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Special Cells, Special Considerations: The Challenges of Bringing Embryonic Stem Cells From the Laboratory to the Clinic

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Abstract

Human embryonic stem (ES) cells are an attractive tool for cell-based therapies because of their limitless capacity for proliferation and their ability to differentiate into all cell types of the body. However, these features introduce certain unique risks that must be considered in developing transplantation strategies for clinical use. We outline these risks and examine current approaches to address them, both before and after transplantation.

In recent years, human embryonic stem (ES) cells have received a great deal of attention as a resource of cells to treat a wide range of diseases. This attention is due largely to two particular features of these cells: their unlimited capacity for proliferation and their pluripotency—the ability to differentiate into any (and all) cell types of the body. These attributes carry with them certain risks and complications that must be addressed when undertaking a transplantation aimed at ameliorating disease. The infinite capacity of ES cells to proliferate, which makes them so attractive for generating the numbers of cells necessary for effective therapy, brings along the risk of uncontrolled growth after transplantation. Pluripotent cells also present the risk of tumorigenicity (*i.e.*, teratoma formation). Furthermore, their ability to differentiate into many different cell types means that they generally *do* become many different cell types—a significant challenge when a proposed therapy calls for only one cell type in particular. Some of these issues have largely been addressed with the development of various methods to enrich for only desired cells and

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CONFLICT OF INTEREST

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elimination of all others. Several such methods will be discussed in the following paragraphs. Yet safe, successful ES cell-based therapies will require more than simply isolating a particular population. Careful attention must also be paid to the behavior of cells after transplantation.

Transplanted ES-derived cells may respond to their host environment in several ways. They may undergo one or more divisions, migrate away from their injection site, or even exhibit plasticity that would allow them to become a different cell type altogether. Furthermore, just as the environment can have an effect on transplanted cells, the cells may be able to have an effect on their environment. Multiple reports have shown that grafted cells can secrete growth or survival factors that elicit a host response.^{1,2} Although interactions between cells and host may prove to be highly beneficial to achieving a therapeutic effect, they open the door for many variables that cannot easily be accounted for or controlled in any transplantation scheme. It may never be possible to predict and avoid all possible outcomes of the introduction of ES-derived cells to a patient, but these issues highlight the critical need for graft monitoring after transplantation.

Isolation of cells to be transplanted

In any cell-based therapy, obtaining a cell population that is as homogeneous as possible is critical for two primary reasons: (i) to ensure that sufficient numbers of the desired cell type are transplanted to achieve a therapeutic benefit and (ii) to eliminate unwanted cells that could have deleterious effects. One of the greatest concerns is that undifferentiated ES cells will persist in a differentiating culture and subsequently form teratomas following transplantation. In theory, even one residual ES cell may be enough to form a tumor. Multiple reports have described tumorigenesis following the transplantation of ES cells or their derivatives.^{3,4} Several techniques are routinely used to isolate a particular cell type and eliminate those that are undesired; they include fluorescence-activated or magnetic activated cell sorting (FACS or MACS) and drug selection.

If the desired cells have one or more well-characterized markers on their surface, they can be sorted away from other cells using FACS or MACS. For example, the cell-surface antigen CD34 has been used to isolate human ES-derived hematopoietic cells.⁵ Conversely, these techniques can eliminate undesired cells, such as undifferentiated ES cells that express SSEA-4 on their surface.⁶ FACS is also effective if the desired (or undesired) cells express a fluorescent reporter gene—*i.e.*, green fluorescent protein (GFP)—under the control of a cell-specific regulatory sequence. Huber *et al.* took this approach to isolate human ES-derived cardiomyocytes that express GFP under the control of the human myosin light chain-2V promoter. This led to the isolation of cardiomyocytes with greater than 90% homogeneity.⁷

Another approach to producing a homogeneous population is to use drug selection. For example, a cell-specific regulatory sequence can be used to drive expression of a drug-resistance gene in the desired cells. This approach was recently used to generate a homogeneous population of lung alveolar epithelial cells from human ES cells in which the neomycin resistance gene was driven by the promoter of the surfactant protein B gene.⁸ Negative selection can also be used. Cells can be engineered to express a “suicide gene,” such as herpes simplex virus thymidine kinase (HSV-TK), under the control of a regulatory sequence that is active in pluripotent cells but inactive in differentiated cells (*e.g.*, the *Oct4* promoter). Cells expressing HSV-TK are killed by the administration of ganciclovir.

Post-transplantation concerns

Among the greatest challenges in developing a successful cell-based therapy are the prediction of engrafted cell behavior and the anticipation of potential problems. Upon

transplantation, cells are placed in an environment that is completely different from their previous *in vitro* culture. Their new host environment may present signals that induce the cells to migrate, proliferate, or further differentiate. Several studies have shown that transplanted cells can migrate widely, especially in response to injury-induced signals.^{1,9} The cells may continue to differentiate *in vivo*, an attribute that might facilitate engraftment but whose outcome is quite difficult to predict and finely control.¹⁰ Roy *et al.* showed that human ES-derived neural progenitors are capable of continued proliferation in the rat brain.⁴ Even if cells do not proliferate to an extent that would lead to tumor formation, just a few divisions after transplantation could be enough to significantly impact the effect of the graft on the recipient. Furthermore, transplanted cells may secrete factors that impact the surrounding host environment. In some cases, such as the secretion of neurotrophic factors by neural stem cells, the secreted factors may also provide a therapeutic benefit.^{1,2}

***In vivo* imaging of transplanted cells**

Once cells have been transplanted, it is critical to track whether they are migrating, proliferating, dying, differentiating, and so on, as well as to verify that they are indeed initially located at the target site. Much has been learned about the behavior of transplanted cells from work done in preclinical animal models. It has become routine to transplant cells that constitutively express a reporter protein, such as GFP or β -galactosidase, that can be used to identify these cells in histologic sections. Such reporters can give single-cell resolution, which has proven invaluable in assessing whether transplanted cells survived, migrated, proliferated, and/or differentiated. However, the utility of these reporters is generally limited to postmortem analysis, and data are therefore obtainable only for one time point in a particular study subject. Because the analysis provides only a “snapshot” without information about the cellular biodynamics, it may be difficult to interpret studies, in particular when using cohorts of animals killed at different time points in model systems with large biological variation (*e.g.*, in experimental autoimmune encephalomyelitis as animal model for multiple sclerosis).

Recently, much progress has been made in the field of noninvasive *in vivo* imaging, which monitors cells in a serial manner in live subjects as a function of time post-grafting. Cells labeled before transplantation with a magnetic resonance (MR) contrast agent, for example, can be readily detected using MR imaging (MRI). Such labeling has the advantage of not requiring genetic manipulation of the cells before transplantation but is limited by cell divisions after labeling. Recent work has demonstrated that the FDA-approved superparamagnetic iron oxide (SPIO) contrast agent Feridex (Bayer Healthcare) can be quickly delivered into cells via electroporation.¹¹ Feridex particles “spoil” the homogeneity of the local magnetic field in the MRI scanner. In the vicinity of these particles, protons become “off-resonance,” which causes them to misalign and become out of sync (“dephase”) when an MRI pulse is delivered. When they return to equilibrium, the MR relaxation time shortens considerably and less signal is received by the MRI coil. The net result is that areas that contain these particles will appear hypo-intense. This approach to creating cellular contrast is very efficient, because many water protons will sense the magnetic field disturbance because of their continuous Brownian motion to and from SPIO-labeled cells.¹² It has been shown, using large magnetic microspheres, that the sensitivity can be at the level of single-cell detection.^{13,14} Following years of animal studies, including MR tracking of stem cells,^{15–19} MRI cell tracking using Feridex has now entered the clinic.^{20,21} A representative example of MR tracking of Feridex-labeled human ES cells is shown in Figure 1a,b. Instead of MRI of protons, one can also image fluorine (¹⁹F); it has the advantage of lack of background signal (there is no endogenous fluorine), and when cells are labeled with fluorine, cellular “hot-spot” imaging is feasible in a fashion similar to radionuclide imaging.²²

Another form of MR cell tracking involves the expression of an artificial reporter gene that encodes a lysine-rich protein (LRP).²³ Contrast is created through the chemical exchange of bulk water protons with (poly)peptide amide protons. The latter spins exhibit a slight, natural off-resonance (due to chemical interactions with the polypeptide backbone and amino acid residues) and, when irradiated with a saturation pulse at the specific off-resonance frequency, lose their capability to create signal. Because the “nulled” protons exchange with the bulk water protons—which always forms the basis of signal in MRI—signal is lost, making it possible to detect cells when they are engineered to express amide-rich polypeptides such as LRP (Figure 1c,d). Because this approach uses an endogenous reporter gene, the MR contrast in the cells is not reduced with each cell division—an advantage over exogenous labels such as Feridex. This is particularly important for assessing the tumorigenicity/teratoma formation of ES cells, in which case rapid cell proliferation occurs.

Other reporter genes that can be used to visualize stem cells *in vivo* include fire-fly luciferase and HSV-TK, which can be monitored via bioluminescence²⁴ or positron emission tomography (PET),^{25,26} respectively. Cao *et al.* demonstrated that these reporter genes can effectively track transplanted mouse ES cells and quantify their proliferation.²⁷ They also made use of the ability of the HSV-TK gene, the reporter used for PET, to become a potent suicide gene upon the administration of ganciclovir. This approach reveals the possibility of an effective fail-safe mechanism for any cell-based therapy: if the transplanted cells should happen to have a deleterious effect on the host, they can be eliminated with pharmacologic levels of ganciclovir (Figure 2).²⁷

Conclusion

It is evident that in recent years impressive progress has been made toward the translation of ES cell research to clinical practice. ES cell-differentiation protocols have become increasingly effective, and several different cell types can now be isolated to (near) homogeneity. The ability to monitor these cells following transplantation, once the absence of tumorigenic ES cells has been clearly proven, has also been greatly enhanced. Noninvasive imaging utilizing reporter genes—whether MR, PET, or bioluminescent imaging—will play an important role in securing regulatory approval for clinical translation of stem cell therapy. When implemented clinically, it is anticipated that the use of MRI will become an essential part of initiating stem cell therapy, because it can accurately determine the exact location of injected cells—even single cells—in real time. However, there are still several challenges yet to be fully addressed. Even the best *in vivo* imaging is unlikely to reveal whether a cell has begun to secrete new factors or has undergone genomic rearrangement. It remains unclear whether it is best to transplant ES-derived cells alone or in combination with other cells that can provide paracrine support. These and other issues will be resolved only through continued research using animal models. Although it may never be possible to fully predict every potential behavior of a transplanted cell, the awareness that many outcomes are indeed possible will help in the design of transplantation procedures to focus on safety as well as effectiveness.

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Figure 1.

Noninvasive imaging. MRI of **(a,b)** Feridex-labeled and **(c,d)** LRP-transfected cells. 5×10^5 Feridex-labeled cells were transplanted into one side of NOD-SCID mouse brain, represented by a hypo-intense area **(a)**, arrow). A 3D reconstruction of the raw data along with color segmentation and surface rendering allows overall visualization of the distribution of cells **(b)**, arrow). 5×10^4 LRP-transfected rat glioma cells were transplanted in the left hemisphere of NOD-SCID mouse brain; cells transfected with empty vector were injected contralaterally. Shown are the anatomical image **(c)** and the CEST signal intensity–difference map overlaid on the anatomical image **(d)**, distinguishing LRP-expressing and control xenografts. (Panels **a** and **b** courtesy of Candace Kerr and Piotr Walczak; panels **c** and **d** reprinted from ref. ²³ with permission.)

Figure 2.

Noninvasive bioluminescent imaging of ES-cell survival, proliferation, and teratoma formation. 1×10^6 ES-DF (double-fusion reporter, containing luciferase and GFP, but lacking truncated thymidine kinase (ttk)) cells were injected subcutaneously into the left shoulder and 1×10^6 ES-TF (triple-fusion reporter, containing luciferase and GFP as well as ttk) cells into the right shoulder of mice. Serial imaging over 2 weeks showed progressive increase in imaging signals (top row). To prevent teratoma formation, ganciclovir treatment was started from week 2 to week 5. At week 5, ES-TF cells carrying the HSV-ttk reporter-suicide gene showed background bioluminescent signal levels, whereas ES-DF cells lacking HSV-ttk showed dramatic increases in signal activity. (Reprinted from ref. ²⁷ with permission.)