Allogeneic Stem Cell–Derived “Repair Unit” Therapy and the Barriers to Clinical Deployment

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Abstract. The development of cellular therapies has been seriously hampered by the paucity of cells available for grafting from living or cadaver donors. This sourcing problem can be resolved, at least in theory, by generating tissue-specific cells from autologous or allogeneic stem cells. Nonetheless, there are formidable barriers to the use of stem cells in the clinic. This review focuses on identifying and surmounting these barriers.

What Can Stem Cells Be Used For?
In the future, adult and embryonic stem cells (AS and ES cells) will be coaxed to become almost whatever cell type the patient needs (Figure 1). Stem cell–derived parenchymal cells can be used to repair organs damaged by disease or injury before or after organ failure.

What Are ES Cells?
These cells, which abundantly express telomerase activity (1), can be propagated indefinitely as undifferentiated cells with a normal karyotype; however, they can differentiate all cell types (2). Unlike other cell types, ES cells can divide indefinitely and differentiate into any mature somatic cell type. In addition, ES cells can readily be cultured in an undifferentiated state for extended periods of time before a stimulus for differentiation is provided. This is advantageous for mass production of stem cell derived therapies. ES cells express stage-specific embryonic antigen (SSEA)-3, SSEA-4, TRA-1-60, TRA-1-80, and alkaline phosphatase (1). ES cells are also present albeit in much lower abundance in fetal tissues.

Where Do the ES Cells Come From?
ES cells, the most versatile (pluripotent) of the various stem cell types, are concentrated in the inner cell mass of the blastocyst stage embryos within the first 5 to 7 d after an egg is fertilized by sperm (1).

What Are AS Cells?
These multipotent cells are less versatile than ES cells, and most AS cells do not divide indefinitely. To date, it has proven very difficult to culture and expand most somatic AS cell types for extended periods of time or to keep them in extended culture in an undifferentiated state. Most known somatic AS cells have a certain degree of lineage commitment (i.e., AS cells derived from muscle tissue most efficiently form muscle cells, whereas stem cells from the brain preferentially differentiate to the neuronal lineage). Nonetheless, certain AS cells long thought to be lineage specific may differentiate into many cell types (3). However, the high degree of plasticity (“transdifferentiation”) that has been reported for several AS cells during recent years (e.g., neuronal stem cells differentiate into blood cells) remains remarkably controversial (4,5). In some cases, AS cells or their progeny fuse with, rather than differentiate into, somatic cells (6). Differing AS cell types are heterogeneous in regard to their plasticity. A rare mesenchymal type bone marrow derived nonhematopoietic stem cell (3) is believed to be unusually versatile and able to transdifferentiate into endothelial and endodermal cells (3). The phenotype of the mesenchymal AS cell type is CD34+, CD44+, CD45+, c-kit+, and SSEA-1+.

Where Do AS Cells Come From?
Hematopoietic stem cells are present in the bone marrow and circulating blood. These cells are readily accessible. Other rare, hard to identify AS cells are scattered throughout the body, and unlike ES cells, AS cells reside in the tissues of young and adult individuals.

Barriers to Clinical Deployment of Allogeneic Stem Cell–Derived Therapies
The stem cell therapies may derive from allogeneic somatic (adult) (4) or ES cell origin (Figure 1). Stem cell–based therapies will likely initially derive from embryonic (1), and hence allogeneic, sources (Figure 1). The use of autologous stem cells...
may involve isolation of stem cells from the patient (Figure 1C) or a more controversial and technically demanding option involving therapeutic cloning/nuclear transfer (7) (Figure 1D).

Creation of an ample source of healthy euploid stem cell lines has been slowed by technical problems, societal concerns, and political constraints. There are at least three formidable barriers to idealized clinical deployment of stem cell–derived replacement therapy. First, some oppose the use of embryonic, but not adult, stem cells because of moral or religious concerns. Even more controversial is the use of nuclear transfer techniques to establish autologous tissues because this method is the basis of efforts to clone individuals. The unique technical aspect of this approach is the subject of another review in this series, and this methodology will not be further analyzed in this review. The other two barriers—barriers in addition to the moral objections—pertain to both embryonic and allogeneic AS cells. To use stem cell–derived replacement cells, we will need a gating procedure to meticulously select and then facilitate expansion of a pure population of the desired cell type. Next, we will need a means to produce tolerance, because it is nearly certain that allogeneic cells will be used for treatment, and reliance on daily immunosuppressive therapy is unacceptable for many potentially important applications.

**Barrier 1: Ethical concerns**

*Who Is Against the Use of ES Cells for Medical Purposes?* Those who believe life begins with conception. In the view of some, gathering cells from disrupted embryos is the moral equivalent of abortion, which they view as murder.

*Do at Least Some Antiabortion Advocates Condone the Use of ES Cells for Critical Patient Needs?* Yes. In vitro fertilization, which often produces more fertilized eggs than are used for conception, is a source of ES cells that some antiabortion advocates find acceptable. Because these fertilized eggs have not implanted in the uterus, some consider in vitro fertilized eggs as “pre-embryos.” Senator Orrin Hatch, a staunch supporter of antiabortion legislation, says he cannot equate “a living child in the womb . . . with an embryo in a freezer” (Boston Globe, July 7, 2001).

*What Are the Moral and Practical Considerations of the Use of ES Cells Versus AS Cells?* Compared with AS cells, ES cells are more versatile (pluripotent versus multipotent), and ES cells, not AS cells (except for nonhematopoietic mesenchymal bone marrow–derived AS cells), can divide indefinitely. Moreover, most AS cells become rapidly senescent as they replicate in culture. Hence, it may prove difficult to produce large-batch quantities of AS cells. Because of the special attributes of ES cells, it should become possible to produce large batches of ES-derived terminally differentiated parenchymal cells and distribute these cells as “repair units” to patients in need before this becomes possible with AS cells.

Although the potential to develop terminally differentiated parenchymal cells as replacement therapies from AS cells is unquestioned, the biologic advantages of ES cells over AS cells should translate to a greatly shortened period of preclinical development for stem cell–derived therapies. If development of ES cells were hampered, provision of cell therapies in the clinic will be delayed. The effect of this delay on the well-being and survival of patients in need of such therapies must be measured against the moral arguments opposing the use of ES cells.

At first glance, it is appealing to develop autologous stem cells as a source of cellular repair units. This approach avoids the need for immunosuppression (Figure 1C). However, AS cells are very scarce. In addition, the logistics of an autologous approach appear daunting, as isolation of the stem cells, GMP production, and quality assurance would have to be done on a per-patient basis, an economic and tactical challenge. The same limitations are valid for therapeutic cloning approaches, which are currently in an experimental stage and subject to intensive and totally justified scrutiny from both moral and technical perspectives.

Currently, all relevant clinically used stem cell grafting therapies are allogeneic and are based on matched donor–derived somatic AS cells (Figure 1A). The best known and best established example is bone marrow transplantation for leukemia patients, and more recently transplantation of hematopoi-
etic stem cells derived from umbilical cord blood. The major problem with this approach for nonhematopoietic AS cell grafting is the very limited availability of donors and the requirement for a highly individualized process, which poses problems in respect to practical needs. The multipotent mesenchymal bone marrow–derived AS cell offers a potential alternative. This rare cell type was found after a very extended period of culture, and it may be difficult to replicate this feat often enough to meet practical considerations.

**Barrier 2: Purity.** Development of a simple and efficient means to produce and propagate pure populations of specific cell types from a complex culture of differentiating stem cells is both a challenge and a necessity for clinical development. Culturing ES cells in suspension and in the absence of differentiation inhibitors, such as leukemia inhibitory factor, results in formation of embryoid bodies (or EB) (8). EB are multicellular structures that spontaneously differentiate to form most ectoderm-, endoderm-, and mesoderm-derived cell lineages. Although the propensity to propagate a cell of a chosen cell lineage in this system can be enhanced by the addition of appropriate growth factors, the degree of purity of the resulting cultures is grossly insufficient for clinical application. More importantly, it is critical that any pluripotent cells are removed from the therapeutic products before clinical use; otherwise, the potential for teratoma formation is great.

A relatively simple enrichment approach has been devised that fulfills many of the requirements needed for clinical use of ES-derived cells (Figure 2). Although this system was initially developed for ES cells, it is equally applicable to multipotent (e.g., adult) stem cell systems. In its initial inception, the approach uses two transcriptional units that are incorporated into a common vector backbone (9). The first transcriptional unit utilizes a promoter that is active in undifferentiated ES cells (e.g., the phosphoglycerate kinase promoter) to drive

![Figure 2](image_url)

*Figure 2.* (A) Schematic diagram of the genetic enrichment approach. The structure of the MHC-neo<sup>r</sup>/pGK-hygro<sup>r</sup> transgene, (B) Hoechst epifluorescence of a nonselected culture of embryonic stem (ES) cells carrying the MHC-neo<sup>r</sup>/pGK-hygromycin transgene 16 d after differentiation was induced. Note the high density of cells present in the field. (C) Antisarcomeric myosin heavy chain immunofluorescence (green signal) of the same field depicted in Panel B. Note that only a small percentage of the cells are cardiomyocytes (arrows demarcate the same group of cells in Panels B and C). (D) Hoechst epifluorescence of a G418-selected culture of ES cells carrying the MHC-neo<sup>r</sup>/pGK-hygromycin transgene 16 d after cardiogenic induction; note the reduction in total cell number compared with the unselected cultures depicted in Panel B. (E) Antisarcomeric myosin immunofluorescence (green signal) of the same field depicted in Panel D. Note that all of the cells present in the G418-selected culture express sarcomeric myosin (arrows demarcate the same group of cells in Panels D and E).
expression of a marker gene suitable for enrichment of cells carrying the transduced DNA (e.g., a cDNA encoding resistance to hygromycin). The second transcriptional unit utilizes a promoter that is active in the desired cell type or types to drive expression of a second marker gene suitable for enrichment of the desired cells (e.g., a cDNA encoding a second drug-resistance protein).

In practice, the engineered DNA is introduced into undifferentiated stem cells, and cells that have been successfully transduced with the DNA vector are selected by virtue of the activity induced by the first transcriptional unit (e.g., hygromycin resistance). The cells are then differentiated either spontaneously or under the influence of growth factor cocktails, which can enhance the yield of the desired cell type. Upon differentiation and by design, the second transcriptional unit is activated only in the desired cell type (which is defined by the cell type specificity of the promoter used). The desired cells are then obtained as a consequence of the activity encoded by the second transcriptional unit. For example, if the second transcriptional unit encodes amino glycoside phosphotransferase, the desired cell type can be obtained by treating the cultures with G418.

This approach was initially tested in a system used to generate cardiomyocytes from differentiating ES cultures. In this experiment, the first transcriptional unit comprised the phosphoglycerate kinase promoter and a cDNA-encoding resistance to hygromycin, and the second transcriptional unit comprised the cardiomyocyte-restricted α-cardiac myosin heavy chain promoter and a cDNA-encoding aminoglycoside phosphotransferase. The DNA was introduced into undifferentiated ES cells, and the successfully transduced cells incorporating the DNA were enriched on the basis of their resistance to hygromycin. Differentiation was then induced, and once an indication of cardiomyogenesis was observed (i.e., spontaneous contractile activity), the cultures were treated with G418. Because the α-myosin heavy chain promoter is active only in cardiomyocytes, only these cells express aminoglycoside phosphotransferase and survive G418 treatment (Figure 2). Immunocytochemical analyses of the resulting cultures indicated that they were >99% pure (9). Moreover, the selected cardiomyocytes were stable in culture for many months.

Recent studies suggest that cellular transplantation provides a potential approach to augment cardiac function in diseased hearts (10). To ascertain whether ES-derived cells were applicable to this potential therapeutic approach, cardiomyocytes derived by the genetic selection approach described above were harvested, and approximately $1 \times 10^4$ cells were delivered into the left ventricular free wall of adult recipient mice. Microscopic examination of the recipient hearts revealed that transplanted cardiomyocytes exhibited normal myocardial topography (Figure 3). Donor ES-derived cardiomyocytes were closely juxtaposed with host cardiomyocytes and displayed

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**Figure 3.** Genetically enriched cardiomyocytes from stable intracardiac grafts. Phase contrast image (A) and antidystrophinimmunofluorescence (B) of the same field from a mdx heart engrafted with G418-selected cardiomyocytes. Dystrophin immune reactivity appears as green signal in Panel B. Arrows indicate myofiber-containing host cells (dystrophin negative); arrowheads indicate myofiber-containing donor cells (dystrophin positive). (C) Transgene-specific PCR amplification of the MHC-neo/PGK-hygro′ transgene from material microdissected from the transplanted hearts. The presence of the anticipated PCR reaction product confirms that the embryonic stem cell–derived cardiomyocytes were stably transplanted.
morphologic attributes suggestive of functional coupling. The transplanted ES-derived cardiomyocytes were detected as late as 7 wk after implantation, the latest time point analyzed. Thus, use of the genetic enrichment approach provides a suitable source of donor cardiomyocytes for cellular transplantation.

Several groups have used this approach to produce pure cardiomyocyte cultures (9,11). It is clear that the enrichment protocol also works in other cell lineages, because pure cultures of neurons (12) as well as insulin-secreting pancreatic beta cells (13) have been obtained by using neuronal- and beta cell–restricted promoters, respectively, to drive expression of the second transcriptional unit. Although the examples presented utilized antibiotic resistance as the basis of the enrichment, a wide variety of analogous marker genes/enrichment protocols can readily be used (e.g., green fluorescence protein, targeted expression of cell surface markers, which could be used in conjunction with FACS protocols).

Barrier 3: Rejection of Allogeneic Cell Grafts. The third barrier that must be overcome to enable routine use of stem cell–derived therapies to heal or rejuvenate tissues is rejection of these allogeneic cell grafts, which will bear foreign histocompatibility antigens. Although antirejection treatment will be needed, a requirement for maintenance immunosuppressive therapy would limit, although not exclude, successful clinical application. For many potential applications, daily immunosuppressive treatment carries more risk than the clinical situation warrants. Creation of immune tolerance would enable use of stem cell–derived therapies for millions of patients. For example, estimates as to the number of patients with type 1 diabetes in the United States hover around 1,000,000. At least as many patients with type 2 diabetes require insulin therapy in the United States. The number of pancreas organ transplants undertaken in the United States will not likely exceed 5000 next year. The need for islets or beta cells is great.

Recent data from our and other laboratories has served to underscore the importance of the pool size of donor-specific alloreactive T cells in the induction of transplant tolerance (14–16). Hosts bearing large donor alloaggressive cell populations are far less amenable to tolerance induction than are hosts whose donor-specific alloaggressive T cell populations are inherently modest in size (good histocompatibility match) (17), or the attacking T cell mass has been trimmed by use of agents that directly kill the alloreactive cell or enhance T cell activation-induced apoptosis (14–16,18–20). Fortunately, the pool size of alloreactive T cells to allogeneic pure stem cell–derived islets or cardiomyocytes is likely to be modest. The ES-derived cell transplant will be devoid of highly immunogenic mature dendritic cells, and these pure parenchymal cell populations will express MHC class I, but not class II, at the time of transplantation. Both of these features should, by comparison with conventional organ transplants, minimize the number of T cell clones able to attack the cell transplant (21). Because of the power of the cell gating and selection procedure, tissue-specific histocompatibility antigens present upon extraneous cell types will not be present to amplify the immune response. It is almost certain that the use of ES cell–derived cellular allografts will be conducted so that multiple lines expressing differing HLA molecules will be available for use. Hence, HLA typing will be used to further minimize the magnitude of the immune response, particularly for class I molecules.

New therapies with particular relevance to the anticipated immune response mounted against ES-derived cell transplants are being tested (22,23). In particular, a highly selective therapy has been designed to negate the role of IL-15 and IL-15 cells in the allograft response. Why? The IL-15Rα chain is coordinately expressed with the IL-2Rα chain (24). This protein is expressed upon recently activated (24) and autoimmune memory type T cells (25), but not by resting T cells (24). Hence, targeting IL-15Rα T cells will not hinder the function of the overwhelming majority of host T cells. Insofar as pure stem cell–derived beta cells will express class I, but not class II, MHC antigens, the direct alloreactive pathway will be dominated by MHC class I reactive CD8+ T cells, whereas the indirect alloreactive pathway will engage both CD4+ and CD8+ T cell activation. The IL-15 system, not the IL-2 system, is critically important for CD8+ T cell proliferation and survival (25,26). Because autoreactive T cells are present in many, if not all, diabetic hosts may contribute to immunologic graft loss, it will be most important to disarm autoreactive, memory-type T cells. Again the IL-15 system, not the IL-2 system, is of particular importance to the maintenance and expansion of memory T cells (25). Although IL-15 and IL-2 drive an identical T cell proliferative signaling pathway, the difference between IL-15– and IL-2–driven T cell activation is a matter of T cell life and death (26,27). IL-15 drives proliferation, but not apoptosis (25–27). IL-2 drives a balanced pattern of proliferation and apoptosis (27). The loss of IL-2 driven apoptosis causes autoimmunity and a resistance in the induction of allograft tolerance. Moreover, IL-15, not IL-2, is responsive for the initial six to seven waves of T cell proliferation in the allograft response (27).

The importance of CD4+ independent CD8+ T cell–dependent rejection is highlighted by data that have evolved in several labs showing that CTLA4-Ig–mediated costimulation blockade, a treatment that is nonlymphoablative, is far less capable of blocking CD4+–independent, CD8+–dependent cytotoxic immune events and synergizes with costimulation blockade; it may play a vital role in rendering tolerant the CD8+–dominated immune response to stem cell–derived islets. More recently, an extremely potent means of tipping the balance of the allograft response from rejection toward tolerance has been achieved through manipulation of the response of activated T cells to both IL-2 and IL-15 (28). The result of such treatment is to create highly selective mass suicide of rejection-causing T cells while strengthening graft-protecting immunoregulatory T cell circuits (28). This extremely potent method, which does not create lymphopenia, may be well suited for the ethical and medical challenges inherent in attempts to create tolerance in the recipients of allogeneic cellular transplants.

Conclusions

It is not unduly optimistic to conceive of near- or intermediate-term testing of stem cell–derived cell transplants. In
accordance with current pharmaceutical industry best-practice considerations, any allogeneic cell preparation for grafting will have to be virtually pure and homogeneous and its application safe. These pharmaceutical and regulatory requirements can only be achieved by efficiently selecting and isolating the desired cells after the differentiation process has been initiated. Therefore, a successful strategy to produce a cell product for desired cells after the differentiation process has been initiated. only be achieved by efficiently selecting and isolating the safe. These pharmaceutical and regulatory requirements can have to be virtually pure and homogeneous and its application accordance with current pharmaceutical industry best-practice standards, such as adjunctive immunosuppressive therapies; solving the engineering and logistics problems that will be encountered during GMP production of cells [feeder layer–free growth].

As a technique, the introduction of the nucleus of a fully differentiated cell into an enucleated regenerative oocyte is a means to produce syngeneic cellular transplant units. To date, this technique has not succeeded in producing healthy cells in a reproducible manner (29). Because this technique forms the basis for creating human clones, there is widespread opposition to this method. For technical and societal concerns, this approach will not succeed in the near future.

In short, it will not be possible to enter clinical trials within 2 to 3 yr from now, but it is likely that clinical testing will commence within 5 yr. Developments may revolutionize the field of transplantation and spawn a new specialty: regenerative medicine.

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