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# Heart Regeneration by Endogenous Stem Cells and Cardiomyocyte Proliferation: Controversy, Fallacy, and Progress

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## Abstract

Ischemic heart disease is the leading cause of death worldwide. Myocardial infarction results in an irreversible loss of cardiomyocytes with subsequent adverse remodeling and heart failure. Identification of new sources for cardiomyocytes and promoting their formation represent a holy grail of cardiac biology and regenerative medicine. Within the past decade, many types of putative cardiac stem cells (CSCs) have been reported to regenerate the injured myocardium by differentiating into new cardiomyocytes. Some of these CSCs have been “translated” from bench to bed with reported therapeutic effectiveness. However, recent basic research studies on stem cell tracing have begun to question their fundamental biology and mechanisms of action, raising serious concerns over the myogenic potential of CSCs. Here, we review the history of different types of CSCs within the past decade and provide an update of recent cell tracing studies that have challenged the origin and existence of CSCs. In addition to the potential role of CSCs in heart regeneration, proliferation of pre-existing cardiomyocytes has recently gained more attention. This review will also evaluate the methodological and technical aspects of past and current studies on CSCs and cardiomyocyte proliferation, with emphasis on technical strengths, advantages, and potential limitations of research approaches. While our current understanding of cardiomyocyte generation and regeneration continues to evolve, it is important to address the shortcomings and

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inaccuracies in this field. This is best achieved by embracing technological advancements and improved methods to label single cardiomyocytes/progenitors and accurately investigate their developmental potential and fate/lineage commitment.

**Keywords**

Cardiac stem cells; cardiomyocyte proliferation; heart regeneration; lineage tracing; myocardial infarction

## Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide, accounting for an estimated 17.9 million deaths annually[1–3]. The most common type of CVD, known as ischemic heart disease, is due to plaque buildup within the coronary arteries that supply the myocardium with oxygen and nutrients. Reduction or blockage of blood flow through the coronaries during myocardial infarction (MI) leads to a dramatic and irreversible loss of cardiomyocytes, with estimates placing that loss to upward of a billion cells[4]. Due to the limited regenerative capacity of the heart, the remaining cardiomyocytes are unable to remuscularize and restore lost cells. Compensatory scarring to replace dead tissue leads to compromised cardiac function and eventually heart failure[5, 6]. A novel approach to combat heart failure has been to stimulate ‘cardiac stem cells’ (CSCs) to generate new cardiomyocytes or to induce the proliferation of existing cardiomyocytes in order to replace the scar tissue. However, the existence of CSCs has not been convincingly established and the proliferative capacity of cardiomyocytes during development, after birth, and in response to injury remains an area of controversy. The technological breakthroughs of the past decade have allowed for the study of stem cell fate and cardiomyocyte regeneration at an unprecedented resolution. Results from these studies[7–9] have led to a paradigm shift of cardiomyocyte generation from endogenous CSCs to the currently accepted model of cardiomyocyte proliferation. Many investigators are exploring different ways to promote the proliferative potential of cardiomyocytes and unlock the cell-cycle arrest of cardiomyocytes in the adult hearts[10]. In this review, we introduce the rise and fall of stem cell theory for cardiomyocyte generation and discuss the accumulating evidence for cardiomyocyte proliferation. We will present detailed technical aspects of each approach with advantages, potential limitations, and future prospects.

## Stem cells for heart repair and regeneration

Cell therapy has been intensely studied over the past two decades as a potential treatment for ischemic heart disease. A wide variety of cells have been evaluated for therapeutic delivery including bone marrow cells, mesenchymal stem cells (MSCs), and endogenous cardiac stem cells[11, 12]. The initial study of bone marrow cells (BMCs) for regenerating injured myocardium has subsequently led to immense basic and clinical investigation to isolate and deliver c-Kit$^+$ bone marrow cells for treating ischemic diseases[13]. Early clinical trials showed inconsistent but slight improvement in cardiac function, generating excitement and hope for patients, clinicians, and scientists for treatment of ischemic heart disease[14, 15].

These earlier studies were often done in smaller Phase I/II cohorts and used more selective post-hoc analyses. Larger and more adequately powered clinical trials as well as meta-analyses subsequently uncovered a minimal or unsustained beneficial effect on heart function[16]. This raised many concerns over the mechanisms of action by transplanted cells, with controversies about how BMCs can transdifferentiate into cardiomyocytes (Figure 1). With the development of new technologies such as genetic lineage tracing, accumulating experimental evidence begins to question the myogenic potential of previously reported stem cells *in vivo*.

## Bone marrow cells for cardiac regeneration

The adult heart has been considered a terminally differentiated organ with no considerable regenerative capacity. In 2001, Piero Anversa's lab began to challenge this view by showing that bone marrow cells regenerate injured myocardium[13]. They isolated c-Kit$^+$ hematopoietic cells from the bone marrow of male GFP mice, and transplanted these cells into the infarcted myocardium of a wild-type female mice[13]. Using GFP and Y chromosome as identification markers, they found that these transplanted cells in the infarcted female heart expressed cardiomyocyte sarcomere markers (Figure 2A). Additionally, these c-Kit$^+$ hematopoietic cells also differentiated into endothelial and smooth muscle cells[13]. In a step further, they utilized the known cytokines to mobilize bone marrow cells to migrate to the infarcted myocardium for cardiomyocyte regeneration, which achieved similar beneficial effect as bone marrow transplantation[17]. This breakthrough report spurred a growing field of research on bone marrow cells for treatment of MI. In 2002, the Anversa group also presented genetic evidence that human bone marrow cells differentiated into cardiomyocytes in the adult stage[18] (Figure 1). They examined autopsy specimens of female hearts transplanted to male host and detected 7–10% of cardiomyocytes and vascular cells in the heart that contained the Y chromosome, some of which showed high proliferative ability[18]. These data demonstrated that in humans, extra-cardiac sources, such as the bone marrow cells, could be mobilized to the heart and differentiate into cardiomyocyte, endothelial cells, and smooth muscle cells. However, co-staining of Y chromosome with a cardiomyocyte marker, as aforementioned, could be severely interfered by non-cardiomyocytes that are interspersed with cardiomyocytes. This would increase the false-positive signals, over-estimating the contribution of bone marrow cells to cardiomyocytes.

In 2004, work from Loren Field and Robert Robbins' labs independently reported that c-Kit$^+$ bone marrow cells do not differentiate into cardiomyocytes to promote cardiac regeneration, but instead, adopt a hematopoietic fate[19, 20]. Both labs utilized the same transplantation strategy as Anversa's lab and transplanted genetic tagged cells from transgenic reporter mice (Figure 2A). They did not find convincing evidence supporting myogenic differentiation of transplanted cells[19, 20]. While hematopoietic cells from the bone marrow did not differentiate into cardiomyocytes, it was later reported that a small proportion could fuse with cardiomyocytes at a very low rate[21, 22]. The promising reports of adult BMC plasticity to generate cardiomyocytes led to a rush of clinical trials[14, 23–25]. A number of pilot clinical trials showed variable outcome in terms of efficacy, with most unable to reproduce the initial favorable outcome in animal studies[26, 27]. It is important to note that considerable heterogeneity exists in the specific bone marrow cells used for the

pre-clinical and clinical studies in this field, with differences in the cell isolation, storage, and enrichment processes[28]. Nonetheless, no study has yet demonstrated a robust generation of engraftable cardiomyocytes after transplantation of BMCs. Subsequent research has shown that the bone marrow-derived cells may secrete angiogenic factors to promote neovascularization of the injured heart after MI[29], which provides reasonable explanation for mechanisms of action for cell transplantation-based therapy. In addition to the paracrine effect, bone marrow cell therapy also causes local inflammation with regional accumulation of distinct macrophage subtypes, which may improve heart function after cardiac injury[30].

## Resident c-Kit⁺ cardiac stem cells for cardiomyocyte contribution

While the myogenic potential of bone marrow cells has been refuted, Anversa group also proposed that the adult heart contains resident stem cells, c-Kit$^+$ CSCs[31]. Through self-renewal and multipotency assays, they demonstrated that isolated c-Kit$^+$ CSCs are multipotent and could expand *in vitro* at a clonal level. Their work demonstrated that single c-Kit$^+$ CSC could differentiate into multiple cell lineages including cardiomyocytes, endothelial cells, and smooth muscle cells. When they injected GFP labeled c-Kit$^+$ CSCs into the border region of infarcted myocardium, they detected new cardiomyocytes that were differentiated from these transplanted cells *in vivo*[31] (Figure 2B). Alternatively, c-Kit$^+$ cells delivered to the coronary arteries could also promote recovery of injured heart through cardiomyocyte differentiation[32] (Figure 2B). The existence of resident CSCs was reported to be evolutionally conserved, as c-Kit$^+$ cells from adult rat, mouse, dog, and human hearts have the ability to regenerate cardiomyocytes[33–35] (Figure 1). Moreover, a clinical trial led by Bolli et al. reported that c-Kit$^+$ CSCs isolated from atrial appendages of patients undergoing surgical revascularization and transplanted into patients with chronic ischemic heart disease led to significantly improved heart function with short-term safety[36]. One caveat of these studies is that most conclusions largely depended on immunostaining, in which artifacts or false-positives may arise. Additionally, cell purification and culture may alter the property of the cells or spur the myogenic potential *in vivo*. Independent studies from different groups showed that transplanted c-Kit$^+$ CSCs into the infarcted adult mouse hearts failed to undergo cardiomyogenic differentiation[37, 38] (Figure 2B). Studies over human heart samples suggested these c-Kit$^+$ cells were mast cells[37, 38].

While the experiments above mainly used a cell transplantation assay, a report in 2013 led by Torella and colleagues utilized a lentiviral system in which a small portion of the Kit gene promoter was used to drive Cre expression to presumably label c-Kit$^+$ CSCs *in vivo* without cell transplantation[39]. However, this approach is not necessarily reflective of *in vivo* genetic lineage tracing studies. By injecting the lentivirus that expresses Cre under the control of c-Kit promoter into the myocardium of *Rosa26-YFP* reporter mice, they showed presence of c-Kit-expressing cells throughout the myocardium (Figure 2C). After isoproterenol-induced heart injury, a substantial number (~10%) of newly formed cardiomyocytes were expressing YFP[39]. Functionally, ablation of these c-Kit$^+$ CSCs resulted in severe cardiomyopathy with significant hypertrophy of the spared cardiomyocytes[39]. These data indicated that endogenous c-Kit$^+$ CSCs are both necessary and sufficient for heart repair and regeneration. A limitation of this study worth noting is that the c-Kit promoter[40] may not be able to reliably recapitulate the endogenous c-Kit

regulatory elements. There is possibility that cardiomyocytes may uptake the lentivirus and subsequent YFP expression would lead to inaccurate assessment of the contribution of non-myocyte c-Kit$^+$ CSCs to newly formed cardiomyocytes. The specificity of a lentiviral approach to trace c-Kit$^+$ cells remains uncertain[41, 42].

To resolve the c-Kit$^+$ CSCs conundrum, van Berlo et al. were the first to generate a transgenic c-Kit mouse for lineage tracing studies by employing a knock-in strategy for Cre expression under the endogenous c-Kit gene promoter[43]. Cre-mediated recombination results in excision of a stop codon that leads to permanent expression of a constitutively active fluorescent reporter, e.g. GFP, in c-Kit-expressing cells and their progeny, even if they differentiate into cardiomyocytes (Figure 2D)[43]. Fate-mapping of c-Kit$^+$ cells revealed very few labeled cardiomyocytes, and from those, many were a result of cell-fusion rather than differentiation[43], indicating that this level of contribution is unlikely to be physiologically significant for cardiac repair, contradicting previous c-Kit studies (Figure 1). Subsequent studies from two other independent laboratories using similar Cre knock-in strategies showed findings consistent with van Berlo, and concluded that c-Kit$^+$ CSCs contribute minimally, if any, to cardiomyocytes during homeostasis and after injury[44, 45]. Additionally, these studies demonstrated that c-Kit was also expressed in a subset of cardiomyocytes, which suggests that lineage tracing of c-Kit$^+$ cells may be confounded by the presence of a subset of c-Kit$^+$ cardiomyocytes. A dual recombinase-activated lineage tracing (DeaLT) was later developed to distinctly label c-Kit$^+$ non-cardiomyocytes from those c-Kit$^+$ cardiomyocytes (Figure 2E), which showed that c-Kit$^+$ non-cardiomyocytes do not contribute to any new cardiomyocytes[46].

In 2018, Vicinanza et al. contended that previous fate mapping results based on Kit-Cre knock-in lines have inherent technical limitations (Figure 1). First, they reasoned that c-Kit Cre tools are not sensitive enough to track all c-Kit$^+$ cells, especially c-Kit$^{low}$ cells[47]. It was argued that those unlabeled c-Kit$^{low}$ cells could be more likely to generate new cardiomyocytes[47]. Secondly, previous gene knock-in targeting strategies unavoidably interrupted the endogenous allele[43–45], leading to haploinsufficiency of c-Kit expression, which was reported to regulate CSCs function and survival of cardiomyocytes[48, 49]. To address these two caveats, two new c-Kit knock-in alleles, *Kit-IRES-Cre* and *Kit-2A-Cre* were generated, such that efficient labeling could be achieved while preserving the endogenous Kit gene[50]. Combined with the DeaLT system, these new Cre-labeled c-Kit$^+$ non-myocytes again demonstrated that no new cardiomyocytes were generated from a c-Kit lineage in the adult heart (Figure 2F). Taken together, these studies utilizing more advanced technologies do not provide scientific evidence supporting the existence of c-Kit$^+$ CSCs[8].

## Sca-1$^+$ cardiac progenitor cells for cardiomyocyte regeneration

In addition to c-Kit, Sca-1 (stem cell antigen-1) is also considered as a cardiomyocyte stem cell marker and has been studied intensively in the last decade (Figure 1). Sca-1, also known as Ly-6a, is a member of Ly-6 gene family expressed in bone marrow stem cells[51]. Sca-1 has also been reported as a stem or progenitor cell marker in other organs such as mammary gland, prostate, lung, and liver[52–55]. In the adult heart, Schneider's group started the adventure of Sca-1$^+$ stem cells, and found its differentiation into cardiomyocytes with

sarcomere structure by performing *in vitro* cell culture assays[56]. Another study showed the *in vitro* potential of Sca-1$^+$ cells from adult hearts to differentiate into mature cardiomyocytes, osteocytes, and adipocytes[57], supporting its multipotency. Furthermore, transplantation of Sca-1$^+$ cells isolated from aMHC-Cre-nLAC mice into R26-reporter mice showed *in vivo* differentiated into cardiomyocytes[56]. Further study revealed that transplantation of sheets of clonally expanded Sca1$^+$ cells ameliorates cardiac function after MI through cardiomyocyte differentiation in addition to paracrine mechanisms[58].

Several studies have highlighted the heterogeneity of Sca-1$^+$ cells residing in the adult heart. Pfister et al. reported that Sca-1$^+$ cells consisted of CD31$^+$ and CD31$^-$ populations, the latter of which have the capacity to differentiate into cardiomyocytes[59]. Subsequent studies confirmed that Sca-1$^+$CD31$^-$ population increased after MI and transplantation of these cells significantly improved heart function through pro-angiogenesis effect[60, 61]. Additional study further defined these populations into four using PDGFRa expression, with the PDGFRa $^+$CD31$^-$Sca-1$^+$ population containing stem cell properties of multiple cardiac lineages[62]. A different study showed that adult Sca-1$^+$ cells contained CSH1 and CSH2 subpopulations, with myogenic capacity residing within the CSH2 cells[63]. Taken together, these studies documented the myogenic potential of Sca-1$^+$ cells (Figure 1). However, most of these studies used cell culture or transplantation assays (Figure 2B), which do not address whether endogenous Sca-1$^+$ cells are capable of differentiating into cardiomyocytes *in vivo*.

To directly address the *in vivo* myogenic potential of Sca-1$^+$ cells, Uchida investigated Sca-1 lineage tracing using tet-off system to trace Sca-1$^+$ cells in tissue homeostasis and after injury (Figure 1). Their fate-tracing tools showed that the labeled Sca-1$^+$ cells contributed to cardiomyocytes in aging heart and after pressure overload injury[64]. However, recent studies from 5 independent labs showed that Sca-1$^+$ cells do not generate new cardiomyocytes[65–69] (Figure 1). Specifically, transplantation of Sca-1$^+$ cells into the infarcted myocardium did not induce their differentiation into new cardiomyocytes (Figure 2B)[69]. Genetic lineage tracing based on Sca-1 knock-in Cre lines (Figure 2D) showed that Sca-1$^+$ cells mainly adopted an endothelial cell fate, but not cardiomyocyte, during homeostasis or after injury[65–68]. Therefore, the beneficial effect from Sca-1$^+$ cell transplantation is unlikely to be due to direct cardiomyocyte differentiation, but more from angiogenesis and paracrine effects. These studies also highlighted the improvement in mouse genetics for lineage tracing and fate mapping that could lead to a change in interpretation of plasticity of stem cells[70].

## Cardiospheres and cardiosphere-derived stem cells

Cardiospheres was first termed based on the observation that isolated undifferentiated cells grew as self-adherent clusters from subcultures of postnatal atrial or ventricular heart tissues[71]. These cardiospheres were thought to possess properties of adult cardiac stem cells such as long-term self-renewal and ability to differentiate into cardiomyocytes after transplantation into infarcted hearts (Figure 1). Since cardiospheres could be readily isolated from human heart biopsy specimens and expanded *ex vivo*, they offered a potential therapeutic strategy to regenerate the damaged myocardium. When endomyocardial samples were plated on tissue culture dishes, it was noted that cardiosphere-derived cells (CDCs)

sprouted from the core. Co-culturing of CDCs with neonatal rat cardiomyocytes resulted in differentiation of CDCs into cardiomyocytes based on gene and protein expression analysis. When CDCs were injected into the infarcted myocardium of mouse, rat, or pig, it was reported that the transplanted cells generated cardiomyocytes and vascular endothelial cells, promoting the recovery of heart function[72–76]. However, considering the limited number of differentiated cardiomyocytes, it was hypothesized that most of the beneficial effects might be derived from the CDCs' paracrine effects after transplantation[77], which provided a variety of growth factors to prevent apoptosis and promote angiogenesis. The initial human clinical trial (CADUCEUS) investigated intracoronary infusion of autologous CDCs obtained through endomyocardial biopsy in patients with acute MI. This trial showed that CDCs treatment reduced scar size by MRI, increased muscle contractility and wall thickness, albeit no significant improvements in the end diastolic or systolic volumes of the left ventricle and the ejection fraction[78, 79]. Due to small sample size and a skewed distribution on the data for statistical analysis, the results from these clinical trials need to be more carefully analyzed and cautiously interpreted[80].

Despite support from many studies regarding the beneficial effects of CDCs in promoting heart function (Figure 1), there are many others that have challenged the efficacy of CDCs in cardiac repair. Several studies failed to show the beneficial effects of CDCs in reducing scar size and improving ejection fraction[81–83]. It has also been suggested that the beneficial effects of CDCs are not likely through differentiation of cardiomyocytes. Recent studies have demonstrated that exosomes from CDCs promoted angiogenesis, cardiomyocyte survival and proliferation[84], which are sufficient to explain the therapeutic effects of CDCs. Additional mechanisms of action may include the direct cell-cell contact between CDCs and cardiomyocytes that may promote their survival and proliferation[85].

## Abcg2$^+$ side population cells for cardiac regeneration

Side population cells were initially defined as a cell fraction with the ability to extrude Hoechst33342 dye and later regarded as a stem cell population[86, 87] identifiable by surface marker Abcg2, also known as Bcrp1[88]. Abcg2$^+$ cells were observed in the adult heart, with studies showing their differentiation into cardiomyocytes *in vitro* (Figure 1)[89]. Injection of side population cells into the rats undergoing heart injury showed their recruitment to the injured regions and ability to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells, which suggested that they may be endogenous cardiac stem cells (Figure 2B)[90]. However, genetic lineage tracing based on *Abcg2-CreER* demonstrated that Abcg2$^+$ cells was only able to differentiate into the multiple cardiac cell lineages during the embryonic stages, but that this ability was lost in adulthood[91, 92] (Figure 2D). The fusion of Abcg2$^+$ cells with preexisting cardiomyocytes was likely to stimulate cardiomyocyte cell cycle entry and proliferation, rather than these cells directly differentiating into cardiomyocytes[93]. Therefore, genetic fate mapping studies have refuted the cardiac stem cell property of adult endogenous Abcg2$^+$ side population and their myogenic regenerative potential *in vivo*.

## Bmi1+ cardiomyocyte stem cells

Bmi1$^+$ cells have been reported to represent another endogenous cardiac progenitors[94, 95], which was supported by genetic lineage tracing studies using *Bmi1-CreER;R26-YFP* transgenic mice (Figure 2D). YFP labeled, Bmi1$^+$ cells could be induced to differentiate into vascular endothelial cells and smooth muscle cells, as well as cardiomyocytes if co-cultured with neonatal rat cardiomyocytes. Additionally, Bmi1$^+$ cells contributed to new cardiomyocytes in this inducible CreER mouse model detected 1 year after tamoxifen labeling, indicating stem cell contribution during homeostasis[94]. Another study showed that after MI, contribution of Bmi1$^+$ cells to cardiomyocytes significantly increased, with *de novo* cardiomyocytes generation reaching to 13.8% after MI compared to 4.7% in age-paired non-infarcted hearts[95]. Such exceedingly high levels of *de novo* cardiomyocytes formation contradicts the consensus in the cardiomyocyte regeneration field, as DNA synthesis detected in cardiomyocytes is an order of magnitude lower than this level[7–9]. Notably, these studies reported that Bmi1$^+$ is a sub-population of Sca-1$^+$ cells. Since Sca-1 lineage tracing studies have documented their inability to generate new cardiomyocytes[65–68], it is less likely that its sub-population (Bmi1$^+$ cells) could then generate cardiomyocytes. It is more likely that the Bmi1-Cre used in the previous studies[94, 95] may also label few cardiomyocytes, which represents a similar caveat as labeling of c-Kit$^+$ cardiomyocytes[45, 46]. Given the perceived lack of rigor in the cardiac stem cell field, such extraordinary findings need to be re-evaluated and independently verified.

## Other CSCs with unknown markers: exist or not?

Since many of the previously reported CSCs such as c-Kit, Sca-1, and side population may not be authentic stem cells for cardiomyocytes, it raises an enduring question as to whether cardiac stem cells actually exist in the adult heart. Conventional Cre-loxP mediated genetic lineage tracing is technically challenging to address this question, as Cre needs to be driven by a known stem cell promoter[96]. Hsieh et al. devised an ingenious ‘pulse-chase’ strategy of genetically labeling pre-existing cardiomyocytes and assessing the dilution of cardiomyocyte percentage during aging and after injury[97]. They showed that the percentage of labeled cardiomyocytes during aging remained stable, indicating that stem or precursor cells did not refresh at a high rate. However, after MI or pressure overload injury, there was a decrease in the percentage of labeled cardiomyocytes, suggestive of an increased renewal rate from stem cells[97]. Subsequent advancement in technology employing multi-isotope imaging mass spectrometry (MIMS) analysis using $^{15}$N isotope incorporation[98] supported cardiomyocyte proliferation and excluded a substantial contribution from stem cells in cardiomyocyte renewal in the uninjured heart[99]. Whether any single cardiomyocyte is generated from CSCs in the adult heart remains technically challenging to answer.

As conventional Cre-loxP strategy (Figure 2D) for tracing CSCs relies on the CSCs gene or marker, the promoters remain unknown for putative CSCs which have not been discovered yet. To circumvent this difficulty, an alternative strategy of addressing CSCs have been proposed. Since CSCs are, by definition, undifferentiated cells, they are not mature cardiomyocytes nor do they express organized sarcomeric structures. To address the existence of CSCs, the challenging question could be addressed by testing whether non-

myocytes in the heart contain CSCs that may differentiate into new cardiomyocytes after heart injury (Figure 2G). To examine this, the dual recombinase-activated lineage tracing (DeaLT) system[46] was utilized to simultaneously and exclusively label pre-existing cardiomyocytes and non-myocytes within the same heart (Figure 2G). Fate mapping results showed that non-myocytes contribute to new cardiomyocytes in fetal but not adult heart[100,101], which was subsequently supported by a lineage tracing study of proliferating cells using Ki67 that shows no evidence for the trans-differentiation of other cell types toward cardiomyocytes[102]. The above studies, without relying on particular stem cell markers, demonstrate that the adult heart lacks an endogenous stem cell for cardiomyocyte regeneration[103]. These above supporting or conflicting the myogenic potential of cardiac stem cells in this review are listed in Figure 3.

## Cardiomyocyte proliferation and heart regeneration

The potential of endogenous CSCs for cardiac regeneration has been disappointing since recent studies suggest that adult mammalian heart does not harbor CSCs for cardiomyocyte regeneration. How can we now rectify the observation of the low level of cardiomyocyte turnover? Considering that the endogenous source for generating new cardiomyocytes seem to derived from self-duplication, more focus should be directed toward this process, of which is known to occur in hearts of many non-mammalian regenerative species. To explore this, an essential basis for finding new approaches in promoting endogenous cardiomyocyte regeneration is to correctly detect and accurately quantify their proliferation by the state-of-the-art technologies.

## Cardiomyocyte proliferation quantified by different methods

### Cell proliferation marker staining

The mammalian heart has long been viewed as a post-mitotic organ with little regenerative capacity. It was previously thought that the total number of cardiomyocytes is established shortly after birth and there is hardly any proliferating cardiomyocyte with aging. However, in the past several years, the quiescent nature of the mammalian heart has been challenged with several reports supporting the generation of new cardiomyocytes after birth. The earliest reports of heart regeneration came from post-mortem histological specimen from newborns who died of diphtheria[104, 105]. To explore whether there is any evidence of cardiomyocyte proliferation in adult human heart, Beltrami et al. examined heart tissues of patients who died from MI, and detected about 4% and 1% of cardiomyocytes that expressed cell proliferation marker Ki67 in the infarct border zone and remote region, respectively[106]. Considering that this Ki67 staining on heart sections reflects only a snapshot of cardiomyocyte proliferation on the day of death (per day), their results, if stands correctly, indicate that, in total, an extremely high level of cardiomyocyte proliferation occurs in a year. However, this contradicts the 2017 consensus statement based on extensive recent experimentation that cardiomyocytes turnover is current estimated at 0.5% to 2% per year in both human and mouse hearts[7–9].

Staining for cell proliferation markers, such as Ki67, pH-H3, PCNA, or Aurora B, have been widely used to study cardiomyocyte proliferation (Figure 4A)[107, 108]. However, given that

staining for these markers represents only a snapshot of cell proliferation and considering the extremely low level of cardiomyocyte proliferation, these studies are unable to reveal the bulk number of cardiomyocyte proliferation in a time window. Additionally, these studies rely on indirect assays of cell division, which are challenging to interpret in the setting of cardiomyocyte polyploidy as well as potential DNA repair upon injury. Furthermore, these proliferation markers are unable to discriminate between karyokinesis and cytokinesis. Another caveat for marker staining is that the proliferation signals from non-myocytes would significantly interfere the unambiguous observation of authentic cardiomyocyte proliferation in a tissue section, where all cells are squeezed and entangled with each other, creating potential for false positive data.

## Isotope incorporation

In 2009, Bergman et al. took advantage of the integration of carbon-14 ($^{14}C$), generated by nuclear bomb tests during the Cold War, into the genomic DNA of human cardiomyocytes[109], and performed retrospective birth dating analysis of $^{14}C$ in cardiomyocyte DNA, which serves as a date mark for when a cell was born (Figure 4B). Their study revealed that about 50% of cardiomyocytes renew during a normal life span, with a 1% annual turnover rate at the age of 25 and 0.45% at the age of 75[109]. Subsequent studies revealed that endothelial cells and fibroblasts have much faster turnover rates than cardiomyocytes[110].

The newly developed technology using MIMS[98] allows one to view and measure stable isotope incorporation with submicrometre resolution on the tissue section, which provides a technical basis for studying *in situ* cardiomyocyte proliferation using isotype incorporation. Taking advantage of $^{15}N$ isotype labeling using MIMS, Senyo et al. detected an increase in $^{15}N$:$^{14}N$ ratio above the natural ratio, indicating DNA duplication in a subset of cardiomyocytes (Figure 4B). Based on MIMS analysis and quantification, about 4.4% of cardiomyocytes in a mouse undergo DNA replication in a year[99]. DNA replication may not necessarily mean complete cell cycle or cell division, as cardiomyocytes may contain multiple nuclei (polyploidy) during their cell cycle. Of note, about 17% of $^{15}N^{+}$ cardiomyocytes were single nucleus and diploid, indicating that these cells resulted from complete cell cycle with cytokinesis, yielding truly new cardiomyocytes[99]. Quantification analysis showed generation of new cardiomyocytes at a yearly rate of 0.76% (4.4% x 17%) in the adult mouse hearts. These above studies based on isotype incorporation assay consistently documented low, discrete rate of cardiomyocyte turnover in the adult mammalian heart, providing valuable quantitative information on cardiomyocyte proliferation.

## Mosaic analysis with double markers and Rainbow reporters

Considering that cardiomyocytes may have multiple nuclei in the adult stage, it is difficult to distinguish newly generated cardiomyocytes that have undergone cytokinesis from those that only completed karyokinesis or incomplete cell cycle events using isotype incorporation or proliferation marker staining techniques. To distinguish these events of cell division, Zong et al. took advantage of inter-chromosome recombination and developed an alternative strategy to distinctly label daughter cells after cytokinesis[111]. The mosaic analysis with double

markers (MADM) strategy utilizes two reciprocally chimeric reporter genes that are knocked into the same location on homologous chromosomes, each containing the N terminus of one reporter and the C terminus of the other reporter interrupted by a loxP-containing intron (Figure 4C). After DNA replication in dividing cells (at the G2 phase), induced inter-chromosome recombination by Cre-loxP creates functional reporter gene expression, e.g. GFP and RFP on two chromosomes[111]. This system enables asymmetric labeling of daughter cells to precisely determine precursor-progeny lineages in various developmental studies[112–114]. Ali et al. utilized MADM system to investigate postnatal cardiomyogenesis, and showed limited, life-long, symmetric division of cardiomyocytes as a rare event in the first month of life in mice and very seldom in the adult stage and that MI did not increase the rate of cardiomyocyte division above the basal level[115] (Figure 4C). While single-labeled cells by MADM are the result of *bona fide* cell division and is an attractive feature for clonal analysis, the inefficiency of inter-chromosome recombination in MADM underestimates the gross number of cell division in the adult hearts.

Subsequently, a stochastic four-color reporter system (Rainbow) was used to retrospectively identify the source of new cardiomyocytes during early mouse development and after injury[116]. Through titration of tamoxifen, this system permits labeling of a small number of cells and their progeny with a distinct fluorescent protein, allowing retrospective tracing of cellular expansion through easily identifiable clones *in vivo* (Figure 4D). Clonal analysis of cardiomyocytes at various timepoints during development showed that a distinct subpopulation of cardiomyocytes may have the potential for proliferation after birth[116]. These studies provide a valuable means for measuring cardiomyocyte division in mammalian heart, which could be iterated and improved in the future to enhance the efficiency of measuring *in situ* cardiomyocyte proliferation.

### Genetic lineage tracing using inducible Cre driven by Ki67

In addition to the cell division analysis at single cell resolution by MADM and Rainbow, recent studies have utilized cell proliferation markers to genetically lineage trace proliferating cardiomyocytes *in vivo*. Clevers et al. took advantage of the widely used cell proliferation marker, Ki67, to study cell proliferation in the intestine, brain, and heart[102, 117, 118]. Distinct from previous studies that utilized marker staining, Clevers used Ki67 based inducible Cre to genetically label proliferating cells with a permanent genetic marker (Figure 4E). Kretzschmar et al. reported that cycling cardiomyocytes were observed shortly after birth, but hardly, if any, in the homeostatic or injured adult myocardium[102]. Most of the cycling cells after injury were non-cardiomyocytes, especially fibroblasts that contribute to fibrosis and which do not transdifferentiate into cardiomyocytes after injury[102]. Quantitatively, only 11 out of an estimated 8 million cardiomyocytes analyzed expressed Ki67 during adult homeostasis within an 18-month time window, reinforcing the notion that adult cardiomyocytes rarely proliferate. However, this number of cell division is much lower than those quantified by other approaches[99, 109]. We reasoned that CreER mouse systems rely on tamoxifen induction, and the continuous induction of CreER activity at high efficiency over a large time window (e.g. months) could be technically challenging to capture those proliferating cardiomyocytes. Since Ki67 inducible Cre only traced the proliferating cardiomyocytes during the period of tamoxifen administration, it is difficult to

trace cardiomyocyte proliferation once tamoxifen levels decline over time. New genetic approaches that could seamlessly record proliferating cardiomyocytes over a long time window is needed for future genetic tracing studies of cardiomyocyte proliferation.

### Technical caveat and room for improvement

While the above studies using isotype incorporation, marker staining, or genetic fate mapping approaches consistently revealed the limited self-renewal capacity of cardiomyocytes, there are obvious discrepancies in the magnitude of cardiomyocyte generation measured from these different methods. Isotype incorporation measures DNA synthesis events, which may capture cells that have undergone karyokinesis, but may not necessarily denote complete cell division. Quantification on the single-nuclear cardiomyocytes would strongly indicate their cell division. Genetic methods based on MADM provides strong evidence for cell division by single color cardiomyocytes. However, some cell division could also exhibit double-labeled color or no color, which could not be distinguished from current MADM strategy. Additionally, the efficiency of inducible Cre for inter-chromosome recombination is too low to target the majority of cardiomyocytes (although an attractive feature for clonal analysis studies), hence a limitation for accurate quantification of the bulk number of cardiomyocyte division. For proliferation marker-driven inducible CreER, almost no proliferating cardiomyocytes were observed in adults, and the reported number is several orders of magnitude lower than isotype incorporation methods. The inducible CreER may encounter difficulty in continuously tracing most cardiomyocyte proliferation for a prolonged time window. It is crucial to explore new methods that may combine the advantages of the above approaches, simultaneously measuring the generic cell cycle (from DNA synthesis to cell division) and also distinguishing cardiomyocytes that have completed cytokinesis from those that have undergone karyokinesis.

## Polyploidy and cardiomyocyte proliferation

While the adult mammalian heart has a limited regenerative ability, teleost fish and urodele amphibians are able to survive after heart apical resection through robust cardiomyocyte proliferation[119, 120]. One notable difference between zebrafish and mammalian cardiomyocytes is the DNA copy number or nuclei number. Fish cardiomyocytes are single nucleated with two sets of homologous chromosomes (diploid), while most adult mouse cardiomyocytes have more than two sets of chromosomes (polyploid), either enclosed within a single nucleus or separated into multiple nuclei[10]. Of note, even in the mouse species, different mouse strains may have different percentages of mononuclear cardiomyocytes, which are highly correlated with cardiomyocyte proliferation and heart function after MI[121]. After surveying 120 inbred mouse strains, Patterson et al. identified *Tnni3k* as one gene that influences variation in the composition of mononuclear diploid cardiomyocytes[121]. This interesting study demonstrated that intrinsic heart regeneration is not limited nor uniform among individuals, but rather is a variable trait dependent on complex genetic background[121]. Recent elegant genetic study in zebrafish provided direct causal-effect relationship between polyploidization and cell proliferation[122]. Zebrafish cardiomyocytes were susceptible to polyploidization upon cytokinesis inhibition mediated by dominant-negative Ect2, and these polyploidy cardiomyocytes failed to regenerate myocardium after

heart injury[122], suggesting that cardiomyocyte polyploidization may be a barrier to heart regeneration. Subsequent studies revealed that thyroid hormone signaling regulates cardiomyocyte polyploidization and also the regenerative ability of adult hearts[123]. It would be worthwhile to explore underlying molecular pathways that promote these rare diploid cardiomyocytes in mammals, or to find new sources other than pre-existing cardiomyocytes for cardiac repair and regeneration.

## Promotion of pre-existing cardiomyocyte proliferation

Many approaches have been reported to stimulate cardiomyocyte proliferation. In this review, we provide a general overview of each approach, as detailed information are available in recent excellent reviews[15, 124–127]. One obvious approach is to stimulate cardiomyocyte mitotic division through overexpression of cell cycle genes[128]. Cyclin D1 promotes cardiomyocyte DNA synthesis[129], and Cyclin A2 induces cardiomyocyte mitosis[130]. Their forced expression in the heart enhanced cardiac function after myocardial infarction in both mouse[131] and pig models[132]. Recent studies combined 4 cell-cycle regulators (CDK1, CDK4, cyclin B1 and D1) to efficiently induce cardiomyocyte division, which significantly improved cardiac function after MI[133]. In contrast, deletion of negative cell-cycle regulator Meis1 was shown to be sufficient for reactivation of cardiomyocyte mitosis in the adult heart[134]. In addition to cell-cycle genes, the Hippo pathway was recently reported to regulate cell proliferation and organ size[135, 136]. Recent studies have shown a critical role of the Hippo tumor-suppressor pathway which controls organ size by coordinating the regulation of cell proliferation and apoptosis. Knockout of Salv or over-expression of YAP significantly increased cardiomyocyte proliferation, reduced fibrosis and improved heart function after MI[137–139]. Additionally, Neuregulin1/ErbB4 and ErbB2, and epicardial derived growth factors such as follistatin-like 1, have been demonstrated to trigger cardiomyocyte proliferation and promote mammalian heart regeneration[107, 140, 141]. In addition to these pathways, microRNAs have been implicated in regulating cardiomyocyte division during normal aging or in response to injury. Several studies have shown that miR-590, miR-199a, miR-99/100 can promote cardiomyocyte proliferation and improve heart function after MI both in mouse and pig models[142–144]. Within the context of neonatal heart regeneration, transient fibroblast senescence was found to induce cardiomyocyte proliferation through senescence-associated secretory phenotype factors and also through p53-mediated pathways[145, 146]. Interestingly, certain physiological conditions such as exercise, pregnancy, and hypoxia have also been suggested to induce cardiomyocyte proliferation through various mechanisms[147–151], which merit further investigations to understand their role in promoting heart regeneration after injury.

## Future Directions

Due to the lack of strong evidence supporting the existence of resident CSCs, efforts are now focused on how to mobilize and promote those few cardiomyocytes that have potential to proliferate in mammalian hearts. While endogenous cardiomyocytes can proliferate at a low frequency, methods that promote cardiomyocyte proliferation may be a vital research field in the future. The aforementioned pathways could be manipulated, optimized, and synergized to achieve better functional improvement after cardiac injury. In addition to inducing

cardiomyocyte proliferation, other alternative approaches could be considered (Figure 5). *In situ* reprogramming of fibroblasts to cardiomyocytes by over-expression of specific transcriptional factors represents a promising direction for future study[152, 153]. Recent studies have also suggested that small molecules can induce reprogramming of fibroblasts to cardiomyocytes *in vivo*, which offers a dual pharmaceutical approach to regenerate cardiomyocytes and also reduce scar formation[154]. Additionally, human embryonic stem cells or induced pluripotent stem cell-derived cardiac progenitors and cardiomyocytes have been successfully transplanted in large animal models[155, 156]. These studies confirmed the survival of transplanted cells within the host myocardium and an improvement in cardiac function after injury. Even without direct cardiomyocyte regeneration, methods that promote neovascularization such as transplantation of MSCs that secrete angiogenic factors, or modulate inflammation after injury[157], may be an alternative approach to improve survival of myocardium, reduce scar formation, and ameliorate inflammation after MI. Furthermore, dual stem cell therapy, such as human iPS cell-derived cardiomyocytes and MSCs, or human ES cell-derived epicardium and cardiomyocytes, synergistically improves cardiac function and augment vascularization in the injured myocardium[158, 159]. Recent advances in engineered epicardial patch containing growth factors[107] or multiple cardiac cell types[160, 161] improved heart function and neovascularization after MI. Additionally, treatment of modified RNA of VEGFA lead to augmented vascular regeneration, partly through cell fate switch of epicardial progenitor cells[162, 163]. With the fallacy of endogenous putative CSCs, it is important to invest efforts and resources toward more promising directions as mentioned above to attain the ultimate goal of heart repair and regeneration.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **Abcg2** | ATP binding cassette subfamily G member 2 |
| **aMHC** | alpha myosin heavy chain |
| **Aurora B** | Aurora kinase B |
| **BMC** | Bone marrow cell |
| **Bmi1** | BMI1 proto-oncogene, polycomb ring finger |
| **CD31** | PECAM1, platelet and endothelial cell adhesion molecule 1 |

| | |
|---|---|
| **CDK** | Cyclin dependent kinase |
| **Ect2** | Epithelial cell transforming 2 |
| **ErbB2** | Erb-b2 receptor tyrosine kinase 2 |
| **ErbB4** | Erb-b2 receptor tyrosine kinase 4 |
| **iPS** | Induced pluripotent stem cell |
| **IRES** | Internal ribosome entry site |
| **Ly-6a** | Lymphocyte antigen 6 complex, locus A |
| **MIMS** | Multi-isotope imaging mass spectrometry |
| **MRI** | Magnetic Resonance Imaging |
| **PCNA** | Proliferating cell nuclear antigen |
| **PDGFRa** | Platelet derived growth factor receptor alpha |
| **pH-H3** | Phosphorylated histone H3 |
| **Salv** | Salvador family WW domain containing 1 |
| **TNNI3** | Troponin I3, cardiac type |
| **Tnni3k** | TNNI3 interacting kinase |
| **TNNT2** | Troponin T2 |
| **VEGFA** | Vascular endothelial growth factor A |
| **YAP** | Yes1 associated transcriptional regulator |

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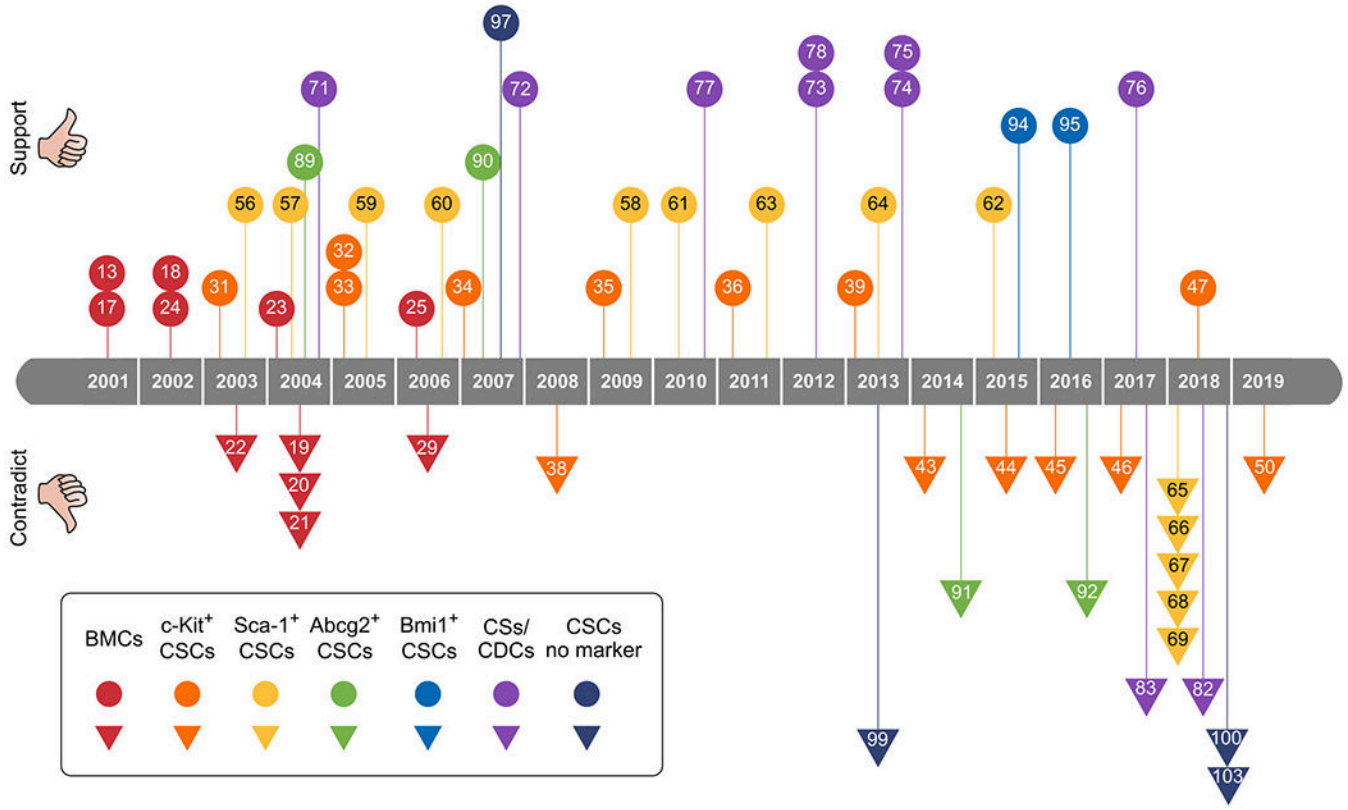


**Figure 1. Timeline of scientific evidence supporting or refuting the notion of myogenic differentiation of stem or progenitor cells.**

BMCs, Bone marrow-derived cells; CSCs, cardiac stem cells; CSs, cardiospheres; CDCs, cardiospheres-derived cells. Number in the box indicating the reference number of this review.

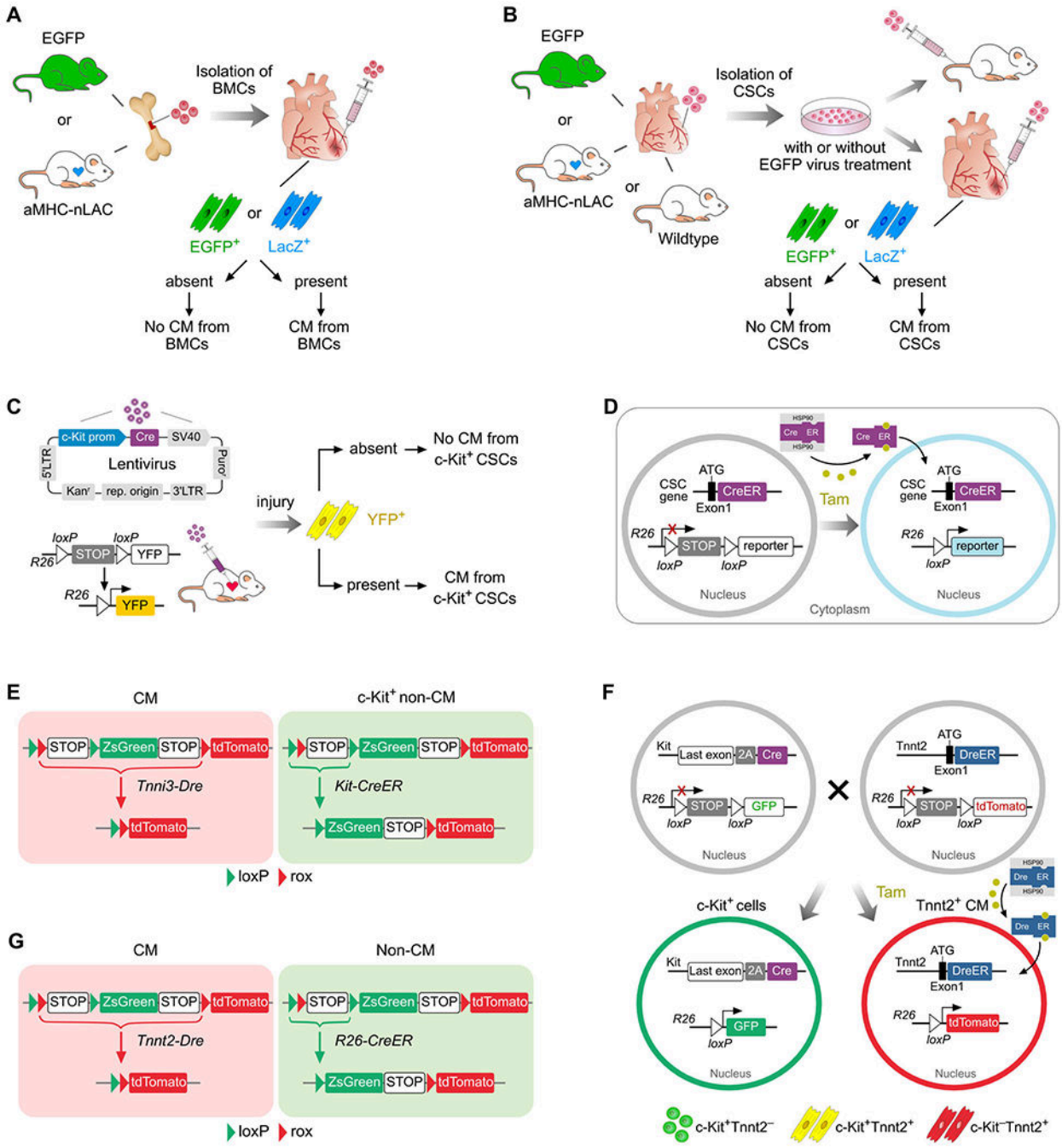


**Figure 2.**

Strategy for studying the myogenic potential of stem cells. **A**, Transplantation of bone marrow cells (BMCs) into MI heart. **B**, Isolation of resident CSCs, and subsequent transplantation via circulation or into myocardium of MI heart. **C**, Injection of lentivirus expressing Cre under c-Kit promoter (prom) into injured myocardium. The Cre-loxP recombination leads to YFP labelling of c-Kit$^{+}$ cells. **D**, Genetic lineage tracing of CSCs using knock-in Cre at the ATG of endogenous CSCs gene. **E**, Dual recombinase-activated lineage tracing (DeaLT) of c-Kit$^{+}$ non-CM (ZsGreen) could distinguish it from pre-existing

CM (tdTomato) that expresses c-Kit by itself. **F**, Lineage tracing of c-Kit$^{+}$ cells using Cre knock-in targeted after last exon before 3' UTR of c-Kit gene maintains c-Kit gene and achieves efficient labelling of c-Kit$^{+}$ cells. DeaLT distinguishes cell fates of c-Kit$^{+}$Tnnt2$^{-}$ non-CM (GFP$^{+}$tdTomato$^{-}$) from c-Kit$^{+}$Tnnt2$^{+}$ CM (GFP$^{+}$tdTomato$^{+}$). **G**, Simultaneous labelling of CM and non-CM by DeaLT. CM, cardiomyocyte; CSCs, cardiac stem cells; R26, Rosa26.

| Cell sources | Support | Ref. | Conflict | Ref. |
|---|---|---|---|---|
| BMCs | • Mouse. BMC transplantation. Lin$^-$c-Kit$^+$ BMC (EGFP$^+$). MI injury model. | 13 | • Mouse. BMC transplantation. Lin$^-$c-Kit$^+$ BMC (β-actin-EGFP or MHC-nLAC). MI injury model. | 19 |
| | • Mouse. Cytokine (CSF) treatment mobilize BMCs to the infarted heart. | 17 | • Mouse. BMC transplantation. EGFP$^+$ BMC. recipient: R26-LacZ MI mice. | 20 |
| | • Human. Autologous BMCs transplantation. MI pattients. Intracoronary transplantation. | 24 | • Mouse. BMC transplantation. EGFP$^+$ BMC. recipient: R26-LacZ MI mice. | 21 |
| | • Human. Heart transplantation. Donor: female; recipient: male. | 18 | • Mouse. BMC transplantation. EGFP+ BMC. recipient: lethally irradiated R26-LacZ mice. | 22 |
| | • Human. Autologous BMCs transplantation. MI pattients. Intracoronary transplantation. | 23 | • Mouse. BMC chimera. c-Kit$^+$ cell relase of angiogenic cytokines to protect cardiac function. | 29 |
| | • Human. Autologous BMCs transplantation. MI pattients. Intracoronary transplantation. | 25 | | |
| c-Kit$^+$ CSCs | •• Rat. Lin$^-$c-Kit$^+$ CSCs transplantation. CSCs (EGFP+). MI injury model. | 31 | • Human. Endomyocardial biopsies. c-Kit$^+$ cell is identified as mast cells. | 38 |
| | • Rat. Lin$^-$c-Kit$^+$ CSCs transplantation. CSCs (EGFP+). MI injury model. | 32 | • Mouse. Genetic lineage tracing. Kit-MerCreMer and Kit-Cre. MI injury model. | 43 |
| | •• Dog. HGF and IGF-1 stimulates resident CSCs to form new cardiomyoctyes. | 33 | • Mouse. Genetic lineage tracing. Kit-MerCreMer. MI injury model. | 44 |
| | •• Human. Lin$^-$c-Kit$^+$ hCSCs transplantation. Immunodeficient MI mice or rats. | 34 | • Mouse. Genetic lineage tracing. Kit-CreER. MI injury model. | 45 |
| | • Human. Lin$^-$c-Kit$^+$ hCSCs transplantation. Immunosuppressed MI rats. | 35 | • Mouse. DeaLT. Tnni3-Dre, Kit-CreER, IR1. MI injury model. | 46 |
| | • Human. Autologous Lin$^-$c-Kit$^+$ CSCs transplantation. MI pattients. | 36 | • Mouse. DeaLT. Tnni3-DreER, Kit-IRES-Cre, Kit-2A-Cre. MI injury model. | 50 |
| | •• Mouse. Genetic lineage tracing. c-Kit-Cre lentivirus. Isoproterenol (ISO) injury model. | 39 | | |
| | •• Mouse. Genetic lineage tracing. Kit-MerCreMer, Kit-Cre. MI injury model. | 47 | | |
| Sca-1$^+$ CSCs | •• Mouse. Lin$^-$Sca-1$^+$CSCs transplantation. MIR injury model. | 56 | • Mouse. Genetic lineage tracing. Sca-1-MerCreMer and Sca-1-Cre. MI injury model. | 65 |
| | • Mouse. Isolation of Sca-1$^+$ CSCs and cultured in vitro. | 57 | • Mouse. Genetic lineage tracing. Sca-1-MerCreMer. MI injury model. | 66 |
| | • Mouse. Isolation of Sca-1$^+$ CD31$^-$CSCs and cultured in vitro. | 59 | • Mouse. Genetic lineage tracing. Sca-1-MerCreMer. MI injury model. | 67 |
| | •• Mouse. Lin$^-$Sca-1$^+$CD31$^-$ CSCs transplantation. CSCs (LacZ$^+$). MI injury model. | 60 | • Mouse. Genetic lineage tracing. Sca-1-CreER. MI injury model. | 68 |
| | •• Mouse. Sca-1$^+$ cell sheet transplantation. Cell sheet (RFP$^+$). MI injury model. | 58 | • Mouse. Lin$^-$Sca-1$^+$ CSCs transplantation. CSCs (ACT-EGFP or MHC-nLAC). MI injury model. | 69 |
| | • Mouse. Lin$^-$Sca-1$^+$CD31$^-$ CSCs transplantation. CSCs (RFP+). MI injury model. | 61 | | |
| | •• Mouse. Lin$^-$Sca-1$^+$ CSCs transplantation. CSCs (RFP+). MI injury model. | 63 | | |
| | • Mouse. Genetic lineage tracing. Sca-1-tTA-IRES-GFP;LC1-Cre. Pressure overload and aging | 64 | | |
| | • Mouse. Lin$^-$Sca-1$^+$ CSCs transplantation. CSCs (mOrange$^+$). MI injury model. | 62 | | |
| Cardiospheres / CDCs | •• Mouse. Cardiospheres transplantation. human or mouse CSs. MI mice. | 71 | • Rat. CDCs transplantation. Human or rat CDCs. MI rat. | 83 |
| | • Mouse. CDCs transplantation. Human CDCs (hCDCs). MI mice. | 72 | • Mouse. CDCs transplantation. Neonatal mouse CDCs. Aged mice. | 82 |
| | • Mouse. CDCs transplantation. hCDCs. MI mice. | 77 | | |
| | • Rat. Allogeneic CDCs transplantation. Human or rat CDCs. MI rats. | 73 | | |
| | • Minipig. Allogeneic CDCs transplantation. Minipig CDCs. MI minipig. | 75 | | |
| | • Rat. Allogeneic CSs transplantation. rat CSs. MI rat. | 74 | | |
| | • Rat. CDCs transplantation. Neonatal rat CDCs. Aged rat. | 76 | | |
| | • Human. Autologous CDCs transplantation. MI patients. | 78 | | |
| Abcg2$^+$ CSCs | • Mouse. Isolation of Abcg2$^+$ side population cells and cultured in vitro. | 89 | • Mouse. Genetic lineage tracing. Abcg2-CreER. Oxidative stress and MI injury model. | 91 |
| | •• Rat. Abcg2$^+$ CSCs transplantation. CSCs (GFP$^+$). Cryoinjury model. | 90 | • Mouse. Genetic lineage tracing. Abcg2-CreER. MI injury model. | 92 |
| Bmi1$^+$ CSCs | • Mouse. Genetic lineage tracing. Bmi1-CreER. Homeostasis. | 94 | | |
| | • Mouse. Genetic lineage tracing. Bmi1-CreER. MI injury model. | 95 | | |
| CSCs no marker | • Mouse. Pulse-chase dilution study of cardiomyocytes by Myh6-MerCreMer. MI injury model. | 97 | • Mouse. Lineage tracing by cardiomyocyte-specific MerCreMer mice. MI injury model. | 99 |
| | | | • Mouse. DeaLT. Tnni3-Dre/Tnnt2-Dre, R26-CreER. MI injury model. | 100 |
| | | | • Mouse. Genetic lineage tracing by Ki67-CreER. MI injury model. | 103 |

**Figure 3. Studies supporting or conflicting the cardiomyocyte differentiation of stem cells.**

BMCs, bone marrow cells; CSCs, cardiac stem cells; CDCs, cardiosphere derived cells; DeaLT, dual recombinase activated lineage tracing. Red dot, transplantation; green dot, cell culture; blue dot, endogenous cells or lineage tracing approach.

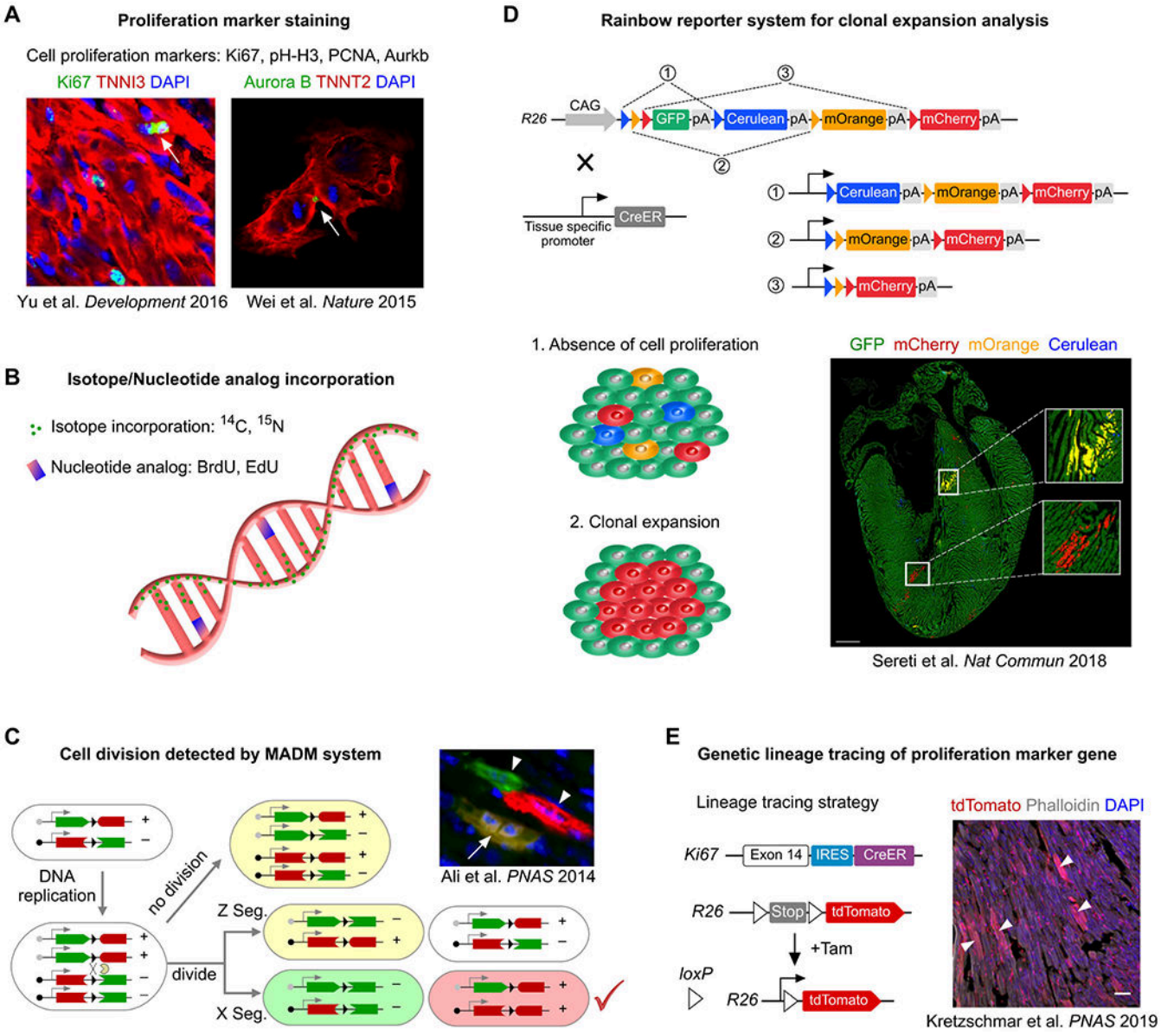


**Figure 4. Cardiomyocyte proliferation detected by different approaches.**

**A**, Immunostaining for antibodies against cell proliferation markers such as Ki67 or Aurora B (Aurkb). Arrow (left panel), Ki67$^{+}$ cardiomyocytes; arrow (right panel), Aurora B expression between dividing cardiomyocytes. **B**, Illustration showing isotope incorporation (such as carbon-14 or nitrogen-15) or nucleotide analog incorporation (BrdU, EdU). **C**, A schematic of the MADM system for detection of GFP$^{+}$ cells and RFP$^{+}$ cells (arrowheads) after G2-X segregation (Seg.). GFP$^{+}$RFP$^{+}$ cells (yellow, arrow) could be results of either cell division after G2-Z segregation or no cell division. **D**, A schematic showing working principle of rainbow reporter for labelling of individual cells with different fluorescent reporters. Rainbow mice carry a cassette of 4 fluorescent genes, inserted in the Rosa26 locus under the control of CAG promotor. Upon Cre mediated recombination, the default GFP expression is replaced by random expression of one of three other fluorescent genes:

mCherry, mOrange, and mCerulean. Immunofluorescent image of heart sections shows mOrange$^{+}$ and mCherry$^{+}$ clones (magnified in boxes) in the heart. **E**, Illustration showing genetic lineage tracing of proliferation marker gene Ki67 using CreER-loxP recombination. Arrowheads, tdTomato$^{+}$ cardiomyocytes that have expressed Ki67 during tracing period.

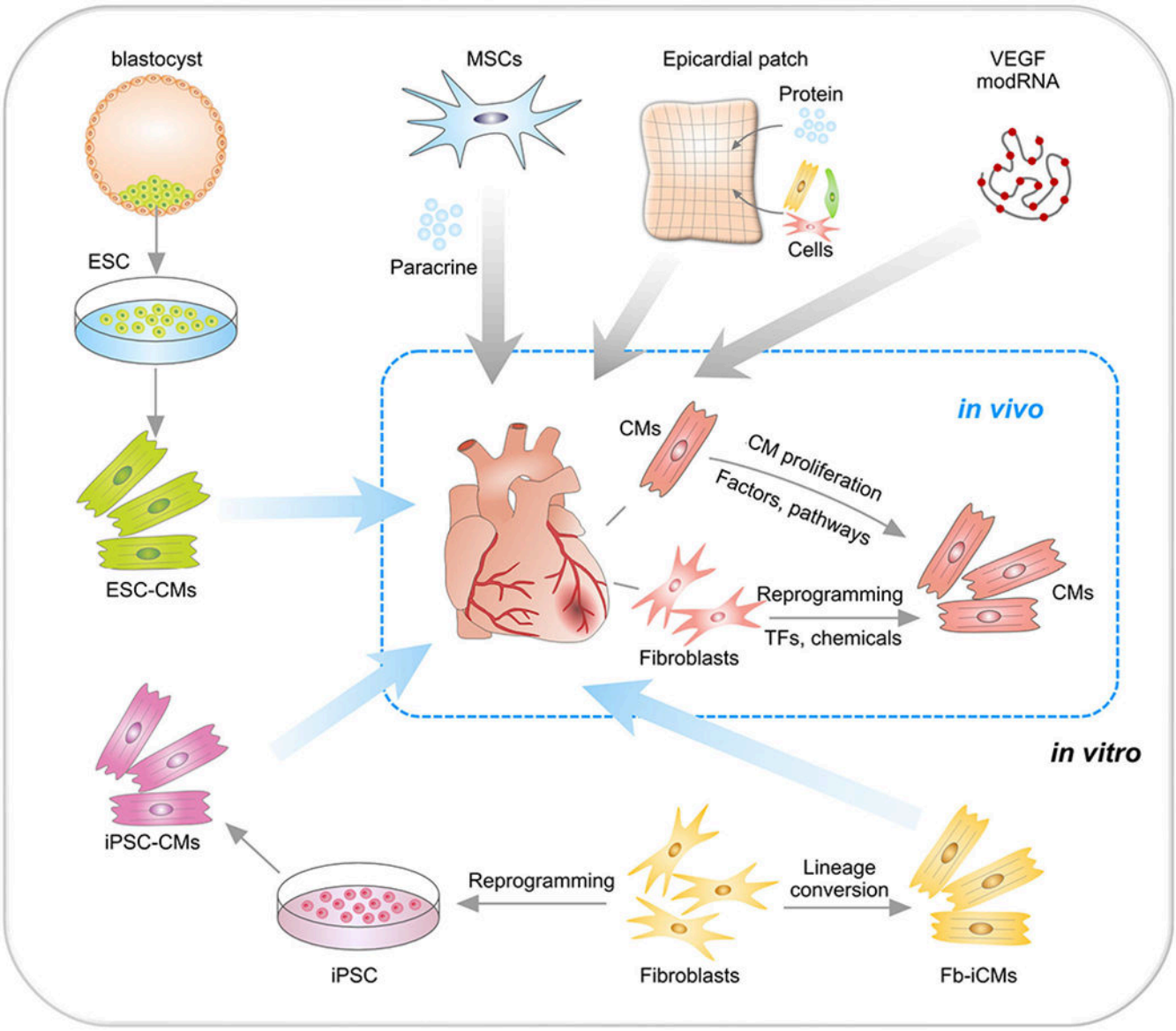


**Figure 5.**

Strategies for treating cardiac repair and regeneration. Transplantation of embryonic stem cell (ESC)-derived cardiomyocytes (CMs) or induced pluripotent stem cells (iPSC)-derived CMs into MI heart. CMs could also be reprogrammed from fibroblasts (Fb) in vitro or by transcriptional factors (TF) or chemicals *in vivo*. Transplantation of mesenchymal stem cells (MSCs) promote neovascularization and cardiomyocyte survival through paracrine mechanism. Epicardial patch containing growth factors or cardiac cells restore heart function after MI. Administration of modified RNA (modRNA) of paracrine factors promote heart function and drive heart progenitor cell fate. CMs could be promoted to proliferation by regulation of factors or signaling pathways.