited cell retention and no evidence for remuscularization[210]. Thus, the

mechanisms of action for ES- and iPS-derived myocyte cell therapy may be multifaceted and not purely due to contractile activity of the injected nascent myocytes.

Direct reprogramming of fibroblasts into myocytes—The discovery that fully differentiated adult fibroblasts could be forced into a pluripotent cell state with defined transcription factors was an exciting recent development in the field. Efforts in the area began with the observation that transfection of cultured embryonic mouse mesoderm with the cardiomyogenic transcription factors Gata4 and Tbx5 along with the chromatic remodeling factor Baf60c was able to redirect non-myogenic mesodermal tissue towards a cardiac fate[211]. This demonstrated that cardiomyogenic capacity could be conferred by defined factors, although whether this was possible in fully differentiated cells or tissues was not known. However, in 2010 a systematic reductionist approach using a collection of candidate transcription factors revealed that combinatorial delivery of Gata4, Mef2c, and Tbx5 (GMT) could reprogram adult fibroblasts isolated from the heart or the tail tip of mice into cardiac myocytes[212]. Moreover, fibroblasts carrying the GMT cocktail expressed cardiac myocyte genes and activated an αMHC-driven GFP reporter when injected into the mouse heart. Myocyte conversion from fibroblasts by GMT was limited to 20% of cells in vitro, suggesting that additional improvements could be made. Addition of the transcription factor Hand2 was shown to synergistically improve fibroblast to myocyte conversion with GMT transduction in vitro[213]. Importantly, in vivo viral-mediated delivery of either GMT[214] or GHMT (GMT+Hand2)[213] ameliorated cardiac dysfunction in a rodent infarct model. However, these data were surprisingly not accompanied by a rigorous assessment of true myocyte conversion rates within the adult heart, making it impossible to conclude that the improvement in cardiac function was due to new myocyte formation in vivo.

While technical challenges remain for adopting this approach as a strategy for new myocyte generation in vivo, direct fibroblast reprogramming has several theoretical advantages (Figure). It avoids the use of an undifferentiated or pluripotent cell state, which should curtail tumorigenicity. The conversion of fibroblasts to myocytes may also serve a dual role of reducing pathological fibrosis while generating new contractile tissue. However, several key questions require further investigation before this approach can be considered for therapeutic myocyte generation. The major limitation presently is that the conversion rate in vivo appears too low to provide sufficient new myocytes to achieve an improvement in cardiac pump function[215]. Moreover, as fibroblast to myocyte conversion appears to occur without inducing additional cellular proliferation, the absolute number of new myocytes generated may be less than what could be achieved with other strategies, particularly in situations such as chronic ischemia where fibroblast activation is still ongoing[216]. Human fibroblasts are also more refractory to reprogramming versus those from mouse, requiring the two additional transcription factors, Mesp1 and Myocardin[217]. Therefore, it is not clear that this approach will work in the adult human heart. Another concern relates to the use of viral gene delivery to achieve reprogramming in vivo. Recent studies have suggested alternative reprogramming approaches by using microRNAs or chemical mediators to indirectly induce the expression of reprogramming factors[218–220] while the use of non-integrating viral constructs could also mitigate some safety concerns[221]. However, any of these potential approaches would most likely need to be made specific to the fibroblasts in

the heart, and no other cardiac cell types or fibroblasts of other tissues. Overall, the ability to reprogram adult fibroblasts into myocytes in vitro or in vivo provides another potential avenue towards achieving meaningful therapeutic myocyte replacement once the molecular pathways have been carefully defined and optimal methods for efficient reprogramming have been determined.

Tissue engineering approaches to new myocyte formation

The collective experience of many labs over the past decades has revealed that injecting isolated cells into an infarcted heart in the hopes they will restore tissue architecture and generate new beating heart muscle is a tall order. To improve the overall situation, the field has increasingly adopted an interdisciplinary approach by integrating cell biology with tissue engineering[222]. Many of the cell types discussed above are now being deployed not in an isolated suspension, but in pre-fabricated biomaterial or chemical scaffolds or as engineered heart tissue patches. Seeding of human ES cell-derived smooth muscle and endothelial cells in a fibrin patch resulted in functional improvement in a swine model of myocardial infarction[223]. This patch lacked ES cell-derived myocytes, suggesting that the beneficial effects were achieved via revascularization or paracrine-mediated effects on the host myocardium. Addition of myocytes, in this case derived from iPS cells, as well as a slow-release delivery of IGF-1, provided a synergistic benefit[224], highlighting the ability to deliver growth factors or protective cytokines via biological scaffolds as an additional advantage.

Delivery of pre-fabricated cardiac tissue patches using hydrogel as the scaffolding material is also under active investigation. Initial proof-of-concept studies using neonatal rat myocytes demonstrated that engineered heart tissues could be constructed in vitro that displayed many of the hallmark features of mature myocardium, including structural maturity of sarcomeres and T-tubules and uniform inotropic responsiveness[225]. Implantation of these tissues onto infarcted rat hearts demonstrated a capacity for electrical coupling, improved heart function, and ameliorated adverse remodeling[226]. Newer iterations of this technology have used human ES cell-derived myocytes[227] or iPS-derived myocytes[228]. These studies have also demonstrated feasibility and functional benefits when grafted onto infarcted hearts. However, the precise mechanisms of functional improvement from engineered heart tissues remain undefined and are likely multifaceted. Remuscularization from patch-derived cells has been observed in some studies[228], suggesting that direct cardiomyogenesis may be occurring to provide functional benefit, but this has not been the case uniformly. Moreover, it remains uncertain how a large cell-derived graft could be completely revascularized when incorporated into the host environment, which would be required for sustained viability of the graft. Of note, a recent analysis of ES cell-derived engineered heart tissues revealed that tissues lethally irradiated prior to transplant (to ablate functional myocytes) were equally protective in a rodent infarction model as their living counterparts[227]. This important control experiment suggests that engineered heart tissues or biomaterial patches may be providing benefits irrespective of myocyte recellularization, such as structural stability or modulation of inflammatory cell activation. Despite this, preliminary clinical assessment in a very small number of patients has begun with several of these technologies, including fibrin patches seeded with ES cells[229]. Still in its infancy, the

merger of cell therapeutics with bioengineering has invariably benefited the field and fueled basic discovery in cardiac repair[230]. The challenge now will be to develop scalable and therapeutically amenable approaches that will reduce or even overcome the inherent limitations of unmodified cell delivery.

Emerging Paradigms and Future Directions for Therapeutic Myocyte Generation

The fundamental questions regarding cardiac myocyte renewal and the search for cardiac regenerative therapies are deeply rooted in the field and have been intensely pursued for over a century[120, 231]. But in the last two decades, new genetic tools, experimental techniques, and a continuing effort to refine measures of myocyte proliferation and cell division[232, 233], combined with an aging heart failure patient population, have fueled an upsurge in attempts to increase myocyte content in the heart. As a result, the field continues to delineate the fundamental molecular pathways that govern or suppress myocyte turnover in various contexts. Here we will briefly touch on emerging and newly uncovered pathways that are likely to provide additional mechanistic insights and may lead to novel therapeutic strategies (Figure).

Role of oxidative and metabolic states in postnatal myocyte generation

Following a newborns first breath, the oxygen content of the arterial blood increases to adult levels. Soon after this event, the mammalian heart undergoes a rapid switch in energy utilization and metabolism, from a largely glycolytic, carbohydrate-driven program to one that primarily utilizes fatty acids[234]. This process is tightly coordinated with myocardial mitochondrial expansion, myocyte terminal differentiation, and cell cycle exit, required for the enhanced pumping requirements of the mature heart[235]. In contrast, lower vertebrates, primarily those living in aqueous environments, retain a more immature, developmental metabolic phenotype[16], raising the question of whether cardiac myocyte plasticity and proliferative capacity are tied to metabolic state and requirements of contractile rigor. To this end, a recent study compared mitochondrial content and ultrastructure across the hearts of zebrafish and neonatal mice from p1 to p7 and found that in mice mitochondrial expansion and cristae formation occurred coincident with the window of time at which myocyte proliferative capacity is lost[236]. In a comprehensive series of experiments, local myocyte hypoxia was shown to promote cell cycle re-entry, and that the expansion of mitochondrial oxidative metabolism and the subsequent reactive oxygen species elevation that occurs postnatally is a driver of reduced myocyte proliferation in vivo[236, 237]. Extending this paradigm, the same group recently reported that systemic gradual hypoxia in adult mice could produce a proliferative response in the heart at baseline or after injury, suggesting that modulating metabolism or tissue oxygen levels could be a novel approach to new myocyte formation. In potentially translating these findings, conformational studies are needed and the potential risks associated with a hypoxic state will need to be considered[238]. Translating these basic findings into a large animal model (Porcine) context would also seem critical.

Role of myocyte nuclear content and ploidy

The majority of terminally differentiated adult cardiac myocytes are binucleated and/or polyploid in rodents, due to karyokinesis without cytokinesis during cell cycle withdrawal[30, 42]. In many regenerative species such as zebrafish, myocytes remain mononucleated and diploid throughout life[10]. Further, mononucleated myocytes in mammals are smaller than binucleated cells, and have been proposed to retain more proliferative potential[239]. To address this possibility, a recent study performed an exhaustive in vivo screen of 120 inbred mouse lines and measured the frequency of mononucleated diploid myocytes in hearts from these lines[240]. Mouse strains selected for analysis that had a greater proportion of mononucleated diploid myocytes showed improved recovery after MI and had increased indices of myocyte cell cycle activity such as EdU uptake. Availability of high-density SNP maps for these lines allowed the group to perform forward genetic screening, from which they identified TNNI3K, a cardiac-specific protein kinase that adversely regulates ischemic injury and pathological hypertrophy in the adult heart[241, 242], as a candidate effector of mononucleated versus binucleated myocytes levels. Regulation of myocyte proliferation by ploidy and nucleation status was also demonstrated in a recent zebrafish study using transgenic mosaicism[243], revealing that diploid myocytes proliferated more readily than those with polyploid nuclei. Moreover, transgenic fish lacking the proper complement of diploid myocytes were unable to mount a regenerative response following heart injury. Taken together these studies suggest that proliferation may proceed more readily in mononucleated diploid myocytes versus those with polyploidy.

Structural cues in provoking or suppressing myocyte proliferation

Perhaps the most thought-provoking and unresolved issue regarding endogenous proliferation of adult cardiac myocytes is that it appears to happen in the setting of an exceptionally rigid and complex cellular architecture; namely, the sarcomere. In order to divide, mammalian cells must rapidly and accurately disassemble and reassemble cytoskeletal and nuclear structural protein complexes. In addition to this intracellular restraint, cell-cell junction and cell-matrix interactions must be disassembled and then properly restored. Cardiac myocyte proliferation during embryogenesis or early postnatal life occurs in an immature and more plastic tissue environment with lower pressures and functional requirements, which is also the case for lower vertebrates such as the zebrafish[12]. To address the question of how adult hearts might achieve a similar feat, or if structural disassembly is required, a recent study took advantage of time-lapse video microscopy to document myocyte proliferation in adult mouse myocytes, cocultured with neonatal rat ventricular myocytes (NRVMs)[244]. Proliferative adult myocytes showed a sequential progression of sarcomere disassembly, induction of cell cycle markers, and then re-organization of sarcomeric structure and restoration of contractility. Mechanistically, physical connection between adult myocytes and NRVMs via connexin 43 was required for this restoration of differentiated myocyte structure[244]. These data suggest that physical interactions are a determinant for coordinated proliferation-differentiation in adult myocytes that undergo cell cycle re-entry. This emerging paradigm is made more evident from a recent series of studies demonstrating key roles for extracellular matrix proteins in promoting endogenous myocyte proliferation. In a pair of reports, coupling of myocytes to the extracellular matrix via agrin, as well as to the dystrophin-glycoprotein (DGC) complex via

dystroglycan 1, were shown to be responsible for regulating the intracellular distribution of YAP and coordinating proliferative vs anti-proliferative signals in the cell[245, 246]. Agrin, via binding to dystroglycan 1, triggers DGC disassembly and release of YAP, promoting myocyte proliferation[245], while the assembled DGC can sequester YAP and confines proliferation, even in the setting of Hippo pathway modulation[246]. Thus, the molecular events underlying complete myocyte proliferation have emerged as even more multifaceted, requiring coordinated steps to both disconnect the dividing cell from its extracellular anchors, and remodel intracellular components to produce functional myocytes as a result. However, the need for dedifferentiation of the adult myocytes in the heart before they are permissive to proliferation poses a known problem related to the loss of functional rigor, as if a significant number of these cells de-differentiated at once, it would surely lead to reduced cardiac functional performance and likely heart failure. Thus, long-term it will be necessary to selectively and temporally control myocyte cell cycle re-entry in coordinating therapeutic regenerative approaches.

Conclusions

Conclusive evidence collected over the last several years across diverse animal models and in humans has demonstrated a clear capacity for new myocyte generation in the adult mammalian heart. Maximal cardiac myocyte turnover during homeostasis in the adult is limited to roughly 1% per year, while in the face of cardiac injury or disease, this turnover slightly increases but cannot compensate for extensive myocyte loss. In both cases, proliferation of pre-existing myocytes is the overwhelming cellular contributor to new myocyte formation.

Proliferation of adult cardiac myocytes involves the reactivation or modulation of numerous signaling pathways central to embryonic and early postnatal heart growth. In contrast, the extent of the intrinsic barriers limiting endogenous proliferation in adult hearts remains unclear, as is the extent to which easily measured DNA proliferative indexes reflect true cellular proliferation with bonafide new myocyte formation. Therapeutic manipulations aimed at enhancing the limited regenerative capacity of the adult heart by directly targeting new myocyte formation can include direct or indirect stimulation of endogenous myocyte cell cycle activity, or the use of exogenous ES cells or pluripotent cells or tissues first directed towards a cardiomyogenic fate. The strategies we have highlighted here are by no means exhaustive, and the broader field of cardiac regeneration is exploring numerous avenues to achieve meaningful heart repair, including direct modulation of tissue fibrosis, stimulating revascularization, and inflammation-based tissue healing and resolution[247–251]. All of these approaches must be thoroughly vetted in small and large animal model systems, and in particular strategies for therapeutic new myocyte generation must be designed with the overarching goal of restoring myocardial tissue that is structurally and functionally equivalent to that which was lost. Achieving this will require uncovering additional cellular and molecular mechanisms that control physiological and pathological cardiac myocyte turnover at the cell, tissue, and whole-organ level.

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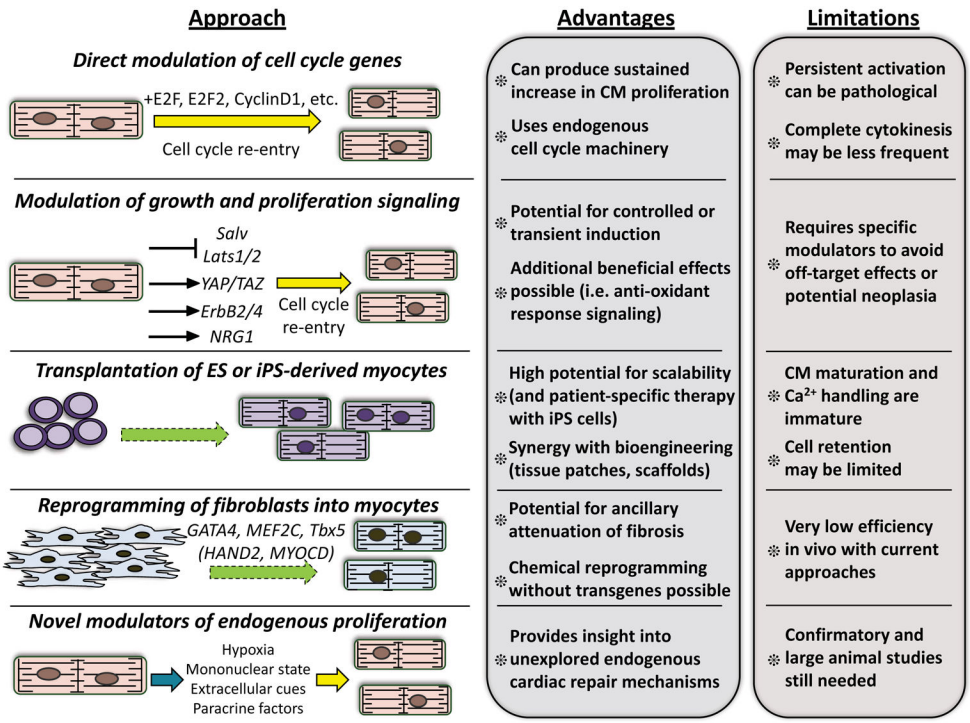

Figure. Therapeutic sources for new myocyte generation in the adult heart

Summary of current and proposed strategies discussed throughout the review that aim to directly replenish cardiac myocytes lost following ischemic injury or in the setting of progressive heart failure. Potential strategies include direct reactivation of the myocyte cell cycle program through genetic or pharmacological manipulations (yellow arrows), reprogramming of non-myocytes by manipulation of cellular states (green dashed arrows), or indirect cell cycle reactivation through myocyte-autonomous or extracellular mechanisms (blue arrow). Therapeutic and mechanistic advantages to each strategy are highlighted, as well as crucial limitations, risks, or other considerations.