Stemming heart failure with cardiac- or reprogrammed-stem cells

Kento Tateishi a, b, Naofumi Takehara a, Hiroaki Matsubara a, b, Hidemasa Oh a, *

a Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan
b Department of Cardiovascular Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Received: February 22, 2008; Accepted: August 20, 2008

Abstract

Despite extensive efforts to control myocyte growth by genetic targeting of the cell cycle machinery and small molecules for cardiac repair, adult myocytes themselves appeared to divide a limited number of times in response to a variety of cardiac muscle stresses. Rare tissue-resident stem cells are thought to exist in many adult organs that are capable of self-renewal and differentiation and possess a range of actions that are potentially therapeutic. Recent studies suggest that a population of cardiac stem cells (CSCs) is maintained after cardiac development in the adult heart in mammals including human beings; however, homeostatic cardiomyocyte replacement might be stem cell-dependent, and functional myocardial regeneration after cardiac muscle damage is not yet considered as sufficient to fully maintain or reconstitute the cardiovascular system and function. Although it is clear that adult CSCs have limitations in their capabilities to proliferate extensively and differentiate in response to injury in vivo for replenishing mature cardiomyocytes and potentially function as resident stem cells. Transplantation of CSCs expanded ex vivo seems to require an integrated strategy of cell growth-enhancing factor(s) and tissue engineering technologies to support the donor cell survival and subsequent proliferation and differentiation in the host microenvironment. There has been substantial interest regarding the evidence that mammalian fibroblasts can be genetically reprogrammed to induced pluripotent stem (iPS) cells, which closely resemble embryonic stem (ES) cell properties capable of differentiating into functional cardiomyocytes, and these cells may provide an alternative cell source for generating patient-specific CSCs for therapeutic applications.

Keywords: cardiac stem cells • heart failure • reprogramming • development

Introduction

Despite recent advances in the treatment of heart failure, cardiovascular diseases are the leading cause of morbidity and mortality throughout the world. Recent experiments with cell transplantation therapies have emerged as a potential strategy to treat ventricular dysfunction secondary to ischaemic cardiac injury [1]. The concept that adult cardiac stem cells (CSCs) may play a significant role in cardiac tissue homeostasis or responses to acute injury has been challenged by a series of experiments by fractionation of resident heart-derived cells, suggesting that adult CSCs could contribute at low levels to regenerate multiple cardiovascular lineages following direct transplantation into injured myocardium [2, 3]. These observations have created a new avenue for studies aimed at repopulating damaged adult hearts by transferring resident CSCs amplified ex vivo; however, the degree of their regenerative potential was not as high as anticipated. Instead, the particular mechanisms by which such CSCs contribute to myocardial regeneration are still under investigation and whether endogenous CSCs are controlled by similar molecular pathways during cardiac development has yet to be determined. In addition, there is no evidence to provide strong support for dedifferentiation from mature cardiomyocytes...
or direct differentiation from relatively undifferentiated stem or progenitor cells as a major regenerative process for resident CSC plasticity during cell turnover under normal circumstances or in response to acute muscle damage [4].

Accumulated evidence suggests that transplantation of bone marrow-derived stem cells (BMCs) or skeletal myoblasts may contribute to the preservation of contractile function in some models of cardiac muscle injury [5], although skeletal myoblasts lack direct cardiomyogenic activity when cultured alone in vitro or myocyte formation in vivo [6]. Most importantly, experimental analyses showed that skeletal myoblasts do not couple electronically with the host myocardium without cellular fusion events [7]. Furthermore, recent studies of adult BMCs in adopting cardiac cell fate has been highly controversial [8–11]. The pathophysiological observations from several studies have indicated that BMCs integrated into the myocardium at very low frequency, if any, attributing to the functional improvement after myocardial infarction through paracrine effectors secretion rather than via direct myocyte differentiation and/or fusion in the transplanted hearts [11–13]. Despite these controversies regarding the efficacy of BMCs and myoblasts for cardiac regeneration, a number of randomized, multicenter clinical trials have conducted using autologous cells via intracoronary infusion or intramyocardial injection (Table 1). The results have shown either no benefit or small improvements in cardiac function, which were conferred unlikely from direct differentiation of the injected cells into cardiomyocytes [14, 15].

In the setting of identifying adult CSCs, several cell surface markers have been associated with cardiac progenitors [2, 3, 16], yet no single marker defines the conclusive pool of CSC population. It is clear that CSCs have more cardiogenic potential than BMCs or skeletal myoblasts, although the contribution of the CSCs to the maintenance or repair of cardiac muscle under physiological and pathologic conditions is not sufficient without further modifications in order to establish CSC transplantation as a new therapeutic strategy. In this regard, efficient engraftment of transplanted cells during cell therapy has emerged as a critical determinant to augment cardiac performance.

Based on these previous observations, investigators have demonstrated that specific cell types of stem/progenitors might be suitable for use in transplantation, but the limited success of cell engraftment requires further understanding of the molecular mechanisms of stem cell migration and proliferation, which are related to the improvement of donor cell survival and differentiation in situ. Autologous human CSC transplantation may hold the possibility to improve contractile function via direct cardiac muscle cell regeneration, of which clinical trials are underway, and may have great therapeutic potential to treat human heart failure.

### Cycling adult myocytes

Recent studies revealed that adult myocytes possess self-replicative capacity, documented by mitosis and cytokinesis under normal and pathophysiological conditions [17, 18], and a small number of new myocytes could be formed after acute cardiac injury [19]. Early hypotheses proposed that new myocytes might be generated via re-entry to the cell cycle from pre-existing, mature myocytes through altered expression of D-type cyclins [20–22], cyclin-dependent kinase inhibitors [23] and pocket proteins [24]; however, the number of cycling cells was relatively low. Further studies demonstrated that forced expression of anti-apoptotic protein bcl-2 [25], proto-oncogene c-myc [26] and telomerase reverse transcriptase [27] may enhance the hyperplastic growth by overriding the determined cellular growth arrest and senescence. Studies using growth factors have suggested that insulin-like growth factor (IGF)-1 [28] and nerve growth factor (NGF) [29, 30] produced myocyte proliferation and might recruit ectopic cells to control cardiac muscle force and cell size during post-natal heart development. The signalling molecules playing important roles in these processes have been postulated through extracellular signal-regulated kinase-1/2- and phosphatidylinositol (PI)-3-kinase-dependent signalling pathways [31–33]. Such studies formed the basis for further investigations of the potential effect of leptin, a product of obesity, on myocyte growth and metabolism in diabetic cardiomyopathy [34, 35].

Innovative strategies targeting single molecules to control myocyte growth are an attractive approach to treat heart failure. The blockade of specific protein kinase p38 has been reported to

### Table 1 Randomized, control, clinical trials of stem/progenitor cell therapy for myocardial infarction

| Cell type     | Study group          | No of cells | Outcome (versus control) |
|---------------|----------------------|-------------|--------------------------|
| BMMNCs        | Wollert et al. [157] | $2.5 \times 10^5$ | 6% greater in LVEF at 6 mo |
| BMMNCs        | Meyer et al. [158]   | $2.5 \times 10^5$ | Not significant in LVEF at 18 mo |
| BMMNCs        | Janssens et al. [159]| $3 \times 10^8$  | Not significant in LVEF at 4 mo |
| BMMNCs        | Lunde et al. [160]   | $8.7 \times 10^7$ | Not significant in LVEF at 6 mo |
| BMMNCs        | Schachinger et al. [161]| $2.4 \times 10^8$ | 2.5% greater in LVEF at 4 mo |
| BMMNCs        | Assmus et al. [162]  | $2 \times 10^8$  | 2.9% greater in LVEF at 3 mo |
| SMBs          | Menasche et al. [163]| $4 - 8 \times 10^6$ | Not significant in LVEF at 6 mo |

BMMNCs: bone marrow mononuclear cells; SMBs: skeletal myoblasts; LVEF: left ventricular ejection fraction; mo: month. BMMNCs were directly infused into coronary arteries. SMBs were injected intramyocardially during open chest surgery.
promote myocyte mitosis and cytokinesis in mice [36]; however, significant improvement in global cardiac function after acute injury required additional growth factor treatment with fibroblast growth factor (FGF)-1 possibly allowing myocyte survival and new blood vessel formation in rat heart [37]. A recent report demonstrated that periostin, an adhesion molecule secreted by cardiac fibroblasts, may modulate the structural integrity of ischaemic myocardium by alterations in the amount of collagen with changes in wall stiffness to support infarct tissue healing [38]. Overexpression of periostin led to cardiac dysfunction, whereas inhibition of periostin has been shown to augment cardiac function by altering myocyte–fibroblast interactions during the process of cardiac remodelling [39]. Surprisingly, injection of periostin induced the re-entry of mature myocytes into the mitotic cell cycle leading to reduced ventricular remodelling and improved cardiac performance in vivo [40]. These findings suggested that altered expression of periostin, which may be dose-dependent, could benefit cardiac remodelling through the activation of PI-3 kinase/Akt and integrins as well as regulating myocyte size and collagen content for prevention of cardiac fibrosis. Furthermore, the promotion of myocardial survival and migration by thymosin-β4, which is involved in actin cytoskeletal organization, acting through integrin-linked kinase and Akt signalling, implies crucial roles for these signalling pathways in myocyte biology [41].

**Intrinsic CSCs**

The recognition of primitive cells in the heart has become an alternative aspect to interpret the mechanisms of the formation of new myocytes, even in the presence or absence of regenerative cues. The identification of cardiogenic progenitor cells has raised the possibility that alternative mechanisms may support cardiac muscle regeneration in the post-natal heart. Within the past few years, many investigators have provided evidence that a subpopulation of progenitor cells exists that possess stem cell-like properties (Table 2) [42]. Recent studies have revealed that during mammalian cardiogenesis, a cell population that expresses the LIM-homeodomain transcription factors Isl1, coexpressed with known early cardiac transcription factors Nkx2-5 and GATA4, arises from the cardiac crescent and marks the secondary heart field [43]. These cells are maintained as a proliferating population throughout early development and then contribute to the formation of the right ventricle and outflow tract [43, 44]. Although cells that expressed Isl1 were recently identified in the post-natal heart of rodents and human beings and gave rise to cardiac myocytes [45], the failure in identification of Isl1+ cells in the adult human heart has limited the clinical use of these progenitors as therapeutic application [46]. Using a tamoxifen-inducible Cre-lox strategy, Isl1 progeny could be specifically recognized and purified at a defined time-point [45]. These cells could be expanded in culture and differentiated into β-adrenergic receptor agonist responsive functional myocytes in vitro. A lineage tracing study was used to document Isl1+ cells in embryonic stem (ES) cells and these cells could be clonally amplified showing unequivocally that a single Isl1+ cell was capable of generating progeny of cardiac, smooth muscle, and endothelial cell lineages [47]. Combinatorial transcriptional signatures of Isl1 with Nkx2-5 [48] and flk1 [49–51], both of which exhibit a differentiation potential for mesodermal lineages such as cardiac muscle during development, revealed that both Isl1+ /Nkx2-5+ and Isl1+/flk1+ populations could serve as more restricted cardiac muscle progenitors. As described above, Isl1+ cells are not the only cardiac progenitor cell population in the heart during development. Unlike the ES cells-derived Nkx2-5+/c-kit+ population that are capable of differentiating into mature cardiomyocytes [48], the Isl1+ population express neither c-kit nor stem cell antigen (Sca)-1, but they do coexpress Nkx2-5 regardless of the cardiogenic progenitors enriched in the sub-fractionation of flk1 [45]. Using Nkx2-5 promoter-driven transgenic mouse lines expressing eGFP specifically in the developing heart, modest expression of c-kit and Sca-1 determined the bipotential cardiovascular progenitors during...
embryonic cardiogenesis [48], suggesting that the developmental regulation of Nkx2-5, Isl1 and flk1 might mutually control the pool of cardiac progenitors during embryogenesis [48, 50]. In addition, recent identification of flk1<sup>low</sup>/c-kit<sup>neg</sup> population in human ES cells that contains cardiovascular progenitors provides an opportunity to investigate the roles of flk1 prior to the expression of Nkx2-5 and Isl1 in mesodermal specification during human cardiac development [51].

The evidence of these distinct cell populations of cardiac progenitors has raised the possibility that multiple primitive cells may coexist in the heart to support myocardial regeneration. Resident CSCs could also be isolated by the expression of protooncogene c-kit, which encodes a receptor tyrosine kinase, maps to the W locus and is activated by stem cell factor-kit ligand. Ligand binding leads to receptor dimerization and activation of multiple downstream signalling pathways involved in target mobilization, anti-apoptosis and cell proliferation [52]. c-kit was used to isolated cardiac mast cells more than a decade ago [53], but they were negatively sorted for blood lineage markers (Lin) including CD45 and CD34 [2]. These c-kit<sup>+</sup>/Lin<sup>-</sup> primitive cells were isolated cells from the adult rat heart with a small subpopulation coexpressing early cardiac transcription factors Nkx2-5, GATA4 and MEF2. As stem cells are defined by their unique capacity to both self-renew in the long-term and to undergo multiple-lineage differentiation, cardiac c-kit<sup>+</sup>/Lin<sup>-</sup> cells were prospectively clonogenic at single-cell level, expandable in cell culture and multipotent to generate the entire cardiovascular system in vitro and in vivo [2, 54]. A less frequency (1%), but detectable, of telomerase active human CSCs have been shown to form chimeric organs upon the introduction of human cells into rodent hearts after infarction with a significant improvement of myocardial function [55]. In mouse heart, most of the CSCs were mitotically quiescent and restored in a cardiac niche, created by supporting cells including myocytes and fibroblasts, coupled with the expression of integrins and connexins [56]. Using BrdU pulse-chasing experiments, nearly 10% of CSCs showed slow-cycling properties, indicating that these primitive cells could become activated in response to stress by injury [19] or in the context of myocyte turnover along with homeostasis [18, 56]. Extrinsic mechanical stretch applied to myocytes also triggers numerous intracellular signals to activate preserved CSCs in vivo [57].

Although c-kit may represent a developmental remnant from a multipotent mesodermal cell population including bone marrow, whether these c-kit<sup>+</sup>/Lin<sup>-</sup> CSCs in adult hearts represent the progeny of cardiac progenitors from the embryos or a distinct population remains unknown. Studies of W locus mutant mice that strongly implicated a loss-of-function in c-kit [58] showed no developmental abnormalities in the heart, suggesting that c-kit might be dispensable for cardiovascular cell proliferation and differentiation during embryogenesis as well as raising the possibility of its alternative function to identify resident mast cells [59]. Although c-kit<sup>+</sup>/Lin<sup>-</sup> CSC populations are associated with high telomerase activity, the absence of cardiac defects in c-kit mutant mice is in sharp contrast to the severe cardiac phenotype observed, ventricular dilation concomitant with cardiac dysfunction, in later generation of telomerase knockout mice [60]. Additional evidence for the crucial role of c-kit<sup>+</sup> cells emerged from experiments on bone marrow reconstitution studies showing that bone marrow-derived c-kit<sup>+</sup>/Lin<sup>-</sup> cells completely rescued the defective cardiac repair in c-kit mutant mice after infarction [61, 62] indicating somatic c-kit<sup>+</sup> cells including cardiac CSCs may be circulating mesodermal progeny of cells residing in the bone marrow and could be mobilized in situ in response to acute injury for myocyte repair. The observation that cardiospheres in human beings and mice (Fig. 1), which contain a population of GSCs, exhibited lower levels or the absence of c-kit expression [63–65] has questioned whether single cell surface markers may be adequate to define all CSCs in the developing heart. Lineage-tracing experiments by targeting c-kit could unveil the functions of these cells in the heart throughout embryonic and post-natal development.

Sca-1, also known as lymphocyte activation protein-6A (Ly-6A), is a glycosyl phosphatidylinositol-anchored cell surface protein and is used widely as an alternative cell surface marker to enrich haematopoietic stem cells (HSCs) in conjunction with Lin<sup>-</sup>/c-kit<sup>+</sup> selection in the presence of interleukin-3 (IL-3) [66]. Studies using reporter gene tracing experiments revealed that foetal Sca-1 expression was first observed at E9 in the ventral dorsal aorta and E11 in the foetal liver [67]; progenitor cells expressing Sca-1 in adult bone marrow-enriched HSCs were capable of generating at least both T and B lymphocytes but lacked myeloid differentiation potential [68, 69]. In adults, Sca-1 is expressed in many somatic tissues including mammary gland [70], prostate [71], lung [72], skeletal muscle [73] and cardiac muscles [3] as a marker of self-amplifying cells within the tissues they reside in. Cardiac Sca-1<sup>+</sup> cells exhibited high levels of telomerase activity similar to that in neonatal myocardium and were negative for the expression of CD45 and CD34, markers of HSCs and endothelial progenitor cells (EPCs). These cells have been shown to be highly coexpressed with side population (SP) cells [3, 74, 75], which also enrich HSCs [76]. The expression of Sca-1 in adult hearts identified a cell population capable of differentiating into cardiac muscle, endothelium, smooth muscle, and contributed to adipogenesis and osteopoiesis in vitro [3, 16]. As with Isl1<sup>+</sup> and c-kit<sup>+</sup> cells in adult myocardium, cardiac Sca-1<sup>+</sup> cells are also potent cardiac progenitors and are able to maintain their own functional population by self-renewal. Studies on cell transplantation after acute myocardial infarction showed that the progeny of resident Sca-1<sup>+</sup> cells adopted divergent fates with the majority of the population differentiating into cardiac muscle and smooth muscle cells along the ischaemic border zone, whereas a minority remained undifferentiated and retained expression of Sca-1. Intravenously transplanted CSCs homed in and migrated into the injured cardiac foci and substantial graft-derived cardiogenic activity persisted in the host myocardium for at least 4 weeks [3, 75].

As described above, no single marker conclusively defines lineage-specific stem/progenitor cells, as seen in the enrichment of bone marrow stem cells and skeletal satellite cells [77]. The heterogeneity of marker expression may also reflect functional differences among CSCs in terms of cardiogenic specification. The expression
of candidate surface markers or transcription factors may just serve as an indicator during the developmental program in different organ systems but might be needed in conjunction with other components to define a lineage-committed cell population of interest. Negative selection of CD31 from the Sca-1 components to define a lineage-committed cell population of interest.serve as an indicator during the developmental program in different
events and parameters may just sufficiently in vitro as compared with CSCs expressing CD31, indicating
small percentage of cardiac Sca-1 that a significant heterogeneity exists even among the relatively small percentage of cardiac Sca-1+ SP cells.

To assess whether clonal CSCs are homogenously multipotent, and if CSCs could be derived from a functionally identical primitive cell population that resided in myocardium, we used single-cell deposition analysis without pre-selection by specific cell surface markers or transcription factors to prospectively isolate and characterize CSCs from adult murine heart [79]. Clonally amplified cell clones arising from intrinsic myocardial cells could be expanded in culture and possessed high telomerase activity as observed in Sca-1+ and c-kit+ CSCs [3, 80]. Nearly 70% of clonal CSCs could be recognized by Sca-1 but were rarely detectable by c-kit, indicating that these markers are provisional rather than definitive indicators. Under undifferentiated conditions, all clones exhibited the characteristics of mesenchymal-like cells, as shown by the marker profile CD105+/CD90+/CD29+/CD44+/CD73+/CD106-, but these cells lacked both CD45 and CD34 expression and expressed the transcription factors Slug and Snail (Fig. 1), which have been suggested to represent mediators of epithelial-mesenchymal transitions (EMT) during neural crest development [81]. These cells could give rise to structurally mature cardiovascular cells regardless of Brachyury expression at baseline, which is a marker for the primitive streak [82]. More detailed study by a retrospective isolation to further delineate the precise identity of CSCs using GFP reporter mice harbouring telomerase reverse transcriptase (TERT) promoter showed that more than 80%, a higher frequency than that of clonal CSCs, of the freshly isolated TERT+ cells in the adult heart expressed Sca-1 [79]. The cardiac niche of telomerase-competent CSCs was marked by TERT expression as previously reported [60, 80] (Fig. 2) and revealed clusters of TERT+ but CD45- cells in both atria and ventricles (left atria: 1.60 ± 0.03 cells/mm², left ventricle: 0.04 ± 0.01 cells/mm², and right ventricle: 0.26 ± 0.02 cells/mm², respectively), most abundantly along the right atria (4.80 ± 0.05 cells/mm²) and outflow tract (4.40 ± 0.03 cells/mm²). The TERT+ CSC distribution was in contrast to the results obtained from c-kit+ CSC growth using a BrdU pulse-chasing study within the cardiac niche, showing atria and apex were preferred residential sites [56]. It remains to be determined how these distinct cell populations are related anatomically and physiologically to control the fate of adult myocyte turnover.

**Epicardial CSCs**

The embryonic heart tube is formed by fusion of the primary heart fields and comprises an outer myocardial layer, including the epicardium, and an inner endocardial layer [83]. Myocardial precursor cells added to the anterior and posterior poles of the linear heart tube are derived from two lineages that segregate early from a common precursor [84, 85]. The epicardium consists of a sub-population of mesothelial cells adjacent to the inflow tract that proliferate and extend into the pericardial cavity. This subpopulation of epicardium-derived cells undergoes an EMT and migrates to give rise to cardiac fibroblasts, vascular endothelium [86], smooth muscle of the coronary system and the epicardium [87]. A recent study has further shown that epicardium-derived c-kit+/CD34+ cells in mouse and human might be a cell population...
of myocardial and vascular precursors [88]. These precursor cells were specified in the subepicardial space and proliferated and differentiated following myocardial infarction. The unique function of these cells was verified by a pericardium open/close system in infarct mice in which the pericardial tissue was kept closed revealed a greater preservation of left ventricular function in contrast to the cardiac function measured in mice where the pericardium was left open during ischaemic injury, in terms of the continuous supply of migrating epicardial progenitor cells towards the ischaemic regions. In addition, c-kit/CD34 epicardial cells represent a population with vasculogenic and angiogenic properties in which the expression of CD31 was absent. Although these CD34 epicardial precursors localized within the subepicardial region, where adipose tissue occupied most of the region, these cells were distinct from circulating blood-derived progenitor cells in terms of their negative expression of CD45 and lower extent of Sca-1 and multi-drug resistance-1 (MDR-1) expression but were positive for CD105 and CD90, suggesting that they could be mesenchymal in origin and had arisen from EMT. This mesodermal mesenchymal supply is critical to provide the heart with vascular and connective tissue progenitor cells [89].

Developmental origin of CSCs

The heart is constructed from cells of a variety of origins including extracardiac sources. The major extracardiac contributions to the heart include cells of epicardial organ and the neural crest [90]. In contrast, the conduction system is not a neural crest derivative but is derived from cardiac myogenic precursors in chicks [91]; a recent study has shown a close relationship between neural crest cells and the developing conduction system in mice using viral and genetic fate-mapping strategies [92]. An additional report has also demonstrated that cardiac neural crest cells originating both from neural tube regions and myogenic precursors invade the myocardium in all segments of the heart including the outflow tract, atrium, atrioventricular junction and ventricle in zebrafish [93]. In the past few years, promoter-reporter constructs or double-transgenic analysis using LacZ or GFP reporter mice and neural crest-specific Cre recombinase has facilitated genetic marking of neural crest cells in mice. Several different transgenic lines carry neural crest-specific Cre recombinase activity including Wnt1 [94] and Pou [95]. The undifferentiated, GFP-labelled neural crest stem cells contain a subset of the SP cell population that reside in the proximal but not distal conduction
system, which could form cardiospheres as we have shown previously (Fig. 1), and maintain neurogenic, gliogenic and cardiovas-
culogenic potentials [92, 95].

Selected aspects of growth factor action on cardiac neural
crest stem cells have been demonstrated recently. Pinch1, which
is composed of five LIM domains, plays an important role in
processes as diverse as stem cell migration, proliferation and dif-
f erentiation. Wnt1-dependent Pinch1-deficient mice displayed
severe cardiovascular defects, including an unusual aneurysmal
common arterial trunk, ventricular septal defects and defective
cushion/valve maturation because neural crest stem cells in the
outflow tract cushion underwent markedly increased apoptosis at
E11.5 to E13.5 caused by decreased transforming growth factor
(TGF)-β signalling [96]. In zebrafish, both Semaphorin (Sema) 3D
and its receptor, Neuropilin 1A, morphants showed defects in the
neural crest and have disrupted cardiomorphogenesis including
hypertrophic cardiomyocytes, decreased ventricular size, and
defects in atrioventricular valve development [97]. Interestingly,
Sema 3A is known to promote the aggregation of neurons into
sympathetic ganglia during early embryogenesis. While Sema 3A
null mice exhibited sinus bradycardia, abrupt sinus slowing and
stellate ganglia defects, cardiac-specific overexpression of Sema 3A
in mice was associated with reduced sympathetic innervation as
well as high susceptibility to ventricular arrhythmias although the
contractile function and myocardial structure were preserved at
baseline levels [98]. These findings suggested that Sema family
members may control the migratory pathways for neural crest
stem cells to regulate formation of the primary heart field and sub-
sequent sympathetic nerve system development. Gain-of-function
and loss-of-function studies have shown that Sonic hedgehog
(Shh) [99] and TGF-β [100] regulate neural crest stem cell differ-
entiation into smooth muscle and neural cells. Moreover, both
TrkC and myocardin-related transcription factor-B are essential for
normal cardiac neural crest stem cell-oriented outflow tract sepa-
tion [101] and smooth muscle differentiation [102]. It will be of
interest to determine if these factors are involved in the cellular
development of other types of CSCs that was not originated from
neural crest cells.

**Signals control CSC proliferation**

Cell-intrinsic properties are not the only determinants of stem cell
fate. CSCs are tightly regulated by their microenvironment and tis-
sue-specific signalling (Table 3). Gain-of-function studies revealed
that activation of canonical Wnt signalling promoted Isl1+ cardiac
progenitor cell expansion with a concomitant increase in FGF sig-
nalling [103, 104].

Studies of loss-of-function and gain-of-function by tissue-
specific gene targeting in mouse models have implicated Wnt-β-
catenin signalling as one of the key regulators of precardiac
mesoderm-specification, Isl1+ CSC self-renewal and subsequent
differentiation. An early study that reported conditional deletion of
β-catenin targeting visceral endoderm led to the formation of
multiple hearts all along the anterior–posterior axis of the
embryo, which is consistent with the regions of ectopic bone
morphogenetic protein 2 (BMP2) expression [105]. However, deletion of β-catenin under the control of MEF2C, which is acti-
vated after initiation of the secondary heart field conducted by
Isl1 expression, led to a severe disruption of outflow tract pat-
terning and growth of the right ventricular and interventricular
myocardium with significant reductions in cyclin D2 and TGF-β2
expression [106]. This finding is precisely consistent with the
cardiac phenotype reported from Isl1 mutant mice [43]. These
observations are also analogous to the report from mice in which
Wnt-β-catenin signalling is reduced by the suppression of the
desmoplakin gene that is responsible for human arrhythmogenic
right ventricular dysplasia/cardiomyopathy (ARVC) [107].

Conditional deletion of Isl1 resulted in a significant reduction
of the number of cardiac progenitors, suggesting that growth fac-
tor signalling may be perturbed. Ablation of β-catenin in the Isl1
locus exhibited embryonic lethality due to the absence of outflow
tract morphogenesis and right ventricular identities with a drastic
reduction in expression of a number of genes including Tbx2/3,
Wnt11, Shh and Prrx2 [108, 109]. Furthermore, the fact that a
significant decrease in the expression of BMP4/7 and FGF8/10 in
Isl1 null mice [43] and the requirement for autocrine FGF8 sig-
nalling for normal formation of the primary and secondary heart
field-derived structures [110] provided a pivotal insight into how
FGFs and BMPs are mutually involved in secondary heart field
proliferation and development as upstream or downstream targets
of Isl1 [111, 112].

c-kit+ CSCs are regulated by a complex network of cytokines
including stem cell factor (SCF), which have specific effects
known to synergize with other cytokines, e.g. G-CSF and Flt-3 lig-
and (FL), to produce rapid expansion and mobilization of progen-
itor cells and resultant differentiation for cardiac repair [113, 114],
whereas later cytokines have broader cytoprotective effects than
direct control of stem cell self-renewal through cell cycle regula-
tion than has been previously appreciated [115]. Studies have
shown a significant cardiac homing of c-kit+ stem cells to the
injured myocardium from bone marrow by intramyocardial delivery
of SCF or bioengineered stromal cell-derived factor-1 (SDF-1), a
chemokine for attracting stem cells [116, 117]. The loss of anti-
apoptotic and angiogenic paracrine activities in c-kit deficient mice
might directly contribute to perturbed cardiac function after infarc-
tion, which could be rescued by c-kit+/Lin− bone marrow cells
reconstitution in the absence of cardiac c-kit+ CSC replenishment
[61, 62]. Although the exact origin of the majority of the stem cells
that spontaneously mobilize to cardiac tissue and differentiate into
functional cardiomyocytes after cardiac injury remains poorly
understood [4], these studies do not exclude the possible role of
intrinsic c-kit+ CSCs participating in myocardial self-repair during
injury. Local injection of ACK2, an antibody that blocks c-kit func-
tion, may further indicate the balance and dependence on intrinsic
and/or extrinsic c-kit+ stem cells as part of the mechanisms of
cardiac regeneration in vivo [118].
The functional involvement of insulin-like growth factor-1 (IGF-1)/IGF-1 receptor and hepatocyte growth factor (HGF)-c/Met systems in c-kit+ CSC senescence and telomere attrition is supported by their effects on CSCs to facilitate migration, proliferation and differentiation in situ to reconstitute the damaged myocardium [60, 119]. Recent work has revealed that local injection of high mobility group box 1 (HMGB1), a nuclear protein that is released by cells undergoing necrosis but not by apoptotic cells, specifically promoted resident c-kit+ CSC migration and proliferation acting through HMGB1 receptor RAGE into the ischaemic border zone. However, HMGB1 injection did not recruit cells from the bone marrow into the systemic circulation [120]. Extracellular HMGB1 has been reported to engage multiple receptors including RAGE to induce nuclear factor (NF)-κB activation dependent on extracellular signal-regulated kinase (ERK) [121]. Most importantly, the endogenous levels of HMGB1 increased during tissue injury, suggesting that the response signals could also be early mediators of inflammation to direct c-kit+ mast cells [122]. The intricate interplay of detrimental effects of HMGB1 has been shown by evidence that treatment with a neutralizing antibody to HMGB1 is associated with reduced phosphorylation of c-Jun NH2-terminal kinase and increased NF-κB DNA binding to reduce damage during liver injury [123].

As with the diminished function and remodelling after myocardial infarction in c-kit mutant mice, Sca-1 deficiency reduced the replicative capacity of clonal CSCs in vitro [79]. It is conceivable that tissue-resident stem cells use tissue-specific signalling, such as Sca-1, to regulate the stem cell balance between self-renewal and growth arrest mediated through different downstream kinases. Eventually, Sca-1 null mice have shown to exhibit age-related bone remodelling and resultant osteoporosis [124], abnormal responses to antigen stimulation in T cells [125], reduction in engraftment of bone marrow transplants [126] and inhibition of myoblast fusion to promote differentiation [127] related to perturbed c-src and Fyn signalling [128, 129]. Evidence from a model of Sca-1 knockout mice revealed that myocardial infarction leads to ventricular remodelling and poor survival; a phenotype that was recapitulated by studies using c-kit mutant mice (Fig. 3) [61, 62]. In the Sca-1 deficient system, activation of Akt in the host myocardium appears to be a crucial regulator for survival of cardiomyocytes. Normal Sca-1 function in CSCs is associated with border zone-specific increase in vascular endothelial growth factor (VEGF) and HGF that could promote myocardial tissue and donor cell survival through autocrine/paracrine mechanisms [79]. Using direct transplantation experiments in Sca-1 deficient mice, we also found that clonal CSCs isolated from wild-type but not from Sca-1-deficient hearts were able to significantly improve survival after infarction, suggesting that perturbed cardiac function after infarction in Sca-1 deficient mice could be partially due to the physiological dysfunction of intrinsic CSCs (K. Tateishi and H. Oh, unpublished observations). It is important that Sca-1 may also define a subset of bone marrow stem cells and functional abnormalities such as mobilization and proliferation in this cell population that may account for the cardiac phenotype observed in this study. Similar results have been reported from the transplantation of Akt-modified mesenchymal stem cells into infant rats, leading to reduced infarct size and prevention of cardiac remodelling through the release of secreted frizzled related protein 2 (Sfrp2) [130, 131]. Furthermore, transgenic experiments with cardiac-specific Akt have been shown to promote myocyte proliferation and resident c-kit+ CSC expansion in vivo via up-regulation of HGF in favour of the expression profile in neonatal myocardium [132]. In addition, fibroblast growth factor 2 (FGF2)-deficient mice have been shown to suffer from an impaired hypertrophic response and dilated cardiomyopathy due to a defective capacity of fibroblasts for releasing growth factors to induce cell growth response in cardiomyocytes [133]. Although our results revealed that clonal Sca-1+ CSCs require FGF2 to proliferate in culture [79], study of FGF2 deficient mice has shown that the Sca-1+...
CSC pool was comparable with wild-type mice [134]. Interestingly, the full process of cardiac differentiation in both in vivo engraftment and ex vivo culture is dependent on FGF2. It seems that certain redundant pathways might involve other FGF family members in vivo to control stem cell growth, or cell adhesion-mediated extracellular matrix desensitization could occur requiring FGF2 activation for CSCs to self-replicate in vitro [135].

Although whether the cardiovasculogenic abilities of epicardial progenitor cells strictly depend on a myocardium-derived signal or they are also sensitive to triggers from epicardial tissue itself in mammalian heart remain to be investigated, several signalling molecules have been implicated in the EMT process including ligand FGF17 and its receptor FGFR2 and FGFR4 in zebrafish heart regeneration [136]. Differentiation into the myocardial or epicardial lineage is mediated by the cooperative action of BMP and FGF signalling in chicken embryos [137]. Of the vascular growth factors that could potentially induce EMT, bFGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-BB and VEGF have been shown to stimulate epicardial cells in coronary morphogenesis [87, 89]. Furthermore, thymosin-β4, a G-actin monomer binding protein involved in reorganization of the actin cytoskeleton, has been shown to promote mobilization and neovascularization not only through cardiac development but also from adult epicardium via direct stimulation of rapid differentiation and migration of Flk-1+ epicardial progenitor cells in terms of an increase in the pro-angiogenic cleavage peptide, N-acetyl-seryl-aspartyl-lysyl-proline [138]. To directly explore whether the embryonic epicardium is a key signalling tissue responsible for transmission of morphogenetic signals during cardiac development, proepicardium-targeted β-catenin-deficient mice have been characterized. Transcription factor GATAs-dependent ablation of β-catenin in the epicardium showed that impaired development of epicardial progenitor growth and concomitant myocardial hypoplasia are secondary to the defective formation of coronary arteries [139]. Although expression of β-catenin in the epicardium did not seem essential for EMT stimulation in this model, it is conceivable that canonical Wnts directly enhance epicardial smooth muscle differentiation in coronary remodelling. It is important to note that α4β1 integrin-mediated cell adhesion functions in the migration of progenitor cells to form the epicardium [140], which is consistent with a previous finding that β-catenin is a bifunctional protein involved in cell–cell adhesion [141].

New advance in cardiac regeneration

Mammalian ES cells, including mouse [142] and human [143], are alternatives for use in cell therapy and were first isolated as undifferentiated cells from the inner cell mass or epiblast of blastocyst-stage embryos. Recent advances in epigenetic reprogramming of somatic cells into ES cells have attracted much attention because of their therapeutic potential allowing the generation of patient-specific pluripotent cell lines to circumvent concerns regarding ethical issues and rejection by the host immune system. Initial studies have attempted to use human ES cells as an alternative to oocytes for reprogramming human somatic nuclei and showed that the hybrid cells generated maintain a stable tetraploid DNA content and the transcriptional state of somatic nuclei can be reprogrammed by cell fusion [144]. This evidence was recapitulated in primate ES cells by nuclear transfer technology and suggested the possibility that differentiated cells can be reprogrammed to an undifferentiated state by trans-acting factors present in the partner ES cells by mechanical reprogramming as well as prompting research to identify specific factors that could mediate this process via molecular reprogramming [145].

Accumulated evidence has provided the stunning result that a simple recipe of just four transcription factors comprising Oct3/4,
Sox2, c-Myc and Klf4, can induce both mouse and human fibroblasts into induced pluripotent stem (iPS) cells, which are indistinguishable from ES cells [146–148]. A previous study has shown that another quartet of reprogramming factors, Oct4, Sox2, Nanog and Lin28, could successfully reproduce the pluripotent characteristics from human fibroblasts [149]. The latest report has shown that a modified protocol without c-Myc, which might increase the incidence of tumour development, could yield functionally similar iPS cells in mice [150]. Regardless of the combinations of transcription factors employed, human IPS cells and their derivatives may hold great promise for stem cell-based cardiac repair. Although the tumourigenity of the retrovirus vectors themselves and the delivered oncogenes has yet to be investigated in long-term observations, elegant outcome from specific cell lineage differentiation such as blood cells as well as successful creation of IPS cells from liver and stomach cells in animal experiments is quite encouraging [151, 152].

Lastly, modulation of the microenvironment for myocardial tissue engineering subsequent to acute injury by any type of cell transplantation requires enhanced survival and resultant differentiation in vivo. Transplantation of differentiated cardiomyocytes [153], human ES cells [154] or undifferentiated mesenchymal stem cells [155] via direct injection with a scaffold or pre-seeded on cell sheet has demonstrated a significantly greater engraftment and improved cardiac function after infarction. Human ES cell research is crucial as a rational basis for investigating how human IPS cells can give rise to functional cardiomyocytes. The combinations of appropriate tissue engineering strategies with the best characterized and the most functional cardiogenic/stem/progenitor cells may potentially enhance cardiac muscle regeneration as clinically applicable and practical approaches to treat patients with severe heart failure.

Conclusions

Within the past 4 years, researchers have succeeded in the isolation of somatic CSCs, genetic creation of iPS cells, the most recently, engineering of a cell-based bioartificial heart as a solid organ for transplantation [156] in experimental or preclinical studies. This review highlights the evidence for the existence of endogenous cardiac progenitor cell populations in adult mammalian heart. A subset of the cell populations are neural crest stem cells in genetic origin, undergo to EMT and acquire mesodermal mesenchyme properties during embryonic development and represents an obvious target for therapeutic intervention for cardiovascular regeneration following heart failure. Recent investigations have not yet unveiled the key elements controlling cardiovascular lineage diversification as well as the orchestrated network conducting cell fate decisions, including self-renewal, migration and differentiation of CSCs. Continued research works may lead to a discovery of the composition of defined factors able to activate CSCs to expand, survive in situ and fully differentiate into the lineages of interest. The rapid progress of tissue engineering technologies may also optimize the efficacy of cell therapy by augmentation of these critical steps during transplantation in patients with cardiac defects or functional failure.

Acknowledgement

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, and by Grants-in-Aid from the Ministry of Health, Labor and Welfare.

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