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REVIEW

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Genetic Modification of Stem Cells for Improved Therapy of the Infarcted Myocardium

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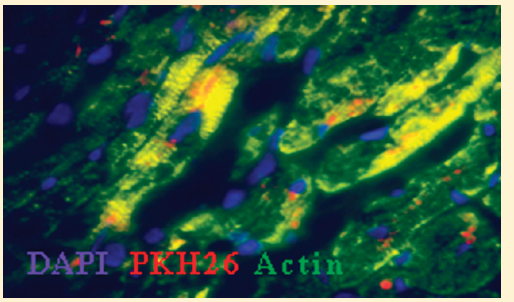
ABSTRACT: The conventional treatment modalities for ischemic heart disease only provide symptomatic relief to the patient without repairing and regenerating the damaged myocardium. Stem cell transplantation has emerged as a promising alternative therapeutic approach for cardiovascular diseases. Stem cells possess the potential of differentiation to adopt morphofunctional cardiac and vasculogenic phenotypes to repopulate the scar tissue and restore regional blood flow in the ischemic myocardium. These beneficial therapeutic effects make stem cell transplantation the method of choice for the treatment of ischemic heart disease. The efficacy of stem cell transplantation may be augmented by genetic manipulation of the cells prior to transplantation. Not only will insertion of therapeutic transgene(s) into the stem cells support the survival and differentiation of cells in the unfavorable microenvironment of the ischemic myocardium, but also the genetically manipulated stem cells will serve as a source of the transgene expression product in the heart for therapeutic benefits. We provide an overview of the extensively studied stem cell types for cardiac regeneration, the various methods in which these cells have been genetically manipulated and rationale of genetic modification of stem cells for use in regenerative cardiovascular therapeutics.



KEYWORDS: angiomyogenesis, gene therapy, heart, stem cells

INTRODUCTION

Coronary artery disease leading to myocardial infarction is a major cause of mortality and morbidity worldwide.[1] The conventional treatment methods for coronary artery disease include lifestyle changes, dietary modification and regular exercise. Although much advancement has been made in pharmacotherapy of myocardial infarction patients, this can barely provide symptomatic relief and leads only to improve quality of life without addressing the root cause of the problem that is characterized by massive loss of functioning cardiomyocytes. In severe cases, invasive surgical intervention such as coronary artery bypass grafting and angioplasty are performed for revascularization of the ischemic myocardium. Despite the recent advances in reperfusion therapy for acute myocardial infarction and pharmacotherapy for post myocardial infarction left ventricular remodeling, the incidence and mortality of post myocardial infarction heart failure is increasing.[2] In order to address the inadequacy of the conventional treatment methods, alternative strategies are being explored. In this regard, gene therapy and stem cell transplantation have emerged as promising approaches to support the inadequate intrinsic repair system of the heart.[3−5] Whereas gene therapy with plasmids encoding for angiogenic cytokines and growth factors may improve regional blood in the ischemic heart via angiogenesis, stem cell transplantation for the heart cell therapy is majorly intended to replace the lost functional myocytes by adopting morphofunctional cardiomyocyte phenotype besides sustaining myocardial angiogenesis and supporting intrinsic repair mechanisms. The field of cell therapy has received a boost with the discovery of induced pluripotent stem (iPS) cells[6,7] and microRNA which serve as critical post-transcriptional regulators of cell functionality including stem cell self-renewal and differentiation.[8,9] Given the complexity of the disease process, it would be prudent to adopt a multimodal treatment strategy which may involve combining stem cell and gene therapy by manipulation of stem cells to enhance their angiomyogenic potential. The present review of literature provides an overview of the progress made in the field of stem cells and gene therapy with special focus on their combined application.

STEM CELLS FOR MYOCARDIAL REPAIR

Stem cells are characterized by the ability of undifferentiated self-renewal through mitotic cell division and differentiation to adopt a diverse range of specialized cell types.[10] Starting almost two decades ago when Marelli et al. provided the first evidence of myogenic potential of skeletal myoblasts (SMs) in an experimental canine heart model of cryoinjury,[11] cells from various tissue sources and origins have been used for potential use in cardiac regenerative medicine (Table 1), each with its own set of advantages and disadvantages.[12] Although the heart has its own endogenous reserve of resident cardiac stem/progenitor cells,[13,14] the number of cardiac stem/progenitor cells is not enough for adequate recovery in case of a massive myocardial infarct, and

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Table 1. Cell Types Used for Heart Cell Therapy in Experimental Animal Models and Clinical Studies

cardiomyocytes
cardiac stem/progenitor cells
skeletal myoblasts
bone marrow derived stem cells
smooth muscle cells
endothelial progenitor cells
cord blood derived stem cells
genetically modified fibroblasts
mesothelial cells
embryonic stem cells
induced pluripotent stem cells

therefore outside intervention is warranted. The transplanted stem cells participate in the myocardial repair process by one or a combination of the mechanisms including transdifferentiation into mature cardiomyocytes which electromechanically couple with the host myocytes,[15,16] by secretion of bioactive molecules as a part of their paracrine activity which has been implicated as the major mechanism for the improvement of left ventricular function *via* proangiogenic and cardioprotective effects.[17−19] Moreover, the paracrine factors thus released by the transplanted stem cells also alter the restoration of extracellular matrix and recruit endogenous stem cells.[19] The first clinical application of stem cells for myocardial repair was reported by Menasche et al. when autologous SMs were injected intramyocardially as an adjunct to routine coronary artery bypass grafting in a 72-year old patient with myocardial infarction.[20] To date, both SMs and bone marrow stem cells (BMSCs) are the most extensively studied types of the cells in various phase I and II studies for myocardial repair as a sole therapy or as an adjunct to routine revascularization interventions.[20−25]

Bone Marrow Stem Cells. BMSCs exhibit multipotentiality, transdifferentiate to adopt multiple phenotypes and have the ability to repopulate many nonhematopoietic tissues, such as neuroectodermal cells, SMs, cardiomyocytes, endothelium, hepatocytes, and lung, gut and skin epithelia.[26−34] A subset of BMSCs referred to as multipotent adult progenitor cells (MAPCs) has been shown to proliferate extensively and differentiate into the cells of all three germ layers.[35] Transplantation studies with BMSCs have demonstrated that endothelial progenitor cells, angioblasts or CD34+ cells incorporated into foci of neovascularization and showed a favorable effect on cardiac function. The more versatile potential of bone marrow derived hematopoietic stem cells was documented with the use of lineage-negative ckit+ cells which differentiated into endothelial cells, SMs and cardiomyocytes in a murine myocardial model.[31] Besides hematopoietic stem cells, bone marrow derived mesenchymal stem cells have been extensively studied for their myocardial regenerative potential.[36,37] Clinical studies with autologous BMSCs documented safety and feasibility of the procedure both in patients with acute myocardial infarction and in those with chronic ischemia and reported a modest, beneficial effect on recuperation of global cardiac function.[25,38,39] However, most of these studies were nonrandomized and lacked a placebo-treated control population. The BOOST study was the first randomized controlled study to report a significant improvement in global left ventricular function after six months, expressed as 6% incremental increase in left ventricular ejection fraction evaluated using MRI in patients who received intracoronary cell infusion.[25] However, yet again the study was not placebo-controlled. Two subsequent double-blind randomized placebo-controlled studies, conducted at the universities of Leuven and Frankfurt,[40,41] addressed some of these confounding variables associated with bone marrow aspiration and a second catheterization. The patients receiving cell transfer had a significantly greater reduction in infarct size for a similar area at risk, as assessed using repeated MRI, and a greater recovery of regional systolic function. Importantly, these beneficial effects were sustained on a longer term basis.[42,43] The modest outcome of BMSCs may be attributed to several factors including the low percentage of adult stem cells isolated from the patient's bone marrow, low delivery and retention efficiency, and poor survival, engraftment and integration of the implanted cells. As most of these clinical studies used autologous stem cells in elderly patients, physiological aging of the cells used for transplantation also remains as one of the prime factors of modest outcome of the procedure. There is no direct evidence for the fate of BMSCs postengraftment in the human patients. The clinical benefits associated with transplantation of BMSCs are largely attributed to factors other than transdifferentiation including paracrine release of bioactive molecules, angiogenic effects, passive mechanical effects, and endogenous responses of resident cardiac stem cells to participate in response to the paracrine factors released from the transplanted cells.[44] For optimum prognosis, the mechanism of action of the transplanted BMSCs needs to be researched in depth. Besides, there are some concerns regarding the differentiation potential of BMSCs to adopt cardiac phenotype[45,46] and safety issues using unselected BMSCs.[47] Direct intramyocardial transplantation of unselected BMSCs may lead to significant calcification in the infarcted myocardium.[47] Likewise, transplantation of undifferentiated mesenchymal stem cells may develop into cardiomyocytes or fibrous scar tissue depending upon the signals from the cytokine rich microenvironment of the ischemic heart.[48] In order to alleviate these concerns of unbridled differentiation into undesired phenotypes, guided cardiogenic differentiation approach may ensure that the transplanted cells only adopt the desired cardiac phenotype post-transplantation in the heart. This approach is based on pretreatment of stem cells with factors that restrict their differentiation potential to the cardiac lineage.[49,50] Some concerns have also been raised regarding arrhythmogenicity of BMSCs postengraftment.[51] The latest development in the use of BMSCs is the application of allogenic mesenchymal stem cells which would pave the way for the development of ready-to-use off-the-shelf availability of stem cells for transplantation in patients. Such an arrangement would alleviate financial implications as well as logistic constraints which are associated with the use of autologous stem cells.[52,53]

Skeletal Muscle Stem Cells. Skeletal muscle fibers have an endogenous reservoir of tissue-committed precursor cells (named satellite cells) that normally lie in a quiescent state under the basal membrane.[54] Following muscle injury, these cells are rapidly mobilized, proliferate and participate in the process of muscle repair through myogenesis.[54] Therapeutic application of skeletal muscle derived stem cells especially focuses on cardiac transplantation of myoblasts which undergo myogenic differentiation at the site of the cell graft.[55] Experimental studies have demonstrated that SMs form myotubules after transplantation, and improve cardiac function.[56] Nevertheless, their inability to form cardiomyocytes[57] and electromechanically couple with the host myocytes though gap junction formation remains a major concern in their clinical application due to the fear of arrhythmias. Therefore, attempts

have been made to improve their cardiomyogenic characteristics *via* transgenic overexpression of connexin-43, which may allow the transplanted SMs to develop gap junctions with the juxtaposed host myocytes.[58,59] Starting with the first ever myoblast transplantation in the human heart as an adjunct procedure to coronary artery bypass grafting,[20] various clinical studies have already shown that SMs transplantation leads to improved cardiac function.[60,61] However, the small size of these studies, their open-label type design and lack of control group of patients made these data inconclusive. Similar to BMSCs, SMs have multiple advantages including safety, easy availability from an autologous source without any ethical or religious concerns, ease of *in vitro* expansion to a very large number and myogenic differentiation without the fear of tumorigenicity. It is also argued that the ischemia resistant nature of SMs can enable their better survival and engraftment rate in the ischemic heart.[62] An important characteristic of SMs is their conditionally immunoprivileged status.[63] We have previously shown that cross-species transplantation of human SMs into a porcine heart model of myocardial ischemia can be supported by transient immunosuppression.[64] These observations led to the first-in-human transplantation of allogenic SMs as an adjunct to coronary artery bypass grafting using transient immunosuppression.[65]

Cardiac Stem Cells. The heart has always been considered as a postmitotic organ and therefore lacks regenerative capacity in the event of myocardial injury. Pioneering work of Anversa and colleagues has shown that, like any other organ in the body, the heart has a resident population of stem cells which are multipotent and can adopt all the required cell phenotypes which are needed to regenerate myocardium in the event of injury.[13,66] Like any other stem cell, cardiac stem/progenitor cells show the properties of self-renewal and differentiation potential and participate in the myocardial repair process by mobilization from cardiac stem cell niches to the site of injury in response to the cues from the cytokine rich microenvironment of the infarcted heart.[67] *In vitro* culture of cardiac-derived cells as three-dimensional cardiospheres has been shown to recapitulate a stem cell niche-like microenvironment which supports their survival and enhances functional benefit after transplantation into the injured heart.[68] Resident cardiac stem/progenitor cells have been successfully isolated from the murine, feline, canine, and human hearts. Two categories of cardiac stem cells, vasculogenic (ckit+/KDR+) and myogenic (ckit+), have been identified in the human heart under physiological as well as pathological conditions.[69] Treatment of the purified Sca-1+ckit- cardiac stem cells from mouse heart with 5-azacytidine induced their myogenic differentiation *in vitro*.[70] Intramyocardial injection of cardiac stem cells showed their functional competence and cardiac regenerative potential in a SCID mouse model of acute myocardial infarction.[71] Eighteen days after cell transplantation, infarction size was significantly reduced with concomitant better preserved anterior wall thickness. Moreover, immunohistology showed that the transplanted cells underwent myogenic differentiation to repopulate the infarcted myocardium and showed functional integration with the surrounding host cardiomyocytes. Experimental animal studies in an immunocompetent rat model of myocardial ischemia reperfusion injury showed that intracoronary delivery of cardiac stem/progenitor cells attenuated left ventricular remodeling and ameliorated left ventricular function.[72] The transplanted cells successfully regenerated the injured myocardium, which resulted in significantly reduced infarct size. Intracoronary delivered cardiac stem cells allowed uniform distribution of the delivered cells in the area at risk in the infarcted myocardium with greater increase in the viable tissue albeit with therapeutic effectiveness similar to if not better than intramyocardial transplantation.[73] Similar results have also been achieved by intramyocardial delivery of Sca-1+ cardiac stem cells complexed with Puramtrix, which incidentally also supported their survival and paracrine activity post-transplantation into the heart.[74] Besides their ability of angiomyogenic differentiation, cardiac stem/progenitor cells also release paracrine factors including soluble vCAM-1, which induced endothelial cell migration as well as protection of cardiomyocytes.[75] Given the encouraging results from these experimental animal studies, University of Louisville, in collaboration with Brigham and Women's Hospital, Jewish Hospital and St. Mary's Healthcare, has initiated a phase I clinical study entitled Cardiac Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO). The study will involve 40 patients between 18 and 75 years of age with less than 40% LVEF and is intended to investigate the safety of intracoronary delivery of cardiac stem cells for myocardial regeneration in patients with myocardial infarction. The primary end points of the study will measure isolation and expansion of cardiac progenitor cells from atrial tissue and the frequency of adverse events. Secondary end points include change in LVEF and change in area of akinesis. The study is scheduled to complete in June 2012 (http://clinicaltrials.gov).

Embryonic Stem Cells. Embryonic stem cells (ES) cells are isolated from inner cell mass of blastocysts and have the ability of unlimited self-renewal in the undifferentiated pluripotent state.[76] ES cells are easily identifiable due to their distinct morphology, a higher nuclear to cytoplasmic ratio and distinctive expression and activity of alkaline phosphatase and a cell surface marker stage specific embryonic antigen-1 (SSEA-1). Given their pluripotent status, ES cells can differentiate into the derivatives of all three embryonic germ layers including cardiomyocytes.[77] The cardiogenic capability of ES cells has been extensively studied in both *in vitro* and *in vivo* experimental animal models.[78,79] A direct *in vitro* comparison of human fetal, neonatal, adult atrial and ventricular myocytes with the ES cell derived cardiomyocyte clusters showed similarity in the expression level and pattern of cardiac specific markers. Moreover, the functional properties of the clusters of cardiomyocyte obtained from the differentiating human ES cells are also similar to the human cardiomyocytes.[78] ES cell derived cardiomyocytes have also been used for cardiac transplantation.[80] These studies provided clear evidence that transplantation of undifferentiated ES cells or their derived cardiomyocytes consistently resulted in repopulation of the infarcted myocardium and improved left ventricle contractile function.[79−81] Besides their ability to repopulate the infarcted myocardium *via* neoangiomyogenesis, ES cells release paracrine factors which contribute to preserve the host cardiac function. A direct comparison of the paracrine activity of ES cells with adult stem cells showed that ES cells release copious amounts of VEGF and IL10 which was significantly higher compared to the adult stem cells.[82] Moreover, the ES cell treated animal hearts had lower levels of proinflammatory cytokine compared to the adult stem cell treated hearts. The paracrine factors thus released, besides reducing the host cardiomyocyte apoptosis, also enhanced angiogenesis in the heart by activation of endogenous $ckit^{+}Flk1^{+}$ endothelial progenitor cells.[83] Strategies are also being developed to prime ES cells prior to transplantation and manipulate their paracrine behavior. Treatment of ES cells with

TGF-β2 significantly enhanced (2−4-fold) the release of cytoprotective factors including IL-10, stem cell factor, tissue inhibitor of matrix metalloproteinase-1, and vascular endothelial growth factor.[84] Treatment of the infarcted heart with TGF-β2 treated ES cells and their conditioned medium significantly reduced the infarct size.[84]

Despite their unquestioned cardiogenic potential, there are numerous problems associated with the use of ES cells which needs to be addressed before their clinical application. Teratogenicity of ES cells raises the major safety concerns regarding their clinical application.[85] The development of teratoma especially in cases where unselected ES cells are transplanted has been extensively reported.[85,86] Additionally, the moral and ethical issues in their availability and the immunological considerations[87] are the major limitations of ES cells in their progress to routine clinical application.

Induced Pluripotent Stem Cells. The ground-breaking findings of Takahashi and Yamanaka raised the possibility to reprogram somatic cells to pluripotent status by retroviral transgenic overexpression of a combination of stemness factors, i.e., Oct3/4, Sox2, cMyc and KLF4.[6] IPS cells are surrogate ES cells which share the same morphological, cultural and differentiation characteristics of ES cells, however, with the advantage that iPS cells can be generated from the patient's own somatic cells and therefore their use is without immunological considerations. Moreover, there are no ethical or moral issues involved in their generation and use. Unfortunately, iPS cells do exhibit teratogenicity similar to ES cells.[88] Strategies are therefore being developed to curtail their tumorigenic potential which include improvement of reprogramming protocols, use of partially differentiated iPS cell derived cardiomyocytes, optimization of cell transplantation protocols etc. Experimentally, functional cardiomyocytes have been differentiated from iPS cells which showed spontaneous contractile activity and expressed cardiac specific protein markers and transcription factors.[89−91] Transplantation of fibroblast derived iPS cells showed extensive repopulation of the infarcted myocardium in an immunocompetent experimental animal model of acute myocardial infarction.[92] Intramyocardially delivered iPS cells (20×10^4 cells suspended in 10 μL) showed extensive survival and were detectable until 2 and 4 weeks after transplantation without any evidence of tumor formation in the heart. Immunostaining of the ischemic myocardium for SSEA-1 revealed rare presence of iPS cell progeny by 4 weeks. Although the authors claimed significantly improved cardiac function in the iPS cell transplanted animal hearts compared to the fibroblast treated animals as control, they did not provide any direct evidence of cardiomyogenic differentiation of the transplanted iPS cells. In one of our recently concluded studies, we have reported reprogramming of SMs to generate iPS cells using a conventional method based on retroviral transduction of Yamanaka's quartet of pluripotency determinant transcription factors.[93] These iPS cells showed spontaneous differentiation into cardiomyocytes under conducive culture conditions. Using an immunocompetent mouse model of acute myocardial infarction, a comparison between iPS cells, their derivative cardiomyocytes and native SMs after intramyocardial delivery showed that all three types of cells significantly improved the infarcted heart function as compared to the basal DMEM treated animal hearts. Nevertheless, the improvement of the heart function was significantly higher in iPS cell derived cardiomyocyte transplanted animals. Moreover, no tumor formation was observed in iPS cell derived cardiomyocyte transplanted animal hearts in comparison to the iPS cell treated animal hearts which showed a significantly higher rate of tumor formation.[88] Immunohistology provided evidence that the transplanted iPS cells and their derivative cardiomyocytes survived in the infarcted myocardium, underwent myogenic differentiation and integrated with the host cardiomyocytes. These study results provided the first evidence regarding the fate of SM derived iPS cells and their derivatives postengraftment in the infarcted myocardium and their functional benefits in terms of improved global contractile heart function.

Table 2. List of Candidate Therapeutic Genes for Stem Cell-Based Delivery to the Infarcted Myocardium

serine/threonine protein kinase (Akt)
proto-oncogene serine/threonine-protein kinase Pim-1 (Pim-1)
B-cell lymphoma-2 (Bcl2)
insulin like growth factor-1 (IGF-1)
vascular endothelial growth factor (VEGF)
hepatocyte growth factor
stromal cell derived factor-1
angiopoietin-1 (Ang-1)

The other stem cell types which are being assessed for their cardiac differentiation potential are umbilical cord derived stem cells, adipose tissue derived stem cells and very small ES like cells. Their cardiogenic potential is being assessed in both *in vitro* and *in vivo* experimental animal models with encouraging results.

COMBINING STEM CELL TRANSPLANTATION WITH THERAPEUTIC GENE DELIVERY

Stem cells are excellent carriers of transgenes and can be easily manipulated *in vitro* for delivery of therapeutic genes to the heart.[64,94−97] Most studies involving genetically modified stem cells have been undertaken with the aim to augment the inherent capabilities of these cells to mediate myocardial repair mechanism. The combined approach of stem cell-based gene therapy has many advantages. First, genetic modification of stem cells is performed *ex vivo*, which allows the researchers to optimize the desired levels of transgene transfer and expression efficiencies. Second, the *ex vivo* gene modification is safer and allows the possibility of regulated expression of the transgene. The choice of the transgene and the method of transgene delivery into the stem cells are influenced by the desired outcome. The therapeutic genes used for stem cell-based delivery to the infarcted heart include the ones encoding for bioactive growth factors, cytokines, and survival signaling molecules either alone or in combination with each[94,97−100] (Table 2). In most cases, stem cells are genetically manipulated to achieve overexpression or inhibition of the gene of interest with high transfection and expression efficiencies using both viral and nonviral strategies (Table 3). In this regard, retroviral and lentiviral vectors lead to long-term expression of the transgene due to its integration into the cellular genome.[101−103] However, the possibility of insertional mutagenesis hampers their human clinical applications.[104] In contrast, adenoviral vectors do not integrate into the cellular DNA, but their ability to induce both humoral and cellular immune response in animals and humans is of concern. More recent development in this regard is the third and fourth generation adenoviral vectors that offer promising alternatives as these constructs only contain minimal residual adenoviral DNA (inverted repeats and packaging signal) resulting in reduced

Table 3. Characteristics of Transgene Delivery Vectors for Genetic Manipulation of Stem Cells

	viral vectors	nonviral vectors
exogenous DNA size limitation	+	−
provocation of immune and inflammatory reactions	+	−
efficiency of gene delivery to variety of cells	+	−
safety issues	+	−
integration into the host genome	+	−
chances of tumorigenic mutations	+	−
generation of active viral particles	+	−
long-term transgene expression	+	−

immunogenicity upon injection.[105] One major advantage associated with the use of these vectors lies in their providing particularly high-level transgene expression for a limited period of time. Additionally, adenoviral vectors are capable of infecting both dividing and quiescent cells with large size of exogenous DNA.[106] Taken together, these advantages make adenovirus one of the most commonly used tools for gene delivery in stem cell research. On the contrary, strategies based on nonviral genetic modification of stem cells are being developed.[107]

Although less efficient compared to the viral vectors, the nonviral approach is safer for human use. Of the nonviral strategies used for gene transfection into the cells, electroporation, microelectroporation and nucleofection are possibly the most efficient to transfect stem cells.[108−111] Besides a plethora of liposome and microvesicle based delivery systems, the use of nanoparticle based delivery system *via* polyplexing of transgenes with polyethylenimine is gaining popularity for both *in vitro* and *in vivo* culture systems.[112,113] Another important new development is the use of transposon-mediated DNA delivery to the cells, which has become progressively more applicable to mammalian cells and has opened the door for the development of a new generation of vectors for human gene therapy.[114] Transposons are more easily controlled, versatile, and safer than viral vectors. In this regard, piggyBac has become the most active and flexible transposon system used for transformation of mammalian cells.[115] Recently, the piggyBac transposon system has been used to create both mouse and human iPS cells.[116]

Whereas progress of the gene therapy approach to routine clinical applications has been interrupted during the last two decades due to realization of the untoward effects in patients due to uncontrolled and nontargeted gene delivery protocols, stem cell-based transgene transfer is developing as an alternative and safer method of transgene delivery in preclinical studies. Although some clinical studies are underway based on a therapeutic gene delivery approach [SERCA gene therapy trial #NCT00534703; ACS gene transfer for congestive heart failure trial #NCT00787059], there are no clinical studies based on stem cell-based gene therapy for the treatment ischemic heart disease.

■ GENETIC MANIPULATION OF STEM CELLS TO PROMOTE SURVIVAL

Poor survivability of the transplanted stem cells in the harsh microenvironment of the infarcted heart is one of the major impediments in the clinical application of stem cell-based therapy. Various strategies have been developed based on preconditioning

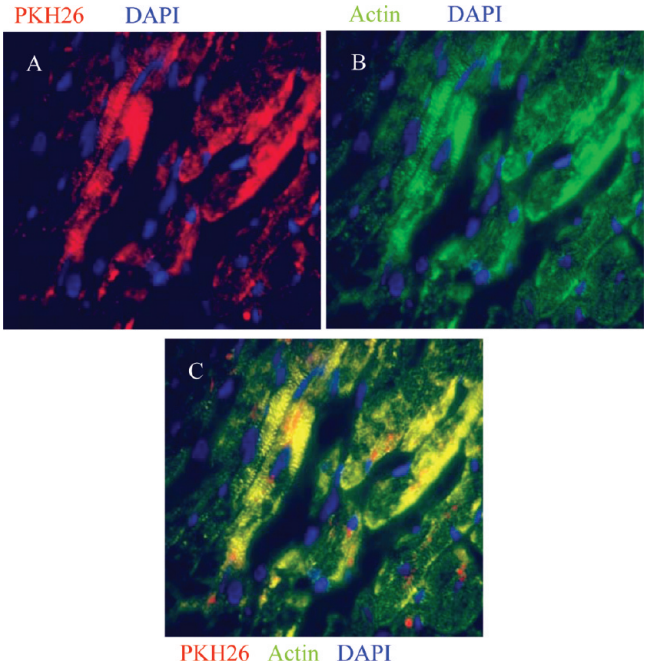

Figure 1. Myogenic differentiation of genetically modified stem cells. Myogenic differentiation of the transplanted mesenchymal stem cells in an experimental rat heart model of acute myocardial infarction. The cells were genetically modified using adenoviral vectors encoding for Akt and angiopoietin-1 to simultaneously overexpress the two transgenes. The animal model was developed by permanent ligation of coronary artery ligation. The cells were labeled with red fluorescent dye PKH26. Extensive myogenic differentiation of the transplanted cells (red fluorescence; A) was observed at 6 weeks after cell transplantation that was observed after fluorescent immunostaining of the histological tissue sections for actin (green fluorescence; B). Nuclei were stained by DAPI staining (blue fluorescence). Panel C depicts the merged imaged.

and reprogramming of stem cells prior to transplantation to address this issue.[117,118] One of the most effective ways to address this issue is to genetically modify these cells with plasmids encoding for survival signaling molecules such as Akt, Pim-1 and Bcl2 to prime these cells.[94,97,119,120] Such manipulations increased the survival of the transplanted cells during acute phase after transplantation and lengthened the duration of engraftment leading to improved cardiac function. Moreover, overexpression of Pim-1 (a known prosurvival and proproliferative kinase)[121] and Bcl-2 has also been reported to increase the differentiation and functional efficacy of stem cells after transplantation.[119,122] Cardiac progenitor cells genetically manipulated for Pim-1 overexpression supported retention of the transplanted cells in the recipient heart for a long term thus resulting in improved cardiac structure and function at 3 and 8 months after pathological challenge as compared to the animals receiving unmodified cells.[119] More recently, a study demonstrated that overexpression of another kinase, serine/threonine kinase glycogen synthase kinase (GSK)-3β, could enhance survival of BMSCs after 8 weeks and promoted their differentiation into cardiac phenotype.[120] Nevertheless, overexpression of a single gene might be insufficient to maximize the antiapoptotic effects on stem cells. Therefore, in one of our studies we combined transduction of Akt with angiocompetent molecule angiopoietin-1.[94] The combined Akt and angiopoietin-1 expression in mesenchymal stem cells not only improved their long-term survival postengraftment in an experimental rat heart model of

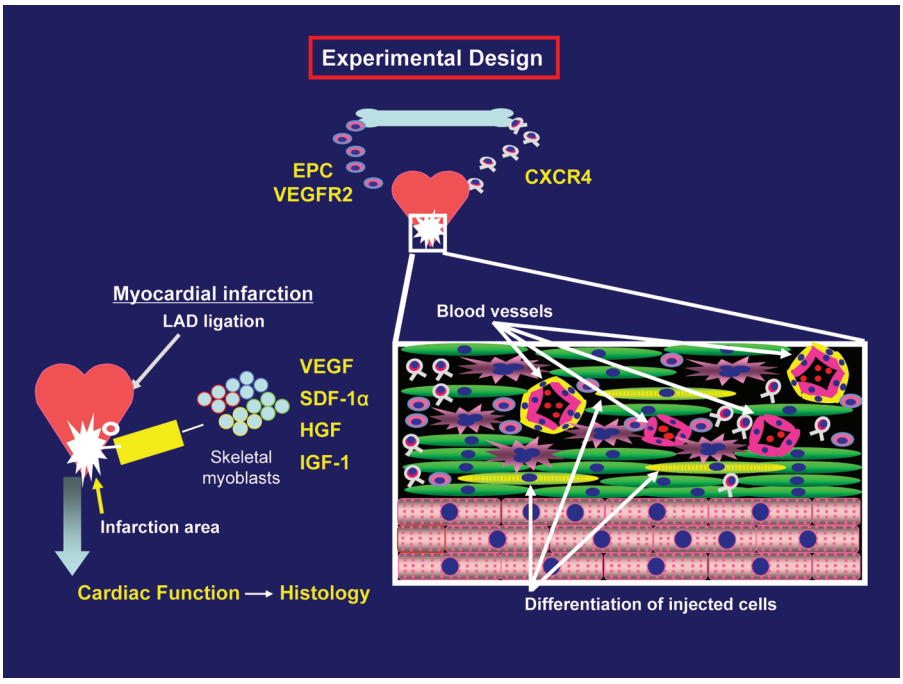

Figure 2. Experimental design of multimodal gene therapy approach in a rat model of permanent coronary artery ligation.

acute myocardial infarction but also promoted their angiomyogenic differentiation to adopt myogenic and endothelial phenotypes[123] (Figure 1).

■ GENETIC MODIFICATION OF STEM CELLS FOR IMPROVED PARACRINE ACTIVITY

Stem cells express secretable bioactive molecules as a part of their paracrine activity, which constitutes an integral part of the mechanism by which the transplanted stem cells improve cardiac function.[17,18,124] The strategy to genetically modify stem cells can be used to modulate their paracrine behavior for enhanced expression of bioactive growth factors or cytokines to achieve desired results. The release of growth factors from the genetically modified stem cells may contribute to preservation of ischemic heart function by multiple mechanisms including protection of cardiomyocytes, improved survival of the transplanted stem cells and their enhanced engraftment and angiomyogenic differentiation.[125−127] We have already shown that overexpression of vascular endothelial growth factor-165 either alone or in combination with angiopoietin-1 in SMs enhanced the paracrine release of angiocompetent molecules and promoted arteriogenesis in the ischemic heart.[125] These molecular changes improved regional blood flow in the ischemic myocardium and improved global heart function. Overexpression of angiocompetent vascular endothelial growth factor-165 also promoted the survival of the transplanted cells.[127] In one of our recent studies, we modulated the paracrine activity of mesenchymal stem cells by transgenic overexpression of insulin-like growth factor-1.[128] Interestingly, mesenchymal stem cells overexpressing insulin-like growth factor-1 secreted significantly higher level stromal cell derived factor-1α, a chemokine which is a known BMSC mobilization and retention factor,[129] as a part of their modified paracrine activity. Transplantation of insulin-like growth factor-1 overexpressing mesenchymal stem cells promoted significant extravasations of BMSCs and their homing-in to the heart by creating a concentration gradient of stromal cell-derived factor-1α in the heart. The mobilized BMSCs improved global cardiac function *via* angiomyogenesis. We have recently adopted a multimodal therapeutic approach by genetically modifying SMs with a select quartet of growth factors including vascular endothelial growth factor, insulin-like growth factor-1, stromal cell-derived factor-1α and hepatocyte growth factor to simultaneously activate multiple signaling pathways, i.e., vascular endothelial growth factor/Flk-1, insulin-like growth factor-1/insulin-like growth factor-1R, stromal cell-derived factor-1α /CXCR4 and hepatocyte growth factor/cMet ligand/receptor interaction[130] (Figure 2). Our novel multimodal stem cell/gene delivery strategy concomitantly involved stem cells from bone marrow, peripheral circulation and resident cardiac stem cells from the heart for the myocardial repair process (Figure 3). The participation of different stem/progenitor cells in the repair process resulted in decreased infarct size and increased angiogenesis in the infarcted heart (Figure 4).

■ GENETIC MODIFICATION FOR CELLULAR REPROGRAMMING

Based on Yamanaka's protocol to reprogram fibroblasts to pluripotent status, unipotent and multipotent stem/progenitor cells can be reprogrammed to pluripotency by genetic modification with a group of stemness factors either alone or in combination with small molecules.[131−133] We have recently demonstrated that SMs which intrinsically express three of the four Yamanaka stemness factors[6] are better candidates for reprogramming to pluripotency.[134] Incidentally, transplantation of the reprogrammed SMs and their derivative cardiomyocytes engrafted well in the infarcted mouse heart and preserved cardiac function. However, like with any other pluripotent stem cells, we observed that the reprogrammed SMs were tumorigenic in the immunocompetent host.[134] However, transplantation of their derivative cardiomyocytes did not develop cardiac tumors (our unpublished data). Srivastava et al. endeavored to directly reprogram committed

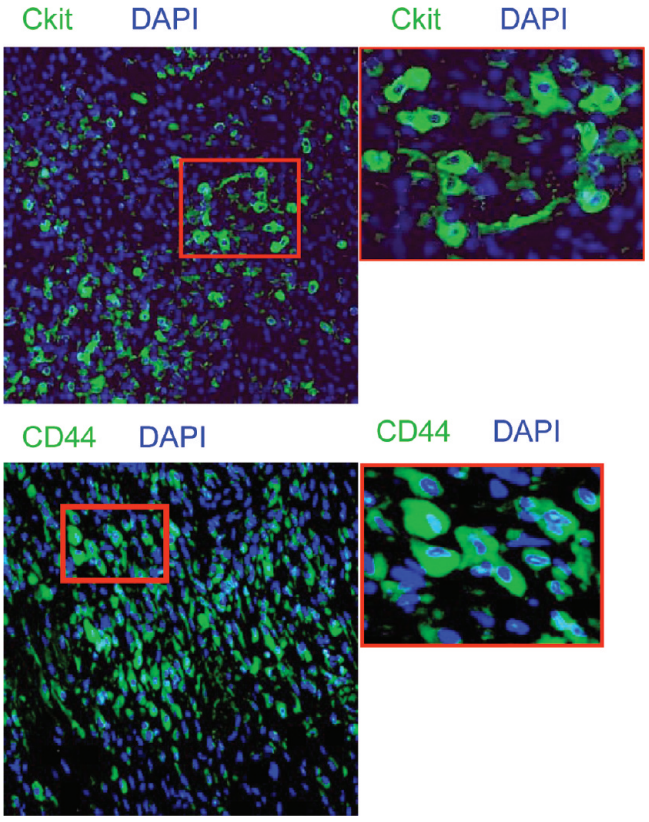

Figure 3. Fluorescence immunostaining of the rat heart tissue sections for ckit and CD44 antigens expression (green) to detect mobilization of stem cells in the infarcted myocardium in response to *ex vivo* transgenic overexpression of a quartet of growth factors including vascular endothelial growth factor, insulin like growth factor-1, stromal cell derived growth factor-1α and hepatocyte growth factor. The animals were euthanized on day 7 after treatment.

fibroblasts to cardiomyocytes and started with a pool of 14 candidate cardiomyocyte-inducing factors.[135] After fine-tuning the cocktail, they carefully winnowed to 3 transcription factors for genetic modification of fibroblasts to induce Gata4, Mef2c, and Tbx5 expression for high efficiency direct induction to become cardiomyocytes. The procedure achieved a remarkable 20% fibroblasts differentiating into cardiomyocytes. In another study Gata4 and Tbx5 combined with Baf60c, a subunit of the Swi/Snf-like BAF chromatin-remodeling complex, successfully differentiated noncardiogenic mouse mesoderm to cardiomyocytes.[136] These data showed that transgenic overexpression of epigenetic modifier may be beneficial for reprogramming. More recently, Ding et al. briefly overexpressed transgenic Oct4, Sox2 and Klf4 in mouse embryonic fibroblast by viral delivery and cultured the cells in a defined solution containing cardiogenic factors.[137] The genetically transformed cells showed expression of cardiac markers such as Mesp1, Nkx2.5, Flk1 and Gata4 during the course of reprogramming.

FUTURE DIRECTIONS

Genetic manipulation of stem cells has advanced our understanding of the basic mechanisms of stem cell survival, proliferation, and differentiation, and senescence while narrowing the gap of translating stem cell-based cardiac repair from the bench to the bedside. Besides, the combinatorial approach of gene therapy and stem cell transplantation has helped to modulate paracrine behavior of stem cells and support their angiomyogenic potential and cardioprotective effects. Stem cell transplantation and gene therapy have always been considered as competitive approaches. However, given the complexity of the disease process in the ischemic heart, none of the two strategies alone can do enough to repair and regenerate the infarcted myocardium. It is therefore prudent to combine these two strategies to exploit the best of these approaches while complimenting their respective

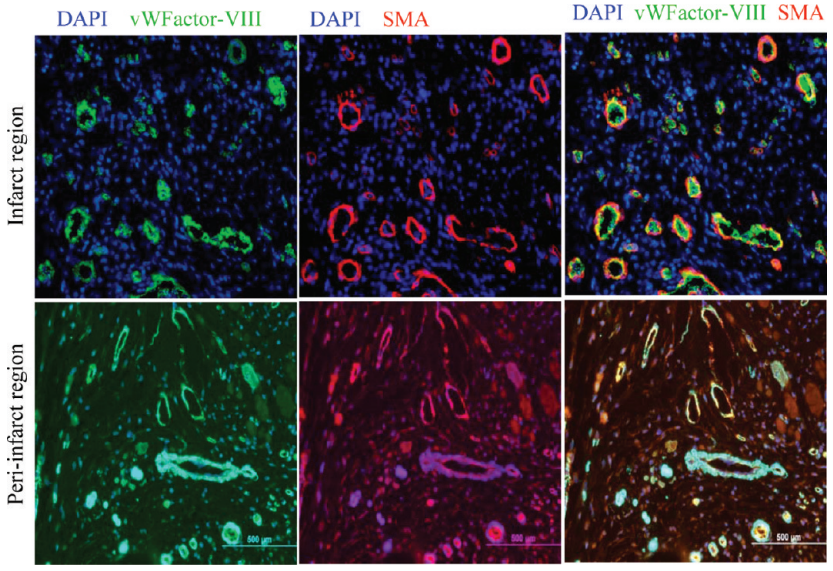

Figure 4. Angiogenesis in infarcted heart and stem cell mobilization. Immunohistological studies on rat heart at 8 weeks after transplantation of SMs overexpressing quartet of growth factors including vascular endothelial growth factor, insulin like growth factor-1, stromal cell derived growth factor-1α and hepatocyte growth factor. The tissue sections were immunostained for vWFactor-VIII (red fluorescence) and smooth muscle actin (green fluorescence) expression to detect angiogenesis and arteriogenesis. Extensive angiogenic response was observed in the animal hearts (both in the infarct as well as peri-infarct regions) which received growth factor expressing SMs as compared to the control animal hearts which received native SMs without growth factor transfection.

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weaknesses. Currently, the required and efficient research tools are available for permanent, reversible, or conditional genetic manipulations of stem cells without altering their stemness characteristics.

Besides the genetic modification approach, the epigenetics of stem cells holds great relevance for stem cell biology and may be alternatively exploited to regulate stem cell functions. Epigenetics deals with heritable changes in gene expression in the cells without alterations in their DNA sequence. However, our understanding and control over epigenetic events (such as chromatin modifications) is still in its infancy and limited. With a deeper insight into epigenetics of stem cells, a strategy based on combined genetic and epigenetic manipulation of stem cells will further refine and improve stem cell behavior post-transplantation. Another latest development is the discovery of very small noncoding post transcriptional regulating RNAs (microRNA) and their critical role as determinants of stem cell functions. MicroRNAs act by translational repression or degradation of their target gene(s).[138] The effect of microRNA manipulation on cells has been exploited from ranging from prodifferentiation to reprogramming to pluripotent status.[139−141] In our recently published study, we have shown that hypoxamir miR-210 was a critical determinant of stem cell survival cultured under lethal anoxia as well as post-transplantation in an experimental model of acute myocardial infarction.[118] We observed that ischemic preconditioning of stem cells initiated survival signaling in which microRNA-210 was critically involved downstream of hypoxia inducible factor-1α activation and microRNA-210 promoted stem cell survival by regulating caspase-8ap2 activity. Our unpublished data clearly demonstrates that the effects of ischemic preconditioning can also be simulated by transfection of microRNA-210 into the stem cells. Transplantation of genetically manipulated stem cells for microRNA-210 expression had salutary effects in terms of preservation of infarcted heart function (our unpublished data). Transgenic overexpression of miR-210 in bone marrow stem cells also improved their survival upon subsequent exposure to lethal anoxia as well as postengraftment in the infarcted heart. In an interesting new development, we have also shown that treatment of stem cells with insulin-like growth factor-1 rather than their genetic modification primed the cells to withstand the rigors of the harsh microenvironment of the ischemic myocardium.[117] The growth factor primed stem cells not only showed extensive survival in the infarcted heart postengraftment, these cells also differentiated to adopt cardiac phenotype and extensively repopulated the infarcted myocardium. While elucidating the mechanism of improved survival, we have shown a critical role of mitochondrial connexin-43 in the survival signaling cascade initiated by growth priming of the stem cells.[142]

In conclusion, a combinatorial approach of stem cell-based therapeutic gene delivery to the heart offers unique advantages over either stem cell transplantation or direct gene transfer into the body. Indeed, the combined stem cell-based gene therapy approach will be important to overcome the limitations and shortcomings of each of these two approaches for better prognosis.

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