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Transplantation of Human Embryonic Stem Cell-Derived Endothelial Cells for Vascular Diseases

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ABSTRACT

Using endothelial cells for therapeutic angiogenesis/vasculogenesis of ischemia diseases has led to exploring human embryonic stem cells (hESCs) as a potentially unlimited source for endothelial progenitor cells. With their capacity for self-renewal and pluripotency, hESCs and their derived endothelial cells (hESC-ECs) may be more advantageous than other endothelial cells obtained from diseased populations. However, hESC-ECs' poor differentiation efficiency and poorly characterized in vivo function after transplantation present significant challenges for their future clinical application. This review will focus on the differentiation pathways of hESCs and their therapeutic potential for vascular diseases, as well as the monitoring of transplanted cells' fate via molecular imaging. Finally, cell enhancement strategies to improve the engraftment efficiency of hESC-ECs will be discussed. J. Cell. Biochem. 106: 194–199, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: HUMAN EMBRYONIC STEM CELLS; ENDOTHELIAL CELLS; DIFFERENTIATION; TRANSPLANTATION; MOLECULAR IMAGING

Cardiovascular disease (CVD) accounts for approximately 30% of all deaths in the United States [Rosamond et al., 2008]. Since CVD is primarily caused by endothelial dysfunction, therapeutic angiogenesis/vasculogenesis holds great promise for a potential cure. The logic behind angiogenesis/vasculogenesis is to improve the spontaneous healing process by supplementation of vascular progenitor cells (VPCs) growth factors [Yang et al., 2004; Li et al., 2007; Yamahara et al., 2008]. VPC transplantation can foster the formation of arterial collaterals and promote the regeneration of damaged tissues. Recently, human embryonic stem cells (hESCs) have generated much interest because of their capacity for self-renewal and pluripotency. In practical terms, hESCs can be cultured indefinitely ex vivo, and can differentiate into virtually any cell type in the adult body [Thomson et al., 1998; Reubinoff et al., 2000]. hESCs are thus an attractive source for the derivation of large numbers of cells to be used in various tissue repair and cell replacement therapies. However, upon transplantation into living organisms, undifferentiated hESCs can spontaneously differentiate into rapidly proliferating teratomas, which are disordered amalgam of all three germs layers. Therefore, safely coaxing hESCs into committed progenitor lineages for therapeutic applications is an innovative and feasible strategy that can minimize the risk of cellular misbehavior and teratoma formation.

The isolation of hESC-derived endothelial cells (hESC-ECs) may have potential therapeutic applications, including cell transplantation for repair of ischemic tissues and tissue-engineered vascular grafts. However, to fully understand the beneficial effects of stem cell therapy, investigators must be able to track the functional biology and physiology of transplanted cells in living subjects over time. At present, most cell therapy protocols are limited by their requirement for histological analysis to determine viable engraftment of the transplanted cells. The development of sensitive, noninvasive technologies to monitor this fundamental engraftment parameter will greatly aid clinical implementation of cell therapy. Moreover, recent research on hESC-based therapy showed poor long-term engraftment of hESC-ECs by serial bioluminescence imaging (BLI) [Li et al., 2008]. Thus, to sustain long-term engraftment of hESC-ECs and realize the full benefits of hESC-EC

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therapies, alternative transplantation protocols with addition of matrix [Cao et al., 2007] or pro-survival factors [Laflamme et al., 2007] to prevent cells death after transplantation must be developed.

ENDOTHELIAL DIFFERENTIATION OF hESC

Due to the limitation of postnatal cell sources and expanding efficiency, endothelial differentiation of hESC provides an alternative source to generate a large supply of healthy, functional cells. hESC-ECs can be generated by two main approaches, spontaneous differentiation of embryoid bodies (EBs) [Levenberg et al., 2002; Wang et al., 2004; Lu et al., 2007; Chen et al., 2007a; Li et al., 2008], also called three-dimensional differentiation (3D), and two-dimensional differentiation (2D) [Kaufman et al., 2001; Wang et al., 2007; Yamahara et al., 2008] (Fig. 1).

For the 3D differentiation, hESCs need to be cultured in low attachment dish for 9–13 days [Levenberg et al., 2002; Wang et al., 2004; Li et al., 2008]. Different mediums have been used with or without growth factors. Whole-mount immunostaining CD31 confirmed that hESCs cultivated as EBs spontaneously differentiated into endothelial cells and formed blood vessel like structure [Levenberg et al., 2002; Li et al., 2008]. Subsequently, EBs were digested into single cells. Differentiated endothelial cells or hemangioblasts were sorted by fluorescence-activated cell sorting (FACS) or magnetic-activated cell separation (MACS). For the 2D differentiation, hESCs were cultured on various mouse fibroblast feeder layers, including mouse embryo fibroblasts (MEF) [Wang et al., 2007], OP9 [Vodyanik et al., 2005; Yamahara et al., 2008], S17 [Kaufman et al., 2001; Vodyanik et al., 2005], MS-5 [Vodyanik et al., 2005], or mouse endothelial cells [Kaufman et al., 2001]. hESCs also can be cultured on collagen IV-coated plates for endothelial differentiation [Gerecht-Nir et al., 2003].

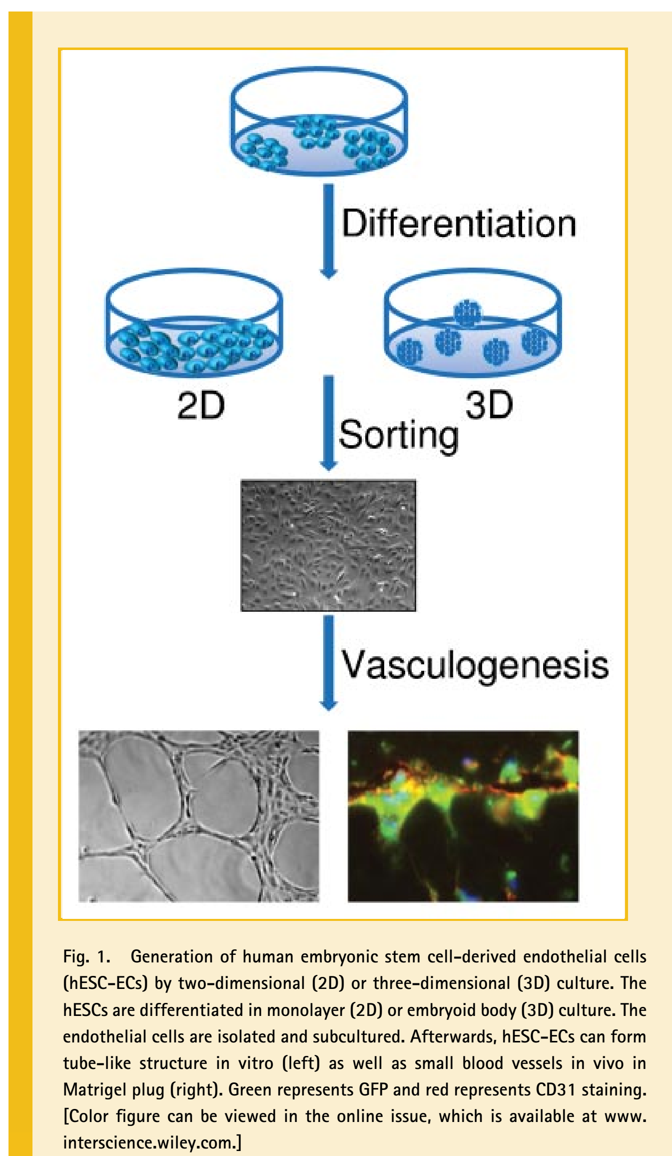

Fig. 1. Generation of human embryonic stem cell-derived endothelial cells (hESC-ECs) by two-dimensional (2D) or three-dimensional (3D) culture. The hESCs are differentiated in monolayer (2D) or embryoid body (3D) culture. The endothelial cells are isolated and subcultured. Afterwards, hESC-ECs can form tube-like structure in vitro (left) as well as small blood vessels in vivo in Matrigel plug (right). Green represents GFP and red represents CD31 staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Both in vitro assays and in vivo transplantation have been used to characterize the hESC-ECs. After in vitro subculturing, these cells express CD31, CD34, Flk-1, VE-cadherin, and vWF. They are capable of DiI-ac-LDL uptake and can generate tube-like structure formation on Matrigel. For in vivo assay, hESC-ECs can form a tube-like structure within Matrigel plug (Fig. 1). Interestingly, postnatal hemangioblast cell markers such as KDR and CD133 are robustly expressed in undifferentiated hESCs [Chen et al., 2007b; Li et al., 2008] and thus are not good markers for endothelial selection of hESC differentiation.

A major challenge of hESC-based therapy is the generation of sufficient numbers of differentiated endothelial cells. The efficiency of endothelial differentiation of the 3D EB system is typically low, ranging from 1% to 3% [Levenberg et al., 2002; Li et al., 2008]. Moreover, it is difficult to get single cells from EBs by enzyme digestion as cell viability is low after this harsh digestion step. Several investigators have introduced modified approaches to bypass the 3D EB formation or serial differentiation procedures to increase differentiation efficiency [Cho et al., 2007; Lu et al., 2007]. Wang et al. [2007] demonstrated that ~10% CD34+ progenitor cells are present by monolayer culturing of hESCs on MEF for 10 days. Lu et al. [2007] developed a two-step differentiation procedure: first, by culturing EBs for 3.5 days, then subculturing EB single cells in methylcellulose for 4–6 days. The team reported that 400 million hES-derived blast cells can be expanded from one 6-well plate of MA01 hESC line, which has hematopoietic and endothelial functions. Cho et al. [2007] introduced a different two-step method by attaching day 9 EBs to dishes, culturing them for 7–9 days, and then mechanically isolating the center region, eventually yielding ~40% cells that are positive for von Willebrand factor (vWF).

To form mature vasculature in vivo, vascular smooth muscle cells and pericytes play critical roles in the structure and functional support of vascular network by stabilizing nascent endothelial vessels during vascular development and blood vessel growth [Hughes, 2008]. Recent studies have demonstrated that VPCs can differentiate into either endothelial cells or smooth muscle cells with the supplementation of vascular endothelial growth factor (VEGF) or platelet-derived growth factor-BB (PDGF-BB), respectively [Sone et al., 2007; Yamahara et al., 2008]. Combined transplantation of hESC-ECs and hESC-derived smooth muscle cells (hESC-SMCs) can augment reparative neovascularization and contribute to newly formed vessels in ischemia model far more effectively than

endothelial cells transplantation alone [Yamahara et al., 2008]. This may represent a major novel strategy for vascular regenerative medicine.

THERAPEUTIC POTENTIAL OF hESC-ECs

Therapeutic angiogenesis/vasculogenesis is a promising option for treating peripheral artery diseases, ischemic heart diseases, and cerebral ischemia. Clinical trials have confirmed that autologous cell therapies using bone marrow-derived or circulating blood-derived progenitor cells are safe and provide beneficial effects [Iba et al., 2002; Tateishi-Yuyama et al., 2002; Huang et al., 2004]. However, impairment of the number and function of patient-derived progenitor cells might limit the efficiency of autologous stem cell therapy [Tepper et al., 2002; Zhou et al., 2006]. Animal experiments have demonstrated promising results of hESC-EC transplantation. For example, hESC-ECs implanted with a mouse mesenchymal precursor cell line (10T1/2) in a fibronectin-collagen gel into the cranial windows of SCID mice could form cord-like networks [Wang et al., 2007]. Following intravenous injection of tetramethylrhodamine-labeled dextran to enhance the contrast of perfused vessels, blood flowing through these engineered vessels was observed, suggesting successful integration into the recipient's vascular tree. In mouse model of hindlimb ischemia, hESC-ECs alone or with hESC-SMCs could improve blood perfusion and limb salvage by facilitating postnatal neovascularization, but adult endothelial cells could not [Yang et al., 2004; Cho et al., 2007; Lu et al., 2007; Sone et al., 2007; Yamahara et al., 2008]. Recently, transplantation of hESC-ECs and mural cells has also been shown to improve therapeutic vascular regeneration and reduce the infarct area after stroke [Oyamada et al., 2008].

These successful animal experiments point to potential applications for hESC-based therapies for various ischemic diseases. However, some issues need to be resolved first, such as animal product contamination and teratoma formation. The majority of hESC lines were generated in the presence of animal serum and animal-derived feeder cells. Such hESC lines that were exposed to animal components should not be used for human therapeutic applications due to the risk of graft rejection and pathogenic transmission from animal sources. Several different types of human feeders, including human adult skin fibroblasts, foreskin fibroblasts, and placental fibroblasts, have been tested with hESC cultures and reported to support prolonged growth of undifferentiated hESCs [Richards et al., 2002; Hovatta et al., 2003; Genbacev et al., 2005]. More recently, a completely defined total animal-free medium TeSR1 has been introduced that does not contain bovine serum albumin (BSA) [Ludwig et al., 2006]. Recent research also demonstrated that poly-D-lysine coating can efficiently support the cell attachment and growth of hESC lines on the culture plate [Harb et al., 2008]. Considering that poly-D-lysine is a widely used synthetic polypeptide that is pathogen-free, inexpensive, and easy to handle, as compared with Matrigel, this new method may provide novel access to the practical animal-free environments critical for hESC-mediated cell therapeutic strategies. Another major risk involving the use of hESCs is the possibility of cell misbehavior following transplantation. This potentially serious complication may occur if any of the transplanted undifferentiated ES cells take on teratoma formation [Li et al., 2007; Li et al., 2008]. Careful and precise protocols for acquiring differentiated cells are needed. However, highly differentiated cells may lose their therapeutic potential, as adult endothelial cells provide no benefits to postnatal neovascularization [Yang et al., 2004; Sone et al., 2007]. Thus, it is a great challenge to identify, characterize, and isolate progenitor populations that will not form teratomas, and yet capable of proliferation and continual differentiation into functional endothelial cells.

IMAGING FATE OF TRANSPLANTED hESC-ECs

For stem cell research to make the next quantum leap, it is imperative to understand the dynamic processes of hESC homing, migration, biodistribution, proliferation, and differentiation in the same subject over time. The development of noninvasive imaging techniques is essential for conducting detailed preclinical studies to optimize the delivery methods and strategies that can enhance cell survival. A number of methods are available to track stem cells by molecular imaging. In general, there are two methods to label the cells: (1) direct labeling method, which physically introduce marker(s) into the cells before transplant; (2) indirect labeling method, which genetically introduce reporter gene(s) into the cells before transplant [Wu et al., 2004].

For direct labeling, detectable probes can be loaded into or attached to the cells during tracking. Examples include labeling with super paramagnetic iron oxide (SPIO) for magnetic resonance (MR) imaging, 18F-fluoro-deoxy-glucose ([^{18}F]-FDG) for positron emission tomography (PET) imaging, [^{111}In]oxine for single-photon emission computerized tomography (SPECT), and quantum dots for fluorescence imaging. These techniques have been used for adult and embryonic stem cell imaging [Zhang and Wu, 2007]. Although direct labeling can be used to evaluate the initial deposition of implanted stem cells, the imaging signals tend to diminish with cell division and proliferation or become undetectable after the decay of radioactive tracers. Another confounding factor with this type of labeling and imaging is that non-viable cells may still generate a robust MR signal, as shown in a recent study comparing iron labeling imaging (direct) versus reporter gene imaging (indirect) [Li et al., 2008].

Indirect imaging such as the reporter gene approach involves inserting reporter gene(s) into stem cells for the purpose of tracking. Products of reporter gene expression generally can be divided into three categories: enzyme-based (e.g., herpes simple virus type 1 thymidine kinase [HSV1-tk] or firefly luciferase [Fluc]), receptor-based (e.g., dopamine type 2 receptor [D2R]), and transporter-based (e.g., sodium-iodide symporter [NIS]). Stable transfection or transduction with reporter genes is useful in assessing kinetic survival status of the implanted cells because the reporter genes can be expressed as long as the cells are alive; the inserted reporter gene(s) can be passed on to daughter cells upon cell division. However, a major disadvantage for future clinical applications of reporter gene approach in cell tracking is the requirement for

genetic manipulations of the cells, which may lead to insertional mutagenesis. Thus, the recent advances in site-specific chromosomal integration mediated by phiC31 integrase may become a useful tool to overcome this obstacle [Thyagarajan et al., 2008].

Among the different reporter gene imaging techniques, BLI is extremely useful because of its high sensitivity, high-throughput screening, and straightforward imaging procedures (Fig. 2). Fluc is a 61-kDa monomeric protein, which reacts with its substrate D-luciferin in the presence of oxygen, Mg^{2+}, and ATP to emit luminescence. The light can be captured by high sensitive CCD camera, such as IVIS imaging system (Caliper Life Sciences, Mountain View, CA). This imaging modality is currently limited to small animal models, because BLI relies on low-energy photons (2–3 eV) that become attenuated within deep tissues. Recent work demonstrated that hESCs can be stably transduced with a lentiviral vector carrying a novel double-fusion reporter gene that consists of firefly luciferase and enhanced green fluorescence protein (Fluc-eGFP) [Li et al., 2008]. Reporter gene expression has shown no significant adverse effects on hESC viability, proliferation, or differentiation into hESC-ECs. After transplantation into the mouse hindlimb, hESC-ECs can be tracked up to 4 week by BLI. Moreover, postmortem histology can be used to confirm engraftment of transplanted hESC-ECs based on GFP immunostaining. Finally, the reporter gene approach may also permit longitudinal monitoring of endothelial differentiation process. Proof-of-principle has been demonstrated with the vascular endothelial cadherin (VE-cadherin) promoter driving the GFP reporter gene in mouse ESCs in vitro [Peiffer et al., 2007]. With further investigation, it may be possible to monitor hESC-EC differentiation in living animals by using either bioluminescence (Fluc) or PET (HSV1-tk) reporter genes.

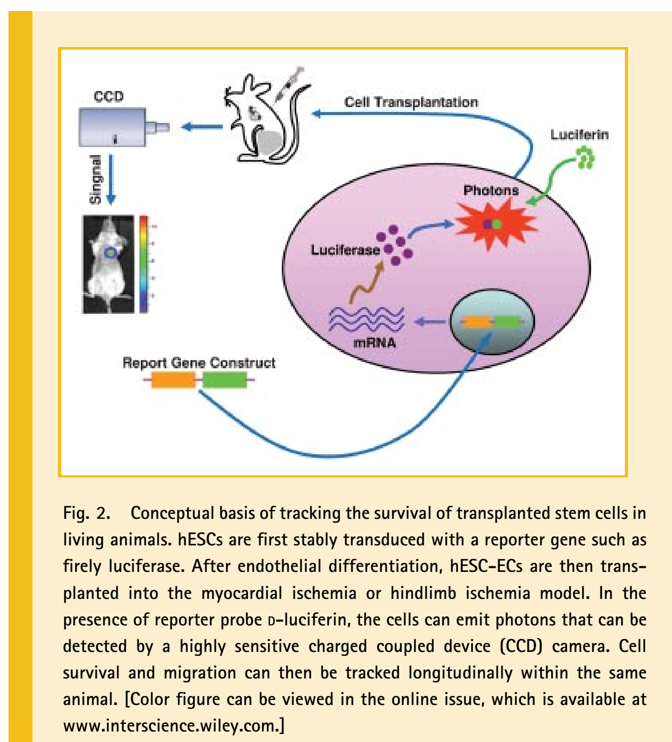

Fig. 2. Conceptual basis of tracking the survival of transplanted stem cells in living animals. hESCs are first stably transduced with a reporter gene such as firely luciferase. After endothelial differentiation, hESC-ECs are then transplanted into the myocardial ischemia or hindlimb ischemia model. In the presence of reporter probe D-luciferin, the cells can emit photons that can be detected by a highly sensitive charged coupled device (CCD) camera. Cell survival and migration can then be tracked longitudinally within the same animal. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CELL-ENHANCEMENT STRATEGIES FOR hESC-EC THERAPY

Stem cell therapy is a promising option for the treatment of CVDs. However, cell therapy is potentially limited by its low rate of engraftment in the ischemic tissue. The pattern of acute donor cell death has been seen in previous studies of neonatal cardiomyocytes [Zhang et al., 2001], mesenchymal stem cells [van der Bogt et al., 2008], bone marrow mononuclear cells [Sheikh et al., 2007], and hESC-derived cardiomyocytes [van Laake et al., 2007; Cao et al., 2008]. Our recent BLI data on transplantation of endothelial cells derived from mouse [Li et al., 2007] and human [Li et al., 2008] ESCs also revealed only 1.5–2.0% survival after 4–8 weeks. The exact pathway leading to acute donor cell death following transplantation is still unknown, but the lack of matrix support, ischemia, and inflammation all probably play major roles. The role of cell anchorage for cell survival is critically important. For example, when cells that normally grow in attachment are kept in suspension, a pathway of cell death called anoikis (Greek: "state of homelessness"), a form of apoptosis, is initiated [Grossmann, 2002; Robey et al., 2008]. Traditional cell preparations for injection involve enzymatically dispersed cells suspended in a protein-free medium, stored for minutes to hours on ice. During this period, important adhesion-related survival signals could be absent and not reinitiated for many hours until the cells find themselves in the context of a recipient tissue; even then, the proper basal surface for the cells may not be present. In addition, injected cells tend to form various sized clumps, so diffusion is the only source of nutrient transport until angiogenesis can provide a vasculature. The ischemia would be exacerbated in the context of host tissue ischemia, for example, following myocardial infarction. The injured tissue is also a highly inflamed environment. In particular, neutrophils are recruited and can produce oxygen-derived free radicals and inflammatory cytokines that can directly damage graft and initiate cell apoptosis [Robey et al., 2008].

Because of these obstacles, the development of a strategy to alleviate apoptotic cell death may be of primary importance for hESC-EC therapy. Enhancing supportive niche function during transplantation could provide a novel and effective strategy for engraftment or accelerate stem cell differentiation, and perhaps reduce the number of stem cells needed for effective tissue reconstitution, as well as promote stem cell self-renewal. Appropriate modifications of signals from the niche could promote stem cell differentiation to favor production of a needed cell type and to recruit host stem cells for tissue regeneration. To counteract the major pathways of cell death after transplantation, two general strategies can be employed. The first is by targeting a specific molecular pathway, for example, through expression of an anti-apoptotic protein or blocking the caspase activation cascade. The second is by inducing a broader spectrum of cytoprotective state, for example, through preconditioning or cell matrix co-transplantation. Figure 3 illustrates the different strategies that may be used to enhance hESC-EC therapy for ischemia heart diseases.

Recent work on hESC-derived cardiomyocyte therapy revealed that Matrigel combined with pro-survival growth factor cocktail, including a cell-permeant peptide from Bcl-XL to block mitochon-

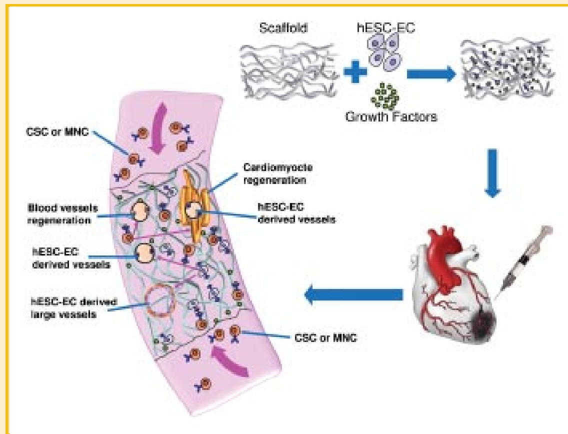

Fig. 3. A combination strategy for increasing the engraftment of hESC-ECs by biomatrix and growth factors. This schematic depicts a composite of injectable and biodegradable scaffolds with hESC-ECs and pro-survival growth factors. The hESC-ECs can differentiate into blood vessels with pericyte. The growth factors can promote cardiac stem cells (CSC) from the peri-infarction region or bone marrow mononuclear cells (MNC) in the circulation to migrate into the infarcted zone to regenerate cardiomyocytes or blood vessels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

drial death pathways, cyclosporine A to attenuate cyclophilin D-dependent mitochondrial pathways, a compound that opens ATP dependent K^+ channels (pinacidil) to mimic ischemic preconditioning, and IGF-1 to activate Akt pathways and the caspase inhibitor ZVAD-fmk, resulted in reliable formation of substantial myocardial grafts and significant functional improvement [Laflamme et al., 2007]. In contrast, single interventions had little impact on engraftment and no impact on cardiac function. Matrigel was shown to make an independent contribution in this research, suggesting anoikis is a significant initiator of cell death. Another study revealed that the combined transplantation of human VPC-derived endothelial cells and mural cells could markedly induce vascular regeneration, compared to the single fraction transplantation of VPC-derived vascular cells (endothelial cells or mural cells alone) [Yamahara et al., 2008]. For hESC-EC therapy, several remedial approaches could be introduced to overcome the menace of apoptotic cell death, including co-delivery of angiogenic genes [Xie et al., 2007], preconditioning by hypoxia exposure before transplantation, and suspension in the various biomatrices to prevent anoikis. Each approach might offer distinct advantages in terms of efficacy, but combined approaches will require further optimization in the future.

SUMMARY

In summary, pluripotent hESCs represent a potentially unlimited source of cells for regenerative medicine. With improved differentiation yield and purity, hESC-ECs could provide a continual source of endothelial cells for treatment of myocardial ischemia, cerebrovascular disease, and peripheral vascular disease. Optimization of cell engraftment conditions with a special emphasis on cell survival strategies may significantly enhance the functional outcome of the procedure. The optimization of these techniques then can be monitored in a high-throughput fashion using the molecular imaging techniques described above.

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