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REVIEW

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Cardiac regeneration by resident stem and progenitor cells in the adult heart

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Abstract Two main pieces of data have created a new field in cardiac research. First, the traditional view on the heart as a postmitotic organ has been challenged by the finding of small dividing cells in the heart expressing cardiac contractile proteins with stem cell properties and, second, cellular therapy of the diseased heart using a variety of different cells has shown encouraging effects on cardiac function. These findings immediately raise questions like "what is the identity and origin of the cardiac progenitor cells?", "which molecular factors are involved in their mobilization and differentiation?", and "can these cells repair the damaged heart?" This review will address the state of current answers to these questions.

Emerging evidence suggests that several subpopulations of cardiac stem or progenitor cells (CPCs) reside within the adult heart. CPCs with the ability to differentiate into all the constituent cells in the adult heart including cardiac myocytes, vascular smooth muscle and endothelial cells have been identified. Valuable knowledge has been obtained from the large number of animal studies and a number of small clinical trials that have utilized a variety of adult stem cells for regenerating infarcted hearts. However, contradictory reports on the regenerative potential of the CPCs exist, and the mechanisms behind the reported hemodynamic effects are intensely debated. Besides directly replenishing cardiac tissue, CPCs could also function by stimulating angiogenesis and improving survival of existing cells by secretion of paracrine factors. With this review we suggest that a better understanding of CPC biology will be pivotal for progressing therapeutic cardiac regeneration. This includes an extended knowledge of the molecular mechanisms behind their mobilization, differentiation, survival and integration in the myocardium.

Key words cardiac stem and progenitor cells – cardiac regeneration – c-kit – side population – cardioblasts

Introduction

The notion that a cardiac stem or progenitor cell (CPC) pool resides in the heart represents a revolutionary new concept in cardiovascular medicine, which might bring the treatment of cardiac diseases into a new era. In this review, we present the accumulated data on this group of cells and discuss the conflicting views on their functional capabilities. For the sake of convenience, we refer to all the cells as CPCs, in spite of evidence supporting the existence of both multipotent stem cells and more committed progenitor cells in the adult heart (for stem cell terminology see BOX 1).

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BOX I. Stem cell terminology

Stem cells are undifferentiated cells with three main characteristics: the ability to (1) self-renew, (2) differentiate into multiple lineages, and (3) reconstitute a given tissue [93]. Stem cells are classified according to their developmental potential. The term *pluripotent* stem cells implies that the cells can give rise to all embryonic tissue, whereas *multipotency* describes the ability to differentiate into multiple organ specific cell types. The so called *progenitor or precursor cells* are cells with limited self-renewal capacities that only differentiate into one defined cell lineage. Stem cell *differentiation* describes the sequential execution of a developmental program to a particular lineage, whereas *transdifferentiation* is a term used to describe irreversible switches of one functional cell type to another [22]. Stem cells are typically characterized by different cell surface markers, e. g. c-kit and Sca-1. The absence of mature surface markers in *lineage negative (lin$^-$)* cells is a characteristic feature of primitive non-committed stem cells.

Stem cell-based therapy in cardiac injury

Within the last few years it has become clear that treatment of ischemic injuries in animal models with an array of different stem cells improves cardiac function. This has resulted in a search for the best stem cell population that would lead to regeneration of lost or diseased myocardium for example by progenitor cell differentiation into functional cardiac myocytes or by stimulating angiogenesis. The hematopoietic stem cell fraction from the bone marrow has been the most frequent source of cells for this purpose ([28, 68] and reviewed by Dawn et al. [13]), but also embryonic stem cells [37, 59], endothelial progenitor cells [2, 34], skeletal muscle cells [27], adipose tissue stroma cells [71], angioblasts [38] and mesenchymal stem cells (MSCs; [78, 85, 86]) and reviewed by Zimmet et al. [97]) have either been differentiated into myocytes *in vitro* or transplanted successfully into hearts in various animal models. Importantly, no significant long-term side effects have been reported.

On the other hand, human trials with unselected bone marrow derived cells have resulted in only modest improvements in cardiac function (reviewed by Dimmeler et al. [16]). Nevertheless, the mechanisms underlying positive results from animal and human studies are the subjects of intense investigation. So far, they appear to involve paracrine secretion of survival factors, improved perfusion due to revascularization and mechanical effects resulting from increased elasticity of otherwise stiff scar tissue. Whether bone marrow cells also transdifferentiate into contracting novel cardiac myocytes is an open question.

The cardiac stem cell phenomenon

Until recently the adult heart was considered a postmitotic organ without regenerative capacity. This view has been challenged by the findings of small cells with cardiac myocyte-like phenotypes undergoing mitosis, especially in relation to diverse forms of cardiac injury [5, 31, 90]. These CPCs can differentiate into all the constituting cells of the heart [4, 58], thus making them theoretically capable of repairing injuries in the heart. Similar 'housekeeper' cells exist in other tissues like the brain [30] and liver [11]. CPCs or their progeny have now been found in hearts of multiple species including mouse [58, 91], rat [4, 14], dog [51], pig (unpublished P. Anversa [4]), and human [58, 90, 92]. Remarkably, in animal studies, administration of *in vitro* expanded CPCs substantially improve cardiac performance. However, CPCs do not *in natura* have the capacity to heal larger structural injuries as e. g. myocardial infarctions show poor functional regeneration. It has been compellingly demonstrated that the CPC pool can differentiate into at least cardiac myocytes, endothelial and vascular smooth muscle cells that are functionally integrated in the heart. [4]. Although these observations are promising, several issues need to be addressed before the CPCs can be applied in human trials. These include establishing methods for isolation from biopsies, culturing and multiplication of the human CPC pool *in vitro*, methods for delivery to the heart, and finding out how to control their growth and maturation once given back to the heart. Such knowledge could potentially also lead to therapeutic principles where CPCs already residing in the heart could be recruited by stimulating their proliferation and differentiation.

Possible mechanisms for beneficial effects of cardiac stem cell transplantation

Most researchers agree that cell transplantation contributes to a better cardiac performance, but the mechanisms are highly debated. Several not mutually exclusive explanations exist as listed in Table 1. To some extent the potential mechanisms underlying the effect on therapy with resident CPCs have to be extrapolated from studies performed with bone marrow derived stem cells, as the knowledge on CPCs is still evolving. However, convincing scientific data suggest that CPCs are able to differentiate into functional myocytes and vessels integrating into the

Table 1 Potential mechanisms behind CPCs' effects in the heart

Effect	Mechanism
Functional regeneration	Differentiation and maturation of CPCs into cardiac myocytes, endothelial cells, and smooth muscle cells
Paracrine signaling	Release of paracrine factors from CPCs that stimulate cell survival and angiogenesis
Therapeutic fusion	Fusion of CPCs with host cells thus conferring survival and proliferation to cardiac myocytes
Mechanical stabilization	Increased elasticity and stability of the cardiac wall by the engraftment of CPCs into scar tissue

diseased myocardium [4, 14, 51, 91]. Increased perfusion has positive effects on remodeling due to myocyte survival and mechanical effects resulting from increased elasticity of otherwise stiff scar tissue [6].

At the molecular level, paracrine secretion of survival and angiogenic factors may also contribute to reported improvement of cardiac function as seen e. g. after stem cell therapy with bone marrow derived stem cells [25, 83, 84]. A recent study utilizing chimeric mice with a background mutation in the c-kit receptor, shows that cells expressing c-kit are recruited from the bone marrow to the infarcted myocardium and rescue the cardiomyopathic phenotype by releasing angiogenic substances including VEGF [20]. Similarly, paracrine secretion of multiple arteriogenic cytokines from MSCs has been shown to enhance proliferation of endothelial and smooth muscle cells, and increase collateral flow and remodeling in a murine hindlimb ischemia model [35, 36]. This attenuated muscle atrophy and fibrosis, without incorporation of MSCs into the vasculature, and involved factors like VEGF and FGF2 [35, 36]. Paracrine secreted hormones could also act as survival factors working directly on the constituting cells of the myocardium or perhaps mobilizing resident CPCs involved in repair mechanisms.

Another proposed mechanism is fusion between donated stem cells and host cardiac myocytes. This might produce a multipotent cell with the ability to proliferate and integrate as functional tissue [57, 79]. Finally, cell therapy could provide thickening and mechanical stabilization to the infarcted myocardium wall, which subsequently could reduce wall tension and remodeling [6].

Resident cardiac stem and progenitor cells with regenerative capacity

Challenging the dogmatic view on the heart as a postmitotic organ

During fetal embryogenesis, the heart consists of rapidly proliferating cardiac myocytes [49]. This growth pattern ceases in late fetal life, and is replaced by hypertrophic growth and binucleation in early postnatal life as part of the myocyte maturation [80]. Until recently, the heart was thus considered terminally differentiated without any significant regenerative potential [82]. This dogmatic view was first challenged by Piero Anversa and coworkers who reported the existence of small proliferating cells in the heart expressing myocyte specific markers [31]. In addition, these cells were up-regulated under pathological cardiac conditions [5, 31].

The notion that cycling myocytes in the heart could originate from stem cells was strongly supported by a study of male patients transplanted with female donor hearts. A number of undifferentiated cells containing Y-chromosomes along with the stem cell-like markers Sca-1, c-kit and MDR1 were found in the female donor heart suggesting that male host cells had infiltrated the heart. These cells seemed to be the first proof of a population of stem cells in the adult heart participating in cardiac homeostasis, though it was not clear if they originated from the bone marrow or invaded the transplanted heart from the remaining atria of the host. Moreover, these cells contributed to the remodeling of female donor hearts constituting a substantial percentage of myocytes, arterioles and capillaries [72]. It has been a matter of debate whether the observed chimerism represents spontaneous cell fusion between host and donor cells, which has been reported to occur at a low rate *in vivo* [64, 96]. So far though, CPCs have generally not been found prone to fuse with host cells in the myocardium after application apart from one study [65]. The observations of chimerism in the hearts and bone marrow transplanted patients [15, 23, 46, 62, 72] together with the recent discovery of resident stem cells in other tissues otherwise considered terminally differentiated (e. g. nervous tissue and skeletal muscle [8, 21]) have subsequently fuelled the search for a cardiac progenitor or stem cell resident in the heart.

At this point, at least three CPC pools residing in postnatal hearts have been reported in different species including mice, rats, dogs and humans. These cells are distinguished by different expression of marker proteins. The three populations include the c-kit$^+$ cells [4, 58], cells from the side population (SP) [26, 55, 70], and the so-called cardioblasts expressing islet-1 [47]. Two other surface antigens have been utilized for isolation and/or characterization of resident CPCs; stem cell antigen-1 (Sca-1) in mice [65], Sca-1-like in other species, and Multi Drug Resistance-like protein-1 (MDR1) [51] (as described below). A degree of overlap between the different CPC subpopulations may exist, and especially three reservations should be borne in mind. First, none of the surface antigens that have been utilized to characterize the cells are specific for CPCs. Second, the expressed surface markers may differ between species and could also change in the course of differentiation from progenitor cell to mature cell. Third, it is difficult to determine if candidate cells from independent studies represent the same cellular lineage on various differential stages or entirely different cell lineages. See Table 2 for a schematic overview of resident CPCs found in the heart.

c-kit positivity defines cardiac stem cells with in vivo regenerative capacity

The current best characterized group of stem cells resident in the heart constitutes a pool of cells that express the receptor for stem cell factor known as *c-kit* [4, 51, 90–92]. This receptor has been widely used as a cell surface marker of pluripotent long-term reconstituting

Table 2 Schematic presentation of resident CPCs found in the heart

Cell definition	Species	Additional markers	Functional characteristics		References
			In culture	In heart	
'c-kit$^+$ Lin$^-$ cells'	Mouse	Sca-1-like$^+$, MDR1$^+$	N/A	Resident cells proliferate and differentiate after injection of IGF1 and HGF; New cardiac myocytes and vessels observed	[91]
	Human	Sca-1-like$^+$, MDR1$^+$	N/A	Possible regeneration in infarct border zones	[90]
	Rat	N/A	Cells are clonogenic, multipotent, and self-renewing, and differentiate into immature cardiac myocytes, smooth muscle and endothelial cells	Cells home to infarctions, and differentiate into small cardiac myocytes, smooth muscle, and endothelial cells, promoting regeneration and restoration of contraction	[4, 14]
	Dog	Sca-1-like$^+$, MDR1$^+$	Cells are clonogenic, multipotent, and self-renewing; differentiate to myocytes (50%), smooth muscle (40%), and endothelial cells (10%)	Growth factor injection promotes cardiac regeneration and improve contraction	[51]
'Cardiospheres'					
c-kit$^+$	Mouse, human	Sca-1$^+$, CD31$^+$, CD34$^+$	Cells are clonogenic, and after culture with EGF, FGF2, cardiotrophin-1, and thrombin, murine cells show spontaneous beating; human cells only beat in co-cultures with myocytes	Injected cells undergo angiogenesis and cardiogenic differentiation, particularly in infarcted hearts	[58]
SP	Mouse, rat	c-kit^{low}, Sca-1^{low}, MDR1$^+$, CD29$^+$, CD44$^+$	Multipotent in monoculture with EGF and FGF2; stem cell-like colonies form from single cells; differentiate to spontaneously beating cardiac myocytes	Cardiosphere-derived cells migrate from region of neural crest to the heart after injection into chick embryos and differentiate into cardiac myocytes	[87]
'SP cells'					
Hoechst expulsion	Mouse	N/A	Stem cell colonies appear when cultured; differentiate and express connexin 43 after co-culture with myocytes	Compensatory activation and subsequent depletion of resident SP cells in animal model of cardiac hypoplasia	[26]
		c-kit^{low}, Sca-1$^+$	Cultured cells give rise to hematopoietic colonies; differentiate to express α-actinin in co-cultures with myocytes	N/A	[55]
		Sca-1$^+$, CD31$^-$	Self-renewal by formation of colonies in culture; cardiac differentiation and spontaneous beating after co-culture with myocytes	Cardiac SP cell pool depletes after infarction, but is reconstituted by local proliferation and homing from bone marrow	[61, 70]
Sca-1$^+$ cells	Mouse	c-kit$^+$ (10%), CD34$^+$ (10%), CD45$^+$ (40%)	Cells are multipotent; 1% beat after oxytocin stimulation	N/A	[56]
		Lin$^-$, CD31$^+$, Flk1$^-$, Flt1$^-$	5-azacytidine stimulation induces expression of sarcomeric proteins	When infused after ischemia/reperfusion, cells home to injured myocardium and express actinin	[65]
'Cardioblasts'					
Isl-1$^+$	Mouse	c-kit$^-$, Sca-1$^-$, MDR1$^-$	Unipotent; cells expand without differentiation in special medium; after co-culture or application of conditioned medium/ cell contact cells differentiate into myocytes	N/A	[47]
heterogeneous non-myocytes	Mouse	c-kitlow, Lin$^-$, Isl-1$^+$	Unipotent; cells are not clonogenic; cardiac differentiation is dependent on specific medium and FGF2	FGF2 is pivotal for homing, engraftment and differentiation in hearts	[76]

murine stem cells (for review see [53]) and has previously been used to define a population of cells from the bone marrow that seemed to be capable of differentiating into functional myocardium when injected into the infarcted mouse heart [69]. Among CPCs resident in the heart only the c-kit$^+$ cells have so far fulfilled all criteria for being stem cells, at least in rats and dogs [4, 51]. It is conceivable that the c-kit$^+$ fraction of stem cells in the heart consists of a mixture of bone marrow derived cells and resident cells, but only a minor proportion of c-kit$^+$ cells in the heart is likely to represent CPCs with cardiomyogenic potential. Thus, the composition of the c-kit$^+$ cell population in the heart is heterogeneous and will depend on the age and presence of diseases in the heart. For instance, bone marrow derived progenitor cells expressing c-kit are chemoattracted in relation to inflammatory stimuli, but their cardiomyogenic potential seems to be limited [20, 94]. The c-kit$^+$ cells from the heart with stem cell abilities do not express cell surface markers of other cell lineages, making them *lineage negative*, they are clonogenic, able to self-renew, multipotent and have been shown to reconstitute the heart after an acute ischemic event by differentiating into cardiac, smooth muscle, and endothelial lineages as well as fibroblasts. However, even c-kit$^+$ CPCs are not a homogeneous population since around 10 % of freshly isolated cells express transcription factors from early myocyte lineages [4].

It is of particular clinical importance that c-kit$^+$ CPCs have been found resident in the adult human heart and seem to be upregulated under pathological conditions including aortic stenosis and infarction [90, 92], and, furthermore, that a depletion of CPCs in chronic infarcts compromise myocardial regeneration and leads to ventricular dilation and reduced cardiac performance [92]. A similar age related depletion of CPCs have been proposed to account for the heart failure sometimes seen with senescence (reviewed in [1]).

Use of c-kit$^+$ cardiac stem cells in stem cell therapy

CPCs could be used to ameliorate cardiac function in two principally different, although not mutually exclusive, ways (see Fig. 1). Either CPCs isolated from the myocardium can be cultured and propagated *ex vivo* before re-administration or resident CPCs neighboring the infarct zone can be boosted to proliferate and differentiate by the injection of survival factors both leading to partial cardiac regeneration and functional improvement. A series of compelling animal experiments have been conducted to analyze the effect of administrating CPCs or stimulating the local CPCs after cardiac injury [4, 14, 51, 91]. The functional competence of CPCs *in vivo* was first demonstrated by injecting CPCs into the border zones of experimentally induced myocardial infarcts in a rat model leading to a substantial band of regenerating myocardium [4]. In addition, in a model of ischemia followed by reperfusion the CPCs homed to the injured rat myocardium after intravenous infusion [14]. In both studies, CPCs turned into phenotypically differentiated cells including capillaries, arterioles and cardiac myocytes, and treated animals exhibited significant improvements in cardiac function as validated by echocardiographic and hemodynamic parameters [4, 14]. Injection of survival factors like hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) into the border zones of the infarcted myocardium in mice [91] and dogs [51], respectively, boosted the repair of damaged cardiac tissue, but still the new myocytes remained immature with cell sizes only a fraction of normal adult cardiac myocytes. This suggests that the myocytes need additional factors to fully differentiate.

These studies have raised important questions regarding the potential and origin of the CPC pool, including: Can the CPCs differentiate into fully mature cardiac myocytes, and if so what is the mechanism? One of the described studies in dogs provides a clue. The

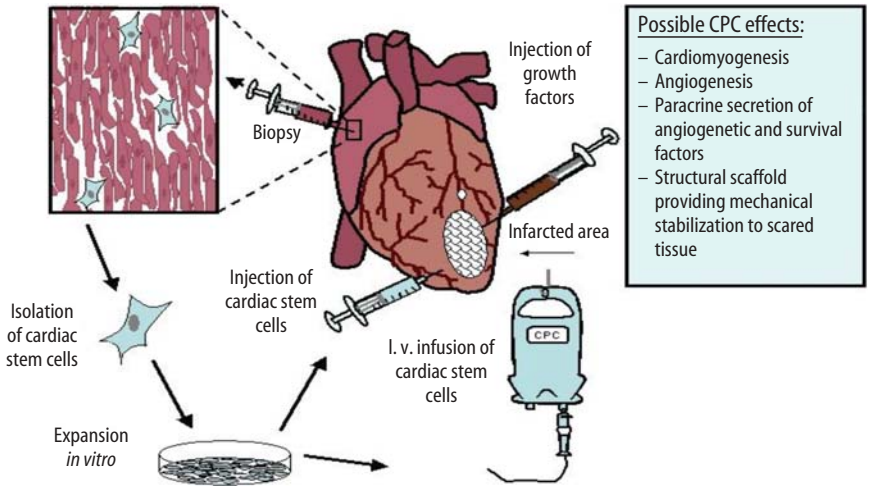

Fig. 1 Schematic presentation of how cardiac stem and progenitor cells (CPCs) can be exploited in the heart and possible mechanisms behind any beneficial effect

CPCs found in the infarct area were small and not fully differentiated, while those found in non-infarcted ventricular tissue were similar to surrounding healthy cardiomyocytes suggesting that the infarcted myocardium is an inadequate milieu for the maturation of CPCs [51]. Taken together with *in vitro* findings that some CPCs are able to differentiate into mature cardiac myocytes when co-cultured with neonatal myocytes [70] these data imply that uncharacterized molecular factors from the myocardium are essential for CPC differentiation.

Sca-1$^+$ cells and the 'side population'

CPCs residing along small capillaries in the mouse heart have been isolated based on the expression of the stem cell surface marker Sca-1 and have been shown to express the enzyme telomerase [65] suggestive of self-renewal capacity [60]. Sca-1 is a well known hematopoietic stem cell marker and has been found on various subpopulations of stem cells [74]. In the study by Oh et al. most Sca-1$^+$ CPCs also expressed the endothelial marker CD31 indicating that these cells were already committed to an endothelial fate. Still, some of the Sca-1$^+$ cells differentiated into cardiac myocytes *in vivo*. Much effort has therefore been put into further characterizing markers for CPCs. The ability to efflux a Hoechst dye by a MDR-like protein has recently been applied to isolate resident CPCs. This feature has previously been used to define multipotent stem cells isolated from the bone marrow [24] and skeletal muscle [29]. Expelling of the Hoechst dye gives a characteristic appearance after fluorescent-activated cell sorting, hence the name 'side population' or 'SP'. The cardiac SP pool differs from the bone marrow isolated SP pool by low or absent expression of c-kit and hematopoietic markers CD34 and CD45 [55, 70]. Interestingly, most of the cardiac SP cells express the stem cell surface marker Sca-1 [65], which together with the absence of CD31 defines a sub-population of the SP cells that possess cardiomyogenic potential and colony forming abilities [70]. This might explain why bone marrow derived SP cells [28] or Sca-1$^+$ CPCs [65], both of which are enriched for CD31, only had modest regenerative potential in an ischemia/reperfusion model. While cardiac SP cells are multipotent and able to self-renew, their ability to differentiate into functional myocardium *in vivo* and restore cardiac contraction has not yet been convincingly demonstrated.

Sca-1$^+$ and SP cells can differentiate into cardiac myocytes in vitro

Reports from several groups confirm that both cardiac Sca-1$^+$ and Sca-1$^+$ /SP cell pools can differentiate into more mature cardiac phenotypes *in vitro* after application of known cardiotrophic compounds or co-culturing with adult cardiac myocytes [26, 55, 56, 65, 70]. For instance, Sca-1$^+$ cells express cardiac transcription factors and some sarcomeric proteins in the presence of 5-azacytidine [65] or oxytocin [56]. In one experiment, a low number of freshly isolated Sca-1$^+$, CD31$^-$ SP cells expressed cardiac transcription factors, but the expression of sarcomeric contractile proteins were dependent on co-culture and subsequent cellular coupling with adult cardiac myocytes as judged by staining for the gap junction protein connexin 43 [70]. Thus, the proper sarcomeric organization and striation in a substantial subset of cells correlated with coupling to cardiac myocytes. The new myocytes exhibited spontaneous contractions and intracellular calcium transients indistinguishable from those of adult cardiac myocytes [70].

The side population including Sca-1$^+$ cells and cardiac regeneration

The SP cell pool may also have cardiac regenerative capabilities [26, 28, 61, 65]. Using a cardiac ischemia-reperfusion protocol, two reports have demonstrated that either SP cells isolated from the bone marrow or cardiac Sca-1$^+$ cells home to the infarct border zone, and that a low number of these cells differentiate into cardiac myocytes and endothelial cells [28, 65]. Using the Cre/Lox marker system to identify differentiated cells, donated cardiogenic Sca-1$^+$ cells carrying αMHC-Cre were shown to home to the infarct border zone, and a large proportion of engrafted cells expressed the sarcomeric protein α-actin after two weeks [65]. Roughly 50 % of the cells differentiating towards a cardiac phenotype expressing αMHC seemed to fuse with native cardiac cells emphasizing the importance of meticulous scrutiny when judging cellular fate under these circumstances [65]. In another study, SP cells in the infarcted myocardium were actively dividing after infarction in mice, which is in agreement with previous indications of these cells' self-renewing capabilities [61]. But effects on cardiac function has not yet been proven.

'Cardiospheres' contain clusters of c-kit$^+$ cells and have cell therapy potential

Cardiospheres are determined as small clusters of primitive c-kit$^+$ cells and have been isolated from both murine and human hearts [58]. These cells are clonogenic and capable of long-term self-renewal, which makes them a possible candidate for cell-based regeneration therapy. After isolation, a minor percentage of cells constituting the cardiospheres express both stem cell (c-kit, Sca-1, and CD34) and endothelial cell (flk-1 and CD31) markers, but after 6 days in culture mainly the expression of c-kit is preserved indicating that cells expressing this marker might be responsible for their continued proliferation [58]. *In vitro*, murine cardio-

sphere-derived cells beat spontaneously, whereas human cells require co-culture with rat cardiac myocytes to obtain this trait. *In vivo*, human cardiosphere-derived cells can differentiate into cardiac myocytes, endothelial and smooth muscle cells after injection into infarcted hearts of immunodeficient mice [3, 58]. These data are consistent with the existence of a multipotent cardiac stem cell resident in the adult human heart, which for the first time was cultured from cardiac biopsies. In another study, 'cardiospheres' were grown from the cardiac SP population of both neonatal and adult rats and mice under conditions normally applied for the culture of neurospheres [87]. Also cardiospheres grown under these conditions expressed low levels of c-kit and Sca-1 and were found to be clonogenic and multipotent, because they could differentiate into cardiac myocytes, smooth muscle cells, glial cells and neurons. A fraction of the cardiospheric cells began spontaneous beating after 14 days in culture. The low levels of Sca-1 seem to distinguish cardiospheres from the previous cell populations.

'Cardioblasts' are embryonic myocyte progenitor cells

Two recent papers characterize populations of cardiac progenitor cells dubbed "cardioblasts" that were isolated from the neonatal mouse heart by means of their expression of the LIM homeodomain transcription factor Islet-1 (Isl-1) [47, 76]. Cardioblasts can be expanded and show distinct cardiomyogenic potential *in vitro*; however, their clinical application is unclear. Isl-1 is a marker for undifferentiated myocytes but this protein is lost upon differentiation [7]. Most likely, Isl-1 together with GATA4 serves as a transcriptional enhancer of the myocyte transcription factor MEF2C [17]. The Isl-1$^+$ cells have been termed 'cardioblasts' as they probably represent a subpopulation of cells constituting the embryonic heart and disappear soon after birth [47]. In the first study, the cardioblasts isolated from early postnatal hearts could be expanded *in vitro* and expressed early markers of cardiac mesoderm, Nkx2.5 and GATA4, but no sarcomeric proteins [47]. In co-culture experiments with neonatal cardiac myocytes some Isl-1$^+$ cells differentiate into a more mature phenotype expressing cardiac myocyte specific proteins and with a subset of cells showing contractile activity and electromechanical coupling to neighboring cells [47].

In the second study, a heterogeneous population of cells from the non-myocyte fraction of mouse hearts was isolated and shown to express several stem cell markers like Isl-1, c-kit and Sca-1 [76]. After three weeks of differentiation some cells showed a phenotype of mature cardiac myocytes and spontaneous beating. Furthermore, precursor cells were multipotent but not clonogenic being able to differentiate into adipocytes, skeletal muscle cells and osteblasts. *In vivo*, the precursor cells homed to the myocardium presenting characteristics of differentiated cardiac myocytes [76]. It was not determined which subpopulation of the non-myocyte cell fraction was accountable for the observed differentiation, but the presence of Isl-1 points to 'cardioblasts'.

The origin and fate of CPCs

From where do the CPCs originate?

At a glance, the CPCs resident in the adult heart could either constitute remaining endogeneous cardiac stem cells dormant in the myocardium or alternatively, home to the heart from another organ during development or in response to injury.

CPCs might originate from the bone marrow or neural crest

Deb et al. showed that CPCs may originate from stem cells in the bone marrow capable of homing and differentiating to cardiac myocytes, since cardiac chimerism was found without evidence of cellular fusion in gender mismatched bone marrow transplanted patients [15]. Two papers show that both the c-kit$^+$ cells and the cardiac population of SP cells might be derived from the bone marrow [20, 61]. In the first paper, genetic tagging experiments showed that c-kit$^+$ cells from the bone marrow homed to the myocardium after acute ischemia and secreted VEGF resulting in angiogenesis. No differentiation into myocytes was found [20]. In the second paper, labeled SP cells from the bone marrow were shown to constitute up to 25 % of the cells in the infarcted myocardium after one week [61]. In addition to this, several reports have documented that various bone marrow derived stem cells can transdifferentiate into functional cardiac tissue when injected into the infarcted heart [28, 33, 68].

It is reasonable to assume that only a minor fraction of bone marrow derived cells is eligible for surveillance of organs like the heart and recruitment in the case of injury. At least one study indicates that a distinct population of 'tissue committed' stem cells existing within the bone marrow exhibits early cardiac markers (transcription factors), is mobilized to the blood stream after myocardial infarction, and is recruited to the site of injury by locally secreted chemokines such as SDF-1 [94]. Myocardial infarction results in an almost immediate mobilization of mononuclear cells expressing specific cardiac, endothelial and muscle markers [94]. They reside in the non-adherent and non-hematopoietic CXCR4$^+$, Sca-1$^+$, lin$^-$, CD45$^-$ mononuclear cell fraction in mice and CXCR4$^+$, CD34$^+$, AC133$^+$, lin$^-$, CD45$^-$ mononuclear cell fraction in humans [43].

Interestingly, multipotent SP cells residing in the heart have been shown likely to originate from the neural crest [87]. These cells form cardiospheres, express markers of undifferentiated neural precursor cells and migrate from the neural crest region to the heart after injection into a chick embryo. Furthermore, when cardiospheres are cultured from the hearts of transgenic mice which have a genetic marker in cells derived from the neural crest, the resulting cardiospheres are indeed labeled [87].

Could different CPCs originate from the same cell?

Although it has been suggested that the various resident progenitor cells from the heart represent different lineages, several of the previously mentioned CPCs could in fact be derived from one common cardiac ancestor caught at different time-points in its differentiation. It has been suggested that special microenvironments dubbed 'cardiac niches' exist in the heart that protect and support the self-renewal and early commitment of CPCs [89]. In stem cell niches a variety of stem and progenitor cells are believed to exist in a developmental continuum [63], perhaps accounting for the difference in potency and cell markers among CPCs. In addition, differences in the observed expression of surface proteins could be due to loss of surface markers during isolation, differentiation or change in microenvironment of CPCs. For instance, culturing hematopoietic stem cells makes them lose CD34 expression [18]. This might also happen *in vivo* and with different surface markers as has been demonstrated by the loss of CD45 among bone marrow derived stem cells upon homing to infarcted myocardium [61]. Expansion of cells *in vitro* might also change other characteristics of the stem cells, e. g. MSCs have been shown to lose their homing capabilities after culturing [75]. Finally, it has been reported that the digestion process to purify resident cardiac cells may have changed the expression of c-kit [70], while others claim that this is not a concern [65].

It is reasonable to think that CPCs isolated on the basis of either the expression of c-kit, the expulsion of a Hoechst dye (SP cells) or the ability to grow in cardiospheres under certain culture conditions all derive from one common progenitor cell of early mesenchymal origin. They are all small with a scanty cytoplasm and large nucleus and grow readily on plastic surfaces; a typical trait of MSCs. Until recently, the SP cells were primarily characterized by the ability to reconstitute the hematopoietic compartment, but it now seems that early MSCs can also be found in the side population of the bone marrow [9, 67] or heart [56]. In skeletal muscle, myogenic progenitor cells also belong to the $CD31^-$, $CD45^-$ SP cells and show some mesenchymal lineage markers [88]. The expression level of c-kit and Sca-1 will likely reflect progenitor cells in different stages of their development and perhaps with various potential for differentiation.

Do CPCs differentiate into mature cells in the heart?

This question is the subject of the controversy that has divided the stem cell research community. While most researchers agree on likely beneficial effects on myocardial survival and angiogenesis due to paracrine secretion of factors from CPCs after myocardial application, the differentiation into new functional myocytes is still questioned due to the discrepancy between the phenotype of adult myocytes and that of differentiated CPCs. Various explanations to the reported plasticity of CPCs can be found in Table 3. The differentiation of stem like cells to functional mature cells in the myocardium must depend on the effectuation of a complicated developmental program in an orderly sequence. The execution of a cardiac differentiation program is likely to rely on the distinct expression of some genes, while other genes need to be silenced, a process that will probably be secondary to changes in chromatin configuration by epigenetic factors like DNA methylation and histone modifications (reviewed by French et al. [22]). Such nuclear reprogramming will likely depend on both cell-to-cell contact with neighboring cells as well as paracrine/autocrine secretion of various factors in a microenviron-

Table 3 Possible explanations behind the reported CPC plasticity in the heart

Reported CPC plasticity due to	Mechanism
true multipotency	CPCs differentiate into myocytes, endothelia, smooth muscle cells, etc.
transfer of donor cell tracers	Transfer of cell tracers (GFP, Cre-recombinase, etc.) from donated stem cells to host cells via e. g. gap-junctions or nanotubuli resulting in wrongful interpretation of stem cell potential
nuclear reprogramming	Incomplete activation of a cardiac program resulting in biphenotypic donor cells appearing like e. g. immature myocytes
cell fusion	Donated stem cells fuse with host cells making them look fully differentiated
histological artifacts	Autofluorescent host myocytes can be mistaken for GFP labeled donor stem cells. Donor stem cells in close proximity to host cells can be misinterpreted as differentiated CPCs
heterogeneity	Donated CPCs could contain a mixture of different lineage progenitor cells if not subcloned

ment mimicking fetal cardiogenesis. Whether such conditions can ever be remade in the adult myocardium remains to be proven.

How to determine CPC differentiation

The differentiation of stem cells in the myocardium or in culture is most often based on the presence or absence of cell surface markers, transcription factors, and cytoplasmic proteins. Thus, an undifferentiated stem cell would express stem cell markers like c-kit, MDR1, Sca-1-like proteins or expel Hoechst dye, while being lineage negative and not expressing transcription factors or cytoplasmic proteins of cardiac cells. Progenitor cells would correspond to cells that express stem cell markers in conjunction with transcription factors specific for cardiac cells. Finally, more differentiated cells will be expected to have lost their stem cell antigens and express cytoplasmic proteins specific for cardiac cells like myocytes, endothelia or smooth muscle cells. To rely on this approach in order to demonstrate complete differentiation of stem cells could pose some problems: First, the expression of a few cardiac transcription factors and even sarcomeric proteins as the CPC matures *in vitro* or *in vivo* does not necessarily reflect the activation of the developmental program of the stem cell. Thus, it might be an intrinsic property of undifferentiated cells in the myocardium to acquire a phenotype that resembles the environment surrounding them and therefore express certain cardiac transcription factors and their translated proteins. Such changes could come about by paracrine stimulation, intercellular connections or cell fusion. Second, so far the demonstration of stem cell differentiation has relied heavily on technical procedures like immunohistochemistry and fluorescent-activated cell sorting, which are very dependent on the specificity of the applied antibody. Furthermore, concern has been raised to many other possible procedural artifacts, e. g. autofluorescence, overlying cells and isolation of a heterogeneous pool of stem cells. Thus, a meticulous approach is highly important during histological evaluation.

When nuclei from small or disintegrated cells lie in close proximity to e. g. cardiac myocytes or inflammatory cells with sarcomeric debris, this could be mistaken for a myocardially differentiated cell unless properly stained [45]. When GFP labeled cells are administered after myocardial injury, which increases autofluorescence in the heart, this may wrongfully lead to the assumption that full integration and differentiation of donor cells have taken place [45].

The Cre/Lox system has also been criticized for its inaccuracy due to the possibility of donor Cre spreading to neighboring cells via intercellular junctions thereby falsely giving the impression of cellular fusion [48]. If true, the recent discovery of ultra fine intercellular connections between endothelial progenitor cells and cardiac myocytes *in vitro*, allowing the exchange of proteins and organelles, only adds to the complexity of determining individual cell progeny *in vivo*, e. g. allowing the exchange of GFP from donor to recipient cells [41, 77]. This could wrongfully point toward differentiation of donated cells due to GFP labeling of recipient cells. It seems that stringent methodologies are needed in order not to misinterpret the differential capacity of progenitor cells perhaps using a wider variety of, or later-stage, cardiac markers.

Future challenges for successful cardiac regeneration

Isolation of CPCs and in vitro enrichment

As previously discussed, the regenerative capacity of resident CPCs could be exploited in two different ways (Fig. 1), either by the boosting of surviving CPCs after injury like myocardial infarction or by *ex vivo* enrichment of progenitor cells obtained directly from the heart by means of a biopsy before re-application. The culture of human CPCs have already been successfully accomplished after biopsy [58]. However, the need for an invasive procedure is potentially hazardous and delays treatment, increases scar formation and thus reduces the efficacy of stem cell mediated regeneration. No published reports have yet determined if early resident CPCs have the same immunological tolerance as MSCs and therefore could be utilized from a common source. This would of course make the treatment more clinically applicable. Much effort is being put into the understanding of factors necessary for *in vitro* enrichment and optimization of the subsequent cardiac differentiation *in vivo*, but the pieces of the puzzle are just beginning to come together. Several problems seem imminent: How can culture conditions be optimized in order to obtain a homogenous and multipotent stem cell population? Should CPCs be differentiated *in vitro* and to what degree before re-administration? Can the extent of regeneration be optimized by the concomitant injection of different subsets of stem cells with different lineage potential? What is the minimum number of cells that will give the maximal amount of regeneration? When is the optimal time point for the injection of CPCs after a myocardial infarction?

The greatest challenge for stem cell mediated regeneration will be to control the inhospitable environment in the sick heart that differs enormously from the conditions during cardiogenesis in the developing embryo. A complicated ensemble of agonistic and antagonistic factors of autocrine and paracrine origin and the necessity for direct intercellular coupling with myocytes all seem to be important for cardiac differentiation (see

next section). New data show that MSCs are able to sense the elasticity of the surrounding microenvironment, which then determines the fate of differentiation. While a soft matrix seems to mimic neural tissue and directs the stem cells toward a neuronal fate, a stiffer matrix is myogenic [19]. Thus, to recreate such a complex microenvironment both *in vitro* and in the diseased heart with scar tissue and inflammation might prove to be a difficult task, and could pose a major obstacle for the implementation of stem cell therapy. In addition, cells in culture seem to modify important characteristics in the time frame necessary for cell expansion. Nevertheless, the data obtained so far with resident CPCs inspires and gives hope for this new treatment modality.

Application of CPCs and maturation in vivo

When utilizing CPCs enriched *in vitro*, the route of delivery to the ischemic heart becomes of outmost importance in order to ensure the greatest number of viable donor cells. No consensus about the preferred method has yet been obtained. Although intravenously administered CPCs have demonstrated the ability to home to the heart in animal studies, this have only lead to inadequate regeneration [14, 65, 76].

The intramyocardial injection of stem cells into a hostile inflamed environment will most likely result in only a few surviving donor cells [81]. Some effort has been focused on increasing the survival of such grafted cells. The overexpression of the survival factor Akt in MSCs transplanted to ischemic myocardium resulted in almost complete regeneration of the injured heart [54]. The direct application of survival and homing factors into the myocardium has been shown to increase survival of resident CPCs after infarction and boost regeneration [51, 91]. The coincidental administration of stem cells and survival factors like IGF-1 has also increased regeneration when utilized with embryonic stem cells [39]. New approaches could include the application of fibrin patches seeded with donor cells onto the infarcted myocardium [52].

Another problem seems to be the lacking ability of CPCs and other stem cells to differentiate into functional cardiac myocytes with an adult phenotype in the inflamed tissue of infarcted hearts. This could of course signify that these cells do not hold true potential for differentiation to mature cardiac tissue. Interestingly, a recent study demonstrated how CPCs differentiated into an adult phenotype when they engrafted into the myocardium at a certain distance from the infarcted tissue [14]. Thus, it seems that either the milieu in the inflamed tissue hinders the maturation of CPCs or that perhaps the destruction of organized cardiac tissue prevents cellular coupling with adult cardiac myocytes necessary for the functional differentiation of CPCs [55, 70, 95]. It might therefore be necessary to determine which factors in the microenvironment govern cardiac differentiation and provide these factors *in vivo* in relation to the injection of CPCs or *in vitro* during cell expansion to obtain a mature cardiac phenotype. One recent study has shown substantial maturation of resident CPCs over a period of 4 months, after the activation of resident CPCs by the injection of two growth factors (HGF and IGF-1) into the infarcted rat myocardium [91]. Multiple autocrine and paracrine factors like TGF-β, VEGF and FGF secreted from the host myocardium could also be important for the differentiation and maturation of CPCs into functional myocardium and this might also be exploited in order to obtain a higher survival among donated cells and more complete regeneration [40, 50]. Recently, the possibility of controlling stem cell growth and differentiation by means of targeting microRNA has emerged as an intriguing new treatment modality [42, 44].

As the infarcted myocardium is succeeded by scar tissue this might pose a physical barrier, which puts a stop for further regeneration [73]. Some data on CPCs hold promise to the regeneration of scarred myocardium. CPCs or growth factors activating CPCs were injected into a one month old scarred infarct leading to substantial regeneration, which seemed to be mediated by the secretion of matrix metalloproteinases [32]. Still, many obstacles are to be solved before cardiac regeneration will be clinical applicable as a standard treatment.

Durability and sustainability of CPCs after treatment

Another point of uncertainty is the long-term sustainability of donor cells in the heart. Long-term data regarding CPCs are scarce. One animal study following the destiny of bone marrow derived MSCs over a period of 6 months after injection into the ischemic rat myocardium showed only transient improvement in cardiac performance during this time period and an immature myofibril organization in differentiated MSCs at the end of the study [10]. Similar problems might be expected for cardiac regeneration mediated by CPCs. It has been hypothesized that CPCs have the ability to repair minor injuries on a daily basis, but is an inadequate source for repair when the tissue loss is of larger scale [4]. It has been demonstrated that native CPCs can differentiate when grown in conditioned medium on a layer of pre-fixed cardiac myocytes [47]. Large infarctions quickly disrupt the cardiac scaffold and change the extracellular environment. Thus, the efficiency of CPCs for tissue repair could depend on the timing of cellular application compared to the destruction of necrotic myocytes as well as various factors in relation to the inflammation itself [66]. The forthcoming challenge will be to control the complete regeneration of a scarred

myocardium with fully mature and functional cardiac myocytes embedded in a richly vascularized environment.

Concluding remarks

In conclusion, the exiting news is that the adult myocardium harbors several subsets of progenitor cells with the ability to differentiate into all major constituting cells of the heart and repair minor damage. However, challenges lie ahead controlling the growth and maturation of CPCs in an infarcted and scarred tissue. New studies must focus on the functional competence of transplanted cells and not only rely on the expression of a few cardiac transcriptions factors and sarcomeric proteins as these might only represent an acquired biphenotype due to a partial nuclear reprogramming. The differentiation of CPCs towards an adult cardiac phenotype might prove to be a very complex process demanding a sophisticated interplay with surrounding cells and matrix relying on direct cell surface interaction, intercellular connections, and paracrine factors. This differentiation might be difficult to obtain in simple cell cultures *in vitro* and exploitation of techniques from the scientific field of tissue engineering could be a solution (reviewed by Davis et al. [12]). Theoretically, a true cardiac progenitor cell could be the ideal cell type for repair of a broken heart. A possible future approach might be to apply different subtypes of stem and/or progenitor cells to the damaged myocardium in a sequential fashion starting with stem cells particularly prone for angiogenesis securing blood supply for the subsequent cell transplantations. Fortunately, a steadily increasing amount of evidence reinforces the hope for a new era, where myocardial regeneration by stem cells will be the cornerstone in the treatment for some of the most prevalent deadly diseases in the western world. Only time and hard work will show if CPCs resident in the patients' own myocardium will prove to be the most suitable cells for this purpose.

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