Stem Cells as a Source of Regenerative Cardiomyocytes

Keiichi Fukuda, Shinsuke Yuasa

Abstract—The realization of regenerative cardiac medicine depends on the availability of cardiomyocytes in sufficient numbers for transplantation of cardiac tissue and the accompanying blood vessels. Embryonic stem (ES) cells, bone marrow (BM) stem cells, and tissue-derived stem cells are all potential cell sources. Although ES cells are highly proliferative and suitable for mass production, an efficient protocol is yet to be established to ensure selective cardiomyocyte induction using these cells. Recent advances in developmental biology have clarified the involvement of critical factors in cardiomyocyte differentiation, including bone morphogenic protein and Wnt signaling proteins, and such factors have the potential to improve the efficiency of stem cell induction. Initial studies of the intracoronary administration of BM mononuclear cells after myocardial infarction has yielded promising results; however, intensive investigation of the underlying molecular mechanisms at play as well as double-blinded clinical trials will be necessary to establish the extent of both migration of the BM stem cells into the damaged cardiac tissue and their differentiation into cardiomyocytes. Several types of cardiac tissue stem cells have also been reported, but an accurate and extensive comparison of these cells with regard to their characteristics and multipotency remains to be done. An integrative study involving developmental biology, stem cell biology, and tissue engineering is required to achieve the full potential of cardiac regeneration. (Circ Res. 2006;98:1002-1013.)

Key Words: cardiomyocyte ■ stem cell ■ differentiation ■ tissue engineering ■ cell transplantation

Since 1999, when we first reported that bone marrow (BM) mesenchymal stem cells (MSCs) could differentiate into cardiomyocytes in vitro,1 research in regenerative medicine has advanced in an explosive manner.2,3 In addition to BM MSCs (BM-MSCs), embryonic stem (ES) cells, cardiac tissue stem cells, and adipose tissue stem cells have been found to undergo myocardial differentiation, and additional cell types may also prove to have cardiac differentiation ability. An early-phase clinical trial of the direct infusion of BM mononuclear cells and peripheral blood mononuclear cells into coronary arteries and the myocardium has also been reported in tandem with the discovery of endothelial progenitor cells.4,5 However, there is a vast gap between demonstrating that a cell type can differentiate into myocardium, and translating this into clinical practice. Major challenges to the therapeutic use of stem cells include the effective harvesting and in vitro expansion of cells to sufficient numbers and of sufficient purity, the refinement of methods to enable selective differentiation into cardiomyocytes, optimization of route of administration of stem cells, and the specific induction of heart tissue regeneration. This review focuses on the advantages and disadvantages each stem cell type as a source of...
regenerative cardiomyocytes and provides an overview of the current status of stem cell therapy in cardiac therapeutics.

ES Cells

The first mouse ES cell line was established by Evans in 1981, and its superior proliferative ability and pluripotency led to the establishment of a number of developmental model systems and knockout mice. When implanted under the skin, ES cells form teratomas and can differentiate into a variety of tissues in vitro when differentiation inhibitors are eliminated. In the mid-1990s, ES cell research slowly moved to using experimental animals as in vivo models for cell transplantation therapy. In 1998, human ES cells were established by Thomson et al, and although ethical issues remained, they quickly attracted significant attention as a source for regeneration therapy. In 2001, Wakayama et al established murine ES cells by applying nuclear transfer techniques to nuclei derived from somatic cells. These findings raised the possibility of using somatic cells from patients to produce their own ES cells and by a technique that would avoid immune rejection in humans.

When mouse ES cells were first differentiated into cardiomyocytes in vitro in 1985, it was well established that such differentiation occurs at a constant rate, the same as other organs. In 1996, Field et al reported that the stable transfection of ES cells with a fusion gene consisting of the α-cardiac myosin heavy chain promoter and a cDNA encoding aminoglycoside phosphotransferase generated relatively pure cultures of cardiomyocytes when differentiated in vitro. This study provided the impetus for the use of ES cells in the clinical setting. The development of methods for differentiating ES cells into cardiomyocytes more selectively and efficiently using a variety of factors progressed steadily in the late 1990s. Cardiac anomalies occur in mice lacking the receptor for retinoic acid (RA), a vitamin A derivative, or when vitamin A is deficient during development, implicating the growth factors expressed in these regions or in the surrounding areas at the relevant developmental stage in cardiomyocyte induction. BMPs are expressed in the lateral plate mesoderm, including the anterior lateral plate, and gene targeting of BMP-2 causes heart defects in early-stage embryos. Application of BMP-2-soaked beads in vivo has also been used to induce the ectopic expression of cardiac transcription factors, and the administration of soluble BMP-2 or BMP-4 to explant cultures induces full cardiac differentiation in stage 5 to 7 anterior medial mesoderm, a tissue that is normally not cardiogenic. These findings strongly suggested a critical role for BMPs in the induction of cardiomyocytes. Additionally, we observed that simple stimulation with BMP-2/BMP-4 did not augment or suppress cardiomyocyte induction from ES cells. Interestingly, in the vertebrate nervous system, Noggin and other BMP inhibitors (Chordin and Follistatin) are involved in neural differentiation, patterning during embryonic development and adult neurogenesis in a context-dependent fashion. In Xenopus gastrula-stage embryos, Noggin and other BMP inhibitors are secreted by the Spemann organizer and induce neural tissue from dorsal ectoderm by inhibiting ectodermal BMPs. Also in the developing neural tube, BMP can specify the dorsal fates of neural progenitor cells. BMP inhibitors are also expressed in ventral somites and in the notochord, suggesting a role in the countergradient of BMP activity along the dorsoventral axis.

We hypothesized that BMP antagonists may also be involved in cardiomyocyte induction. Whole-mount in situ hybridization with probes for various BMP antagonists on mouse embryos at different gastrulation stages revealed the BMP antagonist Noggin to be transiently but strongly expressed in the heart-forming area. This phenomenon was also observed in early embryos of chick and Xenopus, suggesting a conserved mechanism in heart development. Based on the analogy to the central nervous system, we suspected that the context-dependent differential action of BMPs in cardiomyocyte induction might be explained by the local action of Noggin and other BMP inhibitors and accordingly developed a protocol to induce cardiomyocytes from ES cells by transiently expressing Noggin concomitant with the native expression. Administration of Noggin before the em-
bryoid body (EB) formation stage mimicked the transient and strong expression of Noggin at the early gastrulation stage. Administration at −3 days and 0 days from EB formation led to a marked increase in beating EB incidence at 10 days. The cardiomyocyte-inducing activity of Noggin was found to be restricted to the period from 3 days before to 1 day after EB formation.46

The Wnt/β-catenin pathway (canonical Wnt pathway) regulates cell adhesion, morphology, proliferation, migration, and structural remodeling.49–51 The major components of this network are the Wnt ligands, which bind to frizzled receptors at the cell surface. The Wnt ligands comprise ≥ 17 members, with some activating the canonical pathway and others activating the noncanonical pathway.52 The cytosolic β-catenin associates with cadherins at the intracellular side of the plasma membrane to promote cell adhesion and with the actin microfilament network to promote cell shape. Activation of Wnt signaling downregulates the intracellular degradation of β-catenin, allowing translocation to the nucleus and transcription factor activation in conjunction with coexpression factors Lefs and Tcfs. The noncanonical Wnt pathway has no role in β-catenin degradation but can activate c-Jun N-terminal kinase (JNK) and other signaling pathways, although the other signaling pathways remain unknown. Wnt signaling has also been implicated as an inhibitor of cardiomyocyte induction.32–34 Marvin et al reported in the chick that the Wnt inhibitors Crescent and Dkk-153 are expressed in anterior endoderm during gastrulation, inducing the formation of beating heart muscle, and that ectopic Wnt signals can repress heart formation from anterior mesoderm in vitro and in vivo.32 In addition, the forced expression of either Wnt3A or Wnt8 promoted the development of primitive erythrocytes from the precardiac region.32 Schneider and Mercola also reported that in Xenopus, the same Wnt antagonists, but not Frzb or S1, can induce heart formation in explants of ventral marginal zone mesoderm, and that Wnt3A and Wnt8, but not Wnt5A or Wnt11, inhibited endogenous heart induction.33 These findings indicated that inhibition of Wnt signaling by diffusion of Dkk-1 and Crescent from the organizer promotes heart formation in the anterior lateral mesoderm, whereas active Wnt signaling in the posterior lateral mesoderm promotes blood development.

In contrast to the above findings, recent reports cite Wnt members in promoting cardiomyocyte differentiation from ES cells. In chick embryo, Wnt11 is expressed in early mesoderm in a pattern that overlaps with the precardiac regions, and exogenous Wnt11 can promote cardiac differentiation in noncardiogenic tissue.54,55 Subsequent loss- and gain-of-function experiments showed that Wnt11 is required for heart formation in Xenopus embryos and is sufficient to induce a contractile phenotype in embryonic explants via noncanonical Wnt signaling, which is independent of β-catenin but involves protein kinase C and JNK.56 Moreover, they reported that treating the mouse embryonic carcinoma stem cell line P19 with murine Wnt11-conditioned medium triggers cardiogenesis, suggesting that the function of Wnt11 in heart development is conserved in higher vertebrates. In addition, Terami et al57 reported that exogenous Wnt11 promotes the differentiation of murine ES cells into cardiomyocytes. Together, these results indicated that cardiac development requires noncanonical Wnt signal transduction.

The involvement of Wnts and β-catenin in mammalian cardiac myogenesis was examined using a pluripotent mouse embryonic carcinoma cell line (P19CL6),58 which can be induced to differentiate into cardiomyocytes by 1% DMSO, with spontaneous beating.17 The administration of DMSO induced Wnt3A and Wnt8A, which, in turn, activated Wnt/β-catenin signaling and thereby induced cardiac myogenesis in the P19CL6 cells.58 These findings indicated that Wnt/β-catenin signaling is also associated with the inception of mammalian cardiac myogenesis and is indispensable for cardiac differentiation, at least in this pluripotent model system. The Wnt pathway appears to play a crucial role in cardiomyocyte induction, although the complex puzzle of when and how these factors are involved in the induction remains undetermined and need further analysis.

The Notch pathway is involved in differentiation and lineage decision in fetal and postnatal development as well as in self-renewing organs.59,60 The transmembrane Notch receptor is activated at the cell surface by 2 ligand families: Jagged and Delta. Mouse and humans have 4 types of Notch receptors (Notch1 through Notch4) and 5 ligands (jagged1, jagged2, delta-like-1, delta-like3, and delta-like-4).61,62 Notch signaling is initiated through ligand–receptor interactions from neighboring cells. After activation, the receptor undergoes proteolytic cleavage by γ-secretase, releasing the Notch intracellular domain, which, in turn, translocates into the nucleus and trimerizes with the transcription-factor recombination signal sequence binding protein, J-κ (RBP-Jκ) to convert it from a transcriptional repressor to an activator.63 Timmerman reported that Notch activity promotes epithelial–mesenchymal transition during cardiac development and that Notch functions via lateral induction to promote a selective TGF-β–mediated epithelial–mesenchymal transition that leads to cellularization of developing cardiac valvular primordia.36 Schroeder et al further reported that RBP-J–deficient ES cells gave rise to mesoderm with increased cardiomyogenesis and that repression of the cardiac pathway was restored by expressing RBP-J in the RBP-J–deficient ES cells.64 These findings indicated that Notch signaling via RBP-J is a negative regulator for cardiomyocyte induction and might induce mesodermal cells into other cell lineages (noncardiomyocyte). Further characterization of the role of Notch signaling in cardiac development is therefore warranted to progress the potential of cardiac regenerative medicine.

During the development of many organ systems, there is no single growth factor that acts throughout the process of induction; instead, these factors act in a time- and context-dependent fashion. One single factor, such as BMP, Wnt, Notch, and Hedgehog,65,66 can exert opposite actions in different developmental processes. The in-context use of growth factor combinations will enable specific differentiation of cardiomyocytes from ES cells in the near future. The current status of the differentiation cascade for cardiomyocytes and other cell types is summarized in Figure 1, and the
major signaling pathways involving these critical factors in cell differentiation are shown in Figure 2.

In 2001, it was reported that human ES cells, like mouse ES cells, could differentiate into cardiomyocytes with cardiac-specific structural and functional properties. The same group recently revealed that transplanted human ES cell–derived cardiomyocytes can act as a pacemaker in porcine ventricles in which a complete atrioventricular block was artificially generated. In this study, the human ES cell–derived cardiomyocytes made successful electrical connection with the recipient cardiomyocytes in large animals. As demonstrated by such studies, research into ES cells as a source for regenerative medicine has progressed rapidly in recent years, probably because of their high proliferative activity and pluripotency compared with other types of stem cells. Although ethical issues remain with the use of ES cells, the clinical application of these cells in transplantation therapy has definitely become feasible for cardiomyocytes and a host of other cell types.

Besides ethical issues, several problems associated with ES cells, including tumorigenicity, ectopic differentiation, and transplantation issues, need to be overcome for the clinical application of these cells. The use of mouse embryonic fibroblasts as a feeder layer for ES cells and the potential contamination of ES cell lines with animal viruses may hinder the clinical implementation of ES cells for cardiac repair and need to be addressed.

**BM Stem Cells**

Friedenstein first reported the existence of MSCs in BM in 1966 and called them bone formation progenitors. Subsequently, MSCs were reported to comprise ~0.001% to 0.01% of the total nucleated cells in BM, and this is far less than that of hematopoietic stem cells (HSCs). BM-MSCs were initially believed to be a stem cell for osteoblasts, chondroblasts, adipocytes, and connective tissues. In recent years, BM-MSCs have also been reported to differentiate into neurons, skeletal muscle cells, and cardiomyocytes both in vitro and in vivo. BM-MSCs are found in the stromal cell fraction, which can be easily separated from hematocytes in culture. These stem cells were initially isolated from the BM stromal cells by their characteristic proliferative activity and multipotency. Recent advances in fluorescently activated cell sorting (FACS) techniques have enabled the prospective isolation of HSCs by cell surface antigen expression and fluorescent dye efflux characteristics. Matsuzaki et al reported that FACS-derived CD34+c-kit+Sca-1+Lin−tip-side population (SP) cell fractions contain the HSC population in mice. c-kit is a stem cell factor receptor, Sca-1 is a stem cell antigen specifically expressed in various stem cells (only in mice), and Lin is a...
mixture of antibodies against lineage markers for hematopoietic cells (mouse: Gria-1, Mac-1, B220, CD3 and Ter119; human: CD3, CD4, CD8, CD19, CD33 and Glycophylin A). Despite some reports, cell surface markers to isolate MSCs have yet to be determined. CD29, CD44, CD105, and Sca-1 (only in mouse) are widely accepted cell surface markers for MSCs, but other markers differ among researchers.

In 1999, we induced the differentiation of MSCs in vitro by treating cardiomyocytes that had begun to spontaneously beat with 5-azacytidine, which demethylates methyl-cytosine and can induce transcription of critical transcription factors by demethylating CpG islands of promoter regions. This was considered a very surprising phenomenon because at the time, BM cells were thought to form only blood cell lineages or bone cells. This finding was later pursued using a variety of approaches, revealing the potential of BM cells to differentiate into a variety of tissues, including cardiomyocytes. In 2001, Beltrami et al observed cardiomyocyte mitotic figure in human hearts after a myocardial infarction (MI). Their report sparked controversy regarding cardiomyocyte induction and investigated whether the cells, thought to be terminally differentiated, had acquired the ability to proliferate or whether immature cardiomyocytes differentiated from stem cells into cardiomyocytes, then began to proliferate, or whether mature cardiomyocytes acquired the ability to proliferate by fusing with cells that had retained the proliferative ability of stem cells. In 2001, Orlic et al reported cardiomyocyte differentiation by transplanting c-kit^+^Lin^-^ BM cells into peri-infarct tissue after MI. They demonstrated directly that BM cells become cardiomyocytes in vivo. However, c-kit^+^Lin^-^ BM cells are predominantly HSCs, and even if BM cells differentiate into a variety of cells, including cardiomyocytes, various debates remain regarding, for example, whether HSCs actually transdifferentiate or MSCs differentiate. Moreover, in 2002, fluorescence in situ hybridization analysis revealed the presence of numerous cardiomyocytes that seemed to be recipient derived after human heart transplantation, and conversely, in 2003, numerous BM-derived cardiomyocytes were shown to be present in the recipient heart after BM transplantation. In contradictory experiment, Wagers et al examined a variety of organs after transplanting green fluorescent protein (GFP)-labeled single HSCs (c-kit^+^, Lin^-^, Sca-1^-^) into irradiated mice and demonstrated that if HSC transdifferentiation does occur, it is extremely rare, and that cardiomyocyte differentiation did not occur as a result of MI or induced injury. Goodell et al transplanted highly enriched HSCs into lethally irradiated mice subsequently rendered ischemic by coronary artery occlusion for 60 minutes followed by reperfusion and reported that transplanted BM cells differentiated into cardiomyocytes in the peri-infarct region at a prevalence of 0.02%. In 2004, Balsam et al investigated whether the c-kit^+^ HSCs in BM are capable of differentiating into cardiomyocytes by directly injecting BM cells into myocardial tissue.
instead of transplanting BM after irradiation as other groups had done. Importantly, they conducted their study in an experimental system that excluded irradiation because of the possibility that invasive treatment, including irradiation, contributed to a fusion phenomenon. They concluded that c-kit⁺ HSCs do not include cells capable of differentiating into cardiomyocytes. Murry et al investigated this differentiation ability in a similar manner by directly infusing c-kit⁺Lin⁻HSCs into the heart and, as expected, showed that HSCs are unable to differentiate into cardiomyocytes. That same year, we also reported on the differentiation ability of HSCs by using a c-kit⁺Sca-1⁺CD34⁻Lin⁻SP (CD34⁻KSL-SP) of HSCs.88 When we transplanted whole BM cells, which include both HSCs and MSCs, from GFP-transgenic mice into lethally irradiated mice and induced an MI, we found very few GFP⁺ (BM-derived) cardiomyocytes. Interestingly, granulocyte colony stimulating factor (G-CSF) enhanced the number of GFP⁺ cardiomyocytes and nonmyocytes in the infarcted- or border zone–area (Figure 3). In contrast, when we performed HSC transplantation followed by induction of MI and administration of G-CSF, cardiomyocytes were rare in the group transplanted with HSCs alone, although fibroblast-like cells were observed, and G-CSF increased their number (Figure 4). Moreover, we confirmed the presence of predominantly GFP⁺ cardiomyocytes derived from MSCs in the group transplanted with cardiomyogenic cells, which were purified MSCs (Figure 5). It should be emphasized that in these BM transplantation experiments, the

Figure 5. Evidence of mobilization and differentiation of BM-MSCs into cardiomyocytes. a, GFP-marked MSCs were transplanted into the intratibial BM space. b through d, The transplanted MSCs differentiated into adipocytes (b) and osteoblasts (c), and some remained as stem cells (d). e through g, The recombinant plasmid-containing EGFP driven by a myosin light chain-2v promoter was stably transfected into MSCs. The cells (cardiomyogenic [CMG]-ME cells) expressed EGFP on differentiation into cardiomyocytes (f and g), h through j, The intra-BM transplantation was performed using the CMG-ME cells. Then, MI was induced and G-CSF was administered. EGFP-positive cardiomyocytes were observed in the infarcted and border zone areas. Modified with permission from Kawada et al.88
radiation dose must be carefully determined because the radiation sensitivity of MSCs is much higher than that of the HSCs. We proposed that the differentiation by whole BM cells into organs (cells) other than hematopoietic populations is attributable to the MSCs and not to the HSCs, and that MSCs are mobilized from the BM into the bloodstream the same as HSCs.

In 2002, Terada et al suggested that a cell fusion phenomenon had to be considered with regard to the plasticity of the BM cells reported thus far.89 Their coculture of adult animal BM cells with ES cells induced cell fusion naturally in the presence of interleukin-3, and although the karyotype was tetraploid, the cells acquired pluripotency and proliferative ability. More recently, the transplantation of whole BM cells into lethally irradiated mice resulted in fused cardiomyocytes but no transdifferentiation.90 In addition, the same study aimed to identify cell lineages in whole BM responsible for cell fusion by transplanting CD45-Cre mouse BM into R26R mice. Fused cardiomyocytes were observed in this experimental system, and BM-derived leukocyte lineage cells were found to be responsible for the fusion. The lack of a clear definition for cell plasticity has led to confusion with several reports failing to demonstrate that a single cell can indeed differentiate into multiple lineages at significant levels. The plasticity of adult stem cells is reviewed by Lakshmipathy and Verfaillie91 and Gorve et al.92 Lakshmipathy and Verfaillie used 3 criteria to define stem cell plasticity: (1) differentiation of a single cell into multiple cell lineages, (2) functionality of differentiated cells in vitro and in vivo, and (3) robust and persistent engraftment of transplanted cells.

In 2002, Jiang et al reported pluripotent BM-derived cells and referred to them as multipotent adult progenitor cells.93 These cells had the potential to differentiate into the 3 germ layers in vitro and in vivo when transplanted into blastocysts. These multipotent adult progenitor cells were maintained using a low-density culture method, making it difficult to examine the independent corroboration of the findings by other laboratories. In 2005, Yoon et al identified a subpopulation of human BM stem cells (hBMSCs) that did not belong to the previously described class of BM-derived stem cells.94 These cells were CD29-CD44-CD73-, demonstrated minimal expression of CD90, CD105, and CD117, and could differentiate into 3 germ layers. Intramyocardial transplantation of hBMSCs after MI resulted in robust engraftment of transplanted cells, which exhibited smooth muscle cell identity and colocalization with markers of cardiomyocytes and endothelial cells, consistent with differentiation of hBMSCs into multiple lineages in vivo. Coculture of hBMSCs with cardiomyocytes revealed that phenotypic changes of hBMSCs result from both differentiation and fusion. Other laboratories have identified other multipotent, CD45-negative, nonhematopoietic BM-derived cells.4,5,95,96 The relationship between the BM-derived stem cells reported from different laboratories needs to be clarified.

The pluripotency of BM-MSCs and their ability to differentiate into cardiomyocytes has been demonstrated, but the steps required for clinical application are as yet unknown. A great deal of clinical research up until 2005 involved harvesting mononuclear cells from BM or peripheral blood and infusing them through a catheter into a coronary artery to treat acute MI. Strauer et al97 first reported transplantation of BM mononuclear cells 4.8 to 13.5 days after an MI in 10 acute MI patients, which resulted in a slight decrease in left ventricular end-systolic dimension and infarct region, and an increase in the left ventricular ejection fraction (EF) and regional function. The TOPCARE-AMI trial98–100 allocated 20 patients with reperfused acute MI to receive intracoronary infusion of either BM-derived or circulating blood–derived progenitor cells into the infarct artery at 4.3±1.5 days. The results from this trial indicated a significant increase in global left ventricular EF, improved regional wall motion, and reduced end-systolic left ventricular volumes at the 4-month follow-up investigation. At the 1-year follow-up investigation, the transplanted group revealed an increased EF, reduced infarct size, and absence of reactive hypertrophy without significant complication, suggesting functional regeneration of the infarcted ventricles. The BOOST trial101 and Fernandes-Aviles et al102 also transplanted BM mononuclear cells 4.8 to 13.5 days after an MI in acute MI patients. In most of the studies, transplantation resulted in similar results. The intracoronary autologous bone marrow cell transplantation (IACT) Study transplanted BM mononuclear cells via catheter in patients with chronic MI and reported that functional and metabolic regeneration of infarcted and chronically avital tissue could be realized.103 The presence of HSCs and vascular endothelial cell progenitors in BM fluid and peripheral blood can adequately explain the successful angiogenesis.4,5 Taking into account the absence of MSCs in blood and their low frequency in BM fluid, it is unlikely that they are capable of adequate myocardial regeneration. It is possible that MSCs require in vitro expansion for effective myocardial regeneration. Further research is required to precisely unravel the scientific basis for clinical results.

The limitations of using BM-MSCs lie mainly in the lack of well-defined cell markers for isolating populations in vitro and the inability at present in obtaining adequate numbers of cells by in vitro culture to apply them clinically. Although it is possible to culture mouse cells and still maintain their multipotency, and to some extent with human cells, no clinical evaluations have yet been made. A third problem is that specific culture methods for differentiating BM cells are only available for some target cells. Specific differentiation is possible for osteoblasts, chondroblasts, and adipocytes. The use of 5-azacytidine is effective for cardiomyocyte differentiation but is clinically toxic. No methods have been established for cardiomyocytes using physiological growth factors, cytokines, or nontoxic chemical compounds. BM-MSCs were originally known as bone progenitor cells, reflecting their tendency to readily differentiate into bone, and these cells will form bone when transplanted into tissue in pellets. Because pellet culture is actually necessary to cause MSCs to form cartilage, it is considered a convincing phenomenon.

MSCs from human and other species have a cell surface phenotype that is of low immunogenicity.104,105 Several research groups demonstrated long-term allo-MSC engraftment in a variety of noncardiac tissues in the absence of immunosuppression.106–108 Pittenger and Martin reported that allo-MSC engraftment, initially in rats and later in swine, in
the myocardium occurs without evidence of immunologic rejection or lymphocytic infiltration, even in the absence of immunosuppressive therapy. If these results are true, allo-MSCs have advantages for cellular cardiomyoplasty because they are readily available and administered with immunologic acceptance by the recipient.

**Resident Cardiac Tissue Stem Cells**

A variety of studies have been conducted to ascertain whether tissue repair is a viable phenomenon in the heart in pathological states, such as cardiac hypertrophy and MI. Stem cells are present in many other organs and contribute to the maintenance of physiological conditions and to repair mechanisms in pathological states, but the existence of stem cells in the heart was completely unknown. Recent advances in techniques such as FACS and in genetic engineering are beginning to elucidate the presence and properties of stem cells in the heart.

In 2003, Beltrami et al of the same group reported that the c-kit+ cells present in the heart have pluripotency and proliferative ability in vitro. These cells can differentiate not only into cardiomyocytes but also smooth muscle cells and endothelial cells. Transplantation of these stem cells into a murine MI model confirmed their ability to differentiate into cardiomyocytes in vivo, implying the existence of a tissue repair mechanism by stem cells in the heart, as exists in other organs.110

In 2003, Oh et al reported cells expressing the stem cell marker Sca-1 in adult mouse heart, suggesting the presence of cardiomyocyte stem cells. These cells were Lin-c-kit and CD34 negative, but ≥93% of the SP fraction was Sca-1+. These cells are capable of differentiating into cardiomyocytes when exposed to 5-azacytidine in vitro and by administering them to a murine model of myocardial ischemia.111

Recently, Messina et al isolated myocardial stem cells capable of proliferating in vitro from the hearts of adult humans and mice. After dissociating the heart by enzyme treatment, they added a supplement (EGF, basic FGF, cardiotoxin-1, thrombin) under low serum conditions and showed the cell population that formed spheroids on a poly-d-lysine–coated culture dish to be stem cells with proliferative and differentiation ability.112 Immediately after spheroid formation, the stem cell surface markers c-kit, KDR, and CD34 were expressed, and transplantation into heart tissue indicated that these stem cells were proliferation and differentiation competent in vivo (cardiomyocytes, endothelial cells, and smooth muscle cells).

In 2005, Laugwitz et al reported that Isl-1 (an LIM homeodomain transcription factor), which is known as a secondary heart field marker, can also be used as a cardio-

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**Figure 6.** Neural crest–derived cells contribute to the intracardiac tissue stem cells. a and b, Migration of neural crest cells into the heart. Most of the cells reside at the outflow tracts, and some move into the intramyocardium. c, Cardiospheres were obtained by neurosphere culture. Cardiosphere-forming cells expressed stem cell markers, nestin, and Musashi-1. d, Migration and differentiation of neural crest–derived cells and its contribution to cardiac stem cell numbers. e through g, Lineage analysis of neural crest–derived stem cells using P0-CRE-EGFP-double transgenic mice. Neural crest–derived cells formed aortic and pulmonary artery and differentiated into cardiac valves (e). Some of them remain in the intramyocardium and express nestin. h and i, Neural crest–derived cardiac stem cells differentiated into cardiomyocytes in adults (10 weeks). j through l, Rat cardiospheres were marked with Dil and transplanted into the cardiac neural crest of chick embryos. The transplanted cardiosphere migrated into the outflow tract of the heart. m, The red cells demarcate the migrated rat cardiospheres that were transplanted. n, The red cells indicate the transplanted Dil-labeled rat cardiospheres forming cells that were transplanted. AV indicates aortic valve; IVS, interventricular septum; CNC, cardiac neural crest; NT, neural tube; OV, otic valve, Rm, rhombomere; A, atrium; V, ventricle; CT, conotruncus; DLR, dorsolateral pathway; VR, ventral pathway. Modified with permission from Tomita et al.115
myocyte progenitor in the postnatal heart. These cells are present throughout the entire heart, in the following ratio order: right atrium, left atrium, right ventricle, septum, and left ventricle, and were not present in the SP fraction.

In contrast, Pfister et al reported that among cardiac SP cells, the greatest potential for cardiomyogenic differentiation is restricted to cells negative for CD31 expression and positive for Sca-1 expression (CD31−/Sca-1+), and that these cells are capable of both biochemical and functional cardiomyogenic differentiation into mature cardiomyocytes.

We recently reported that rodent cardiac SP cell fractions formed clonal spheroids in serum-free medium in the presence of epidermal growth factor and FGF-2. These clones expressed nestin, Musashi-1, multi-drug resistant gene-1, and p75 nerve growth factor receptor, markers of undifferentiated neural precursor cells (Figure 6). After differentiation, the precursor markers were lost, and differentiation into neurons, glia, smooth muscle cells, and cardiomyocytes was observed. Thus, neural crest–derived cells have stem cell characteristics in their capacity to proliferate and differentiate into various types of cells, including sensory and sympathetic neurons, glial cells in the peripheral nervous system, and smooth muscle cells in the blood vessels. Because some neural crest–derived cells in adults express nestin, Musashi1, and p75, and can proliferate and differentiate into multiple lineages in vitro, they are regarded as stem cells (neural crest stem cells). Thus, we transplanted rodent cardiosphere-derived cells into chick embryos and observed their migration to the truncus arteriosus and cardiac outflow tract, where they contributed to dorsal root ganglia, spinal nerves, and aortic smooth muscle cells. Lineage studies using double transgenic mice encoding P0-Cre/Floxed-enhanced GFP (EGFP) revealed undifferentiated and differentiated neural crest–derived cells in the fetal myocardium. Undifferentiated EGFP+ cells expressed GATA4 and nestin, but not actinin, in the fetal or neonatal stage, whereas the differentiated cells were identified as cardiomyocytes in adult. These results suggest that cardiac neural crest–derived cells migrate into the heart, remain there as dormant multipotent stem cells, and under the appropriate conditions differentiate into cardiomyocytes and typical neural crest–derived cells, including neurons, glia, and smooth muscle.

Although the claims of studies reviewed herein vary considerably, clearly there are cells with proliferative ability and differentiation ability into cardiomyocytes resident in the heart. Multipotency was proved in c-Kit+ cells and SP cell–derived cardiosphere (neural crest stem cell), indicating their status as stem cells. From the viewpoint of multipotency, Sca-1− cells and Isl-1+ cells might be cardiac progenitor cells. More detailed studies are needed to define whether this is a heterogeneous cell population or a single cell type that can switch phenotype in response to various signals. It will be interesting to test whether these cells can be expanded and perhaps differentiated in vitro and then transplanted into a diseased heart to improve function. Although these studies may not lead to immediate clinical applications, they are a first major step toward a potentially remarkable new field of cardiac medicine.

Conclusion

The enormous clinical potential of cardiac regeneration has generated great expectations in both clinicians and patients. Regenerative medicine in the field of skin, cartilage, bone, adipose tissue, and cornea is already well established and used clinically. In contrast, cardiac regeneration has to overcome some hurdles to be clinically viable, including developing methods to increase the selectivity of cardiomyocyte induction, ensuring mass production, and the construction of transplantable tissue, including the vascular system. The realization of cardiac regeneration depends on the outcome of basic research and its application to the clinic. Careful clinical trials using the various stem cell sources may significantly advance this field but should be performed in tandem with further intensive investigation of the basic mechanisms at play.

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