Characterization of embryonic stem cell transplantation immunobiology using molecular imaging

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CHAPTER 4

Molecular Imaging of Human Embryonic Stem Cells: Keeping an Eye on Differentiation, Tumorigenicity, and Immunogenicity

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ABSTRACT

Human embryonic stem cells (hESCs) are capable of differentiation into every cell type of the human being. They are under extensive investigation for their regenerative potential in a variety of debilitating diseases. However, the field of hESC research is still in its infancy, as there are several critical issues that need to be resolved before clinical translation. Two major concerns are the ability of undifferentiated hESCs to form teratomas and the possibility of a provoked immune reaction after transplantation of hESCs into a new host. Therefore, it is imperative to develop non-invasive imaging modalities that allow for longitudinal, repetitive, and quantitative assessment of transplanted cell survival, proliferation, and migration in vivo. Reporter gene-based molecular imaging offers these characteristics and has great potential in the field of stem cell therapy. Moreover, it has recently been shown that reporter gene imaging can be combined with therapeutic strategies. Here, we provide an outline of the current status of hESC research and discuss the concerns of tumorigenicity and immunogenicity. Furthermore, we describe how molecular imaging can be utilized to follow and resolve these issues.

INTRODUCTION

Adult stem cells have great promise as potential treatments for a variety of intractable diseases. However, these cells are generally limited in their plasticity. Therefore, it would be ideal to obtain or create a cell line that is truly able to differentiate into every cell of the body. The first such cell line was derived in the 1960's and originated from teratomas that developed spontaneously in male mice of the 129 strain. These "embryonal carcinoma" (EC) cells were capable of teratoma formation after transplantation of single cells into a new host,1 confirming their ability to differentiate into progeny of all three germ layers (ectoderm, mesoderm and endoderm)--a phenomenon known as 'pluripotency'.2 Further research led to the first isolation of murine embryonic stem cells (mESCs) in 1981 from the epiblast of blastocyst-stage mouse embryos,3, 4 followed by the establishment of the first human embryonic stem cell (hESC)-line in 1998.2 To date, there are more than 300 hESC lines, but only 22 hESC lines are commercially available and registered in the "NIH Human Embryonic Stem Cell Registry".5

Although most studies using hESCs in disease models show auspicious results, there are several concerns about hESC transplantation. First, the pluripotent character of hESCs is somewhat a double-edged sword. They are an attractive candidate for cell based therapies, but their pluripotency can also lead to risk of teratoma formation after transplantation. Second, since it is presently impossible to transplant hESCs syngeneically, the possibility that hESCs might provoke an immune reaction following allogeneic transplantation must be considered. This review will discuss these issues and how molecular imaging can help resolve them.

DERIVATION, MAINTENANCE, AND DIFFERENTIATION

Traditionally, hESCs are isolated from the inner cell mass of the human blastocyst, or as recently shown, can be derived from single blastomeres.[6] The isolated cells can be expanded *in vitro*, with an average doubling time of 30-35 h.[7] However, strict homeostatic culture conditions and the addition of inhibiting compounds are necessary to keep the hESCs in an undifferentiated state, a condition required for maintaining a normal karyotype and an unlimited capacity for self-renewal. This is possible by growing hESCs on a cellular feeder layer. Inactivated murine embryonic fibroblasts prove to be an effective feeder layer for the undifferentiated growth of hESCs because they secrete differentiation-inhibiting factors.[8] Due to the risk of cross-species retroviral infection, however, this is an unattractive option in the long term. Recently, several reports have described the culture of hESCs in animal-free conditions, using human feeder cells consisting of foreskin,[9] pure human fibroblast populations,[10] or uterine endometrial cells and serum-free medium.[11] Generally, these undifferentiated hESCs express transcription fac-

tors OCT-3/4, Sox-2, and NANOG; surface markers CD9, CD133, and SSEA-3/4; proteoglycans TRA-1-60/81 and TRA-2-54; and enzyme alkaline phosphatases and telomerase.[12, 13]

Following withdrawal of inhibitory factors, hESCs will aggregate into three-dimensional clusters of cells in an early stage of differentiation, thereby losing pluripotency. These clusters, named Embryoid Bodies (EBs),[14] form the first step of further differentiation into any type of progeny. Within the EBs, a microenvironment exists in which various signals will promote differentiation into all three germ layers. Although differentiation generally occurs spontaneously, much effort currently focuses on stimulating directed differentiation to achieve sufficiently large populations for clinical use. The generation of pure, differentiated cultures is indispensable for developing cell based therapies, and will help us better understand cellular developmental processes and test pharmacological strategies.

Differentiation into Mesoderm Lineages

While reports have been published of hESCs differentiating into various mesodermal lineages, including kidney, muscle, bone and blood cells,[15] it is cardiomyogenesis that has received the most attention. Cardiomyogenesis typically manifests as a beating area within the EBs around 5 days after EB-formation, the surface of which increases gradually with time.[16] Kehat and colleagues were the first to show that hESC-derived cardiomyocytes within these beating EBs actually resemble the structural and functional properties of early stage human cardiomyocytes.[16] Since then, several other methods have been tested to improve the efficiency of *in vitro* differentiation of hESCs into cardiomyocytes with moderate success.[17-19]

Differentiation into Ectoderm Lineages

Using different growth factors and stimulating environments, hESCs can also be driven to differentiate into brain, skin, and adrenal derivates.[15] The potential of hESC-derived cultures for the treatment of neurodegenerative disorders is under intensive investigation. While many groups have described neuronal differentiation within the EB,[20-22] factor-induced neuronal differentiation seems to be limited to the addition of retinoic acid (RA) and nerve growth factor (betaNGF),[21] or the use of serum-free, conditioned medium.[23] The coculture of hESCs with murine skull bone marrow-derived stromal cells also seems to induce neuronal differentiation.[24]

Differentiation into Endoderm Lineages

From the beginning, hESCs were shown to be capable of differentiating *in vitro* into liver and pancreatic cells when exposed to a variety of growth factors.[15] The creation of insulin-secreting pancreatic cell populations has generated much interest, as this might ultimately provide a cell-based therapy for patients with type I diabetes.[25] However, the identification of insulin-producing cells has proven to be difficult and susceptible to artifacts.[26] Thus, the *in vitro* pancreatic differentiation from hESCs remains a challenging multi-step culturing procedure at present.[27]

In summary, although much is being done to improve the efficacy of *in vitro* differentiation systems, little is known about the cellular interactions that occur during natural differentiation. Most of the *in vitro* differentiation methods are to some extent dependent on EB formation. The process of *in vitro* EB formation mimics the natural transcriptional pathways occurring in the developing embryo, leading not only to the differentiation into the desired cell type, but also to the production of undesired cells. The most dangerous example of the latter is undifferentiated hESCs that retain the ability to form teratomas. Until we understand the precise pathways of pluripotent differentiation, the acquisition of desired, transplantable cell types can only rely on stimulating known pathways and the pre-transplantation selection of the desired cell type.

Teratoma formation

At present no selection method exists that can yield a 100% pure population, which is a major obstacle for clinical translation. When transplanted in immunodeficient mice, hESCs form teratomas consisting of human tissues from all germ layers.[2, 28] The formation and composition of teratomas seem to be influenced by several factors, including graft site,[29] transplanted cell number, and developmental phase of the host,[24] as described next.

One factor influencing hESC-based teratoma formation is the site of transplantation, which affects both growth and composition of the tumor. As recently shown by Cooke and colleagues, teratomas rising from hESCs will grow faster and contain more undifferentiated cells when transplanted in the *liver* of nude mice, as compared to *subcutaneous* transplantation.[29] It is of major interest why hESCs differentiate at the subcutaneous site but remain undifferentiated in the liver. The authors hypothesize that this was due to the well vascularized, growth factor-rich, immune-privileged porous structure in the liver.[29] These findings are not only a stimulant for further research on graft site and teratoma formation, but also indicate the importance of *in vivo* experiments, as there may be factors present in the liver that could help maintain hESCs in an undifferentiated state *in vitro*.

Another factor influencing teratoma formation is the number of undifferentiated hESCs that are viable after transplantation. As discussed earlier, transplantation of pure undifferentiated hESC inevitably leads to teratoma formation.[2, 28] Interestingly, transplantation of selected hESC-derived cells in a more developed phase will not automatically lead to teratomas, even when this population is not 100% pure.[30] However, there are no extant studies that assess the minimal cell number needed for teratoma formation, or stated otherwise, the maximum percentage of contamination with undifferentiated hESC. Our laboratory is actively investigating these issues.

Recently, Muotri and colleagues have studied undifferentiated hESC after *in utero* transplantation into the lateral brain ventricle of day-14 mouse embryos.[24] The results showed that hESCs integrated into the brain, giving rise to neuronal and glial lineages, but not to teratomas. These findings suggest that hESCs are susceptible to environmental cues that can

modulate its differentiation and tumorigenic potential, as was suggested in earlier studies with hESCs in chick embryos.[31]

Taken together, these observations and questions are not only a stimulant for further research on teratoma biology, but also indicate the importance of developing novel imaging techniques to track their growth *in vivo* longitudinally, repetitively, and quantitatively.

Immunogenicity

Another hurdle facing clinical transplantation of hESCs is the potential immunologic barrier. The immune response generated after transplantation is directed towards alloantigens, which are antigens presented on the cell surface that are considered non-self by the recipient immune system.[32] The major system of alloantigens responsible for cell incompatibility is the major histocompatibility complex (MHC). In humans, MHC-I molecules are expressed on the surface of virtually all nucleated cells and present antigens to CD8+ cytotoxic T cells, while MHC-II molecules are normally more restricted to antigen presenting cells such as dendritic cells and macrophages, and are selectively recognized by CD4+ helper T cells.[33]

It has been shown that hESCs express low levels of MHC-I in their undifferentiated state.[34, 35] In one study, the MHC-I expression increased two to four-fold when the cells were induced to spontaneously differentiate to EB, and an eight to ten-fold when induced to differentiate into teratomas.[34] In contrast, a different group observed MHC-I downregulation after differentiation induced with retinoic acid or on Matrigel or in extended cultures.[35] In both studies, MHC-I expression was strongly upregulated after treatment of the cells with interferon-γ, a potent MHC expression-inducing cytokine known to be released during the course of an immune response. MHC-II antigens were not expressed on hESCs or hESC derivatives.[34] The latter finding confirms that, in contrast to tissue allografts, hESC transplants are devoid of highly immunogenic mature dendritic cells, or any other type of specialized antigen presenting cells. Thus, the transplanted cells may not express MHC-II molecules required for effective priming of alloreactive CD4+ T cells through direct recognition.

Previously, our group tested allogeneic undifferentiated mESCs for their ability to trigger alloimmune response in a murine model of myocardial infarction.[36] We found progressive intragraft infiltration of inflammatory cells mediating both adaptive (T cells, B cells, and dendritic cells) and innate (macrophages and granulocytes) immunity. Cellular infiltration progressed from mild infiltration at two weeks to vigorous infiltration at four weeks, leading to rejection of the mESC allograft. Moreover, we found an accelerated immune response against mESCs that had differentiated *in vivo* for 2 weeks, suggesting that mESC immunogenicity increases upon their differentiation.[36]

Although it was previously reported that hESCs failed to elicit immune responses during the first 48 hours after intramuscular injection of immunocompetent mice,[37] a recent report using a similar model found hESCs to be completely eliminated at 1 month post-transplantation.[38] Thus, questions regarding the exact character and intensity of immune responses

towards allogeneic hESCs and their derivatives remain. Solutions that reduce or eliminate the potential immunological response to transplanted allogeneic hESCs are urgently needed. Possible strategies to minimize rejection of hESC transplants have been extensively reviewed elsewhere.[39, 40] Examples of these strategies include: (1) forming HLA isotyped hESC-line banks; (2) creating a universal donor cell by genetic modification; (3) inducing tolerance by hematopoietic chimerism; or (4) generating isogeneic hESC lines by somatic nuclear transfer. To optimize these techniques in the future, it is crucial to develop sensitive and reliable imaging methods for monitoring the viability of transplanted cells *in vivo*.

Molecular imaging

To date, most studies on stem cell therapy have relied on conventional reporter genes such as GFP[41] and β-galactosidase (lacZ) to monitor cell survival and differentiation. However, these reporter genes cannot be used to reliably track *in vivo* characteristics of transplanted cells due to poor tissue penetration and the need for extrinsic excitation light, which produces an

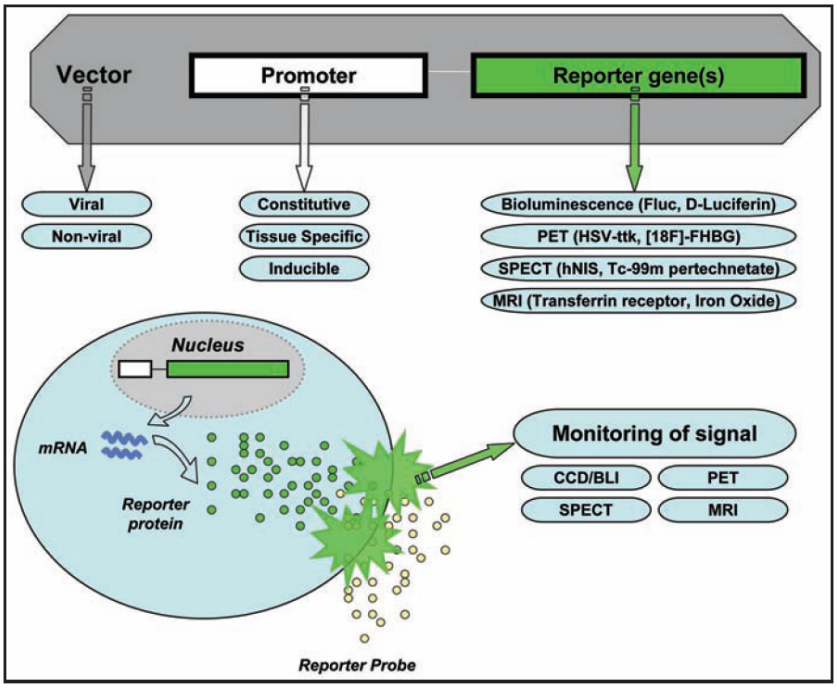

Figure 1: ***Schematic overview of molecular imaging.*** Outline of a vector containing a DNA reporter construct with the reporter gene(s) driven by a promoter of choice. Transcription and translation lead to production of mRNA and reporter protein, respectively. After administration of a reporter probe systemically, the reporter probe will be catalyzed by specific cells that have the reporter proteins. This amplification process can be detected by a sensitive imaging device. Examples of reporter genes and their specific reporter probes are listed per imaging modality. Abbreviations: Fluc, Firefly luciferase; PET, positron emission tomography; HSV-ttk, herpes simplex virus truncated thymidine kinase; [^{18}F]-FHBG, 9-(4-[^{18}F]-fluoro-3hydroxymethylbutyl) guanine; SPECT, single photon emission computed tomography; hNIS, human sodium/iodide symporter; MRI, magnetic resonance imaging; CCD, charged coupled device; BLI, bioluminescence imaging.

unacceptable amount of background signal. Instead, GFP-labeled cells are typically identified histologically, which provides only a single snapshot representation rather than a complete picture of cell survival over time. To solve these shortcomings, our group has been developing reporter gene-based molecular imaging techniques.[42]

Molecular imaging can be broadly defined as the *in vivo* characterization of cellular and molecular processes.[43] The backbone of reporter gene-based molecular imaging technique is the design of a suitable reporter construct. This construct carries a reporter gene linked to a promoter/enhancer, which can be inducible, constitutive, or tissue specific. The construct can be introduced into the target tissue by molecular biology techniques using either viral or non-viral techniques. Transcription of DNA and translation of mRNA lead to the production of reporter protein. After administration of a reporter probe, the reporter protein reacts with the reporter probe, giving rise to signals that are detectable by a charged-coupled device (CCD) camera, positron emission tomography (PET), single photon emission computed tomography (SPECT), or magnetic resonance imaging (MRI) (**Figure 1**). For thorough review, please refer to other relevant articles.[43, 44]

Imaging ESC Transplantation, Tumorigenicity, and Immunogenicity

A major advantage of reporter gene imaging is the incorporation of the reporter construct into the cellular DNA. This ensures that the reporter gene will only be expressed by living cells and will be passed on equally to the cell's progeny. Thus, this imaging modality can provide significant insight into cell viability and proliferation. As discussed earlier, monitoring cell viability is a critical requirement to assess immunogenicity, as a provoked immune reaction can kill transplanted cells. Monitoring cell proliferation is another important feature, considering the tumorigenic potential of undifferentiated hESCs. Moreover, the ability to image the whole-body will allow us to track cell migration in other organs. This is a major advantage when compared to tissue biopsies using GFP-labeled cells.

In addition, multiple reporter genes can also be introduced into the same cell for multimodality imaging. Recently, our group has tested the efficacy of mESC with a self-inactivating lentiviral vector carrying triple-fusion (TF) construct containing firefly luciferase (Fluc), monomeric red fluorescent protein (mRFP), and herpes simplex virus truncated thymidine kinase (HSV-ttk).[45] The mRFP in the construct facilitates the imaging of single cells by fluorescence microscopy and allows for the isolation of a stable clone population by fluorescence activated cell sorter (FACS). The Fluc can be used to perform high throughput bioluminescence imaging (BLI) for assessment of cell survival, proliferation and migration at relatively low costs. Finally, the HSV-ttk allows for deep-tissue PET imaging of gene expression in small animals[45, 46] as well as in patients.[47, 48] After transplantation into the hearts of athymic nude rats, mESCs could be successfully followed for 4 weeks using BLI and PET imaging. Between week 2 and 4, both BLI and PET reporter gene signals increased rapidly, indicating teratoma formation. This was confirmed by histological analysis.[45]

Because of the risk of teratoma formation, it would be ideal to have an *in vivo* imaging modality in combination with a fail-safe suicide-gene mechanism. Using the antiviral drug ganciclovir, which is toxic against cells expressing HSV-ttk, Cao and colleagues were able to ablate teratoma formation and follow this progress non-invasively.[49] This study reveals the excellent potential of reporter gene imaging for future use with hESC transplantation. In fact, preliminary studies in our lab suggest that as low as 100 undifferentiated hESCs (H9 line) can cause teratoma formation after subcutaneous injection (**Figure 2**). Whether lower cell numbers (e.g. 1, 10, 50), other graft sites (e.g. intramuscular, intravenous), or different cell lines (e.g., federally and non-federally approved) have similar kinetics of teratoma formation will need to be determined carefully in the future.

Finally, a very critical question with regard to reporter genes is whether they will affect ESC differentiation and hamper efforts for clinical applications. A previous study from our lab has shown that the TF reporter genes affect <2% of total genes of mESC using transcriptional

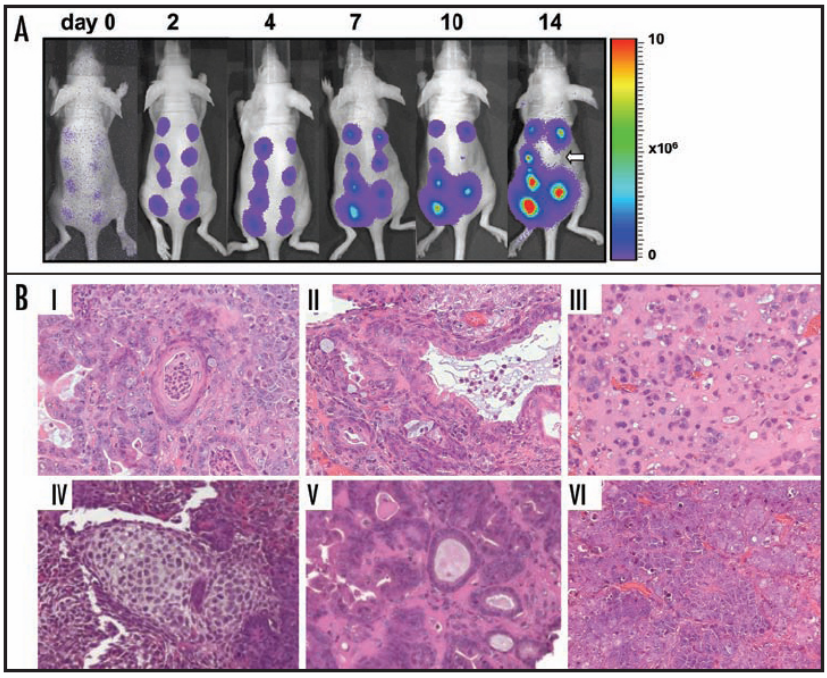

Figure 2: ***In vivo bioluminescence imaging of teratoma formation after transplantation of 100 hESCs.*** **(A)** Bioluminescence image showing longitudinal follow up after transplantation of 100 hESCs stably expressing a double fusion reporter gene (Fluc-GFP). Faint imaging signals were seen as early as 2 hrs after transplant, which became progressively stronger over 2 weeks. Histology at 8 weeks confirmed teratoma formation. Note one of the hESC transplanted sites did not successfully engraft (arrow) as there were no detectable signals by 2 weeks. **(B)** Histology from a representative explanted teratoma showing hESCs that have differentiated into Histology from a representative explanted teratoma showing hESCs that have differentiated into derivates from different germ layers. (I) squamous cell differentiation with keratin pearl; (II) respiratory epithelium with ciliated columnar and mucin producing goblet cells; (III) osteoid (non-mineralized bone) formation; (IV) cartilage formation; (V) gland cells; and (VI) rosette consistent with neuroectodermal differentiation (400x magnification).

profiling analysis.[50] A more recent follow up study using proteomic analysis show that there were no significant differences between control mESCs versus mESCs with reporter genes.[51] Importantly, reporter probes such as D-Luciferin (for Fluc) and [^{18}F]-FHBG (for HSV-ttk) had no adverse effects on mESC viability and proliferation as well.[45] Ongoing studies are evaluating the effects of reporter gene expression and reporter probes on various hESC cell lines.

CONCLUSION

Clearly, the capacity of hESCs to differentiate into almost all human cell types highlights their promising role in regenerative therapies for the treatment of heart disease, Parkinson's disease, leukemia, diabetes, and other degenerative disorders. But the pluripotency of hESCs may also pose major risks such as teratoma formation. Likewise, hESCs might not be immunoprivileged and could trigger host immune responses, leading to decreased cell survival or acute rejection. These are issues that can become significant barriers to future clinical application of hESC-based therapies. To meet these challenges, researchers must gain a better understanding of the *in vivo* behavior of transplanted hESCs. This review outlines the burgeoning application of molecular imaging to track transplanted hESCs *in vivo*. Continuing research merging molecular imaging and hESC biology will likely lead to significant advances in the future, both scientifically and medically.

REFERENCES

1. Kleinsmith LJ, Pierce GB, Jr. Multipotentiality Of Single Embryonal Carcinoma Cells. *Cancer Res.* Oct 1964;24:1544-1551.
2. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* Nov 6 1998;282(5391):1145-1147.
3. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* Jul 9 1981;292(5819):154-156.
4. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A.* Dec 1981;78(12):7634-7638.
5. NIH. National Institutes of Health - Human Embryonic Stem Cell Registry.
6. Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R. Human embryonic stem cell lines derived from single blastomeres. *Nature.* Aug 23 2006.
7. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol.* Nov 15 2000;227(2):271-278.
8. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol.* Oct 2001;19(10):971-974.
9. Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, Itskovitz-Eldor J. Human feeder layers for human embryonic stem cells. *Biol Reprod.* Jun 2003;68(6):2150-2156.
10. Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol.* Sep 2002;20(9):933-936.
11. Lee JB, Lee JE, Park JH, Kim SJ, Kim MK, Roh SI, Yoon HS. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. *Biol Reprod.* Jan 2005;72(1):42-49.
12. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. *Nat Biotechnol.* Jun 2005;23(6):699-708.
13. Wei H, Juhasz O, Li J, Tarasova YS, Boheler KR. Embryonic stem cells and cardiomyocyte differentiation: phenotypic and molecular analyses. *J Cell Mol Med.* Oct-Dec 2005;9(4):804-817.
14. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol.* Jun 1985;87:27-45.
15. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A.* Oct 10 2000;97(21):11307-11312.
16. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest.* Aug 2001;108(3):407-414.
17. Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, de la Riviere AB, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells.* Jun-Jul 2005;23(6):772-780.
18. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res.* Sep 20 2002;91(6):501-508.

19. Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, Ding S. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci U S A.* May 2 2006;103(18):6907-6912.

20. Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T. Neural progenitors from human embryonic stem cells. *Nat Biotechnol.* Dec 2001;19(12):1134-1140.

21. Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, Benvenisty N. Induced neuronal differentiation of human embryonic stem cells. *Brain Res.* Sep 21 2001;913(2):201-205.

22. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol.* Dec 2001;19(12):1129-1133.

23. Schulz TC, Palmarini GM, Noggle SA, Weiler DA, Mitalipova MM, Condie BG. Directed neuronal differentiation of human embryonic stem cells. *BMC Neurosci.* Oct 22 2003;4:27.

24. Muotri AR, Nakashima K, Toni N, Sandler VM, Gage FH. Development of functional human embryonic stem cell-derived neurons in mouse brain. *Proc Natl Acad Sci U S A.* Dec 20 2005;102(51):18644-18648.

25. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. *Diabetes.* Aug 2001;50(8):1691-1697.

26. Rajagopal J, Anderson WJ, Kume S, Martinez OI, Melton DA. Insulin staining of ES cell progeny from insulin uptake. *Science.* Jan 17 2003;299(5605):363.

27. Segev H, Fishman B, Ziskind A, Shulman M, Itskovitz-Eldor J. Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells.* 2004;22(3):265-274.

28. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol.* Apr 2000;18(4):399-404.

29. Cooke MJ, Stojkovic M, Przyborski SA. Growth of teratomas derived from human pluripotent stem cells is influenced by the graft site. *Stem Cells Dev.* Apr 2006;15(2):254-259.

30. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, Huber I, Satin J, Itskovitz-Eldor J, Gepstein L. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat Biotechnol.* Oct 2004;22(10):1282-1289.

31. Goldstein RS, Drukker M, Reubinoff BE, Benvenisty N. Integration and differentiation of human embryonic stem cells transplanted to the chick embryo. *Dev Dyn.* Sep 2002;225(1):80-86.

32. Janeway CA, Jr. The role of self-recognition in receptor repertoire development. Members of the Janeway Laboratory. *Immunol Res.* 1999;19(2-3):107-118.

33. Lechler RI, Lombardi G, Batchelor JR, Reinsmoen N, Bach FH. The molecular basis of alloreactivity. *Immunol Today.* Mar 1990;11(3):83-88.

34. Drukker M, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, Reubinoff B, Mandelboim O, Benvenisty N. Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci U S A.* Jul 23 2002;99(15):9864-9869.

35. Draper JS, Pigott C, Thomson JA, Andrews PW. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat.* Mar 2002;200(Pt 3):249-258.

36. Swijnenburg RJ, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, Lebl DR, Caffarelli AD, de Bruin JL, Fedoseyeva EV, Robbins RC. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. *Circulation.* Aug 30 2005;112(9 Suppl):I166-172.

37. Li L, Baroja ML, Majumdar A, Chadwick K, Rouleau A, Gallacher L, Ferber I, Lebkowski J, Martin T, Madrenas J, Bhatia M. Human embryonic stem cells possess immune-privileged properties. *Stem Cells.* 2004;22(4):448-456.

38. Drukker M, Katchman H, Katz G, Even-Tov Friedman S, Shezen E, Hornstein E, Mandelboim O, Reisner Y, Benvenisty N. Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells. *Stem Cells.* Feb 2006;24(2):221-229.

39. Boyd AS, Higashi Y, Wood KJ. Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation. *Adv Drug Deliv Rev.* Dec 12 2005;57(13):1944-1969.

40. Drukker M. Immunogenicity of human embryonic stem cells: can we achieve tolerance? *Springer Semin Immunopathol.* Nov 2004;26(1-2):201-213.

41. Ro S. Magnifying stem cell lineages: the stop-EGFP mouse. *Cell Cycle.* Oct 2004;3(10):1246-1249.

42. Sheikh AY, Wu JC. Molecular imaging of cardiac stem cell transplantation. *Curr Cardiol Rep.* Mar 2006;8(2):147-154.

43. Blasberg RG, Tjuvajev JG. Molecular-genetic imaging: current and future perspectives. *J Clin Invest.* Jun 2003;111(11):1620-1629.

44. Wu JC, Tseng JR, Gambhir SS. Molecular imaging of cardiovascular gene products. *J Nucl Cardiol.* Jul-Aug 2004;11(4):491-505.

45. Cao F, Lin S, Xie X, Ray P, Patel M, Zhang X, Drukker M, Dylla SJ, Connolly AJ, Chen X, Weissman IL, Gambhir SS, Wu JC. In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation.* Feb 21 2006;113(7):1005-1014.

46. Wu JC, Chen IY, Sundaresan G, Min JJ, De A, Qiao JH, Fishbein MC, Gambhir SS. Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. *Circulation.* Sep 16 2003;108(11):1302-1305.

47. Jacobs A, Voges J, Reszka R, Lercher M, Gossmann A, Kracht L, Kaestle C, Wagner R, Wienhard K, Heiss WD. Positron-emission tomography of vector-mediated gene expression in gene therapy for gliomas. *Lancet.* Sep 1 2001;358(9283):727-729.

48. Penuelas I, Mazzolini G, Boan JF, Sangro B, Marti-Climent J, Ruiz M, Ruiz J, Satyamurthy N, Qian C, Barrio JR, Phelps ME, Richter JA, Gambhir SS, Prieto J. Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology.* Jun 2005;128(7):1787-1795.

49. Cao F, Drukker M, Lin S, Sheikh A, Xie X, Li Z, Weissman I, Wu J. Molecular Imaging of Embryonic Stem Cell Misbehavior and Suicide Gene Ablation. *Cloning and Stem Cells - in press.*

50. Wu JC, Spin JM, Cao F, Lin S, Xie X, Gheysens O, Chen IY, Sheikh AY, Robbins RC, Tsalenko A, Gambhir SS, Quertermous T. Transcriptional profiling of reporter genes used for molecular imaging of embryonic stem cell transplantation. *Physiol Genomics.* Mar 13 2006;25(1):29-38.

51. Wu JC, Cao F, Dutta S, Xie X, Kim E, Chungfat N, Gambhir SS, Mathewson S, Connolly AJ, Brown M, Wang EW. Proteomic analysis of reporter genes for molecular imaging of transplanted embryonic stem cells (in press). *Proteomics.* 2006; ( ).