

# MUSCLE

## Fundamental Biology and Mechanisms of Disease

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# Mammalian Myocardial Regeneration

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The adult heart is among the least regenerative organs in the body, posing one of the greatest challenges in regenerative biology. Each year millions of patients die of heart failure, which at the most fundamental level is caused by inadequate number and function of cardiomyocytes (1). It is estimated that during a typical myocardial infarction, one billion or more cardiomyocytes are lost (2). In the short term, surviving myocytes may adapt and take on a greater work load to sustain cardiac function. However, these adaptations cause cardiomyocyte dysfunction and additional cardiomyocyte death. The resulting vicious cycle causes a “cardiomyopathy of overload”, leading to heart failure and death (3,4). Current therapy is directed at interrupting the neurohumoral factors that sustain the vicious cycle, and thus mitigate but do not reverse progressive heart failure.

Fundamentally changing the natural history of heart failure requires generating new cardiomyocytes. Until quite recently, it was accepted dogma that cardiogenesis, the birth of new cardiomyocytes, occurs in mammals only during fetal development. This conventional view held that post-natal cardiac expansion under physiological or pathological conditions results from cardiomyocyte hypertrophy (increase of cell size) rather than via hyperplasia (increase of cell number through division or differentiation of progenitors) (5). However, ground-breaking studies over the past decade have challenged this dogma and shown that the adult mammalian heart indeed possesses intrinsic, albeit limited, regenerative capacity. These findings have focused efforts on augmenting the heart's natural regenerative mechanisms. In this chapter, we will critically review studies on the endogenous regenerative capacity of the adult heart. Transplantation of exogenous cell populations has also been studied for use in therapeutic myocardial regeneration, but this extensive literature is outside of the scope of this review. The reader is referred to excellent recent reviews on this topic (2,6).

The field of cardiac regeneration is replete with controversy and contradictory findings. Not surprisingly, this arises from technical factors that limit our ability to

accurately and precisely measure the birth of new cardiomyocytes. Thus, a nuanced understanding of the literature requires knowledge of the technical challenges, the methods used to overcome the challenges, and the limitations of our approaches. In this review we cover these experimental facets to equip readers to best grasp the burgeoning literature in this field.

## NORMAL MYOCARDIAL GROWTH AND CELL CYCLE ACTIVITY

The fetal myocardium grows by cardiomyocyte proliferation. Post-natally, cardiomyocyte cell division largely stopped by 3 days after birth in rats (7). Subsequently, cardiomyocyte number was constant, but cardiomyocyte volume increased 2.5-fold between day 3 and 12, indicating that post-natal myocardial growth occurs primarily by increasing cardiomyocyte size.

One hallmark of cellular proliferation is DNA synthesis, and therefore cardiomyocyte DNA synthesis has been exhaustively studied (reviewed in (8)). In rodents, intense DNA synthesis peaked at post-natal day 10 (P10) and declined to adult levels by P20 (7,9). Between P3 and P12, cardiomyocytes no longer underwent cell division (cytokinesis) but continued to synthesize DNA and to undergo nuclear division (karyokinesis), a form of endoreduplication known as acytokinetic mitosis. As a result, by day 12 cardiomyocytes reached their adult level of binucleation of 90% (7,10). Acytokinetic mitosis was associated with formation of stable, highly ordered and functional sarcomeres, suggesting that the organized contractile apparatus impairs cytoplasmic division (11,12).

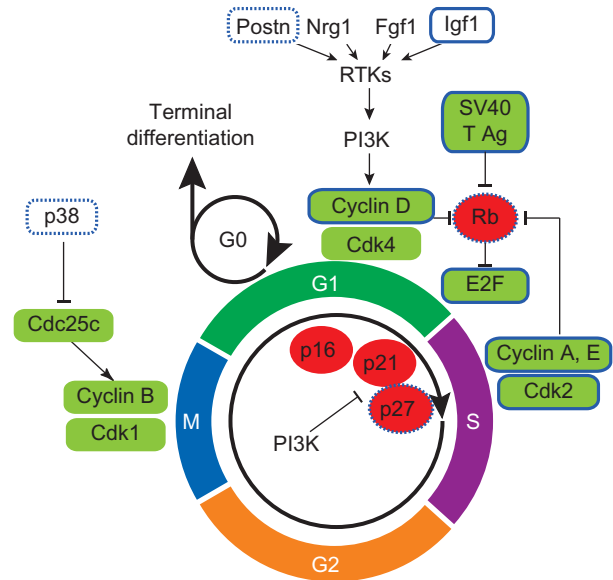
In addition to increased ploidy from multinucleation, cardiomyocytes can become polyploid through DNA synthesis in the absence of karyokinesis, a form of endoreduplication known as endocycling or endomitosis. The extent of endocycling was measured in mouse by FACS sorting of cardiomyocyte nuclei (13). Murine fetal cardiomyocyte nuclei were mononuclear and diploid. At birth, only 65% of cardiomyocyte nuclei remained diploid, with

most of the remaining nuclei being tetraploid. The fraction of diploid nuclei reached a stable level of  $\sim 55\%$  by P21 (13), corresponding to the time by which intense DNA synthesis had halted (7). Importantly, endoreduplication is stimulated by cardiomyocyte stress and activation of specific signaling pathways (14,15), making it essential for experiments that address upregulation of cell cycle markers in myocardial injury models to demonstrate productive formation of new cardiomyocytes rather than more limited forms of cell cycle activity.

In the human heart, cardiomyocyte growth similarly changes from hyperplasia to hypertrophy in infancy, although the timing of this transition is less clearly defined (16). From infancy to adulthood, the number of cardiomyocytes in the normal human heart was constant (17,18). However, unlike myocytes from mice, rats, dogs, and pigs, mononucleated cells predominated and binucleated cells were in the minority (77% and 22%, respectively) (18). This proportion did not change with age or ischemic or hypertrophic heart disease (18). Polyploidization through endocycling continued in humans up to 10 years of age, considerably longer than observed in rodents (19). As with rodents, myocardial injury was observed to stimulate endomitosis and to increase cardiomyocyte ploidy (20).

The newborn heart also grows through expansion of the non-myocyte compartment. In mice, fetal and neonatal myocardium contains few non-myocytes. Post-natally, the non-myocyte fraction expands rapidly from 13% on post-natal day 1 (P1) to 80% at P20 (10). In adult mice, the non-myocyte cell number fraction is  $\sim 85\%$ . This expansion involves fibroblast expansion as well as rapid growth of the vascular bed, which increases by more than four-fold during post-natal cardiac growth (17).

A series of proteins promote or inhibit cell cycle progression (Figure 39.1, reviewed in (21)). To study the mechanisms governing post-natal cardiomyocyte cell cycle exit, the expression of cell cycle regulators was investigated in human and rodent heart (reviewed in (11,22)). Cell cycle regulators that promote cell cycle activity, including Cyclins A, B, D1/D2/D3, and cyclin-dependent kinases (CDKs) CDK1 (also known as CDC2) and CDK2, were highly expressed in fetal heart and markedly downregulated in adult heart (10,13,23–25). The E2F family of transcription factors, pivotal regulators of the G1/S phase transition, were also markedly downregulated between neonatal and adult cardiomyocytes (26). Activity of E2F factors is normally held in check by the pocket protein family, containing the retinoblastoma susceptibility gene (Rb) and its relatives p107 and p130. During hyperplastic heart growth, CDK2/CyclinE/CyclinA and CDK4/CyclinD complexes phosphorylate pocket proteins, releasing inhibition of E2F and driving cell cycle activity. During hypertrophic heart growth, downregulation of these kinases leads to pocket protein



**FIGURE 39.1 Cell cycle regulation.** Cell cycle genes that promote cycling are shown in green, and those that inhibit cycling are shown in red. RTK, receptor tyrosine kinase; PI3K, phosphoinositol-3-kinase. Symbols with solid blue outline have been overexpressed in mouse heart, while those with dashed blue outlines have been knocked out in mouse heart.

hypophosphorylation and inhibition of the cell cycle promoting activity of E2F (27,28). The CDK inhibitors p21 and p27 act as important brakes on cell cycle activity by inhibiting Cdk2/CyclinE/CyclinA and Cdk4/CyclinD activity and thereby repressing E2F. p21 and p27 were markedly upregulated during the transition from hyperplastic to hypertrophic growth (25,29). In summary, cardiomyocyte expression of cell cycle regulators is carefully regulated, so that during hyperplastic growth the profile of factors favors cell cycling. During hypertrophic growth, the profile is reversed, leading to cell cycle exit. Important future directions will uncover the molecular pathways that coordinately regulate the profile of cell cycle regulator expression.

Based on changes in expression of cell cycle regulators during the transition from hyperplastic to hypertrophic cardiomyocyte growth, concerted efforts were made to promote adult cardiomyocyte cell cycle reentry by direct manipulation of cell cycle regulators (Figure 39.1). Knockout of the cell cycle inhibitor p27 and the redundant pocket protein genes Rb and p107 increased heart size, cardiomyocyte number, and adult cardiomyocyte DNA synthesis (28,30). Transgenic overexpression of SV40 T antigen robustly stimulated cardiomyocyte cell cycle reentry, but these mice showed extensive cardiac pathology and died before weaning (31). Ectopic cardiomyocyte expression of the E2F family member E2F1 resulted in increased DNA synthesis, but unfortunately

caused cardiomyocyte apoptosis and death (32). Forced cardiomyocyte expression of Cyclin B and Cdk1 drove adult cardiomyocyte cell cycle reentry in cell culture (33), although extension of this observation *in vivo* has not been reported. D-cyclins are regarded as sensors of the extracellular environment that link mitogenic pathways to the cell cycle machinery, and cyclins D1-3 are required for fetal cardiomyocyte proliferation (34). Transgenic overexpression of cyclin D1, D2, or D3 promoted cardiomyocyte DNA synthesis and multinucleation without affecting the cardiomyocyte differentiation (24,35). Cardiomyocyte-specific cyclin D2 overexpression increased the fraction of cardiomyocytes labeled by <sup>3</sup>H-thymidine by over ~500-fold in adult heart. Immediately following experimental left anterior descending coronary artery (LAD) ligation, infarct size in cyclin D2 transgenic mice was not distinguishable from littermate controls. However, 2 and 6 months after infarction, infarct size was markedly smaller in transgenic mice, indicating substantial myocardial repair (35,36). Likewise, transgenic cyclin A2 overexpression enhanced early post-natal cardiomyocyte cell cycle activity. Although this effect was not sustained in normal adult heart, myocardial infarction elicited new cardiomyocyte

formation that improved ventricular function compared to controls (37). The promising results from transgenic cyclin D2 and A2 mice provide proof of concept that driving cardiomyocyte cell cycle reentry may be a viable strategy for stimulating cardiac regeneration.

## THE EXTENT OF ENDOGENOUS MYOCARDIAL REGENERATION (TABLE 39.1)

### Myocardial Regeneration in Lower Vertebrates

Some lower vertebrates such as newt and zebrafish are able to regenerate many organs, including the heart. Understanding the regenerative process in these model organisms will lead to insights in mammalian heart regeneration by defining regenerative mechanisms and regulatory pathways. Zebrafish is a well-developed platform for both forward and reverse genetic approaches, and as a result has emerged as a major model for studying myocardial regeneration. After amputation of as much as 20% of the heart ventricle, adult zebrafish fully regenerated the heart without residual scar (38). Shortly after amputation

**TABLE 39.1** Endogenous Myocardial Regeneration

Species	Detection Method	Injury	Regeneration Response	Regeneration Source	Caveats	References
Zebrafish	Genetic lineage tracing	Amputation	Repair without scar	Differentiated CMs	Expression of differentiated CM marker in CPCs?	40,41
Newt	Morphology; Dil or adenoviral label	Amputation squeeze	Fibrosis; CM de-differentiation and proliferation	Differentiated CMs	Cannot exclude other sources of CMs	42–45
Mouse	Dilution of genetic pulse labeled differentiated CMs	None	None detected	NA	Cannot exclude new CMs from differentiated CMs	52
	Dilution of genetic pulse labeled differentiated CMs	MI; TAC	Up to 18% of CMs in borderzone arise from CPCs	Undifferentiated CPCs	Cannot exclude new CMs from differentiated CMs. CPC source of new CMs not determined	52
	Genetic fate mapping of normal and Hccs-deficient cells	Fetal loss of Hccs <sup>-</sup> CMs	Regeneration of 40% of CMs by birth	Differentiated CMs	Cannot exclude new CMs also from CPCs	46
Human	<sup>14</sup> C cardiomyocyte birth dating	Undefined	~1% CM turnover per year	Not determined	Did FACS isolation procedure yield a biased population of nuclei?	53,54
	Confocal microscopy: mitotic figures, Ki67, IrdU uptake	ICM; MI	All CM turned over every 4.5 years; greater turnover with injury	Differentiated CMs	Results have not been reproducible in other laboratories	54,56,57

Abbreviations: CM, cardiomyocyte; CPC, cardiac progenitor cell; NA, not applicable; MI, myocardial infarction; TAC, transverse aortic constriction.

of the ventricular apex, a blastema formed around the amputation site. Progenitor cells within the blastema proliferated, expressed pre-cardiac markers, and underwent differentiation (39). Initial studies suggested that new cardiomyocytes of the regenerating myocardium arose from differentiation of cardiac progenitors (39). More recently, reports from the Poss and Belmonte groups demonstrated that new cardiomyocytes of the regenerating myocardium arose from preexisting cardiomyocytes (40,41). This conclusion was based on application of Cre/loxP genetic fate mapping technology to zebrafish. The tamoxifen-activated Cre recombinase CreERT2 was expressed only in differentiated cardiomyocytes in fish that also contained a Cre reporter transgene. This transgene expresses Green Fluorescent Protein (GFP) after Cre recombination. Treatment of these fish with a pulse of tamoxifen selectively and heritably labeled differentiated cardiomyocytes with GFP. After apex amputation (in the absence of tamoxifen), regenerating cardiomyocytes were observed to express GFP (40,41), indicating that regenerating cardiomyocytes originate from differentiated cardiomyocytes. While these data appear to be at odds with initial studies suggesting that new cardiomyocytes arise from undifferentiated cardiac progenitors, the results may be reconciled if differentiated cardiomyocytes proceed through a stage of initial de-differentiation prior to formation of regenerating cardiomyocytes. Future studies promise to address this question and to probe the signaling pathways responsible for arresting cardiomyocyte proliferation in the normal adult heart and for stimulating cardiomyocyte proliferation after heart injury.

Newts are capable of limited myocardial regeneration. Amputation of the ventricular apex leads to scar formation, although cardiomyocytes populated the scar and were likely the product of new myocyte formation (42). Cardiac repair was enhanced when the amputated apex was returned as a minced cardiac muscle graft, which coalesced to form a regenerated ventricular wall composed primarily of cardiac muscle (43). After cardiac injury, adult newt cardiomyocytes de-differentiated, downregulating sarcomeric genes such as myosin heavy chain (44). The de-differentiated cardiomyocytes then proliferated and re-expressed cardiomyocyte sarcomeric genes, contributing to functional regeneration of the heart (45). Consistent with cardiomyocyte de-differentiation yielding multipotent progenitor-like cells, cardiomyocytes injected into regenerating newt limbs de-differentiated and contributed to limb regeneration by redifferentiating into skeletal muscle. In contrast, cardiomyocytes injected into intact newt limbs maintained their identity as cardiomyocytes (44). Elucidation of the mechanisms that stimulate cardiomyocyte de-differentiation and proliferation in injured heart and limbs is an important direction for future studies.

## Myocardial Regeneration in Mice

The fetal mammalian heart has robust intrinsic regenerative capacity. This was demonstrated recently in a genetic mouse model in which the fetal heart's regenerative capacity compensated for an effective loss of 50% of cardiac tissue (46). Holocytochrome c synthase (HCCS) is an enzyme essential for normal mitochondrial respiration. The *Hccs* gene is located on the X chromosome. Males and homozygous females with cardiac-specific inactivation of *Hccs* died between E10.5 and E14.5 (E, embryonic day, indicates gestational age) due to cardiac mitochondrial abnormalities. In contrast, female *Hccs* heterozygotes survived normally. This was surprising because early in development of female embryos, one of the two X chromosomes is inactivated, and the inactivated state is heritably maintained through successive cell divisions. Therefore female *Hccs* heterozygotes were expected to be genetic mosaics, with roughly 50% of cells being *Hccs* deficient. Indeed, mitochondrial complex III activity was reduced by 50% in E12.5 female *Hccs* heterozygotes, and the expected 50:50 distribution of normal and *Hccs*-deficient cardiomyocytes was observed at E10.5. However, the proportion of normal cardiomyocytes progressively increased thereafter, reaching 90:10 at birth. This expansion of normal cardiomyocytes was due to their selective proliferation, thus indicating a regenerative response of healthy cardiomyocytes. Enhanced cardiac differentiation of normal cardiac progenitor cells was not excluded as a potential mechanism. This study revealed the regenerative capacity of mammalian fetal heart. Signaling pathways that regulate fetal cardiomyocyte regeneration may be therapeutically applied to the post-natal heart, and thus this study opened new avenues to approach the problem of adult heart regeneration.

The window during which mice are able to regenerate lost myocardium appears to extend into early post-natal life. Sadek and colleagues showed that 1-day-old mice could regenerate myocardium removed by amputation of the heart apex, restoring heart shape and mass without fibrosis (47). Lineage tracing studies showed that, as in zebrafish, regenerated cardiomyocytes arose predominantly by proliferation of preexisting cardiomyocytes. However, this regenerative capacity was lost quickly over the first week of life, so that apex amputation of 7-day-old mice led to scarring rather than formation of new myocardium. The period of regenerative competence coincided with the window of post-natal cardiomyocyte proliferation, which ends at 3 days of life (7), suggesting that loss of cardiomyocyte proliferative capacity and loss of regenerative ability are linked.

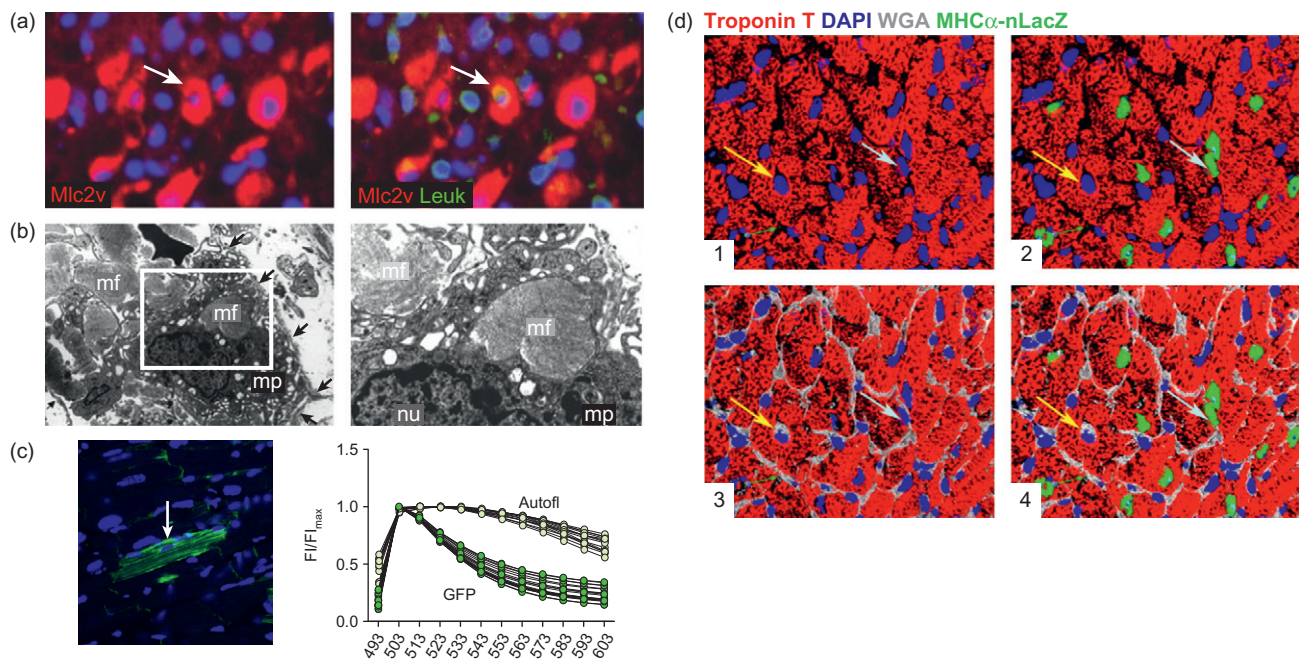
The regenerative capacity of the adult mammalian heart has been investigated in detail over the past decade. These studies have largely overturned the dogma that this



organ is devoid of regenerative capacity, but the extent of endogenous cardiomyocyte turnover and regeneration remains controversial (2,48). The controversy is directly related to the technical challenge of measuring infrequent cardiomyocyte proliferation in the background of non-myocyte proliferation. By definition, cardiomyocyte proliferation increases cardiomyocyte number. However, because of measuring absolute cardiomyocyte number is technically difficult, it is rarely done. Rather, scientists typically measure the fraction of cardiomyocytes expressing markers of cell proliferation. Non-myocytes constitute at least 80% of the cells of the adult heart (10), and these cells may proliferate to a greater extent than cardiomyocytes. Thus measurement of the fraction of proliferating cardiomyocytes is susceptible to many artifacts (Figure 39.2). There must be robust and objective means to distinguish between myocyte and non-myocyte proliferation (49). Some investigators have used transgenic cardiomyocyte markers to conveniently and objectively identify cardiomyocytes (2), while others have suggested

that this is not necessary (48). To add to the difficulty, myocytes undergo endoreduplication, yielding multiple nuclei per cell and also greater than two genomic copies per nucleus. Thus, increased DNA synthesis, cell cycle activity, and even nuclear division does not necessarily indicate birth of a new myocyte. Additional important artifacts to address are overlapping signals in tissue sections that may lead to the mistaken impression of co-expression unless three-dimensional analysis is performed (50); auto-fluorescence leading to inaccurate signal identification (2,50); fusion of progenitor cells to cardiomyocytes to give the inaccurate appearance of transdifferentiation (51); and the effect of inflammatory cells, which may localize within cardiomyocyte cytoplasm (2).

The research group of Richard Lee performed an important study that addressed the extent to which cardiac progenitors contribute to the normal and injured myocardium. This study was based on a genetic “pulse-chase” approach, using mice engineered to contain both MHC $\alpha$ -MerCreMer and Z/EG transgenes (52). MHC $\alpha$ -MerCreMer



**FIGURE 39.2** Examples of pitfalls in measuring adult cardiomyocyte proliferation. (a,b) Inflammatory cells, particularly prevalent after MI, can be found within cardiomyocytes. Arrow in (a) seems to show a cardiomyocyte nucleus. On staining with leukocyte markers, the nucleus belongs to a leukocyte within a cardiomyocyte. (b) is a transmission electron micrograph of post-MI myocardium showing a necrotic macrophage containing myofibrils (mf) adjacent to a macrophage (mp). Higher magnification of the boxed area shows that the macrophage has engulfed some myofibrils and its nucleus (nu) could inadvertently be inferred to belong to a cardiomyocyte. (c) Autofluorescence in normal mouse myocardium. One cell with strong green fluorescent signal is present within a field of non-fluorescent cells. This could be inferred to be a cell specifically expressing GFP. However, emission spectroscopy shows that the fluorophore spectrum is consistent with autofluorescence (Autofl) and not GFP. (D) Improved identification of cardiomyocyte nuclei by staining for the basal lamina (using WGA) and a transgenic marker of cardiomyocyte nuclei (MHC $\alpha$ -nLacZ). The yellow arrowhead indicates a non-myocyte nucleus surrounded by myocyte cytoplasm. WGA-staining suggests that it may be a non-myocyte nucleus (image 3), and this is clear using the transgenic marker (images 2,4). The pale blue arrowhead indicates a cardiomyocyte nucleus at the edge of a cardiomyocyte. WGA enhances its identification as a cardiomyocyte nucleus (image 3), and this is confirmed using the transgenic marker (images 2, 4). (Panels a and b adapted from Laflamme and Murry, 2005 (2); panel c from Zhou et al., 2011 (74); panel d from Ang et al. (2010) (49); with permission.)

expresses a tamoxifen-inducible Cre fusion protein (MerCreMer) only in differentiated cardiomyocytes. The Z/EG transgene expresses GFP only after recombination by Cre. Thus, administration of a tamoxifen “pulse” transiently activated Cre and irreversibly and heritably labeled a high fraction of differentiated cardiomyocytes with GFP. During the “chase” period, no tamoxifen was given. If cardiomyocytes were generated by differentiation of progenitor cells, then the fraction of GFP-labeled cardiomyocytes would decline during the chase. On the other hand, if no new cardiomyocytes were generated, or if new cardiomyocytes were generated by proliferation of existing cardiomyocytes, the fraction of labeled cardiomyocytes would not change. During the 1-year chase period, no dilution of the genetic label was observed, indicating that cardiac progenitors do not substantially contribute to normal homeostasis of the murine heart. This conclusion is based upon the critical assumption that cardiac progenitors are not labeled by MHC $\alpha$ -MerCreMer. Reasonable evidence supporting this assumption was provided, although the possibility cannot be completely excluded because of the lack of consensus on the proper progenitor population to study.

The same genetic pulse-chase approach was used to investigate endogenous myocardial regeneration after myocardial injury. In the face of myocardial injury from myocardial infarction, the fraction of GFP<sup>+</sup> cardiomyocytes decreased from  $82.8 \pm 1.5\%$  to  $67.5 \pm 2.0\%$  in the MI borderzone over three months (52). These results suggest that about 18% ( $(82.8 - 67.5)/82.8$ ) of cardiomyocytes in the MI borderzone are newly born from unlabeled cardiomyocyte precursors. In regions of myocardium remote from the infarct, label dilution was less substantial but still significant, and consistent with about 7% of cardiomyocytes arising from unlabeled cardiomyocyte precursors. Control experiments suggested that preferential cell death of GFP<sup>+</sup> cells or decline in GFP<sup>+</sup> cardiomyocyte proliferation were unlikely causes of dilution. However, this approach could not completely exclude label dilution arising by other means, such as differential responses of GFP<sup>+</sup> and GFP<sup>-</sup> cardiomyocyte populations to myocardial injury. Another limitation of the experimental strategy is that it did not consider myocardial regeneration from proliferation of MHC $\alpha$ -expressing cardiomyocytes, and therefore may underestimate the extent of new myocyte birth during myocardial injury. Collectively, the study provided evidence for birth of new myocytes from progenitors in the setting of myocardial injury, but did not inform us of the identity of these progenitors. This result also suggests that the mechanisms underlying adult and neonatal heart regeneration are distinct, with the former resulting from progenitor cell expansion and the later arising from cardiomyocyte proliferation (47,52).

## Myocardial Regeneration in Humans

In humans, isotopic cardiomyocyte birth dating provides evidence supporting ongoing cardiomyocyte turnover (53). As a result of above-ground nuclear testing, bioavailable carbon-14 (<sup>14</sup>C) globally increased sharply from 1955 to 1963. Cessation of above-ground nuclear testing after 1963 permitted exponential clearance of bioavailable <sup>14</sup>C. The <sup>14</sup>C content of a cell's DNA correlates with bioavailable <sup>14</sup>C at the time of the cell's birth. Thus, measurement of human cardiomyocyte DNA <sup>14</sup>C concentration allows retrospective birth dating. In subjects born before 1955, cardiomyocyte <sup>14</sup>C concentrations were higher than environmental levels at the time of their birth, consistent with significant birth of cardiomyocytes post-natally (53). In the five oldest individuals studied, cardiomyocyte DNA <sup>14</sup>C concentration remained below present-day levels, indicating that a substantial fraction of cardiomyocytes persist from early life even in the elderly (53). Quantitative modeling suggested annual cardiomyocyte turnover rates of 0.2–2%, with a negative correlation to age. According to this modeling, at age 50 about half of cardiomyocytes remain from the time of the individual's birth, while the rest were generated later (53).

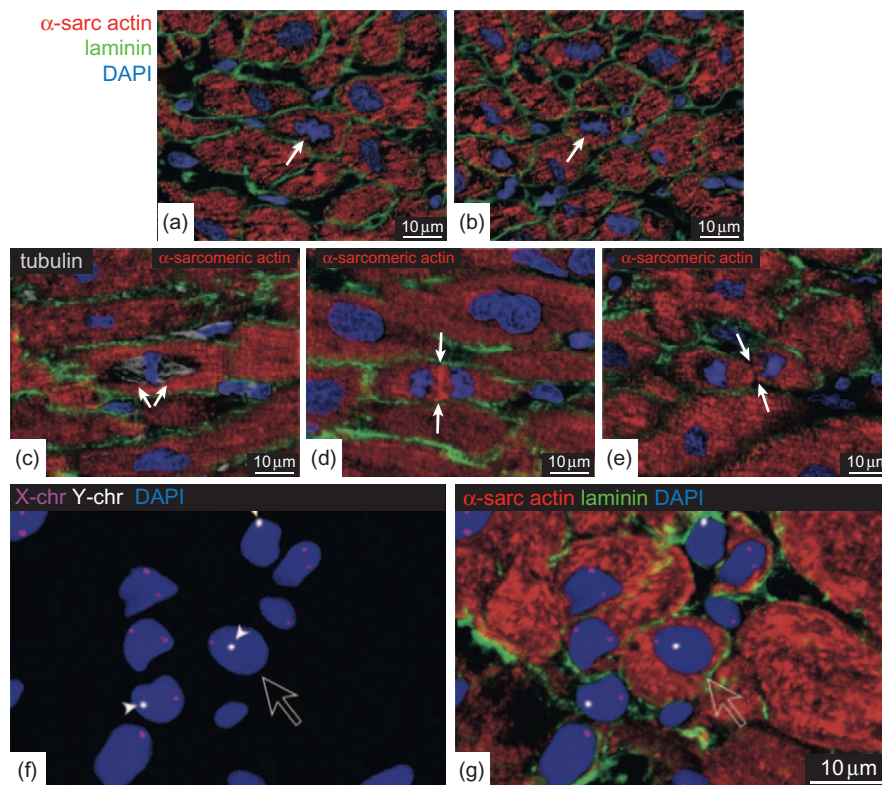
While this elegant study represents perhaps the best assessment of cardiomyocyte turnover in the human heart, it has limitations that perpetuate uncertainty about the extent of human cardiomyocyte turnover. An important technical hurdle in this experiment was isolating purified cardiomyocyte DNA. This was achieved by fluorescence-activated cell sorting (FACS) of cardiomyocyte nuclei, which were identified by the presence of the sarcomere proteins TNNT2 or TNNI3. Although predominantly cytoplasmic, sufficient TNNT2 or TNNI3 was present in nuclei for FACS. The sorted nuclei were highly enriched for cardiomyocyte markers and depleted for non-myocyte markers, and cardiomyocyte nuclei were judged to be 96% pure (53). However, Anversa and colleagues have argued that the TNNI3<sup>+</sup> nuclear fraction was biased toward senescent cardiomyocytes due to increased nuclear permeability and therefore underestimated the turnover of younger cardiomyocytes that may be more proliferative (54). Another limitation is that the study measured the timing of DNA synthesis, which may be dissociated from cardiomyocyte birth by DNA repair, multinucleation, and polyploidy. The authors accounted for these factors and argued that they are unlikely to significantly impact their findings (53). The study also did not investigate the effect of myocardial injury, which substantially stimulated myocardial regeneration in mouse (52), and the study did not provide insights into the source of the new cardiomyocytes. In summary, this important study showed that there is a low but significant contribution of cardiomyocyte turnover to homeostasis of the normal human heart.



Other groups, most notably the one led by Piero Anversa, have argued that cardiomyocyte turnover is far more extensive (48,55). These investigators stained human heart sections for cardiomyocyte markers (sarcomeric actin) and markers of proliferating cells. Their quantitative analysis identified 14 myocytes with mitotic figures per million myocytes in normal hearts, and this increased to 152 mitotic myocytes per million myocytes in ischemic cardiomyopathy (56) (Figure 39.3a–e). Similarly, staining for Ki-67, a marker of actively cycling cells, identified 500 cycling myocytes per million myocytes in normal hearts, and 42,000 cycling myocytes per million myocytes in myocardium bordering a myocardial infarct (57). Based on these data and assumptions about the duration of mitosis, the Anversa group calculated that in a normal adult heart containing  $\sim 5 \times 10^9$  myocytes,  $3 \times 10^6$  new myocytes are born daily. At this rate, the entire myocyte population would be exchanged every 4.5 years, and the myocytes lost in a myocardial infarction could be replaced in 18 days (48,55). By this estimate, the heart has copious regenerative capacity, and the limitation to myocardial recovery after injury is not regeneration *per se* but barriers to regeneration imposed by the competing processes of inflammation, ischemia, and scarring (48,55).

While highly provocative, these results have been difficult to reproduce. Field et al. used transgenic mice expressing nuclear localized lacZ to permit unambiguous

identification of cardiomyocyte nuclei (15). In uninjured heart, after 60 hours of exposure to  $^3\text{H}$ -thymidine, only one in 180,000 myocyte nuclei was identified as having undergone DNA synthesis and taken up the label. By this measure, at most 5 myocytes per million actively synthesize DNA in a 60-hour period (15). A recent study using immunostaining and DNA content analysis of FACS-sorted troponin T<sup>+</sup> cardiomyocyte nuclei independently confirmed cell cycle exit by post-natal day 21 (13). Estimated cell cycle activity from  $^3\text{H}$ -thymidine uptake was at least 350-fold lower than the 14 mitotic myocytes per million (56), because of the relative difference in the labeling periods (60 hours versus an estimated 30 minute duration for mitosis). After injury, the  $^3\text{H}$  thymidine uptake assay also confirmed increased cardiomyocyte cell cycle activity, although the level of activity remained low: 14 myocytes per million actively synthesized DNA in a 60-hour period (15), a level that is 1,300-fold lower than the mitotic activity estimated by Anversa and colleagues (56). The difference in results may stem from technical difficulties in measuring myocyte proliferative activity, such as establishing thresholds to classify signals as positive or negative, distinguishing cardiomyocyte from non-myocyte nuclei in sections (49), and resolving forms of endoreduplication from cardiomyocyte proliferation (20) (Figure 39.2). It is possible that human myocardium, where the highest values of cardiomyocyte cell



**FIGURE 39.3 Proliferation of human adult cardiomyocytes.** (a,b) Meta-phase chromosome in acromegaly (a) and diabetes (b). (c) Mitotic spindle within a dividing cardiomyocyte. (d) Actin condensation at the contractile ring. (e) Cardiomyocyte undergoing cytokinesis. (f,g) Cardiomyocyte chimerism in sex-mismatched transplantation. Host (male) nuclei within the female donor heart can be identified by staining for X and Y chromosomes. The large arrow points to a cardiomyocyte, identified by staining for  $\alpha$ -sarcomeric actin. (Adapted from Anversa et al., 2007 (48), with permission.)

cycle reentry have been reported, has intrinsically greater regenerative capacity than rodents, perhaps reflecting the greater fraction of mononuclear cardiomyocytes and the far greater lifespan of humans. However,  $^{14}\text{C}$  birth dating of human cardiomyocytes suggests this possibility is unlikely (53).

In summary, multiple approaches concur that there is detectable post-natal birth of new cardiomyocytes, and myocardial injury strongly stimulates cardiomyocyte cell cycle activity. However, there is no agreement on the extent of new cardiomyocyte production, with estimates ranging from a trivial level inadequate to support myocardial regeneration to a robust level competent for regenerating injured myocardium. The preponderance of evidence supports the former, but each approach has limitations that could lead to errors in absolute quantitation of cardiomyocyte turnover.

## CARDIAC PROGENITOR CELLS

There are two fundamental mechanisms that might regenerate myocardium. First, the adult animal may contain cardiac progenitor cells (CPCs) capable of self-renewal and cardiomyocyte differentiation. These progenitors may be located within the heart itself (resident CPCs) or located at distant sites (e.g. the bone marrow) and disseminated to the heart via the blood stream. Second, a subset of differentiated cardiomyocytes may not be terminally differentiated, and may be able to reenter the cell cycle to generate new cardiomyocytes. These two mechanisms are not mutually exclusive, and both could potentially contribute to myocardial regeneration, even within the same process. For instance, cardiac progenitors have been proposed to be slowly cycling, multipotent stem cells that generate transiently amplifying immature cardiomyocytes (55). These immature myocytes are proposed to be small, proliferating, mononuclear cells that express some cardiomyocyte markers. In this section we review reported cardiac progenitor cell populations, which can be subgrouped as resident and non-resident progenitors.

### Resident Cardiac Progenitor Cells (see Table 39.2)

Several resident CPCs have been reported in the adult heart (reviewed in (58)). In general, these stem cell populations have been discovered by expression of key regulators of cardiogenesis in the developing heart, or by markers used to identify stem cell populations in other tissues. The use of developmental markers allows lessons of heart development to be applied to the biology of adult CPCs, while this can be more challenging for markers not grounded in normal heart development. Moreover, genetic fate mapping has become the best available method to

confirm *in vivo* cardiogenic activity of a putative progenitor population. This approach is problematic for progenitors defined by markers expressed by a heterogeneous population of cells within and outside of the heart.

Perhaps the most thoroughly characterized CPC is defined by expression of the transcription factor *Isl1*. In the developing mouse embryo, at embryonic day 7.5 (E7.5) a crescent of cells express differentiated cardiac markers such as troponin T (see Chapters 3 and 4 for a review of cardiogenesis in normal heart development). By E8.0, these cells fuse in the ventral midline to form a tube of differentiated cardiomyocytes encompassing a second endothelial tube, the endocardium. The cells of this initial heart tube and their precursors have been described as the “first heart field” (59). Work over the last decade has shown that this initial heart tube elongates primarily by continued addition of cardiomyocytes that differentiate from progenitors located at either pole of the heart tube (59–63). These “second heart field” progenitors, marked by expression *Isl1*, contribute to the outflow tract, right ventricle, part of atrium and a subset of left ventricle (62). As these progenitor cells differentiate, *Isl1* is downregulated, and it is no longer expressed in differentiated cardiomyocytes. Clonal analysis showed that these *Isl1*<sup>+</sup> cells are multipotent, i.e. an *Isl1*<sup>+</sup> progenitor can differentiate into cardiomyocyte, smooth muscle, and endothelial lineages (64). *Isl1*<sup>+</sup> cells remain in the post-natal heart, where they retain self-renewal ability and adopt cardiomyocyte fates (65). However, the role of *Isl1*<sup>+</sup> cells in post-natal heart growth and injury response remains unclear, particularly since these progenitors appear to be more relevant to the biology of the atria, right ventricle, and outflow tract rather than the left ventricle, the principle pumping chamber.

Another important progenitor population is marked by expression of the cardiac transcription factor *Nkx2-5*. *Nkx2-5*<sup>+</sup> progenitors contribute to both first and second heart fields (66,67). Although *Nkx2-5* is expressed in both cardiac progenitors and differentiated cardiomyocytes, an enhancer of *Nkx2-5* is preferentially active in progenitors. Wu et al. isolated progenitors in transgenic mice and ES cells in which the enhancer drives expression of GFP, and clonal analysis showed that these cells differentiate into cardiomyocyte and smooth muscle lineages (68). In addition to cardiomyocyte and smooth muscle lineages, Cre-loxP fate mapping approaches in developing embryos showed *Nkx2-5*-lineage cell contribution to endocardium (67,69). Intriguingly, infrequent cells in the neonatal and adult heart continue to exhibit activity of the *Nkx2-5* progenitor enhancer, and their role in the normal and injured post-natal heart is under active investigation (S.M. Wu, unpublished).

Epicardial progenitors are vital to the developing heart and can be found on the adult heart. Epicardial progenitors originate from the proepicardium, an outpouching from the

**TABLE 39.2** Resident Cardiac Progenitor Cells

Cell Type	Multipotency <i>in vitro</i>	Differentiated Lineages <i>in vivo</i>	Resident in Adult Heart	Role in Therapeutic Regeneration	References
Isl1 <sup>+</sup>	CM, SM, EC	CM, SM, EC	Not reported in adult. In atria and OFT of neonatal heart	Not determined	64,65
Nkx2-5 progenitor enhancer	CM, SM	CM, SM, EC	Yes	Not determined	68
WT1 <sup>+</sup> or TBX18 <sup>+</sup> epicardial progenitor	CM, SM	CM, SM, EC, fibro	Adult epicardium	Normal expansion after MI. EPDC injection and EPDC conditioned media reduced infarct size and improved function after MI. TB4 priming may permit EPDC differentiation to cardiomyocytes	71,72,74,77,106
c-Kit <sup>+</sup> Lin <sup>-</sup>	CM, SM, EC	CM, SM, EC. Not reproducible in all labs.	Myocardial clusters	After MI, myocardial injection gave bands of regenerating myocardium and functional improvement	78–80
Sca1 <sup>+</sup>	Unknown	CM	Yes	Sca1 <sup>+</sup> cells engrafted and differentiated to CM after MI. Functional improvement not demonstrated	51
Cardiac SP	Unknown	CM	Yes	After cryoinjury, SP cells home to injury site and differentiate into cardiomyocytes. Functional improvement not demonstrated	81–84
Cardiospheres	CM, SM, EC	CM, SM, EC	Yes	After myocardial infarction, cardiosphere derived cell infusion into the coronary arteries decreased infarct size and improved ventricular function	86,107

Abbreviations: CM, cardiomyocyte; SM, smooth muscle cell; EC, endothelial cell; EPDC, epicardium-derived cell; MI, myocardial infarction.

septum transversum located below the atrioventricular groove (70). Proepicardial cells migrate onto the surface of the heart to form the epicardium, a mesothelial sheet overlying the myocardium. Progenitor cells in the epicardium undergo epithelial to mesenchymal transition (EMT) to form mesenchymal cells that migrate into the myocardium and differentiate into smooth muscle, endothelial, interstitial, and cardiomyocyte cells (71,72). Consistent with their cardiomyogenic potential, epicardial progenitors are descended from precursors that express *Nkx2-5* and *Isl1* (71). In addition, epicardial cells engage in extensive bi-directional signaling with the underlying myocardium, promoting myocardial growth and coronary vessel development (reviewed in (73)). Thus, epicardium may participate in regenerative responses both by generating key differentiated cardiac lineages and by conditioning a microenvironment favorable for myocardial repair.

Epicardial progenitors are marked by expression of the transcription factors WT1 and TBX18 (71,72). In the adult heart, the epicardium is quiescent and downregulates WT1,

TBX18, and other genes actively expressed in fetal epicardium. However, myocardial infarction stimulates reactivation of fetal epicardial genes including WT1, and proliferation and expansion of the epicardial layer through an EMT-like process (74). Unlike fetal heart, adult epicardium-derived cells remained on the surface of the heart and were not observed to differentiate into cardiomyocytes. The cells largely differentiated into fibroblasts, myofibroblasts, and smooth muscle cells. These cells actively produce secreted factors that promote angiogenesis and that have protective effects in myocardial infarction (74). Similar to epicardial activation by MI in mice, apex amputation in zebrafish stimulated reactivation of a fetal gene expression program (39). Remarkably, apex amputation activated a *gata4* transcriptional enhancer in a subpopulation of cardiomyocytes located just below the epicardium, and cardiomyocytes that repopulated the amputated apex originated from this select subepicardial cardiomyocyte subpopulation. These data suggest a key role of epicardial signaling in the regenerative process (40).

The nature of the epicardial signals and the function of the mammalian epicardium in conditioning a subepicardial microenvironment conducive to regeneration is an area of intense interest. One leading candidate signal is thymosin beta4 (TB4), a small G-actin monomer binding protein that promotes cardiomyocyte survival and myocardial repair after experimental infarction (75). Although the mechanisms of TB4 action remain unclear, at least in part TB4 stimulates expansion, mobilization, and differentiation of adult epicardial progenitors (76). Remarkably, Riley and colleagues found that administration of TB4 prior to MI allowed a small fraction of epicardial cells of the adult heart to differentiate into cardiomyocytes (77), suggesting that TB4 may also influence the plasticity of cardiac progenitors.

CPCs have also been identified using markers of stem cells identified in other systems. The most prominent such progenitor population are the c-Kit<sup>+</sup>, hematological lineage negative (Lin<sup>-</sup>) cardiac progenitors, which are found in the adult mouse heart in clusters suggestive of stem cell niches. These c-Kit<sup>+</sup> cells were reported to possess the stem cell properties of self-renewal, clonogenicity, and multipotency, and differentiated into cardiomyocyte, smooth muscle, and endothelial cell lineages *in vitro* (78,79). Moreover, when injected into an injured heart, these cells formed functional blood vessels and cardiomyocytes in regenerated myocardium (78,79). An independent group, using mouse lines with transgenic cardiomyocyte markers, confirmed that a subset of neonatal c-kit<sup>+</sup> cells had cardiomyogenic potential (80). However, their data indicated that adult cardiac c-Kit<sup>+</sup> cells possessed little to no cardiomyogenic potential (1 event in 56,000 cells tested) (80). The factors underlying the discrepant results are uncertain, but may relate to changes in cell properties with prolonged expansion of c-Kit<sup>+</sup> cells in culture. Further studies are required to understand the cardiogenic activity of adult c-Kit<sup>+</sup> cells, and the signals that regulate their expansion and lineage commitment.

The stem cell antigen Sca1 has also been used to identify a population of adult heart-derived cardiac progenitor cells. Sca1<sup>+</sup> CPCs express cardiac transcription factors GATA4, MEF2c, and SRF, but not markers of differentiated cardiomyocytes. Under differentiation conditions in cell culture, cardiac Sca1<sup>+</sup> cells upregulated markers of differentiated cardiomyocytes, and when injected intravenously after heart injury, they were recruited to the injury site and differentiated into cardiomyocytes (51). The cardiac Sca1<sup>+</sup> population is related to cardiac “side population” (SP) cells (81,82), defined by their enhanced efflux of the fluorescent DNA-binding dye Hoechst 33342 through an ATP-binding cassette transporter, because 93% of cardiac SP cells expressed Sca1 (51). Cardiac SP cells also underwent cardiac differentiation on methylcellulose or by co-culture with cardiomyocytes, and

cardiomyogenic potential was limited to the SP subpopulation that expressed Sca1 but not CD31 (83). When cocultured with adult rat ventricular cardiomyocytes, these SP cells adopted a mature cardiac phenotype and exhibited contractions and calcium transients in response to stimulation (83). In the uninjured heart, systemically injected cardiac SP cells were not efficiently recruited to the myocardium, and they did not differentiate into the cardiac lineage. However, recruitment was substantially enhanced by cardiac cryoinjury, and 4% of the recruited cells in the injury borderzone expressed the cardiomyocyte marker TNNT2, while the remainder expressed endothelial and smooth muscle markers (84). Another related CPC population characterized by expression of c-Kit and Sca1 has been isolated from primary cultures of human cardiac biopsies as spontaneous cell aggregates known as cardiospheres. Cardiosphere cells are self-renewing, and differentiate into cardiomyocytes when co-cultured with neonatal rat cardiomyocytes (85,86). Human cardiosphere cells injected into the borderzone of murine myocardial infarcts engrafted, reduced infarct size, and improved left ventricular function (86).

In summary, a number of cardiac progenitor populations have been defined that differentiate and express cardiomyocyte markers *in vitro* and *in vivo*. The ability of these progenitor populations to differentiate into functional, mechanically, and electrically integrated myocardium needs to be verified in independent laboratories using definitive *in vivo* fate mapping techniques. The extent to which these populations represent different subsets of a common progenitor remains to be determined.

## Non-resident Cardiac Progenitor Cells

Cells with cardiogenic potential do not necessarily need to originate from the heart, and in fact extra-cardiac progenitors may have greater translational value due to improved accessibility. Perhaps the strongest evidence supporting noncardiac progenitors arises from studies of human heart chimerism. In male recipients of female heart transplants, substantial numbers of cardiomyocytes bearing the Y chromosome were reported in the female donor heart, strongly suggesting migration of primitive cells from the recipient to the grafted heart (87) (Figure 39.3f–g). Consistent with this work, studies of sex-mismatched bone marrow transplant recipients established the human bone marrow as a source of extra-cardiac progenitor cells capable of *de novo* cardiomyocyte formation (88). While work from several labs supports cardiomyocyte chimerism in sex-mismatched transplantation, the frequency of this finding has been controversial, with some investigators rarely identifying them after controlling for fusion, autofluorescence, and intracytoplasmic circulating cells (2,50,88–90).



The potential use of bone marrow as a source of cardiac progenitor cells was established in a landmark study by Anversa and colleagues (91). They reported that injected bone marrow cells were recruited to infarcted myocardium and robustly transdifferentiated into cardiomyocytes. Consistent with this result, in mice transplanted with GFP-marked bone marrow, bone marrow-derived cells were recruited to infarcted myocardium, where they differentiated to cardiomyocytes and endothelial cells (92). However, the ability of bone marrow cells to differentiate into cardiomyocytes could not be reproduced by two other groups, who used genetic markers to lineage trace the injected cells and to identify cardiomyocytes (93,94). Fusion of transplanted cells with host cardiomyocytes and microscopy artifacts might potentially explain the transdifferentiation observed in the Anversa study (2,95,96). However, follow-up studies from the Anversa group continued to indicate the ability of transplanted bone marrow cells to differentiate into cardiomyocytes within infarct region, independent of cell fusion (97).

Spurred on by reports of beneficial effects of bone marrow injection in mouse myocardial infarction models and by reports of striking cardiomyocyte chimerism in sex-mismatched transplant patients, a number of human clinical trials were executed to test the hypothesis that bone marrow stem cells would improve outcome after myocardial infarction (6). Detailed review of the results of these studies is outside of the scope of this chapter, but in short the results were mixed and when benefits were seen they were not sustained. The injected cells did not efficiently engraft, leading to the conclusion that what benefits were seen were likely due to undefined paracrine mechanisms (98,99).

In summary, it remains contentious whether or not bone marrow progenitor cells are recruited to the heart and transdifferentiate into cardiomyocytes. While evidence of chimerism in the human heart is certainly suggestive, difficulty in reproducibly demonstrating homing, engraftment, and differentiation of bone marrow cells into cardiomyocytes in mouse models has led to doubts about the conceptual underpinnings of bone marrow progenitor therapy for myocardial injury. This has been reinforced by disappointing engraftment and minimal sustained impact on clinical endpoints in initial therapeutic trials. Clearly additional basic understanding of the underlying biology is required.

## PROLIFERATION OF DIFFERENTIATED CARDIOMYOCYTES

In addition to amplification and differentiation of cardiac progenitors, endogenous and therapeutic myocardial regeneration can occur by proliferation of differentiated cardiomyocytes. This model has been pushed to the fore

by the recent demonstration that regenerating myocardium in zebrafish originates from differentiated cardiomyocytes (40,41). In mammals, it appears that adult cardiomyocytes can also be induced to reenter the cell cycle by manipulation of appropriate signaling pathways (Figure 39.1). Insulin-like growth factor 1 (Igf1) is a major driver of physiological heart growth, and Igf1 stimulation was reported to increase cardiomyocyte number and cell cycle reentry (100). p38 mitogen-activated protein (MAP) kinase promoted cell cycle exit and cardiomyocyte differentiation and hypertrophy. Conversely disruption of p38 MAPK activity permitted fibroblast growth factor 1 (Fgf1) stimulated adult cardiomyocyte cell cycle reentry both *in vitro* and *in vivo*, in part by upregulating Cyclins A2 and D2 (12,101). Periostin, a ligand for  $\alpha$ V $\beta$ 3 and  $\alpha$ V $\beta$ 5 integrins, is among the most highly upregulated genes following myocardial injury. Periostin-induced adult cardiomyocyte cell cycle reentry through integrin signaling pathways, and recombinant periostin treatment improved cardiac function after myocardial infarction (102). However, this result has been disputed because it has not been supported by experiments in mice with genetic gain or loss of periostin function (103). Neuregulin 1 (Nrg1) was also reported to stimulate cardiomyocyte cell cycle reentry by signaling through the ErbB4 receptor tyrosine kinase (104). Interestingly, it appeared that Nrg1 preferentially stimulated cell cycle activity in mononuclear cardiomyocytes, some of which appeared to pass through the cell cycle at least twice. The greater proliferative capacity of smaller mononuclear cardiomyocytes has been noted by other groups (105), leading to the idea of a transiently amplifying population derived from cardiac progenitors that expresses cardiomyocyte markers (55). The phosphoinositide 3-kinase (PI3K) pathway is downstream of all four of the extracellular signals (Igf1, Fgf1, periostin, and Nrg1) known to stimulate cardiomyocyte cell cycle reentry, and thus the PI3K pathway may be central for this process. Collectively, these data indicate that adult cardiomyocytes can be stimulated to reenter the cell cycle through manipulation of intracellular signaling pathways, suggesting a promising avenue for therapeutic myocardial regeneration.

## CONCLUSIONS

While considerable evidence has overturned the decades-old dogma that the heart is devoid of regenerative capacity, the extent of endogenous cardiomyocyte turnover remains uncertain. Tantalizing studies suggest that the limited regenerative capacity of the mammalian myocardium can be therapeutically augmented to achieve clinically significant cardiac repair, a Holy Grail of cardiovascular medicine. However, attainment of this

goal will require surmounting considerable obstacles. Future work will need to achieve reproducibility between laboratories and to develop consistent definitions of cardiac progenitor populations. We need to understand lineage choices of cardiac progenitors and the signals that regulate them. We must have greater insight into the myocardial niches that foster myocardial regeneration. We need learn how new myocardium generated from progenitors is mechanically and electrically integrated with existing myocardium. With this knowledge in hand, we will be positioned to translate our expanding knowledge of myocardial regenerative mechanisms to clinical application.

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