

Review Article

Molecular and cellular basis for cardiac regeneration

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It was generally believed that the mammalian heart is a post-mitotic organ and therefore lacks the ability to regenerate or self-renew. However, this notion has been challenged by recent studies suggesting that the adult heart has the capability to create new muscle cells and that there exist cardiac stem/progenitor cells with the potential to differentiate into multiple cell types in the heart. It is also known that lower vertebrates such as amphibians or fish readily regenerate the injured heart. Elucidation of the molecular and cellular mechanisms of heart regeneration will eventually lead to the novel therapeutic approaches to enhance the regenerative capacity of the diseased human heart.

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In contrast to lower vertebrates such as amphibians and fish, the mammalian heart has been traditionally viewed as a post-mitotic organ that is incapable of self-renewal and regeneration. This notion was based on the observation in the first quarter of the 20th century that there was no mitotic figures in the hypertrophic human hearts¹⁾, and supported by clinical observations that the heart is unable to recover from large myocardial infarction and that the infarct area is replaced by non-contractile scar tissue. However, recent studies challenged this view and it is now recognized that mammals have limited capacity to regenerate cardiac muscle cells that is insufficient to effectively restore contractile function after extensive cardiac injury. In this review we first discuss about the heart regeneration in lower vertebrates. We then summarize the evidences of heart regeneration in ro-

dents and humans, and discuss about the possible contribution of resident stem/progenitor cells for heart regeneration in mammals.

Heart regeneration in lower vertebrates

It was well recognized for decades that amphibians have the ability to regenerate the injured heart²⁾. This regenerative capacity appears to be largely attributable to the robust proliferation of cardiac myocytes, because approximately 10% of myocytes were labeled with the marker of S phase following injury whereas few cells were labeled in comparable experiments in adult mammalian hearts^{3,4)}.

More recently, zebrafish proved to be a useful model organism for studying molecular mechanism of cardiac regeneration⁵⁾,

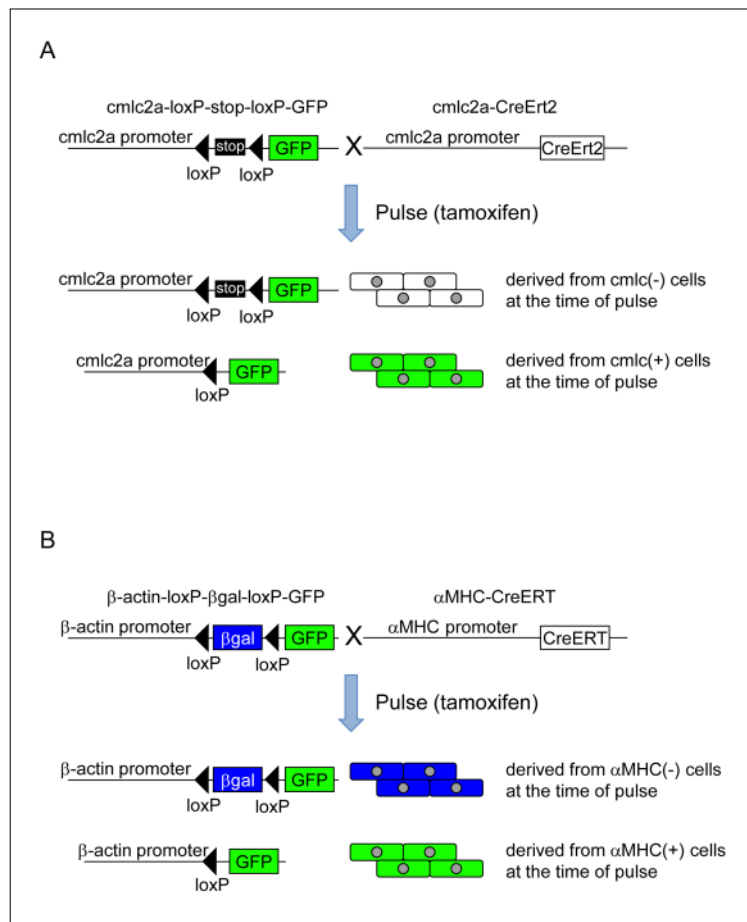


Fig.1 Genetic fate mapping of regenerated myocytes in zebrafish and mice

A : Transgenic zebrafish harboring two transgenes (*cmlc2a-CreErt2* and *cmlc2a-loxP-stop-loxP-GFP*) were treated with tamoxifen and subjected to partial resection of the ventricle. When the regenerated myocytes are GFP(-), those cells are derived from stem/progenitor cells that did not express *cmlc2* at the time of tamoxifen pulse. When the regenerated myocytes are GFP(+), those cells are derived from pre-existing myocytes that expressed *cmlc2* at the time of pulse.

B : Transgenic mice harboring two transgenes (α MHC-CreERT and β -actin-loxP- β gal-loxP-GFP) were treated with tamoxifen and subjected to myocardial infarction or pressure overload. When the regenerated myocytes are β gal(+), those cells are derived from stem/progenitor cells that did not express α MHC at the time of tamoxifen pulse. When the regenerated myocytes are GFP(+), those cells are derived from pre-existing myocytes that expressed α MHC at the time of pulse.

because zebrafish fully regenerate the heart following partial resection of up to 20% of the ventricle and are amenable to genetic manipulation. Like amphibians, zebrafish heart regeneration was associated with increased proliferation of myocytes⁵. Although it was initially proposed that undifferentiated progenitor cells are the source of newly generated myocytes⁶, recent two studies, using genetic fate-mapping techniques, convincingly demonstrated that the pre-existing myocytes are the primary source of regenerated myocytes^{7,8}. In one of these studies, transgenic fish that harbor two transgenes (*cmlc2a-CreErt2* and *cmlc2a-loxP-stop-loxP-GFP*) were treated with tamoxifen 48 hours after fertilization. Tamoxifen treatment results in Cre activation in cardiac myocytes (because CreErt2 is driven by cardiomyocyte-specific cardiac myosin light chain 2a (*cmlc2a*) promoter), which leads to the excision of loxP-flanked stop sequence and the expression of green fluorescent protein (GFP) in cardiac myocytes. Thus, when myocytes are regenerated from progenitors that do not express *cmlc2a*, newly formed myocytes should be negative for GFP. On the other hand, when myocytes are regenerated from

pre-existing myocytes, newly formed myocytes should express GFP (Fig.1A). The results show that cardiomyocytes within the regenerating tissue were uniformly GFP-positive, clearly demonstrating that the pre-existing myocytes are the primary source of newly generated myocytes⁸. It was also shown in this study that proliferating myocytes exhibit limited dedifferentiation characteristics⁸. These results therefore suggest that zebrafish heart regeneration is primarily mediated by dedifferentiation and proliferation of cardiomyocytes.

The precise molecular mechanism by which myocyte proliferation is enhanced during cardiac regeneration is not fully understood. However, it was recently shown that retinoic acid production from the endocardium is required for myocyte proliferation during regeneration in zebrafish⁹. Retinoic acid signaling is also required for cardiomyocyte proliferation during embryonic heart development in mice^{10,11}. Of note, it appears that cardiomyocytes are not the direct target of retinoic acid, because retinoic acid signaling in cardiac myocytes is not required for normal myocyte proliferation during mouse heart development¹²⁻¹⁴. Elu-

citation of the role of retinoic acid signaling during embryonic development may provide insights into the mechanism by which retinoic acid promotes myocyte proliferation during regeneration. It should also be noted that the production of retinoic acid in the endocardium was observed in the heart of another fish species *Polypterus senegalus* that are capable of cardiac regeneration after injury but not in non-regenerative mouse heart⁹, which may in part explain the species differences in regenerative capacity of the heart.

Heart regeneration in rodents

In rodents, cardiac growth during embryonic development is primarily achieved by myocyte proliferation. However, the proliferative capacity of cardiac myocytes is lost soon after birth. In fact, assessment of cardiomyocyte DNA synthesis as measured by thymidine incorporation in the adult mouse heart revealed that labeled nuclei was observed in 0.0005% of myocytes at baseline and in 0.0083% of myocytes in the border zone after injury¹⁵, suggesting that cardiomyocyte DNA synthesis in the adult rodent heart is an extremely rare event. Nonetheless, several approaches were taken to therapeutically enhance the limited proliferative capacity of adult cardiac myocytes, which include the overexpression of cell cycle regulatory factors in cardiac myocytes¹⁶. For instance, overexpression of cyclin D2 in cardiac myocytes in transgenic mice resulted in increased DNA synthesis at baseline and regression of infarct size after permanent coronary ligation¹⁷. Pharmacological approaches to enhance proliferation of adult cardiac myocytes were also tested, which includes periostin¹⁸, fibroblast growth factor plus p38 inhibitor¹⁹, and neuregulin²⁰. In these studies, increased proliferative capacity of cardiac myocytes was associated with reduced ventricular remodeling after myocardial infarction. These genetic and pharmacological studies suggest that cardiomyocyte cell cycle control is a potential target for therapeutic heart regeneration.

Although cardiomyocyte proliferation appears to be a rare event in the adult rodent heart, a recent study showed that the heart of 1-day-old neonatal mice can regenerate after partial resection of the left ventricular apex²¹. A genetic fate-mapping study revealed that this regenerative response was attributable to the proliferation of pre-existing cardiac myocytes, and the regenerative capacity was lost by 7 days of life. The mode of regenerative response of the neonatal mouse heart is quite similar to that of zebrafish, and an important question is how the mammalian heart switches off its regenerative capacity in the neonatal stage.

The studies mentioned above are focused on the proliferative

capacity of pre-existing myocytes and are not designed to test whether resident stem/progenitor cells contribute to cardiac regeneration. To examine this possibility, a genetic fate-mapping study was performed using transgenic mice harboring two transgenes (α MHC-CreERT and β -actin-loxP- β gal-loxP-GFP)²². In these animals, α -myosin heavy chain (α MHC) promoter drives the expression of tamoxifen-inducible Cre recombinase in cardiac myocytes, and the activation of Cre recombinase by tamoxifen treatment induces the excision of loxP-flanked β -galactosidase gene and the expression of GFP in cardiac myocytes (Fig. 1B). Treatment of these animals with tamoxifen for 14 days resulted in GFP expression in $\sim 80\%$ of myocytes. When heart regeneration occurs and new myocytes are formed from stem/progenitor cells that do not express myosin heavy chain, the percentage of GFP-positive myocytes will be reduced. On the other hand, when new myocytes are generated from pre-existing myocytes, the percentage of GFP-positive myocytes will be maintained. When mice were analyzed 1 year after tamoxifen treatment, the percentage of GFP-positive myocytes were unaltered, suggesting that there is no myocyte renewal from stem/progenitor cells during normal aging. However, when mice were analyzed 3 months after myocardial infarction or pressure overload, there was a significant reduction in the percentage of GFP-positive myocytes, suggesting that new cardiac muscle cells were generated in response to injury from stem/progenitor cell population. Together with the observation that cardiomyocyte proliferation in the adult heart is an extremely rare event, these results suggest that the regenerative capacity of the mouse heart depends on new myocyte generation from stem/progenitor cells and is mechanistically distinct from that of zebrafish.

Human heart regeneration

Whether human hearts are able to regenerate or self-renew have been vigorously debated. It was previously reported that in the hearts of patients who died after myocardial infarction, 4% and 1% of myocytes in the border zone and the remote area, respectively, were positive for cell proliferation marker (Ki-67). There were also some Ki-67-positive cells in control hearts, suggesting that significant myocyte proliferation occurs at baseline and is further enhanced following injury²³. It was estimated that the entire heart muscle cells are replaced every 4.5 years²⁴.

A different strategy was also used to estimate the turnover of human cardiomyocytes, which utilized the ^{14}C levels in the nucleus as a marker of a birth date of human cells²⁵. Due to above-ground nuclear testing during the Cold War, atmospheric levels of ^{14}C were dramatically increased, and ^{14}C quickly spread

around the world as $^{14}\text{CO}_2$. With the signing of the Limited Nuclear Test Ban Treaty in 1963, the atmospheric ^{14}C levels gradually and exponentially declined. Because ^{14}C is ultimately incorporated into the DNA of dividing cells and DNA of a cell is stable after its last division, ^{14}C concentration in DNA mirrors the atmospheric levels of ^{14}C at the time of its last division and therefore can be used to retrospectively birth date human cells²⁶. Using this technique, it was shown that the lifespan of cardiomyocytes isolated from patients are generally shorter than the lifespan of patients. Mathematical modeling suggested that $\sim 1\%$ and $\sim 0.4\%$ of myocytes are renewed every year at the age of 20 and 80, respectively, leading to the estimation that $\sim 45\%$ of myocytes are replaced during the normal human lifespan²⁵.

The reason for the discrepancy between the above-mentioned two studies is not clear. However, Ki-67 labeling may over-estimate cardiomyocyte proliferation, because cardiomyocyte sometimes undergo DNA synthesis without nuclear division (endoreduplication) or nuclear division without cytokinesis (binucleation). Taken together, these studies in human specimen provide strong evidences that human heart is capable of self-renewal, albeit with extremely low frequencies. It is also not clear from these studies whether the newly formed myocytes are derived from pre-existing myocytes or from a stem/progenitor cell population, although a genetic fate-mapping study in mice mentioned in the previous section strongly suggests that resident stem/progenitor cells contribute to cardiomyocyte renewal in humans²².

Cardiac stem/progenitor cells

Several groups have identified a population of resident cardiac stem/progenitor cells in the adult heart (Table 1).

1) c-Kit(+) cells

Analogous to hematopoietic stem cells in the bone marrow, cells that are positive for c-Kit and negative for markers of lineage commitment were isolated from adult heart. These cells were shown to differentiate into cardiomyocytes, endothelial cells, and vascular smooth muscle cells *in vitro* after isolation, and to contribute to the regenerated myocardium following *in vivo* transplantation²⁷, although there also exists a study showing the lack of cardiomyogenic potential of c-Kit(+) cells in the adult heart²⁸. A minority of these cells are reported to express early cardiac marker genes such as Nkx2.5, GATA4, and Mef2c. The developmental origin of these c-Kit(+) cardiac progenitor cells is not clear, although bone marrow transplantation experiments suggest that many of the c-Kit(+) cells in the adult heart are of bone marrow origin²⁹.

2) Sca-1(+) cells

Sca-1 is another cell surface antigen that is used for the isolation of hematopoietic stem cells. Accordingly, Sca-1(+) cells were isolated from the adult heart and shown to differentiate into beating cardiomyocytes when isolated and treated with 5-azacytidine or oxytocin^{30,31}. When administered intravenously after myocardial infarction, these cells home to the infarct border zone and differentiate into cardiomyocytes, although fusion between Sca-1(+) cells and host myocytes were also observed. The expressions

Table 1 Characteristics of resident cardiac stem/progenitor cells

cell type	markers	self-renewal	clonogenic	multipotent	effect after transplantation
c-Kit (+) cells	(+) : c-Kit (±) : Nkx2.5, GATA4, Mef2c (−) : lineage markers	Yes	Yes	Yes	myocardial regeneration functional improvement
Sca-1(+) cells	(+) : Sca-1, Nkx2.5, GATA4 (−) : c-Kit, Mef2c	Unknown	Yes	Unknown	differentiation into cardiac myocytes
SP cells	(+) : Sca-1 (±) : Nkx2.5, GATA4, Mef2c (−) : c-Kit	Unknown	Unknown	Yes	differentiation into cardiac myocytes smooth muscle cells endothelial cells
Isl-1(+) cells	(+) : Nkx2.5, GATA4 (−) : c-Kit, Sca-1	Yes	Yes	Yes	Unknown

of early cardiac markers Nkx2.5 and GATA4 but not Mef2c are reported in Sca-1(+) cells.

3) Cardiac side population (SP) cells

A population of cells with stem-like properties has been identified first in bone marrow and subsequently in multiple tissues based on their ability to pump out Hoechst dye^{32,33}. The name side population (SP) is derived from the observation that these cells are visualized as a negatively stained “side population” on density dot plots of fluorescence-activated cell sorting, and SP cells are marked by the expressions of the ATP-binding cassette (ABC) transporters that mediate the efflux of Hoechst dye. SP cells from the adult heart were isolated, and demonstrated to be capable of differentiation into cardiomyocytes, endothelial cells, and smooth muscle cells after transplantation³⁴⁻³⁷. The expressions of Sca-1 and early cardiac markers such as Nkx2.5, GATA4, and Mef2c are reported in these cells.

4) Isl-1(+) cells

Isl-1 (Isl-1) was originally identified as a LIM homeodomain-containing transcription factor that regulates pancreas and motor neuron development, and subsequently it was found that Isl-1 also regulates the development of the second heart field-derived tissues³⁸. Lineage tracing experiments demonstrate that Isl-1(+) cells contribute to cardiomyocytes, endothelial cells, and smooth muscle cells^{39,40}. Isolated Isl-1(+) cells were shown to proliferate and differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells⁴⁰. These cells are reported to express Nkx2.5 and GATA4 but not c-Kit or Sca-1.

Although the study of cardiac stem/progenitor cells is an exciting area in cardiovascular research, it is also highly controversial. For instance, the four populations of cardiac stem/progenitor cells mentioned above appear to be partly overlapping but different from each other in terms of marker expression and differentiation potential, and it is not clear whether these stem/progenitor cell populations are really distinct cells or we are looking at the same population of cells at different stages of development/differentiation. In this regard, the identification of a single cell surface marker that distinguishes cardiac stem/progenitor cells from other cell types is highly desired. In addition, most of the studies are based on cell culture and transplantation experiments, and it is not formally demonstrated whether these resident cardiac stem/progenitor cells contribute to the generation of new cardiovascular cells *in vivo*. Genetic fate mapping studies are mandatory to address this issue.

Perspective

Our understanding of cardiac regeneration and resident cardiac stem/progenitor cells has been dramatically broadened and redefined by recent seminal studies. However, it is still not clinically feasible to induce meaningful cardiac regeneration in patients with severe heart failure. Although manipulation of extrinsic stem cells (e.g., embryonic stem cells, induced pluripotent stem cells, and induced cardiomyocytes) as well as tissue engineering techniques to build three-dimensional functional muscle tissues are also important areas of research, the ultimate goal is the development of pharmacological interventions that enhance the intrinsic regenerative capacity of the heart. Further advances in the developmental and regenerative biology will help us to achieve this goal.

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