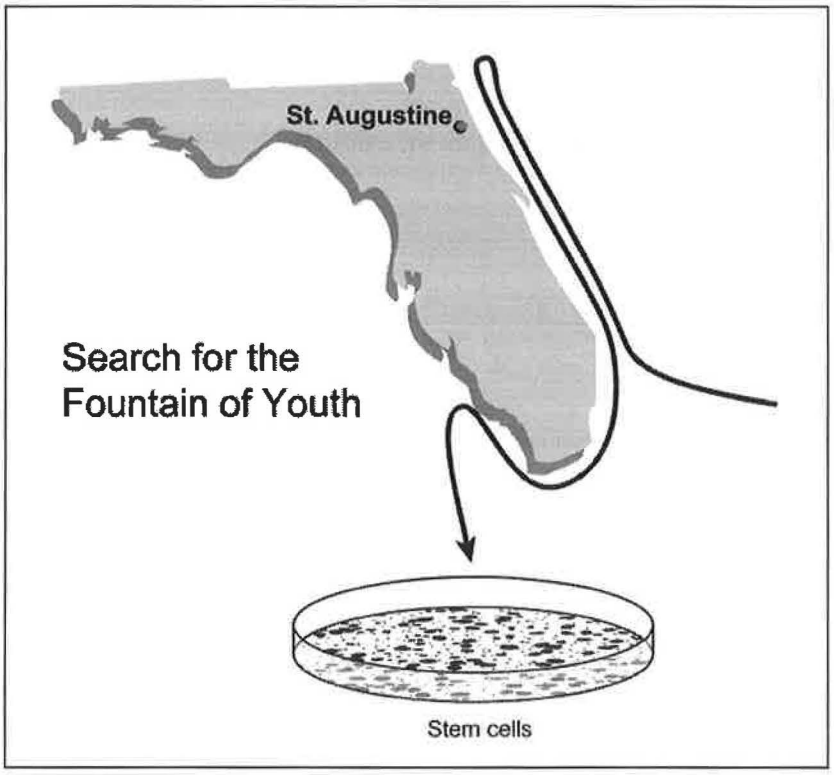
Ponce de Leon's fountain: stem cells and the regenerating heart

Daniel J. Garry, M.D., Ph.D.
Internal Medicine Grand Rounds
University of Texas Southwestern Medical Center at Dallas
February 12, 2004



This is to acknowledge that Dr. Garry has not disclosed any financial interests or other relationships with commercial concerns related directly to this program. Dr. Garry will not be discussing off-label uses in his presentation.

Biographical information:

Daniel J. Garry, M.D., Ph.D.
Assistant Professor
Departments of Internal Medicine and
Molecular Biology
UT Southwestern Medical Center

Interests:

Congestive Heart Failure and Cardiac Transplantation
Stem cell biology, Tissue regeneration and Oxygen metabolism

An age of discovery. As a Spanish explorer, Juan Ponce de Leon (1460-1521) arrived in America with Columbus on his second voyage in 1493. Ponce de Leon later conquered and became governor of Puerto Rico. According to the legend, the natives of Puerto Rico told the explorers that in Bimini, a land to the north, there was a river, spring or fountain where waters had such miraculous curative powers that any elderly person who bathed in them would regain their youth. This legend was not new as Alexander the Great supposedly searched for such waters in eastern Asia and similarly the Polynesians searched for the fountain of perpetual youth in Hawaii. Equipped with three ships, Ponce de Leon set out in search of the fountain of youth and on April 2, 1513 he landed on the eastern coast of present day Florida (Figure 1). Either because the discovery was made during the Easter season or because the coast was lined with flowering magnolia trees he named the country, La Florida (feast of flowers). Despite his searches, Ponce de Leon never found the fountain of youth or secured his immortal youth but he secured recognition as the discoverer of Florida.

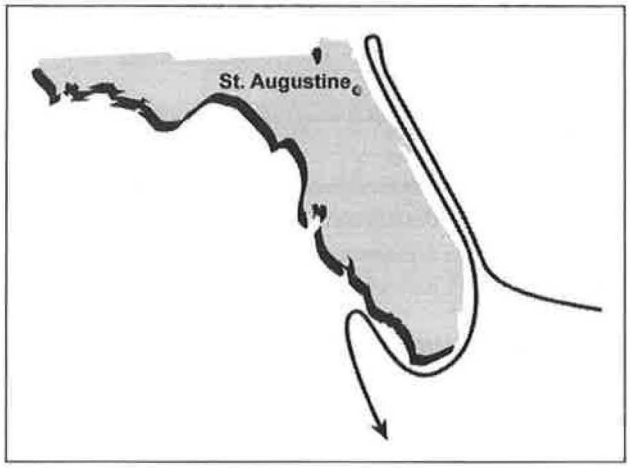


Figure 1. Ponce de Leon's search for the Fountain of Youth.

Today the search for the "fountain of youth" continues and the navigational course includes multiple products directed toward the aging population and those with chronic diseases.

Advanced heart failure is a deadly disorder. Nearly 5 million Americans currently have heart failure with more than 250,000 new cases diagnosed each year in this country (1-3). This disease is costly as it captures more than 250,000 lives each year and accounts for more than a $30 billion dollar annual expenditure for our society (1,3). Pharmacological therapies have decreased the morbidity/mortality associated with this disease but the only definitive therapy for advanced heart failure remains orthotopic heart transplantation (Figure 2).

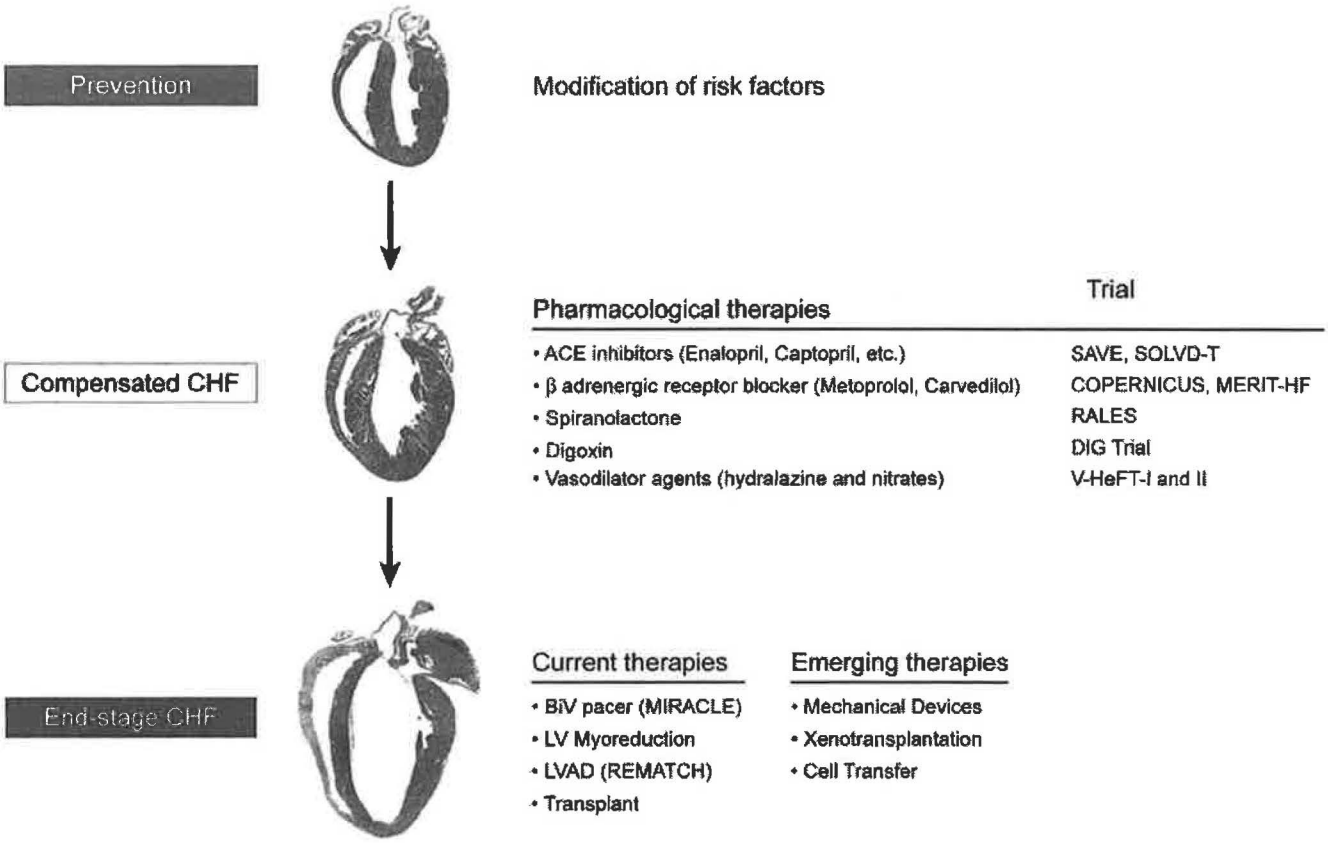


Figure 2. Current and emerging therapies for the treatment of advanced heart failure.

Inadequate organ availability remains the major factor that limits the number of cardiac transplantation procedures performed each year (1-3). For example, 2,200 – 2,500 heart transplant procedures are performed each year, but estimates suggest that more than 60,000 patients would benefit from the procedure each year (1,3). Therefore, there is a disparity between those patients that need such a lifesaving procedure and those that receive such a procedure. At UT Southwestern Medical Center we have performed 308 orthotopic heart transplants with one, five and ten year survival rates of 91%, 81% and 67%, respectively (Figure 3).

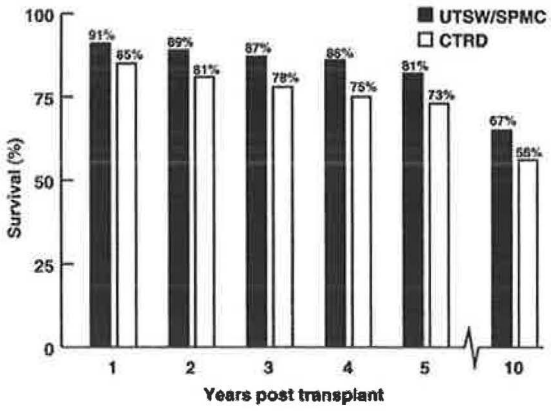


Figure 3. Orthotopic heart transplant survival.
UTSW/University Hospital vs. the Cardiac Transplant Research Database (CTRD) (n = 7,283).

Additionally, we utilize mechanical devices as a bridge to transplantation (42 left ventricular assist devices have been implanted at our center) and we have recently been designated a Medicare approved site for left ventricular assist device (LVAD) destination therapy. Our center was one of 20 institutions that participated in the REMATCH Trial that examined the utility of the LVAD as a permanent form (i.e. destination therapy) of circulatory support (Figure 4C). We currently follow 212 post-transplant recipients in our outpatient transplant clinic.

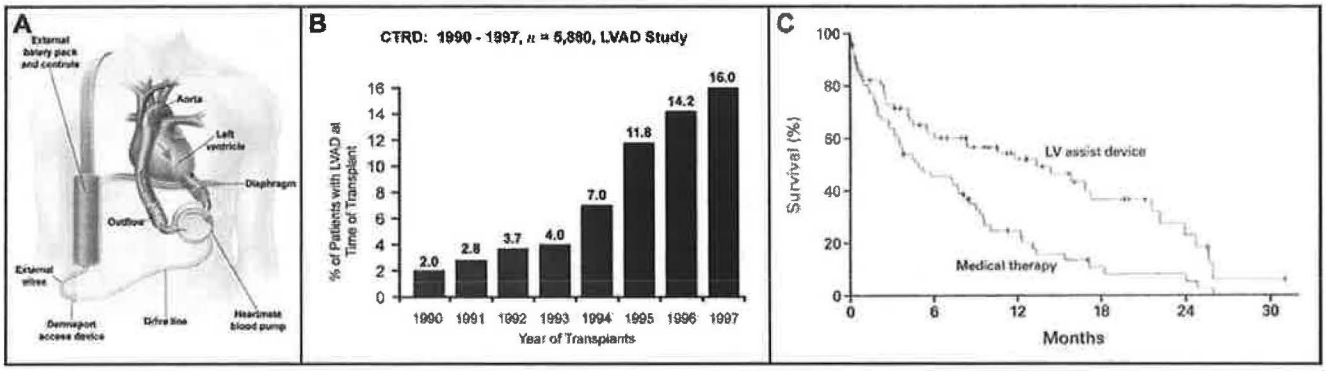
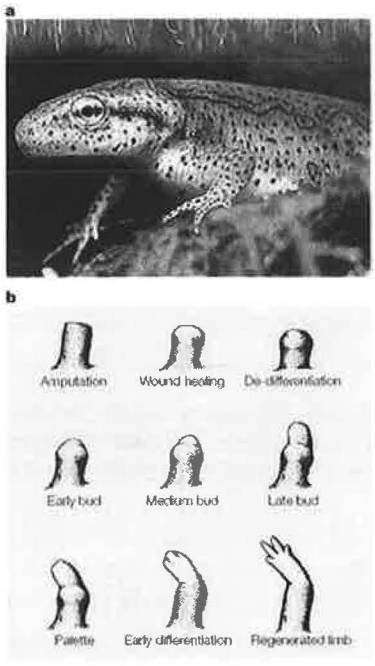


Figure 4. Utilization of a Left Ventricular Assist Device (LVAD) in advanced heart failure. (A) A schematic outlining the placement of the LVAD with the inflow cannula inserted into the apex of the LV and the outflow cannula anastomosed to the ascending aorta. (B) Middle panel reveals the percentage of patients supported with an LVAD as a bridge to transplant. (C) Survival of patients with LVAD vs. medical therapy (REMATCH Trial) (references 4-6).

Limited availability of donor organs for use in orthotopic heart transplantation has prompted the examination of alternative therapies including stem cell based therapies. We will review the current knowledge of regenerative models, stem cell populations and introduce recent studies focused on resident and extracardiac stem cells and their applications for the treatment of advanced heart failure.



Champions of regeneration. In most vertebrates, the regenerative capacity is limited to few tissues including skin, bone, skeletal muscle, hematopoietic or liver. Regeneration of these tissues largely recapitulate the embryonic differentiation from multipotential stem cells. In skeletal muscle, a subpopulation of stem cells are established during embryogenesis for use during postnatal growth and regeneration. The titans or champions of tissue regeneration include amphibians (salamander or newt) that are capable of limb, tail, jaw, retina and heart regeneration (Figure 5) (7-9). This regenerative capacity involves the formation of a local growth zone or blastema of undifferentiated cells (7-9). It is unclear whether blastema formation involves cellular dedifferentiation, the activation of quiescent stem cells or both processes. Nevertheless, discrete cellular mechanisms result in restoration of a complete body plan.

Figure 5. Urodele (newts & salamanders) limb regeneration. a) The red spotted newt. b) Stages of limb regeneration in the newt (reference 7).

Zebrafish (*Danio rerio*) a teleost fish is a well-described genetic model that has been utilized extensively by developmental biologists (Figure 6). The zebrafish is also capable of regenerating multiple structures including fins, scales, heart and spinal cord (10-11). Comparable to the amphibian models, the hallmark of fin or cardiac regeneration is the formation of a blastema (Figure 6C-6E) (11). Unlike the amphibian models, the zebrafish regenerative model is amenable to future genetic screens to identify a regenerative molecular program.

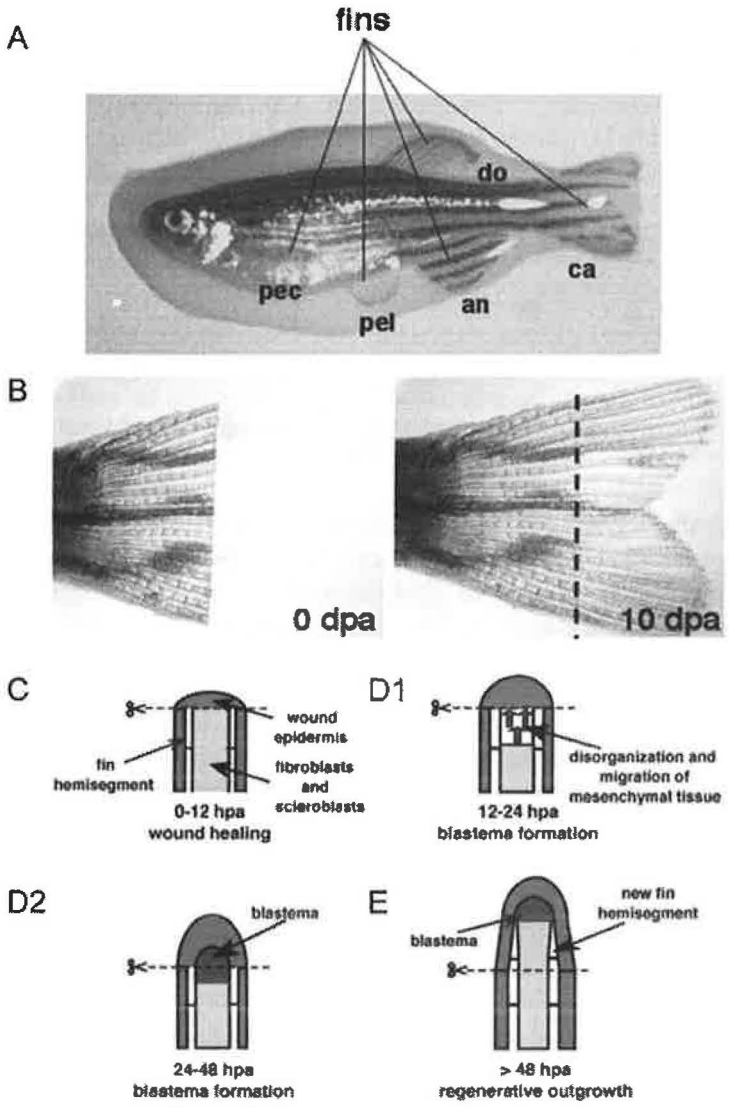


Figure 6. Stages of fin regeneration in zebrafish. A) The adult zebrafish is approximately 3 cm in length and has 5 different fins all capable of regeneration. B) The caudal (ca) fin is capable of regeneration within approximately 10-14 days. (C-E) Discrete stages of fin regeneration (hpa, hours post-amputation) are dependent on the formation of the blastema (11).

Regeneration of the injured adult mouse heart. Wound repair in most vertebrate organs is dominated by a fibroproliferative response that produces a fibrotic scar. By contrast the MRL/MpJ (MRL) mouse strain is capable of ear hole closure with minimal scar formation (12). The ear hole closure is considered to be a result of regeneration and not wound repair because there is the replacement of multiple tissues (i.e. skin, hair follicles, cartilage, sebaceous glands, skeletal muscle, etc.) and there is complete healing. This reparative process has been associated with the formation of a blastema-like structure (a mass of mesenchymal cells from which new tissue is differentiated) and increased vascularization (12). Recent studies suggest that other tissues in the adult MRL mouse may be capable of regeneration including the heart (12,13). Studies undertaken in our laboratory verify the regenerative capacity of ear hole closure in the MRL adult mouse (Figure 7A-7D). In addition, restoration of myocardial function is observed in either the ischemic or cryoinjured MRL mouse heart with decreased scar formation and evidence of myocardiocyte proliferation two months following injury. Moreover, BrdU positive cardiomyocytes are evident in the MRL heart 2 months following myocardial injury suggesting that proliferating stem cells participate in myocardial regeneration. In contrast, the C57BL/6 control mice have persistent myocardial left ventricular dysfunction and evidence of fibrosis (i.e. scar formation) (7E and 7G). Future studies will focus on the definition of the molecular program associated with myocardial regeneration in the zebrafish and mouse models.

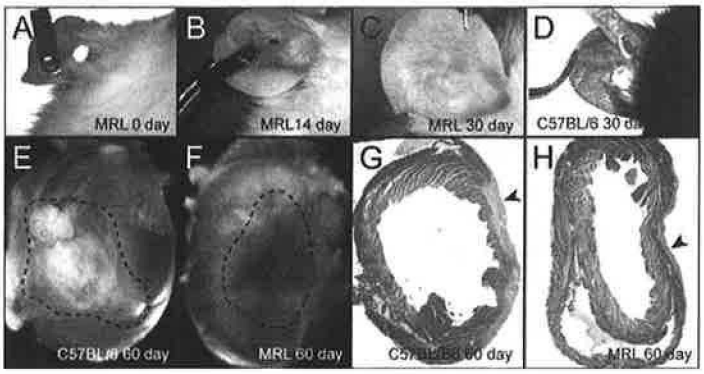


Figure 7. Earhole closure and myocardial regeneration in the injured MRL adult mouse. (A-D) MRL mice are capable of ear hole closure (regeneration) within a 1-month period in contrast to the C57BL/6 mouse, which are not capable of ear hole closure (D). Two months following a myocardial cryoinjury, a fibrotic scar is evident in the C57BL/6 mouse (E and G) but only a highly vascular blastema-like region is evident in the MRL mouse (F and H).

The regenerative response in these animal models is most likely due to either re-entry of cardiomyocytes to the cell cycle or activation of stem cell populations. Two distinct stem cell populations include embryonic and somatic (adult) stem cells.

Embryonic stem cells. Stem cells are characterized by an unlimited proliferative potential, the ability for self-renewal and the capacity to contribute to multiple lineages (multipotency) (14,15). In mammalian systems, total multipotency (totipotency) is limited to the fertilized egg and to the daughter cells of the first few cleavage divisions following fertilization. The embryonic stem (ES) cell represents a pluripotent stem cell that has contributed to our understanding of human disease and stem cell biology.

In 1981, embryonic stem cells were first isolated from the inner cell mass of the 3.5 day mouse blastocyst (16,17). The inner cell mass of the blastocyst contributes to the embryo proper and consists of a transient, pluripotent pool of cells, which are capable of generating derivatives of all three germ layers (endoderm, mesoderm and ectoderm). The genesis of an immortalized pool of embryonic stem cells that maintain a normal karyotype was possible following the culture of the inner cell mass in the

presence of leukemia inhibitory factor (LIF) (14,15,18). Under normal conditions, ES cells are co-cultured with a mitotically inactive (i.e. gamma irradiated) feeder cell layer (primary mouse embryonic fibroblasts or STO fibroblasts) (18,19). These feeder cell layers provide growth factors and chemokines such as LIF, which maintain the ES cells in an undifferentiated, proliferative state. Early studies established that the embryonic stem cells had an unlimited proliferative capacity and were pluripotent but not totipotent as they contributed to the trophectoderm or primitive endoderm lineages only at very low frequencies following injection into host blastocysts (20). The pluripotency of the ES cell allowed for the generation of animals homozygous for specific genetic disruptions (i.e. the generation of knockout mice). Homologous recombination to selectively mutate a gene locus in embryonic stem cells was first described in 1987 (21-23). Subsequently, manipulated (i.e. targeted) ES cells were injected into blastocysts to ultimately yield animals bearing germline mutations capable of transmission to their progeny.

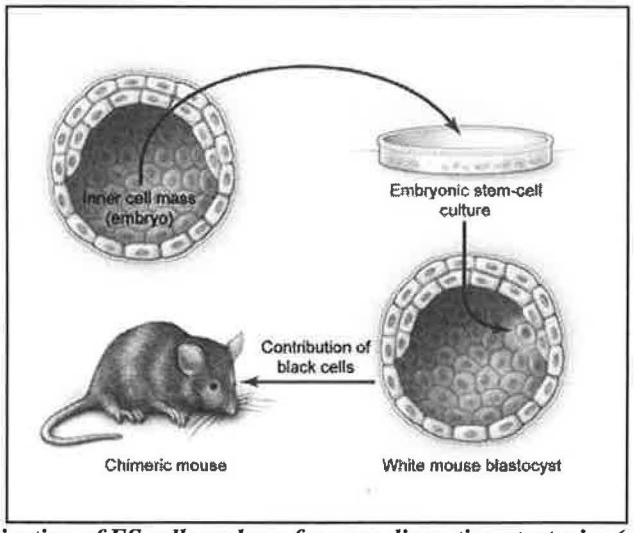


Figure 8. Derivation of ES cells and use for gene disruption strategies (gene knockout) (63).

Human embryonic stem cells. The isolation, characterization and use of murine embryonic stem cells to produce targeted gene disruption and ultimately mutant (knockout) mice provided much of the technology for the isolation of human embryonic stem (HES) cells in 1998 (24). Since then approximately 72 cell lines have been generated for research and therapeutic applications that meet the eligibility criteria for federal funding as outlined in the HES Cell Registry (http://escr.nih.gov) (Figure 9). HES cells, like their murine counterparts, have an unlimited proliferative capacity *in vitro* with retention of a normal karyotype, have high telomerase activity and are capable of forming derivatives of all three embryonic germ layers when injected into immunologically deficient (SCID) mice (24). Furthermore, homologous recombination of selected genes and sustained transgene expression using lentiviral vectors have been successfully undertaken using HES (25,26). Most HES cell lines have been propagated on non-human feeder layers limiting their use for therapeutic applications due to the risk of zoonosis (infections transmitted from non-human species to humans). Recently, Richards et al., reported that the use of human fetal fibroblast feeders for the propagation of HES cells resulted in persistent proliferation and retention of the pluripotency and genetic stability (19,27). While these advances promote enthusiasm for cell transfer strategies, further studies are necessary to comprehensively characterize the plasticity and the molecular signature of the HES cells and fully define the similarities and differences compared to murine ES cells.

STEM CELL RESEARCH: WHAT THE U.S. WILL PAY FOR

The National Institutes of Health has reported 72 lines that may be available for federally financed research. President Bush has said that the government will finance research on existing lines, but not new ones.

LOCATION	INSTITUTION	STEM CELL LINES
Gotenborg, Sweden	Gotenborg University	●●●●● ●●●●● ●●●●● ●●●●
San Diego	CyThera Inc.	●●●●● ●●●●
Menlo Park, Calif.	Geron Corporation	●●●●● ●●●●
Bombay	Reliance Life Sciences	●●●●● ●●
Melbourne, Australia	ES Cell International	●●●●● ●●
Stockholm	Karolinska Institute	●●●●● ●
Madison, Wis.	Wisconsin Alumni Research Foundation	●●●●● ●
Haifa, Israel	Technicon University	●●●●
Athens, Ga.	BresaGen, Inc.	●●●●
Bangalore, India	National Center for Biological Sciences	●●●
San Francisco	University of California	●●

Figure 9. Availability of Human Embryonic Stem Cell Lines [as outlined in the HES Cell Registry (http://escr.nih.gov)].

Adult (somatic) stem cells for the maintenance and regeneration of tissues. While diversification of cell types is largely complete at or shortly after birth, many tissues in the adult undergo self-renewal in response to normal use or acute injury and, accordingly, must establish a lifelong population of somatic stem cells for the maintenance and regeneration of tissues (14,15,19,28). A number of somatic tissue-specific stem cells have been identified including myogenic (satellite stem cells), epidermal (crypt or intestinal), neuronal (nestin-positive cells), hepatic (oval cells) and hematopoietic stem cells (14,15,19,28,29). In contrast to ES cells, these tissue-specific stem cells often exhibit pronounced, but not unlimited, proliferative capability and an ill-defined multilineage potential (Figure 10).

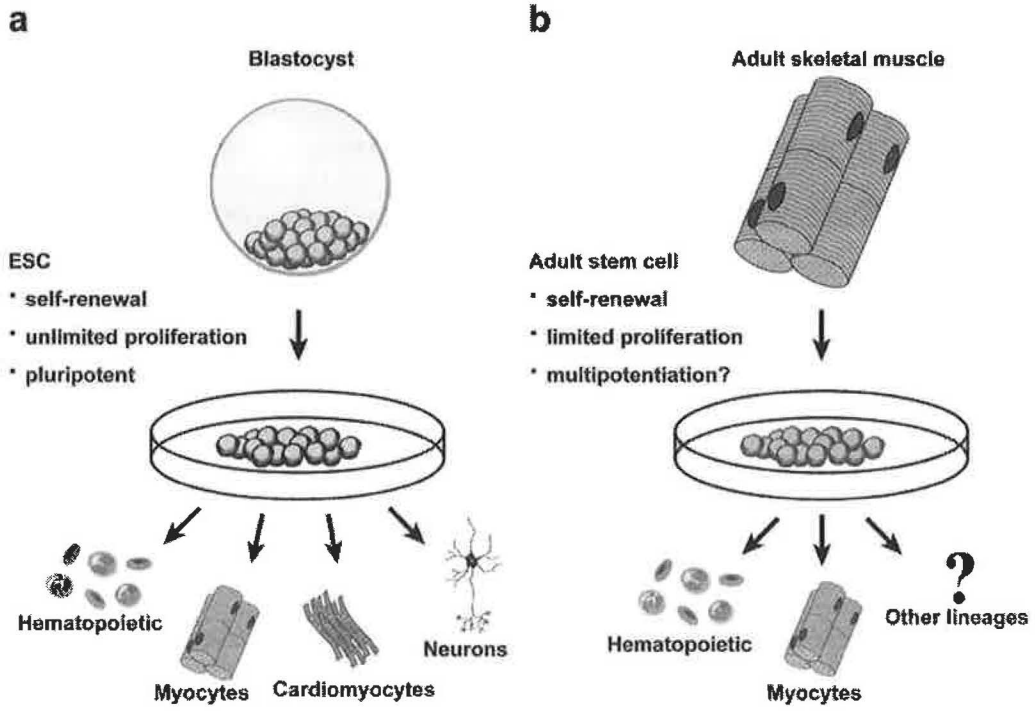


Figure 10. Schematic highlighting similarities and differences of embryonic and somatic (adult) stem cell populations (19).

The most intensely studied adult somatic stem is the hematopoietic stem cell (HSC). The molecular pathways that regulate hematopoietic self-renewal and differentiation have been elegantly and extensively characterized (15,30). These studies have defined the molecular distinctions between HSCs, their progeny, and their progenitors, and have identified the varied growth factors, extracellular matrix ligands, transcriptional regulators, and cell surface proteins necessary for HSCs to interact within their niches for correct differentiation. Several key features of hematopoietic stem cell regulation may be instructive for the general study of somatic stem cells. First, is the linear restriction model of stem cell differentiation (15,30). In this model, a pluripotent stem cell exhibiting transcriptional and phenotypic promiscuity becomes sequentially restricted to a particular fate through the hierarchical activity of specific cytokines and the activation of lineage-specific transcriptional regulators (Figure 11). Other somatic stem cell types follow this basic model during ontogeny.

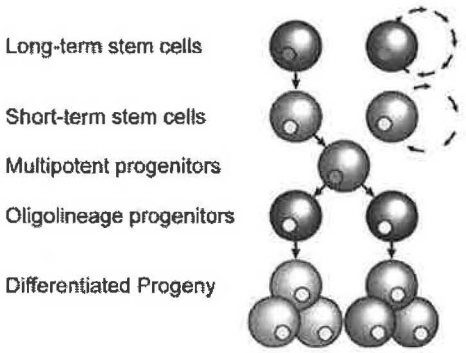
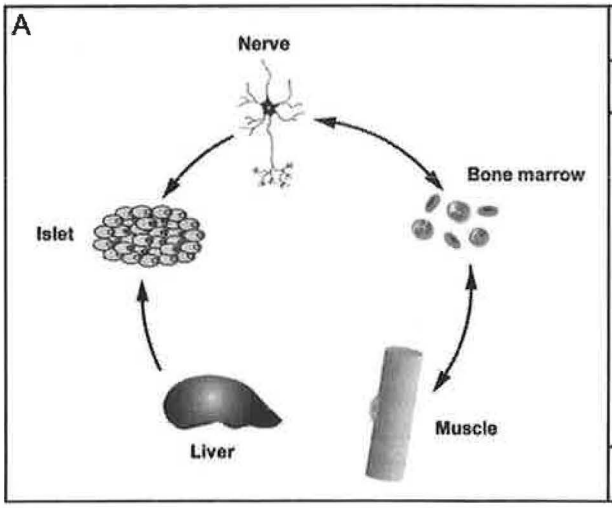


Figure 11. Paradigm for hematopoietic stem cell and progenitor cell lineages. Hematopoietic stem cells are capable of self-renewal and differentiation but differ in the duration of their lifespan (long-term or long-lived vs. short-term or short-lived) as well as their multipotency (long-term stem cells are able to reconstitute the entire organism vs. oligolineage or unilineage progenitor cells that are more limited in their potential) (15).

A second feature of HSC regulation that may have broad application to stem cell biology is the importance of the interaction between stem cells and their environment, or niche (Figure 12). In other stem cell populations, several publications describe studies in which stem cells isolated from an adult organ and then incorporated into blastocysts or injected into irradiated adult animals were able to contribute to multiple lineages *in vivo*, including lineages derived from a different embryonic germ layer than the donor cell (31-39). These studies underscore the importance of milieu in determining stem cell fate. A striking example of this type of study is described by Clarke et al., in which lacZ-expressing neural stem cells derived from the adult brain of Rosa26 mice were found to contribute to derivatives of all three germ layers in chimeric mouse embryos (34). Although the number of embryos containing lacZ-positive cells was low (12% for blastocyst injection of cells), the number of tissues displaying chimerism in positive embryos was extensive, including the heart, somites, mesonephric mesenchyme, and the gastrointestinal tract epithelium (34). The applicability of this type of strategy to human disease has been tested in several mouse models. One such study demonstrated the potential of HSCs to contribute to cardiac repair and functional recovery in a model of myocardial infarction (Figure 12) (38).



B **Potential Plasticity of Stem cells***

Location of Stem Cell	Type of Cells Generated
Brain	Neurons, oligodendrites, skeletal muscle, blood cells
Bone marrow	Endothelial cells, blood cells, cartilage, bone, adipocytes, cardiac muscle, skeletal muscle, neuronal cells, skin, oval cells, gastrointestinal tract cells, thymus, pulmonary epithelial cells
Skeletal muscle	Skeletal muscle, bone, cartilage, fat, smooth muscle
Myocardium	Myocytes, endothelial cells
Skin	Keratinocytes
Liver	Liver cells
Testis and ovaries	Gonads
Pancreatic ducts	Islet cells
Fatty tissue	Fat, muscle, cartilage, bone

* Data are adapted from Kuehnle and Goodell and Tsonis.

Figure 12. Potential plasticity of somatic (adult) stem cells. Many adult tissues have a resident stem cell population that function in maintenance and regeneration of the respective tissues. Recent studies suggest that these adult stem cells may be multipotential when exposed to a permissive environment (reference 63).

These and other cell transfer studies present evidence for differentiation of adult somatic stem cell into cell types of varied lineages, presumably in response to environmental cues or by having the capacity for dedifferentiation (Figure 12) (40). Several caveats should be mentioned in interpreting this data, however. Two recent studies demonstrated the potential for cell fusion between adult stem cells (HSCs, neural stem cells) and ES cells under certain culture conditions (41,42). While the possibility of such fusion events occurring *in vivo* is unlikely, these results do raise concerns regarding systems in which cells are co-cultured prior to injection into host animals. Additionally, many in the field have called for more stringent definitions of donor cell populations and for rigorous functional assessment of engrafted cells in order to more clearly assess cell transfer results (43). Adding to the controversy regarding the results of cell transfer studies are the apparent contradictions between studies that use slightly different methods to explore the same biological question (44,45). Conflicting results may be explained by slight methodological differences that affect both the state of the host niche (the age of animals used or the presence of injury) and the transcriptional accessibility of the donor cell (the criteria used for stem cell isolation). One possible model to reconcile the lineage restriction of adult somatic stem cells with apparent translineage potential demonstrated *in vivo* would invoke the presence of subpopulations within the adult stem cell compartment that are inherently more transcriptionally accessible or niche-responsive than the parent population. A candidate cell population with this type of activity is the SP cell.

Preliminary evidence suggests that the adult heart is capable of repair and limited regeneration. Virtually all somatic postnatal tissues have a resident stem cell population that function in the maintenance and regeneration of the respective tissues (14,15,28). Unlike these postnatal tissues, no such stem cell population has been previously identified in the heart. Due to the absence of a defined cardiac stem cell population and the progression of heart failure, the accepted paradigm predicted that the heart (unlike all other tissues) is incapable of regeneration. Less than a decade ago, the dogma was that the brain and spinal cord were also incapable of repair or regeneration. Therefore, prior therapies

for degenerative neuronal disorders were directed toward limiting the degree of damage. Within the past several years neural stem cells have been successfully isolated from the adult brain and have been shown to participate in neurogenesis following injury (29,33,34,43). Despite the presence of this well documented neural stem cell population in the adult brain, the endogenous repair response is inadequate for replacing lost function. Therefore, present efforts are directed toward the promotion or mobilization of resident neural stem cells to participate in the repair process following a neural injury or insult. *We hypothesize that the postnatal heart (like the brain) has a resident stem cell population that is capable of limited repair but may be inadequate for complete restitution of function following a severe injury.*

Using a mouse model with a labeled bone marrow (SP cells isolated from the ROSA26 mouse, which constitutively express β-galactosidase, were subsequently engrafted in a lethally irradiated mouse), Jackson et al. reported that bone marrow stem cells were able to participate in limited cardiac muscle regeneration following an ischemic insult (46). Recent studies further suggest that myocardial regeneration occurs in the injured human adult heart. Using fluorescent *in situ* hybridization (FISH) techniques to identify Y chromosome positive cells, previous studies have reported the presence of Y chromosome positive myocardiocytes in postmortem specimens of female-to-male adult human cardiac transplants (47-49). While considerable controversy exists regarding the frequency of extracardiac stem cells, which migrate and repopulate the myocardium in the adult (47-49), these results suggest that extracardiac stem cells give rise to cardiomyocytes to participate in myocardial repair. Data from our laboratory (50,51) in addition to others (52) have defined stem cell populations that are resident in the adult heart that are capable of forming cardiomyocytes. We examined the molecular program of the cardiac stem cells in the developing heart as a strategy to identify a cardiac stem cell population that is resident in the adult heart (Figure 13).

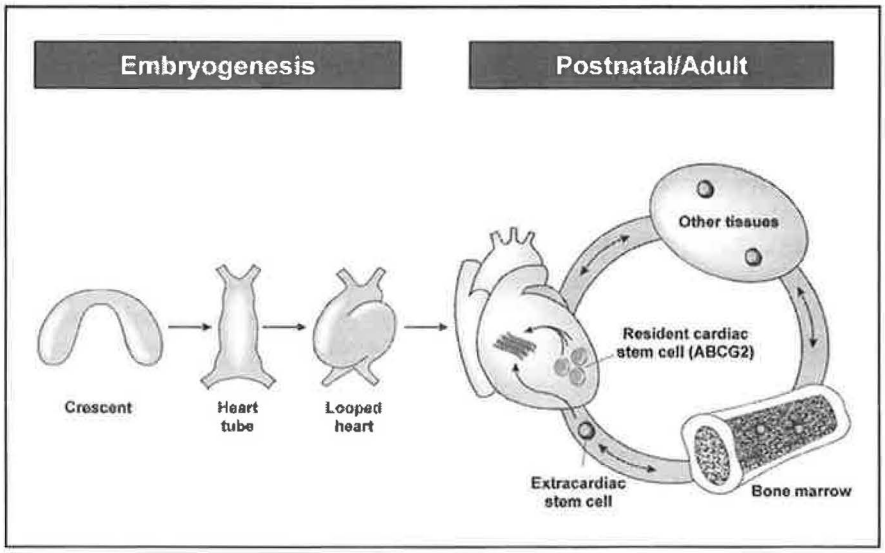


Figure 13. Schematic diagram outlining the strategy to decipher the transcriptional program of the stem cell population in the developing heart as a strategy to identify a postnatal cardiac stem cell population and as a platform for cell based cardiovascular therapies.

Side Population (SP) stem cells. A recently described strategy for the isolation of stem cells from postnatal or adult tissues employs Hoechst 33342 staining and dual-wavelength FACS analysis (Figure 14) (53,54). This isolation protocol defines a subset of cells in embryonic and adult tissues that are capable of adopting alternative fates including the ability to completely reconstitute the hematopoietic lineage in a lethally irradiated mouse (36,37). This latter finding suggests that the SP cells are capable of commitment to alternative lineages, given the right environmental cues (36,37,55-59). Furthermore, the SP cells isolated from adult skeletal muscle have been shown to express stem cell antigens (i.e. Sca-1^+, c-Kit, CD34) and are enriched at least 1,000-fold for *in vivo* reconstitution activity (53,54). Studies undertaken in our laboratory have established that SP cells are present in a subset of embryonic stem cells as well as adult tissues such as bone marrow and skeletal muscle. Additionally, we have demonstrated that skeletal muscle SP cells increase more than 50-fold following injury and these SP cells are able to repopulate injured skeletal muscle further supporting the role of the SP cell as a resident stem cell population.

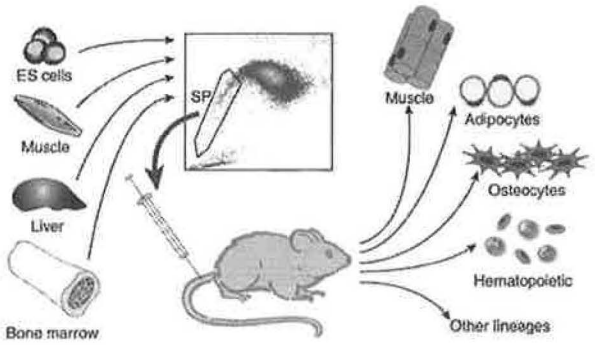


Figure 14. Potentiality of adult skeletal muscle specific stem cells. Purified stem cells (SP cells) are isolated from adult tissues or ES cells and gated, based on their ability to exclude Hoechst 33342 dye. For example, purified muscle SP stem cells home and reconstitute the irradiated bone marrow in its entirety (36,37). These stem cells are then recruited from the bone marrow to repopulate injured skeletal muscle. Therefore, SP stem cells isolated from adult skeletal muscle can contribute to the skeletal muscle lineage and the hematopoietic lineage.

Abcg2 confers the SP cell phenotype in cell lines. We have established that the ability of SP cells to efflux Hoechst 33342 dye is dependent on the expression of Abcg2 (also known as Bcrp1 for breast cancer resistance protein), which is a member of the family of ATP Binding Cassette (ABC) transporters (59-61). The superfamily of ABC transporters were initially characterized based on their ability to participate in multidrug resistance as they were able to efflux (pump out) various structurally unrelated antitumor agents in an energy-dependent manner (62). Abcg2 is a half-transporter molecule with a carboxy-terminal transmembrane segment and an amino-terminal cytoplasmic ATP-binding site (60,61) (Figure 15A). While the functional role of the ABC transporters remains ill defined (59,60), we established that Abcg2 was able to confer the SP phenotype in a striated muscle cell line. C2C12 cells, a cell line derived from immortalized murine satellite cells isolated from skeletal muscle, contains no identifiable SP cells when cultured under growth promoting conditions (Figure 15C). Overexpression of Abcg2 (Figure 15A & 15B) in C2C12 cells confers the SP cell phenotype (i.e. efflux of Hoechst 33342 dye) (Figure 15D) (50). The ability of Abcg2 to efflux the Hoechst dye was further established following the addition of fumitremorgin C (FTC), a specific inhibitor of Abcg2 (Figure 15E) (50). FTC

specifically inhibits the Abcg2-associated ATPase activity resulting in the disappearance of the cells that efflux the Hoechst dye (Figure 15E) (50).

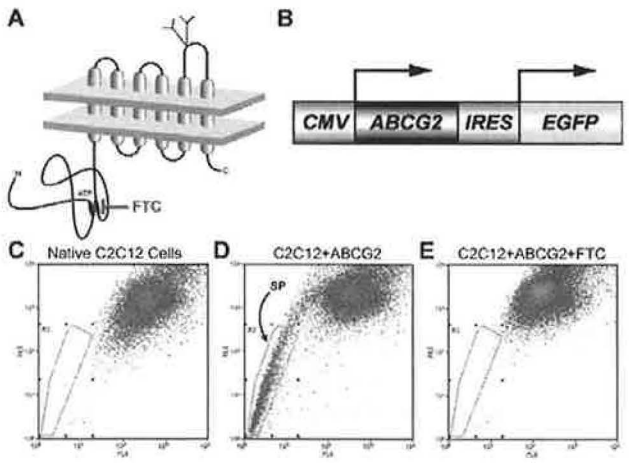


Figure 15. Overexpression of ABCG2 confers the SP cell phenotype in the myogenic C2C12 cell. (A) Schematic of ABCG2 containing six transmembrane domains and a cytoplasmic ATP binding site. (B) Schematic of the bicistronic vector used to transfect C2C12 myoblasts. CMV, cytomegalovirus promoter; IRES, internal ribosomal entry site. (C) The FACS profile of native C2C12 cells reveals an absence of SP cells. (D) Following overexpression of ABCG2, the FACS profile of the transfected C2C12 cells reveals the SP cell phenotype (i.e. efflux of the Hoechst 33342 dye) within the gated region. (E) Inhibition of the SP cell phenotype with the addition of FTC (a specific inhibitor of Abcg2). Following overexpression of the Abcg2 plasmid, the cells were incubated with FTC. Note an absence of cells within the gated region.

Abcg2 is expressed early during cardiac development and identifies a postnatal cardiac progenitor cell population. SP cells have been shown to function as stem cells (60,61) in a number of adult tissues. Using the ABC-transporter, Abcg2, as a specific molecular marker for this cell population (61) we have established the following (50):

- ***SP cells are present early during embryogenesis***
- ***SP cells are present during cardiogenesis and in the adult heart***
- ***SP cells are increased following myocardial injury***
- ***Cardiac SP cells form differentiated cardiomyocytes***

The Abcg2 expressing SP stem cells are present in the adult heart. The Abcg2 cardiac expression observed with *in situ* hybridization and immunohistochemical staining during embryogenesis was further confirmed using semi-quantitative RT-PCR and FACS analyses (50). Both techniques illustrated persistent expression of Abcg2 throughout cardiac development and in the adult. Using Hoechst 33342 staining and dual wavelength FACS analysis, we observed an SP cell population present in the adult heart (Figure 16) (50). To confirm the presence of an Abcg2 expressing SP cell population in the adult heart, a sample from the same cardiac preparation was incubated with FTC, a specific inhibitor of Abcg2. Using this specific inhibitor, we observed a disappearance of the SP cells (96% decrease) when

the cells were incubated with FTC further supporting the presence of an Abcg2 expressing SP cell population in the adult heart (Figure 16) (50).

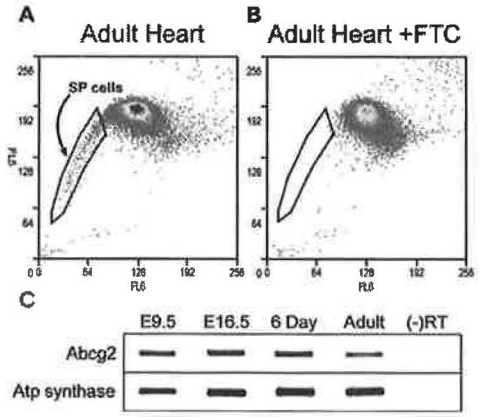
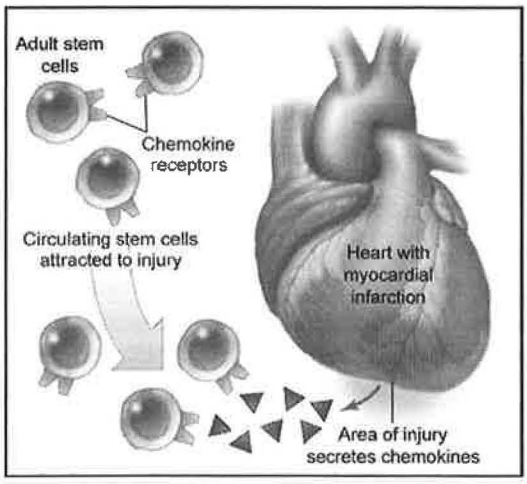


Figure 16. SP cells are present in the adult heart. (A) Using Hoechst 33342 dye and dual wavelength FACS analysis, a SP profile is present in adult murine cardiac tissue (50). (B) The SP profile disappears following the incubation with FTC, a specific Abcg2 inhibitor. (C) Total RNA was isolated from embryonic (E9.5 and E16.5), neonatal and adult isolated hearts and semiquantitative RT-PCR was performed to examine Abcg2 cardiac expression. Using primers that spanned an intron, we observed Abcg2 expression in the developing heart and decreased expression in the adult heart. Atp synthase was used as a loading control.

Perhaps the most successful long-term approach would be the promotion of resident stem cells within the patient's diseased organ to participate in the repair process. Conceivably this strategy would require the administration of mobilizing or activating agents (i.e. growth factors such as insulin-like growth factor, hepatocyte growth factor, etc.) that would prime the resident stem cells to re-enter the cell cycle, proliferate and differentiate to repair the damaged myocardial tissue (Figure 17) (63). Such a strategy would require enhanced understanding of the regulatory mechanisms of the resident stem cells and limited side effects associated with the administration of the priming agents.



Candidate stem cell signaling molecules

- Insulin growth factors
- Hepatocyte growth factor
- Stem cell factor
- Stromal cell derived factor-1

Figure 17. Signaling factors may recruit resident and extracardiac stem cells to the area of injury to participate in myocardial repopulation (63).

Stem cells and congestive heart failure: a paradox. An apparent paradox exists when considering the availability of stem cells in the heart and the prevalence of heart failure. Stem cells provide regenerative potential and should influence the status of the heart, thereby decreasing the incidence of heart failure. So why is heart failure so prevalent? Several competing hypotheses may, in part, explain this paradox and they are schematized in Figure 18. These include, but are not limited to, an inadequate number of resident myocardial stem cells to repopulate injured tissue following a large myocardial infarction. Alternatively, the fibroproliferative response following a myocardial injury produces a fibrotic scar. The scar may function to limit both the access of resident or extracardiac stem cells to the area of injury or it may limit the release (or serve as a barrier) of signals (growth factors such as HGF, IGF, stromal cell derived growth factor, etc.) that recruit stem cells to the site of injury. Additionally, the milieu of the injured myocardium may have a negative (i.e. inflammatory environment) effect on stem cell viability and differentiation. Finally, an active, working, contracting heart may have a limited regenerative response compared to a resting, unloaded heart. Future studies will examine each of these possibilities as a mechanistic strategy to enhance the contribution of resident and extracardiac stem cells for myocardial regeneration.

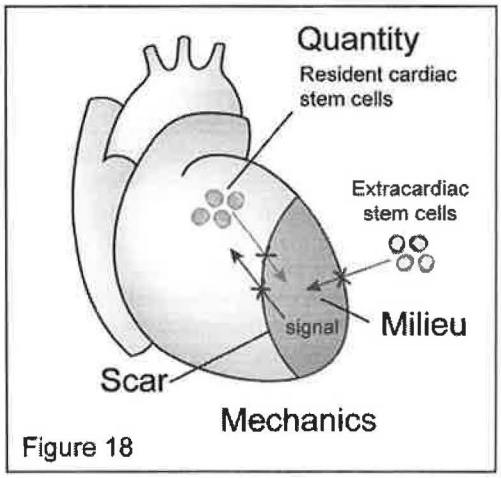


Figure 18

Cell Transfer Strategies as a platform for myocardial repair

Use of stem cells for therapeutic applications. Embryonic and adult (somatic) stem cells have generated intense interest for use in regenerative medicine (14,15,19,28). This interest is largely due to the morbidity and mortality associated with chronic diseases and aging as well as limitations associated with alternative strategies such as orthotopic whole-organ transplantation, xenotransplantation and mechanical or artificial organs. The use of cell transfer strategies using allogeneic or autologous cellular sources has biological plausibility. Furthermore, hematopoietic transplantation strategies are well established cell-based therapies

Bone marrow transplantation as an effective therapy. Allogeneic and autologous stem cell sources have been used successfully used for bone marrow transplantation (BMT) for more than 35 years. The first successful bone marrow transplants were performed in three children with congenital immune deficiency diseases in 1968 (64). An enhanced understanding of immunosuppression therapy, immunological barriers and stem cell biology have collectively resulted in the successful treatment of otherwise lethal diseases. Approximately 50,000 hematopoietic stem cell transplants are performed each year at approximately 400 centers worldwide (Figure 19A & 19B) (last year 17,700 BMT procedures were performed by approximately 200 centers in the US). Approximately two thirds of the patients received autologous transplants (generally obtained from peripheral blood by leukapheresis), 25% received related-donor transplants (HLA-identical sibling donors) and 10% unrelated-donor transplants (65). Posttransplant survival is dependent primarily on the disease but is also influenced by transplant

type, recipient age, conditioning regimen, prophylaxis for graft vs. host disease and disease status at the time of transplantation (Figure 19A-C).

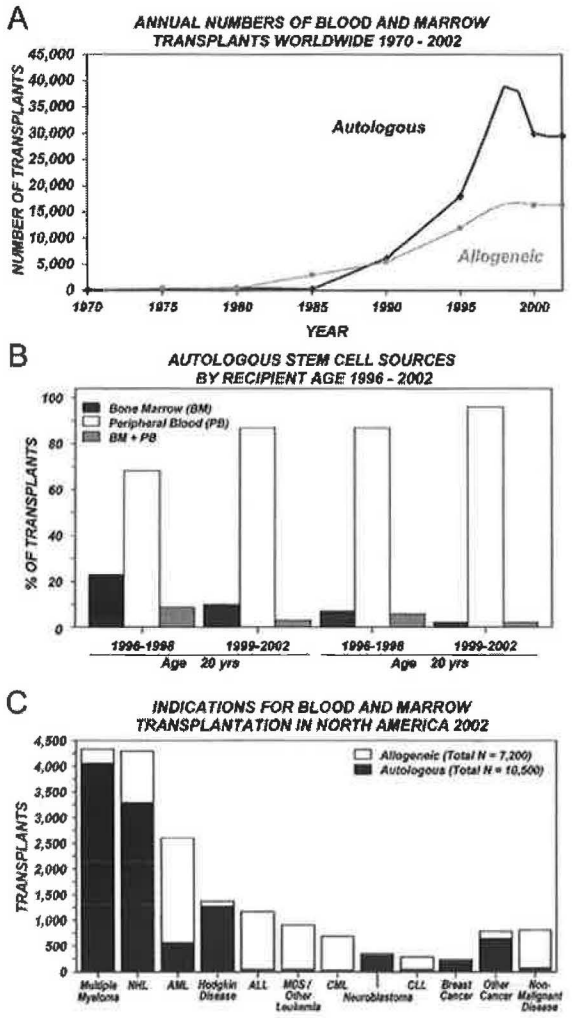


Figure 19. Human Bone Marrow Transplant (BMT) Registry Results. A) Annual numbers of blood and marrow transplants worldwide. B) Autologous stem cell sources worldwide. C) Clinical indications (i.e. disease process) for BMT in North America. Data provided by the International BMT Registry, Milwaukee, WI.

The proven efficacy of this therapy using somatic stem cells (i.e. hematopoietic stem cells) underscores the potential of adult stem cells and provides rationale for the application of cell transfer strategies in the treatment of diseases (Figure 19). This cell transfer strategy has been successful for hematopoietic disorders and may be applied to other systems such as cardiovascular disorders. Recent studies further suggest that myocardial regeneration occurs in the injured human adult heart. Using fluorescent *in situ* hybridization (FISH) techniques to identify Y chromosome positive cells, previous studies have reported the presence of Y chromosome positive myocardiocytes in postmortem specimens of female-to-male

adult human cardiac transplants (see Table 1) (13-16). While considerable controversy exists regarding the frequency of extracardiac stem cells, which migrate and repopulate the myocardium in the adult (13-16), these results suggest that extracardiac stem cells give rise to cardiomyocytes and participate in myocardial repair (see Table 1).

Chimerism of the Human Transplanted heart

No. of Patients	Y-chromosome positive cells in heart	Reference
n=8	9 ± 4% myocyte; 10 ± 3% arterioles; 7 ± 1% capillaries	*Quaini et al.
n=5	0.03% myocytes; (range 0.005% to 0.07%)	**Laflamme et al.
n=6	0% myocytes; 2.6% smooth muscle cells (med/small arteries)	***Glaser et al.

Table 1. Chimerism of the human transplanted heart. Results of published studies that examined the presence of Y-chromosome positive cells (using FISH techniques) in postmortem samples of female donor hearts transplanted into male recipients. Note that the percent of Y-chromosome positive cells in positive controls include *44 $\pm$ 4% myocytes; **53.3% myocytes and ***34.7% myocytes (references 47-49).

Recent Phase I Clinical Trials have been undertaken utilizing autologous stem cell populations delivered into the myopathic human heart (see Table 2). These studies have largely been undertaken by European Institutions. Several preliminary observations can be made based on the results of these studies. The delivery of skeletal myoblasts into the myocardium may modulate the remodeling associated with advanced heart failure (66-68). These myoblasts form skeletal (not cardiac) myocytes that may serve as a source for dysrhythmias. No prospective, randomized studies have been undertaken to examine the efficacy of these cellular sources for the repair of the myopathic heart (66-73). Future studies will be required to define the viability of cells following delivery, the optimal cell source, the potential tumorigenicity associated with the respective stem cell populations, the ideal patient population, or the timing of delivery following an acute injury.

No. of Patients	Clinical Symptoms	Cell type	No. of Cell (10^6)	Delivery mode	Complications	Period of follow-up	Results	References
n=5	CHF after MI	Skeletal MB	196	Transendocardial	1pt NSVT to AICD	6 months	LVEF 36 ± 11% to 45 ± 8% 3/4 pt with viable MB in explanted heart	Smits et al.
n=5	LVAD implant	Skeletal MB	300	IM	2/5 VT	68-191 days		Pagani et al.
n=1	CHF at time of CABG x3V	Skeletal MB	800	IM	-	5 months	LVEF 21% to 30%	Menasche et al.
n=21	LVEF <40%; CAD not ammenable to intervention	BM cells	25 ± 6.3	Transendocardial	-	4 months	LVEF 20 % to 29%	Perin et al.
n=8	LVEF >30%; CAD	BM cells	?	Transendocardial	-	3 months	LVEF 57% with no change	Tse et al.
n=6	CABG	BM (AC133$^+$)	1	IM	SVT; pericardial effusion	9-16 months	LVEF 21% - 47% to 43% - 58% No change LVEF (47% ± 10% vs. 53 ± 6%)	Stamm et al.
n=10	LVEF >30%; CAD	BM	78	Transendocardial	-	3 months		Fuchs et al.
n=20	PTCA after MI	BM vs. Blood derived progen	245 vs. 10	Intra-coronary (IRA)	-	4 months	LVEF 51 ± 10% to 60 ± 9%; Decresed LVEDV	Assmus et al.

Table 2. Results of cell based therapies for the human myopathic heart (references 66-73).

Future directions

Recent studies suggest that embryonic and somatic stem cells hold promise as sources for cellular augmentation and tissue engineering. The use of emerging technologies will further enhance our understanding of these stem cell populations and their molecular regulatory events that promote stem cell characteristics as well as the early events that specify cell fate decisions. Further studies will be needed to mechanistically define the regenerative capacity of resident cardiac stem cell populations as well as the safety and efficacy of cell based therapies for the treatment of the myopathic heart. Autologous (the donor and recipient are the same individual) sources would require the harvesting of a resident stem cell population (i.e. hematopoietic stem cells from the bone marrow or myogenic stem cells from skeletal muscle, etc.) followed by *in vitro* expansion and the use of exogenous agents to direct the differentiation to a specific lineage prior to delivery. An autologous source of somatic stem cells would eliminate the use of immunosuppression agents as well as any issues pertaining to donor availability, as the donor and recipient are one and the same. Timing of the delivery of the cellular source, the limited proliferative capacity of somatic stem cells, the ability to efficiently direct differentiation to a particular lineage and the efficiency of cell transfer strategies are all issues that require optimization or definition before this strategy could be applied in the clinical setting (14,15,19).

References:

1) Jessup, M. and Brozena, S. Heart failure. *New Eng J Med* 348:20:2007-2018, 2003.
2) Packer, M., Bristow, M. Cohn, J., Colucci, W., Fowler, M., Gilbert, E., Shusterman, N. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *New Eng J Med* 334:21:1349-1355, 1996.
3) Sharpe, N. and Doughty, R. Epidemiology of heart failure and ventricular dysfunction. *Lancet* 352:S1:3-7, 1998.
4) Goldstein, DJ, Oz, MC, Rose EA. Implantable left ventricular assist devices. *New Eng J Med* 339:1522-1533, 1998.
5) Jaski, BE, Kim, JC, Naftel, DC, Jarcho, J, Costanzo, MR, Eisen, HJ, Kirklin, JK, Bourge, RC. Cardiac translant outcome of patients supported on LVAD vs. intravenous inotropic therapy. *J Heart Lung Transplant* 20:449-456, 2001.
6) Rose, EA, Gelijns, AC, Moskowitz, AJ, et al. Long-term use of a LVAD for end-stage heart failure. *New Eng J Med* 345:1435-1443, 2001.
7) Brockes, JP, Kumar A. Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nature Rev Mol Cell Biol* 3:566-574, 2002.
8) Kumar, A, Velloso, CP, Imokawa, Y, Brockes, J. Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. *Dev Biol* 218:125-136, 2000.
9) Brockes, JP, Kumar, A, Velloso, CP. Regeneration as an evolutionary variable. *J Anat* 199:3-11, 2001.
10) Poss, KD, Wilson, LG, Keating, MT. Heart regeneration in zebrafish. *Science* 298:2188-2190, 2002.
11) Poss, KD, Keating, MT, Nechiporuk, A. Tales of regeneration in zebrafish. *Dev Dyn* 226:202-210, 2003.
12) Clark, L., Clark, R.K. and Heber-Katz, E. A new murine model for mammalian wound repair and regeneration. *Clin Immunol Immunopath* 88:35-45, 1998.
13) Leferovich, J., Bedelbaeva, K., Samulewicz, S., Zhang, X., Zwas, D., Lankford and Katz, E. Heart regeneration in adult MRL mice. *Proc Natl Acad Sci* 98:9830-9835,2001.
14) Fuchs, E., Segre, J.A. Stem cells: a new lease on life. *Cell* 100:143-155, 2000.
15) Weissman, I.L. Stem Cells: units of development, units of regeneration and units in evolution. *Cell* 100:157-168, 2000.

16) Evans M, Kaufman M. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;292:154-156.
17) Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Nat Acad Sci USA* 1981;78;7634-7638.
18) Rossant J. Stem cells from the mammalian blastocyst. *Stem Cells* 2001;19:477-482.
19) Garry, DJ, Masino , AM, Meeson, AP, Martin, CM. Stem cell biology and therapeutic applications. *Current Opin Nephrol and Hyperten* 12:447-454, 2003.
20) Beddington R, Robertson E. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 1989;105:733-737.
21) Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 1987;51:503-512.
22) Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* 1988;336:348-352.
23) Doetschman T, Maeda N, Smithies O. Targeted mutation of the Hprt gene in mouse embryonic stem cells. *Proc Natl Acad Sci USA* 1988;85:8583-8587.
24) Thomson JA, Itskovitzeldor J, Shapiro S, et al. Embronic stem cell lines derived from human blastocysts. *Science* 282;1145-1157.
25) Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. *Nat Biotechnol* 2003;21:319-321.
26) Ma Y, Ramezani A, Lewis R, Hawley RG, Thomson JA. High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 2003;21:111-117.
27) Richards M, Fong C-Y, Chan W-K, et al. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nature Biotechnol* 2002;20:933-936.
28) Hawke, T., Garry, D.J. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol* 91:534-551, 2001.
29) McKay R. Stem cells in the central nervous system. *Science* 1997;276:66-71.
30) Weissman, I.L. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 287:1442-1446, 2000.
31) Jiang Y, Jahagirdar BN, Reinhardt R, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41-49.
32) Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001;105:369-377.
33) Bjornson C, Rietze R, Reynolds B, Magli MC, Vescovi A. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science 1999; 283:534-537.
34) Clarke, D.L., Johansson, C., Wilbertz, J., Beress, B., Nilsson, E., Karlstrom, H., Lendahl, U., Frisen, J. Generalized potential of adult neural stem cells. *Science* 288:1660-1663, 2000.
35) Ferrari G, De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528-1530.
36) Gussoni, E., Soneoka, Y., Strickland, C., Buzney, E., Khan, M., Flint, A., Kunkel, L., Mulligan, R. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401:390-394, 1999.
37) Jackson, K., Mi, T., Goodell, M. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *PNAS* 96:25:14482-14486, 1999.
38) Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D., Leri, A., Anversa, P. Bone marrow cells regenerate infarcted myocardium. *Nature* 410:701-705, 2001.
39) Malouf M, Coleman W, Grisham JW, et al. Adult derived stem cells from the liver become myocytes in the heart in vivo. *Am J Pathol* 2001;158:1929-1935.
40) Odelberg SJ, Kollhoff A, Keating MT. Dedifferentiation of mammalian myotubes induced by msx1. *Cell* 2000;103:1099-1109.
41) Terada N, Hamazaki T, Oka M, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion *Nature* 2002:416: 542-545.

42) Ying Q-L, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. *Nature* 2002; 416; 545-548.
43) Anderson DJ, Gage FH , Weissman IL. Can stem cells cross lineage boundaries? *Nat Med* 2001;7:393 – 395.
44) Krause DS, Thiese ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone-marrow derived stem cell. *Cell* 2001; 105:369-370.
45) Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoetic stem cells. *Science* 2002;297:2256-2259.
46) Jackson, K.A., Majka, S.M., Wang, H., Pocius, J., Hartley, C.J., Majesky, M.W., Entman, M.L., Michael, L.H., Hirschi, K.K., Goodell, M.A. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 107:1395-1402, 2001.
47) Quaini, F., Urbanek, K., Beltrami, A.P. Finato, N., Beltrami, C.A., Nadal-Ginard, B., Kajstura, J., Leri, A., Anversa, P. Chimerism of the transplanted heart. *N Eng J Med* 346:5-15, 2002.
48) Laflamme, M.A., Myerson, D., Saffitz, J.E., Murry, C.E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res* 90:634-640, 2002.
49) Glaser, R., Lu, M.M., Narula, N., Epstein, J.A. Smooth muscle cells, but not myocytes of host origin in transplanted human hearts. *Circulation* 106:17-19, 2002.
50) Martin, C.M., Meeson, A.P., Robertson, S.M., Hawke, T.J., Richardson, J.A., Bates, S., Goetsch, S.C., Gallardo, T.D., Garry, D.J. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev Biol* 265:262-275, 2004.
51) Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L., Behringer, R., Garry, D.J., Entman, M., Schneider, M.D. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci USA* 100:12313-12318, 2003.
52) Beltrami, A., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B. & Anversa, P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114:763-776, 2003.
53) Goodell, M., Brose, K., Paradis, G., Conner, A., Mulligan, R. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183:1797-1806, 1996.
54) Goodell, M., Rosenzweig, M., Kim, H., Marks, D., DeMaria, M., Paradis, G., Grupp, S., Sieff, C., Mulligan, R., Johnson, R. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nature Med* 3:12:1337-1345, 1997.
55) Asakura, A., Rudnicki, M.A. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. *Exp Hem* 30:1339-1345, 2002.
56) Preffer, F.I., Dombkowski, D., Sykes, M., Scadden, D., Yang, Y-G. Lineage negative side population (SP) cells with restricted hematopoietic capacity circulate in normal human adult blood: Immunophenotypic and functional characterization. *Stem Cells* 20:417-427, 2002.
57) Lechner, A., Leech, C.A., Abraham, E.J., Nolan, A.L., Habener, J.F. Nestin-positive progenitor cells derived from adult human pancreatic islets of langerhans contain side population (SP) cells defined by expression of the ABCG2 ATP binding cassette transporter. *Biochem Biophys Res Com* 293:670-674, 2002.
58) Uchida, N. Fujisaki, T., Eaves, A.C., Eaves, C.J. Transplantable hematopoietic stem cells in human fetal liver have a CD34+ side population (SP) phenotype. *J Clin Invest* 108:1071-1077, 2001.
59) Sharenberg, C.W., Harkey, M.A., Torok-Storb, B. The ABCG2 tranporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99:507-512, 2002.
60) Bunting, K. ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 20:11-20, 2002.
61) Zhou, S., J. Schuetz, K. Bunting, A. Colapietro, J. Sampath, J. Morris, I. Lagutina, G. Grosveld, M. Osawa, H. Nakachi, B. Sorrentino. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nature Med* 7:9:1028-1034, 2001.
62) Litman, T., T. Druley, W. Stein, S. Bates. From MDR to MXR: new understanding of multidrug resistance systems, their proerties and clinical significance. *Cell. Mol. Life Sci.* 58:931-959, 2001.

63) Rosenthal, N. Prometheus's vulture and the stem-cell promise. *New Eng J Med* 349:267-274, 2003.
64) Goldman, J.M., Horowitz, M.M. the international bone marrow transplant registry. *Int J Hematol* 76:393-397, 2002.
65) Horowitz, MM, Loberiza, FR, Brdeson, CN, Rizzo, JD, Nugent ML. Transplant registries: guiding clinical decisions and improving outcomes. *Oncology* 15:1-16, 2001.
66) Smits, PC, van Geuns, R, Poldermans, D, Bountioukos, M, Onderwater, E, Lee, CH, Maat, A, Serruys, PW. Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure. *J Am College Cardiol* 42:2063-2069, 2003.
67) Pagani, FD, DerSimonian, H, Zawadzka, A, Wetzel, K, Edge, AS, Jacoby, DB, Dinsmore, JH, Wright, S, Aretz, Th, Eisen, HJ, Aaronson, KD. Autologous skeletal myoblasts transplanted to ischemia damaged myocardium in humans. *J Am College Cardiol* 41:879-888, 2003.
68) Menasche, P, Hagege, AA, scorsin, M, Pouzet, B, Desnos, M, Duboc, D, Schwartz, K, Vilquin, JT, Marolleau, JP. Myoblast transplantation for heart failure. *Lancet* 357:279-280, 2001.
69) Perin, EC, Dohmann, H, Borojevic, R, Silva, SA, Sousa, A, Mesquita, CT, Rossi, M, carvalho, AC, Dutra, HS, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation* 107:2294-2302, 2003.
70) Tse HF, Kwong, YL, Chan J, Lo, G, Ho, CL, Lau, CP. Angiogenesis in ichaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet* 361:47-48, 2003.
71) Stamm C, Westphal, B, Kleine, HD, Petzsch, M, Kittner, C, Klinge, H, Schumichen, C, Nienaber, CA, Freund, M, Steinhoff, G. Autologous bone marrow stem cell transplantation for myocardial regeneration. *Lancet* 361:45-46, 2003.
72) Fuchs, S, Satler, LF, Kornowski, R, Okubagzi, P, Weisz, G, Baffour, R, Waksman, R, Weissman, NJ, Cerqueira, M, Leon, MB, Epstein, SE. Catheter based autologous bone marrow myocardial injection in no option patients with advanced coronary artery disease. *J Am College of Cardiol* 41:1721-1724, 2003.
73) Assmus, B, Schachinger, V, Teupe, C, Britten, M, Lehmann, R, Dobert, N, Grunwald, F, Aicher, A, Urbich, C, Martin, H, Hoelzer, D, Dimmeler, S, Zeiher, AM. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106:3009-3017, 2002.