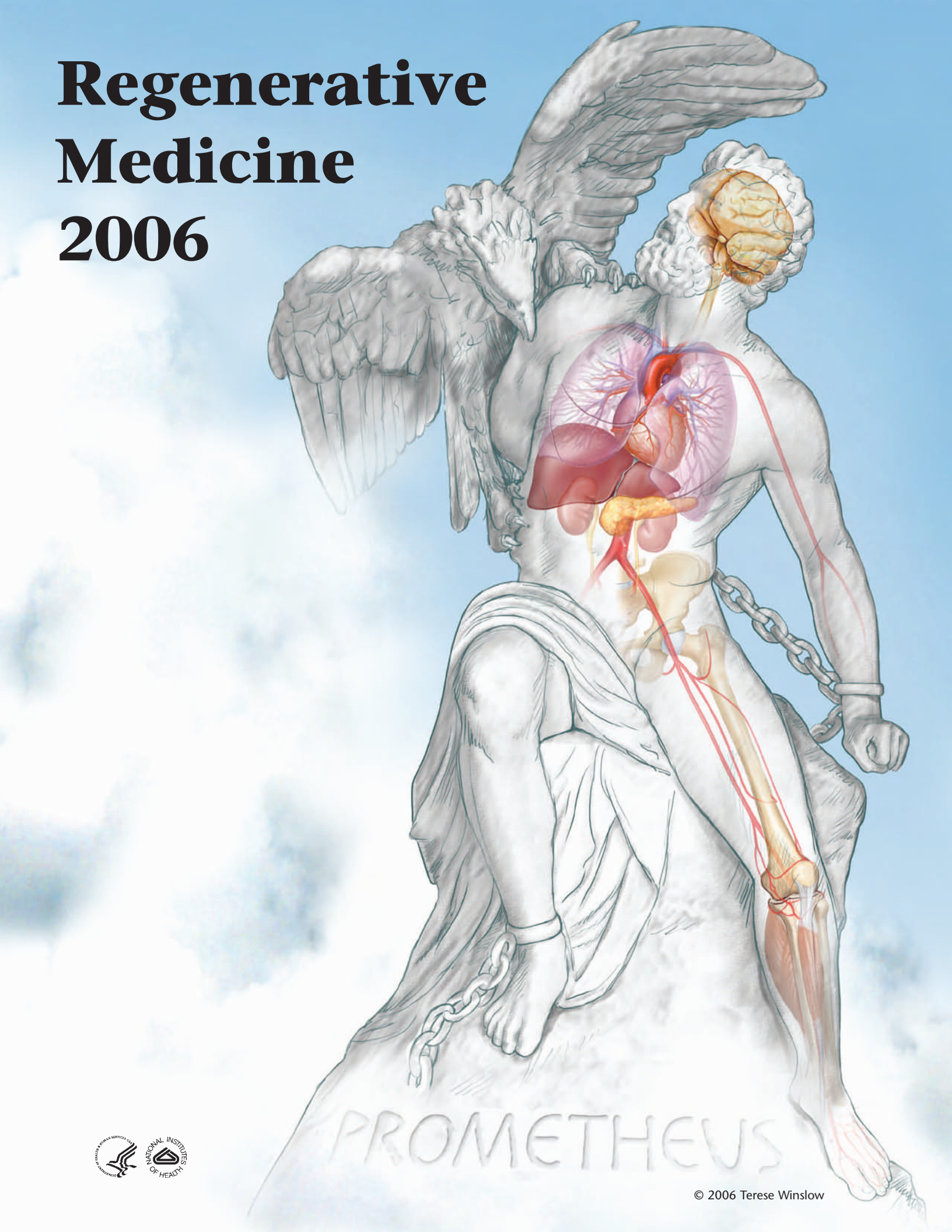
Regenerative Medicine 2006



INTRODUCTION

The Greek Titan, Prometheus, is a fitting symbol for regenerative medicine. As punishment for giving fire to Humankind, Zeus ordered Prometheus chained to a rock and sent an eagle to eat his liver each day. However, Prometheus' liver was able to regenerate itself daily, enabling him to survive. The scientific researchers and medical doctors of today hope to make the legendary concept of regeneration into reality by developing therapies to restore lost, damaged, or aging cells and tissues in the human body.

This report features chapters written by experts in several areas of enormous potential for regenerative medicine. Drs. Junying Yu and James A. Thomson explain the basic features of embryonic stem cells, how they are being used in research, and how they may lead to human therapies. Drs. Jos Domen, Amy Wagers, and Irving Weissman describe the historical origins of blood-forming stem cell research, basic features of these adult stem cells, progress on using these cells for human therapies, and future possibilities. Dr. David Panchision explores ways to use cell-based therapies to restore lost function in the human nervous system. Dr. Thomas Zwaka explains how stem cells may be used for gene therapy, and Dr. Mark L. Rohrbaugh explains the current state of intellectual property issues associated with research using human embryonic stem cells.

[This page intentionally left blank]

1. EMBRYONIC STEM CELLS

by Junying Yu and James A. Thomson***

Human embryonic stem (ES) cells capture the imagination because they are immortal and have an almost unlimited developmental potential *(Fig. 1.1: How hESCs are derived).* After many months of growth in culture dishes, these remarkable cells maintain the ability to form cells ranging from muscle to nerve to blood — potentially any cell type that makes up the body. The proliferative and developmental potential of human ES cells promises an essentially unlimited supply of specific cell types for basic research and for transplantation therapies for diseases ranging from heart disease to Parkinson's disease to leukemia. Here we discuss the origin and properties of human ES cells, their implications for basic research and human medicine, and recent research progress since August 2001, when President George W. Bush allowed federal funding of this research for the first time. A previous report discussed progress prior to June 17, 2001 (http://stemcells.nih.gov/info/scireport/.)

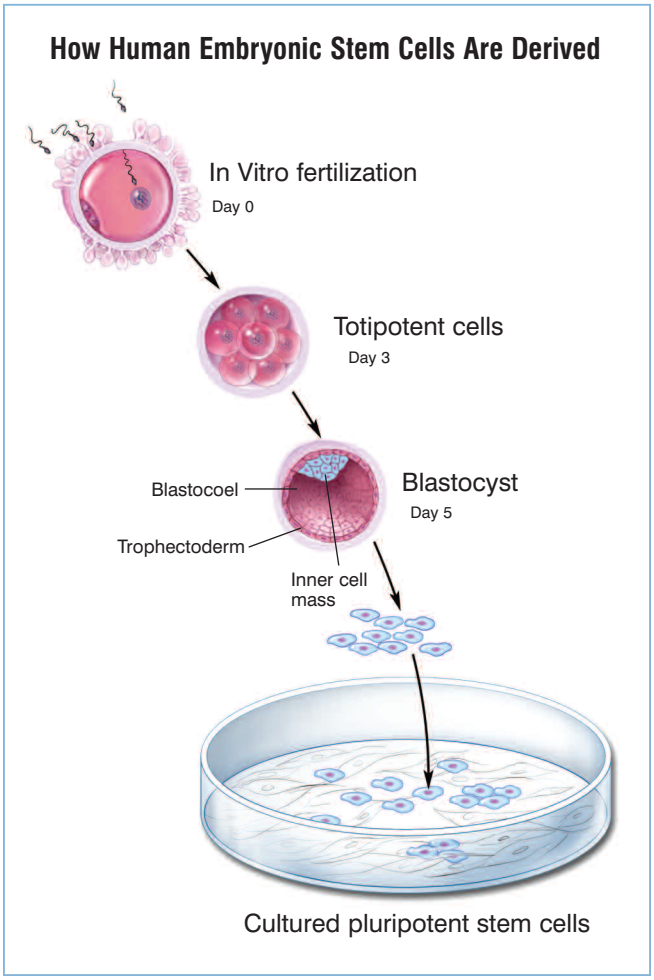


Figure 1.1. How Human Embryonic Stem Cells are Derived

WHAT ARE EMBRYONIC STEM CELLS?

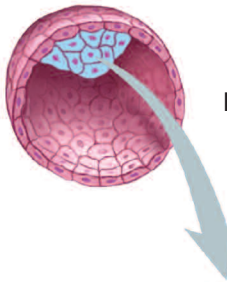
Embryonic stem cells are derived from embryos at a developmental stage before the time that implantation would normally occur in the uterus. Fertilization normally occurs in the oviduct, and during the next few days, a series of cleavage divisions occur as the embryo travels down the oviduct and into the uterus. Each of the cells (blastomeres) of these cleavage-stage embryos are undifferentiated, *i.e.* they do not look or act like the specialized cells of the adult, and the blastomeres are not yet committed to becoming any particular type of differentiated cell. Indeed, each of these blastomeres has the potential to give rise to any cell of the body. The first differentiation event in humans occurs at approximately five days of development, when an outer layer of cells committed to becoming part of the placenta (the trophectoderm) separates from the inner cell mass (ICM). The ICM cells have the potential to generate any cell type of the body, but after implantation, they are quickly depleted as they differentiate to other cell types with more

* Genetics and Biotechnology Building, Madison, WI 53706, *Email:* jyu@primate.wisc.edu

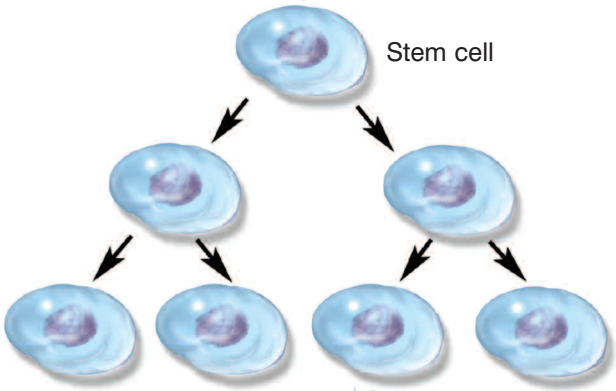
** John D. MacArthur Professor, Department of Anatomy, University of Wisconsin–Madison Medical School, The Genome Center of Wisconsin, and The Wisconsin National Primate Research Center, Madison, WI 53715, *Email:* thomson@primate.wisc.edu

Characteristics of Embryonic Stem Cells

1. Origin:
Derived from pre-implantation or peri-implantation embryo



Blastocyst

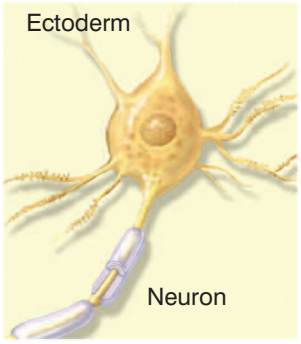


Stem cell

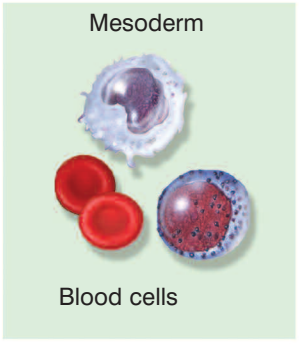
2. Self-Renewal:
The cells can divide to make copies of themselves for a prolonged period of time without differentiating.

3. Pluripotency:
Embryonic stem cells can give rise to cells from all three embryonic germ layers even after being grown in culture for a long time.

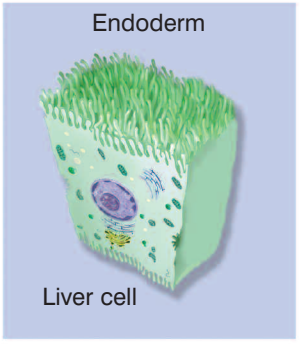
The three germ layers and one example of a cell type derived from each layer:



Ectoderm gives rise to: brain, spinal cord, nerve cells, hair, skin, teeth, sensory cells of eyes, ears nose, and mouth, and pigment cells.



Mesoderm gives rise to: muscles, blood, blood vessels, connective tissues, and the heart.



Endoderm gives rise to: the gut (pancreas, stomach, liver, etc.), lungs, bladder, and germ cells (eggs or sperm)

Figure 1.2.Characteristics of Embryonic Stem Cells.

limited developmental potential. However, if the ICM is removed from its normal embryonic environment and cultured under appropriate conditions, the ICM-derived cells can continue to proliferate and replicate themselves indefinitely and still maintain the developmental potential to form any cell type of the body (*"pluripotency"; see Fig. 1.2: Characteristics of ESCs*). These pluripotent, ICM-derived cells are ES cells.

The derivation of mouse ES cells was first reported in 1981,[1,2] but it was not until 1998 that derivation of human ES cell lines was first reported.[3] Why did it take such a long time to extend the mouse results to humans? Human ES cell lines are derived from embryos produced by *in vitro* fertilization (IVF), a process in which oocytes and sperm are placed together to allow fertilization to take place in a culture dish. Clinics use this method to treat certain types of infertility, and sometimes, during the course of these treatments, IVF embryos are produced that are no longer needed by the couples for producing children. Currently, there are nearly 400,000 IVF-produced embryos in frozen storage in the United States alone,[4] most of which will be used to treat infertility, but some of which (~2.8%) are destined to be discarded. IVF-produced embryos that would otherwise have been discarded were the sources of the human ES cell lines derived prior to President Bush's policy decision of August 2001. These human ES cell lines are now currently eligible for federal funding. Although attempts to derive human ES cells were made as early as the 1980s, culture media for human embryos produced by IVF were suboptimal. Thus, it was difficult to culture single-cell fertilized embryos long enough to obtain healthy blastocysts for the derivation of ES cell lines. Also, species-specific differences between mice and humans meant that experience with mouse ES cells was not completely

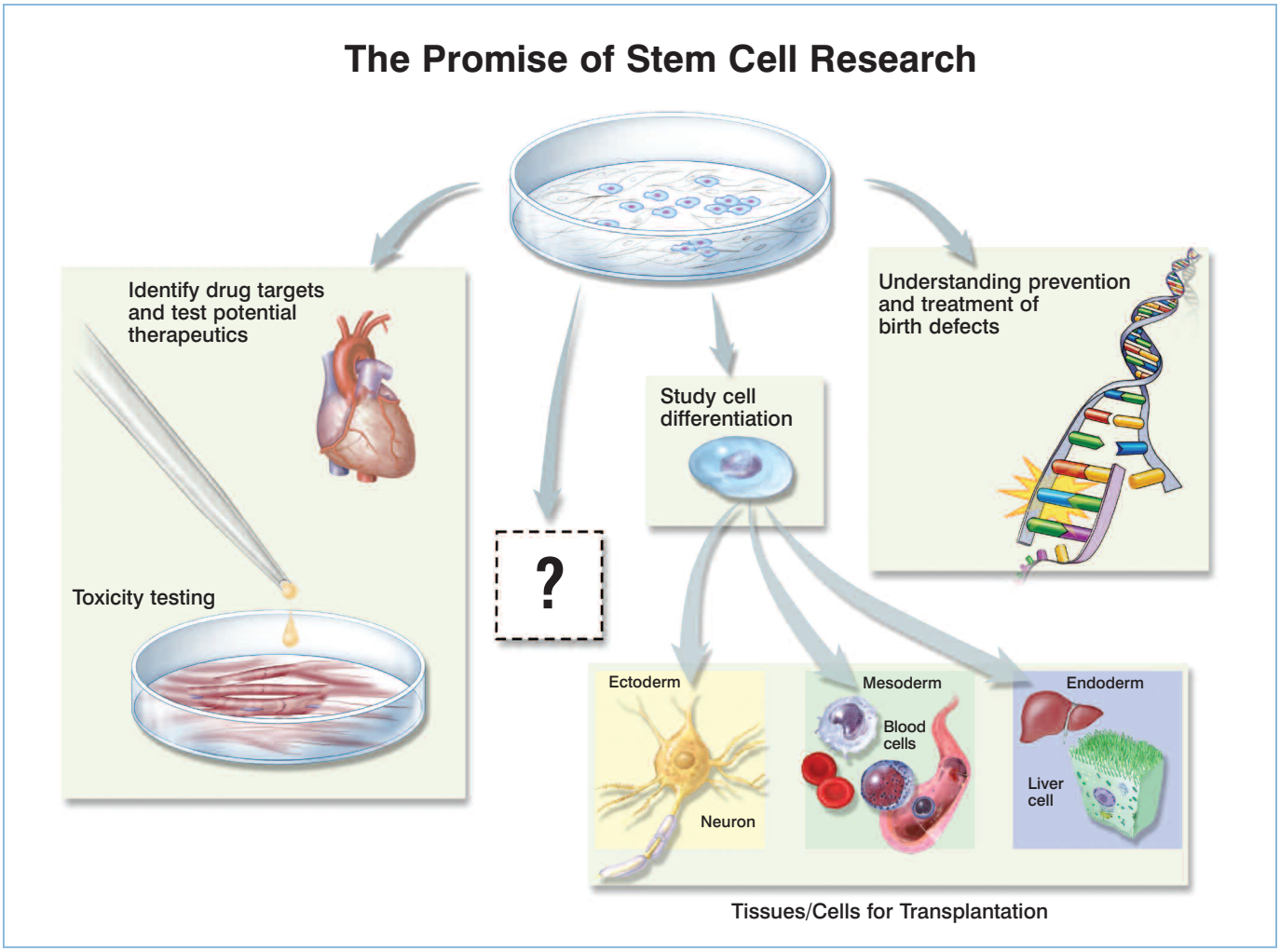


Figure 1.3: The Promise of Stem Cell Research

applicable to the derivation of human ES cells. In the 1990s, ES cell lines from two non-human primates, the rhesus monkey[5] and the common marmoset,[6] were derived, and these offered closer models for the derivation of human ES cells. Experience with non-human primate ES cell lines and improvements in culture medium for human IVF-produced embryos led rapidly to the derivation of human ES cell lines in 1998.[3]

Because ES cells can proliferate without limit and can contribute to any cell type, human ES cells offer an unprecedented access to tissues from the human body. They will support basic research on the differentiation and function of human tissues and provide material for testing that may improve the safety and efficacy of human drugs *(Figure 1.3: Promise of SC Research).*[7,8] For example, new drugs are not generally tested on human heart cells because no human heart cell lines exist. Instead, researchers rely on animal models. Because of important species-specific differences between animal and human hearts, however, drugs that are toxic to the human heart have occasionally entered clinical trials, sometimes resulting in death. Human ES cell-derived heart cells may be extremely valuable in identifying such drugs before they are used in clinical trials, thereby accelerating the drug discovery process and leading to safer and more effective treatments.[9-11] Such testing will not be limited to heart cells, but to any type of human cell that is difficult to obtain by other sources.

Human ES cells also have the potential to provide an unlimited amount of tissue for transplantation therapies to treat a wide range of degenerative diseases. Some important human diseases are caused by the death or dysfunction of one or a few cell types, *e.g.,* insulin-producing cells in diabetes or dopaminergic neurons in Parkinson's disease. The replacement of these cells could offer a lifelong treatment for these disorders. However, there are a number of challenges to develop human ES cell-based transplantation therapies, and many years of basic research will be required before such therapies can be used to treat patients. Indeed, basic research enabled by human ES cells is likely to impact human health in ways unrelated to transplantation medicine. This impact is likely to begin well before the widespread use of ES cells in transplantation and ultimately could have a more profound long-term effect on human medicine. Since August 2001, improvements in culture of human ES cells, coupled with recent insights into the nature of pluripotency, genetic manipulation of human ES cells, and differentiation, have expanded the possibilities for these unique cells.

CULTURE OF ES CELLS

Mouse ES cells and human ES cells were both originally derived and grown on a layer of mouse fibroblasts (called "feeder cells") in the presence of bovine serum. However, the factors that sustain the growth of these two cell types appear to be distinct. The addition of the cytokine, leukemia inhibitory factor (LIF), to serum-containing medium allows mouse ES cells to proliferate in the absence of feeder cells. LIF modulates mouse ES cells through the activation of STAT3 (signal transducers and activators of transcription) protein. In serum-free culture, however, LIF alone is insufficient to prevent mouse ES cells from differentiating into neural cells. Recently, Ying *et al.* reported that the combination of bone morphogenetic proteins (BMPs) and LIF is sufficient to support the self-renewal of mouse ES cells.[12] The effects of BMPs on mouse ES cells involve induction of inhibitor of differentiation (Id) proteins, and inhibition of extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinases (MAPK).[12,13] However, LIF in the presence of serum is not sufficient to promote the self-renewal of human ES cells,[3] and the LIF/STAT3 pathway appears to be inactive in undifferentiated human ES cells.[14,15] Also, the addition of BMPs to human ES cells in conditions that would otherwise support ES cells leads to the rapid differentiation of human ES cells.[16,17]

Several groups have attempted to define growth factors that sustain human ES cells and have attempted to identify culture conditions that reduce the exposure of human ES cells to non human animal products. One important growth factor, bFGF, allows the use of a serum replacement to sustain human ES cells in the presence of fibroblasts, and this medium allowed the clonal growth of human ES cells.[18] A "feeder-free" human ES cell culture system has been developed, in which human ES cells are grown on a protein matrix (mouse Matrigel or Laminin) in a bFGF-containing medium that is previously "conditioned" by co-culture with fibroblasts.[19] Although this culture system eliminates direct contact of human ES cells with the fibroblasts, it does not remove the potential for mouse pathogens being introduced into the culture via the fibroblasts. Several different sources of human feeder

cells have been found to support the culture of human ES cells, thus removing the possibility of pathogen transfer from mice to humans.[20–23] However, the possibility of pathogen transfer from human to human in these culture systems still remains. More work is still needed to develop a culture system that eliminates the use of fibroblasts entirely, which would also decrease much of the variability associated with the current culture of human ES cells. Sato *et al.* reported that activation of the Wnt pathway by 6-bromoindirubin-3'-oxime (BIO) promotes the self-renewal of ES cells in the presence of bFGF, Matrigel, and a proprietary serum replacement product.[24] Amit *et al.* reported that bFGF, TGFβ, and LIF could support some human ES cell lines in the absence of feeders.[25] Although there are some questions about how well these new culture conditions will work for different human ES cell lines, there is now reason to believe that defined culture conditions for human ES cells, which reduce the potential for contamination by pathogens, will soon be achieved*.

Once a set of defined culture conditions is established for the derivation and culture of human ES cells, challenges to improve the medium will still remain. For example, the cloning efficiency of human ES cells — the ability of a single human ES cell to proliferate and become a colony — is very low (typically less than 1%) compared to that of mouse ES cells. Another difficulty is the potential for accumulation of genetic and epigenetic changes over prolonged periods of culture. For example, karyotypic changes have been observed in several human ES cell lines after prolonged culture, and the rate at which these changes dominate a culture may depend on the culture method.[26,27] The status of imprinted (epigenetically modified) genes and the stability of imprinting in various culture conditions remain completely unstudied in human ES cells**. The status of imprinted genes can clearly change with culture conditions in other cell types.[28,29] These changes present potential problems if human ES cells are to be used in cell replacement therapy, and optimizing medium to reduce the rate at which genetic and epigenetic changes accumulate in culture represents a long-term endeavor. The ideal human ES cell medium, then, (a) would be cost-effective and easy to use so that many more investigators can use human ES cells as a research tool; (b) would be composed entirely of defined components not of animal origin; (c) would allow cell growth at clonal densities; and (d) would minimize the rate at which genetic and epigenetic changes accumulate in culture. Such a medium will be a challenge to develop and will most likely be achieved through a series of incremental improvements over a period of years.

Among all the newly derived human ES cell lines, twelve lines have gained the most attention. In March 2004, a South Korean group reported the first derivation of a human ES cell line (SCNT-hES-1) using the technique of somatic cell nuclear transfer (SCNT). Human somatic nuclei were transferred into human oocytes (nuclear transfer), which previously had been stripped of their own genetic material, and the resultant nuclear transfer products were cultured *in vitro* to the blastocyst stage for ES cell derivation.[30]*** Because the ES cells derived through nuclear transfer contain the same genetic material as that of the nuclear donor, the intent of the procedure is that the differentiated derivatives would not be rejected by the donor's immune system if used in transplantation therapy. More recently, the same group reported the derivation of eleven more human SCNT-ES cell lines*** with markedly improved efficiency (16.8 oocytes/line vs. 242 oocytes/line in their previous report).[31]*** However, given the abnormalities frequently observed in cloned animals, and the costs involved, it is not clear how useful this procedure will be in clinical applications. Also, for some autoimmune diseases, such as type I diabetes, merely providing genetically-matched tissue will be insufficient to prevent immune rejection.

Additionally, new human ES cell lines were established from embryos with genetic disorders, which were detected during the practice of preimplantation genetic diagnosis (PGD). These new cell lines may provide an excellent *in vitro* model for studies on the effects that the genetic mutations have on cell proliferation and differentiation.[32]

---

* **Editor's note:** Papers published since this writing report defined culture conditions for human embryonic stem cells. See Ludwig et al., Nat. Biotech 24: 185-187, 2006; and Lu et al., PNAS 103:5688-5693, 2006.08.14.

** **Editor's note:** Papers published since the time this chapter was written address this: see Maitra et al., Nature Genetics 37, 1099-1103, 2005; and Rugg-Gunn et al., Nature Genetics 37:585-587, 2005.

*** **Editor's note:** Both papers referenced in 30 and 31 were later retracted: see Science 20 Jan 2006; Vol. 311. No. 5759, p. 335.

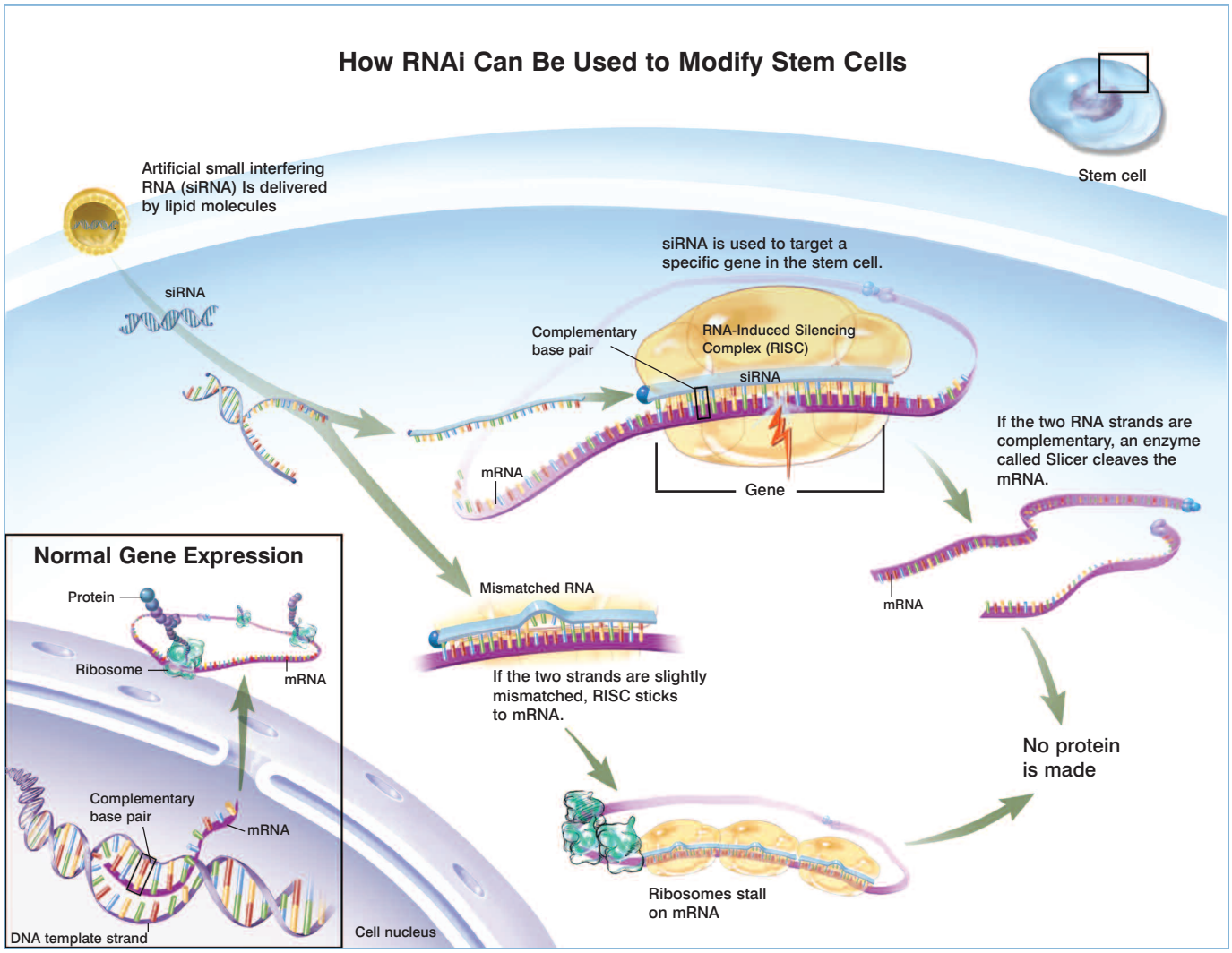


Figure 1.4. How RNAi Can Be Used To Modify Stem Cells

To date, more than 120 human ES cell lines have been established worldwide,[33]* 67 of which are included in the National Institutes of Health (NIH) registry (http://stemcells.nih.gov/research/registry/). As of this writing, 21 cell lines are currently available for distribution, all of which have been exposed to animal products during their derivation. Although it has been eight years since the initial derivation of human ES cells, it is an open question as to the extent that independent human ES cell lines differ from one another. At the very least, the limited number of cell lines cannot represent a reasonable sampling of the genetic diversity of different ethnic groups in the United States, and this has consequences for drug testing, as adverse reactions to drugs often reflect a complex genetic component. Once defined culture conditions are well established for human ES cells, there will be an even more compelling need to derive additional cell lines.

PLURIPOTENCY OF ES CELLS

The ability of ES cells to develop into all cell types of the body has fascinated scientists for years, yet remarkably little is known about factors that make one cell pluripotent and another more restricted in its developmental potential. The transcription factor Oct4 has

* **Editor's note:** One recent report now estimates 414 hESC lines, see Guhr et al., www.StemCells.com early online version for June 15, 2006: "Current State of Human Embryonic Stem Cell Research: An Overview of Cell Lines and their Usage in Experimental Work."

been used as a key marker for ES cells and for the pluripotent cells of the intact embryo, and its expression must be maintained at a critical level for ES cells to remain undifferentiated.[34] The Oct4 protein itself, however, is insufficient to maintain ES cells in the undifferentiated state. Recently, two groups identified another transcription factor, Nanog, that is essential for the maintenance of the undifferentiated state of mouse ES cells.[35,36] The expression of Nanog decreased rapidly as mouse ES cells differentiated, and when its expression level was maintained by a constitutive promoter, mouse ES cells could remain undifferentiated and proliferate in the absence of either LIF or BMP in serum-free medium.[12] Nanog is also expressed in human ES cells, though at a much lower level compared to that of Oct4, and its function in human ES cells has yet to be examined.

By comparing gene expression patterns between different ES cell lines and between ES cells and other cell types such as adult stem cells and differentiated cells, genes that are enriched in the ES cells have been identified. Using this approach, Esg-1, an uncharacterized ES cell-specific gene, was found to be exclusively associated with pluripotency in the mouse.[37] Sperger *et al.* identified 895 genes that are expressed at significantly higher levels in human ES cells and embryonic carcinoma cell lines, the malignant counterparts to ES cells.[38] Sato *et al.* identified a set of 918 genes enriched in undifferentiated human ES cells compared with their differentiated counterparts; many of these genes were shared by mouse ES cells.[39] Another group, however, found 92 genes, including Oct4 and Nanog, enriched in six different human ES cell lines, which showed limited overlap with those in mouse ES cell lines.[40] Care must be taken to interpret these data, and the considerable differences in the results may arise from the cell lines used in the experiments, methods to prepare and maintain the cells, and the specific methods used to profile gene expression.

GENETIC MANIPULATION OF ES CELLS

Since establishing human ES cells in 1998, scientists have developed genetic manipulation techniques to determine the function of particular genes, to direct the differentiation of human ES cells towards specific cell types, or to tag an ES cell derivative with a certain marker gene. Several approaches have been developed to introduce genetic elements randomly into the human ES cell genome, including electroporation, transfection by lipid-based reagents, and lentiviral vectors.[41–44] However, homologous recombination, a method in which a specific gene inside the ES cells is modified with an artificially introduced DNA molecule, is an even more precise method of genetic engineering that can modify a gene in a defined way at a specific locus. While this technology is routinely used in mouse ES cells, it has recently been successfully developed in human ES cells *(See chapter 5: Genetically Modified Stem Cells),* thus opening new doors for using ES cells as vehicles for gene therapy and for creating *in vitro* models of human genetic disorders such as Lesch-Nyhan disease.[45,46] Another method to test the function of a gene is to use RNA interference (RNAi) to decrease the expression of a gene of interest *(see Figure 1.4: RNA interference).* In RNAi, small pieces of double-stranded RNA (siRNA; small interfering RNA) are either chemically synthesized and introduced directly into cells, or expressed from DNA vectors. Once inside the cells, the siRNA can lead to the degradation of the messenger RNA (mRNA), which contains the exact sequence as that of the siRNA. mRNA is the product of DNA transcription and normally can be translated into proteins. RNAi can work efficiently in somatic cells, and there has been some progress in applying this technology to human ES cells.[47–49]

DIFFERENTIATION OF HUMAN ES CELLS

The pluripotency of ES cells suggests possible widespread uses for these cells and their derivatives. The ES cell-derived cells can potentially be used to replace or restore tissues that have been damaged by disease or injury, such as diabetes, heart attacks, Parkinson's disease or spinal cord injury. The recent developments in these particular areas are discussed in detail in other chapters, and Table 1 summarizes recent publications in the differentiation of specific cell lineages.

The differentiation of ES cells also provides model systems to study early events in human development. Because of possible harm to the resulting child, it is not ethically acceptable to experimentally manipulate the postimplantation human embryo. Therefore, most of what is known about the mechanisms of early human embryology and human development, especially in the early postimplantation period, is based on histological sections of a limited number of human embryos and on analogy to the experimental embryology of the

mouse. However, human and mouse embryos differ significantly, particularly in the formation, structure, and function of the fetal membranes and placenta, and the formation of an embryonic disc instead of an egg cylinder.[50–52] For example, the mouse yolk sac is a well-vascularized, robust, extraembryonic organ throughout gestation that provides important nutrient exchange functions. In humans, the yolk sac also serves important early functions, including the initiation of hematopoiesis, but it becomes essentially a vestigial structure at later times or stages in gestation. Similarly, there are dramatic differences between mouse and human placentas, both in structure and function. Thus, mice can serve in a limited capacity as a model system for understanding the developmental events that support the initiation and maintenance of human pregnancy. Human ES cell lines thus provide an important new *in vitro* model that will improve our understanding of the differentiation of human tissues, and thus provide important insights into processes such as infertility, pregnancy loss, and birth defects.

Human ES cells are already contributing to the study of development. For example, it is now possible to direct human ES cells to differentiate efficiently to trophoblast, the outer layer of the placenta that mediates implantation and connects the conceptus to the uterus.[17,53] Another use of human ES cells is for the study of germ cell development. Cells resembling both oocytes and sperm have been successfully derived from mouse ES cells *in vitro*.[54–56] Recently, human ES cells have also been observed to differentiate into cells expressing genes characteristic of germ cells.[57] Thus it may also be possible to derive oocytes and sperm from human ES cells, allowing the detailed study of human gametogenesis for the first time. Moreover, human ES cell studies are not limited to early differentiation, but are increasingly being used to understand the differentiation and functions of many human tissues, including neural, cardiac, vascular, pancreatic, hepatic, and bone *(see Table 1)*. Moreover, transplantation of ES-derived cells has offered promising results in animal models.[58–67]

Although scientists have gained more insights into the biology of human ES cells since 2001, many key questions remain to be addressed before the full potential of these unique cells can be realized. It is surprising, for example, that mouse and human ES cells appear to be so different with respect to the molecules that mediate their self-renewal, and perhaps even in their developmental potentials. BMPs, for example, in combination with LIF, promote the self-renewal of mouse ES cells. But in conditions that would otherwise support undifferentiated proliferation, BMPs cause rapid differentiation of human ES cells. Also, human ES cells differentiate quite readily to trophoblast, whereas mouse ES cells do so poorly, if at all. One would expect that at some level, the basic molecular mechanisms that control pluripotency would be conserved, and indeed, human and mouse ES cells share the expression of many key genes. Yet we remain remarkably ignorant about the molecular mechanisms that control pluripotency, and the nature of this remarkable cellular state has become one of the central questions of developmental biology. Of course, the other great challenge will be to continue to unravel the factors that control the differentiation of human ES cells to specific lineages, so that ES cells can fulfill their tremendous promise in basic human biology, drug screening, and transplantation medicine.

Table 1. Publications on Differentiation of Human Embryonic Stem Cells since 2001

Cell types	Publications	References
Neural	8	61, 66, 68-73
Cardiac	6	9-11, 74-76
Endothelial (Vascular)	2	77, 78
Hematopoietic (Blood)	8	79-86
Pancreatic (Islet-like)	2	87, 88
Hepatic (Liver)	3	89-91
Bone	1	92
Trophoblast	2	17, 53
Multilineages	9	16, 57, 93-99

ACKNOWLEDGEMENT

We thank Lynn Schmidt, Barbara Lewis, Sangyoon Han and Deborah J. Faupel for proofreading this report.

REFERENCES

1. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 1981;292:154-156.

2. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA.* 1981;78:7634-7638.

3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282:1145-1147.

4. Hoffman DI, Zellman GL, Fair CC, et al. Cryopreserved embryos in the United States and their availability for research. *Fertil Steril.* 2003;79:1063-1069.

5. Thomson JA, Kalishman J, Golos TG, et al. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA.* 1995;92:7844-7848.

6. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biol Reprod.* 1996;55:254-259.

7. Bremer S, Hartung T. The use of embryonic stem cells for regulatory developmental toxicity testing in vitro — the current status of test development. *Curr Pharm Des.* 2004;10:2733-2747.

8. Rolletschek A, Blyszczuk P, Wobus AM. Embryonic stem cell-derived cardiac, neuronal and pancreatic cells as model systems to study toxicological effects. *Toxicol Lett.* 2004;149:361-369.

9. He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res.* 2003;93:32-39.

10. Mummery C, Ward-van Oostwaard D, Doevendans P, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation. 2003;107:2733-2740.

11. Vanderlaan RD, Oudit GY, Backx PH. Electrophysiological profiling of cardiomyocytes in embryonic bodies derived from human embryonic stem cells. Circ Res. 2003;93:1-3.

12. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell.* 2003;115:281-292.

13. Qi X, Li TG, Hao J, et al. BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA.* 2004;101:6027-6032.

14. Daheron L, Optiz SL, Zaehres H, et al. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells.* 2004;22:770-778.

15. Humphrey RK, Beattie GM, Lopez AD, et al. Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells.* 2004;22:522-530.

16. Pera MF, Andrade J, Houssami S, et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *J Cell Sci.* 2004;117:1269-1280.

17. Xu RH, Chen X, Li DS, et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol.* 2002;20:1261-1264.

18. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol.* 2000;227:271-278.

19. Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol.* 2001;19:971-974.

20. Amit M, Margulets V, Segev H, et al. Human feeder layers for human embryonic stem cells. *Biol Reprod.* 2003;68:2150-2156.

21. Lee JB, Lee JE, Park JH, et al. Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. *Biol Reprod.* 2004.

22. 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.* 2002;20:933-936.

23. Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells.* 2003;21:546-556.

24. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* 2004;10:55-63.

25. Amit M, Shariki K, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod.* 2004;70:837-845.

26. Draper JS, Smith K, Gokhale P, et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol.* 2004;22:53-54.

27. Inzunza J, Sahlen S, Holmberg K, et al. Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. *Mol Hum Reprod.* 2004;10:461-466.

28. Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod.* 2000;62:1526-1535.

29. Mann MR, Lee SS, Doherty AS, et al. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development.* 2004;131:3727-3735.

30. Hwang WS, Ryu YJ, Park JH, et al. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science.* 2004;303:1669-1674.

31. Hwang WS, Roh SI, Lee BC, et al. Patient-specific embryonic stem cells derived from human SCNT blastocysts. *Science.* Jun 17 2005;308(5729):1777-1783.

32. Verlinsky Y, Strelchenko N, Kukharenko V, et al. Human embryonic stem cell lines with genetic disorders. *Reprod Biomed Online.* 2005;10:105-110.

33. Stojkovic M, Lako M, Strachan T, Murdoch A. Derivation, growth, and applications of human embryonic stem cells. *Reproduction.* 2004;128:259-267.

34. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet.* 2000;24:372-376.

35. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell.* 2003;113:643-655.

36. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003;113:631-642.

37. Tanaka TS, Kunath T, Kimber WL, et al. Gene expression profiling of embryo-derived stem cells reveals genes associated with pluripotency and lineage specificity. *Genome Res.* 2002;12:1921-1928.

38. Sperger JM, Chen X, Draper JS, et al. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc Natl Acad Sci USA.* 2003;100:13350-13355.

39. Sato N, Sanjuan IM, Heke M, Uchida M, Naef F, Brivanlou AH. Molecular signature of human embryonic stem cells and its comparison with the mouse. *Dev Biol.* 2003;260:404-413.

40. Bhattacharya B, Miura T, Brandenberger R, et al. Gene expression in human embryonic stem cell lines: unique molecular signature. Blood. 2004;103:2956-2964.

41. Eiges R, Schuldiner M, Drukker M, Yanuka O, Itskovitz-Eldor J, Benvenisty N. Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Curr Biol.* 2001;11:514-518.

42. Gropp M, Itsykson P, Singer O, et al. Stable genetic modification of human embryonic stem cells by lentiviral vectors. *Mol Ther.* 2003;7:281-287.

43. Lakshmipathy U, Pelacho B, Sudo K, et al. Efficient transfection of embryonic and adult stem cells. *Stem Cells.* 2004;22:531-543.

44. 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.

45. Urbach A, Schuldiner M, Benvenisty N. Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells. *Stem Cells.* 2004;22:635-641.

46. Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. *Nat Biotechnol.* 2003;21:319-321.

47. Matin MM, Walsh JR, Gokhale PJ, et al. Specific knockdown of Oct4 and beta2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells.* 2004;22:659-668.

48. Vallier L, Rugg-Gunn PJ, Bouhon IA, Andersson FK, Sadler AJ, Pedersen RA. Enhancing and diminishing gene function in human embryonic stem cells. Stem Cells. 2004;22:2-11.

49. Velkey JM, O'Shea KS. Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. *Genesis.* 2003;37:18-24.

50. Castellucci M, Scheper M, Scheffen I, Celona A, Kaufmann P. The development of the human placental villous tree. *Anat Embryol (Berl).* 1990;181:117-128.

51. Luckett WP. The development of primordial and definitive amniotic cavities in early Rhesus monkey and human embryos. *Am J Anat.* 1975;144:149-167.

52. Luckett WP. Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. *Am J Anat.* 1978;152:59-97.

53. Gerami-Naini B, Dovzhenko OV, Durning M, Wegner FH, Thomson JA, Golos TG. Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. *Endocrinology.* 2004;145:1517-1524.

54. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature.* 2004;427:148-154.

55. Hubner K, Fuhrmann G, Christenson LK, et al. Derivation of oocytes from mouse embryonic stem cells. *Science.* 2003;300:1251-1256.

56. Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci USA.* 2003;100:11457-11462.

57. Clark AT, Bodnar MS, Fox M, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. *Hum Mol Genet.* 2004;13:727-739.

58. Bjorklund LM, Sanchez-Pernaute R, Chung S, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinsonian rat model. *Proc Natl Acad Sci USA.* 2002;99:2344-2349.

59. Chiba S, Ikeda R, Kurokawa MS, et al. Anatomical and functional recovery by embryonic stem cell-derived neural tissue of a mouse model of brain damage. *J Neurol Sci.* 2004;219:107-117.

60. Kim D, Gu Y, Ishii M, et al. In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cells. *Pancreas.* 2003;27:e34-e41.

61. Lee DH, Park S, Kim EY, et al. Enhancement of re-closure capacity by the intra-amniotic injection of human embryonic stem cells in surgically induced spinal open neural tube defects in chick embryos. *Neurosci Lett.* 2004;364:98-100.

62. Marchetti S, Gimond C, Iljin K, et al. Endothelial cells genetically selected from differentiating mouse embryonic stem cells incorporate at sites of neovascularization in vivo. *J Cell Sci.* 2002;115:2075-2085.

63. Min JY, Yang Y, Converso KL, et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Appl Physiol.* 2002;92:288-296.

64. Miyagi T, Takeno M, Nagafuchi H, Takahashi M, Suzuki M. Flk1+ cells derived from mouse embryonic stem cells reconstitute hematopoiesis in vivo in SCID mice. *Exp Hematol.* 2002;30:1444-1453.

65. Nishimura F, Yoshikawa M, Kanda S, et al. Potential use of embryonic stem cells for the treatment of mouse parkinsonian models: improved behavior by transplantation of in vitro differentiated dopaminergic neurons from embryonic stem cells. *Stem Cells.* 2003;21:171-180.

66. Park S, Kim EY, Ghil GS, et al. Genetically modified human embryonic stem cells relieve symptomatic motor behavior in a rat model of Parkinson's disease. *Neurosci Lett.* 2003;353:91-94.

67. von Unge M, Dirckx JJ, Olivius NP. Embryonic stem cells enhance the healing of tympanic membrane perforations. *Int J Pediatr Otorhinolaryngol.* 2003;67:215-219.

68. Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp Neurol.* 2001;172:383-397.

69. Park S, Lee KS, Lee YJ, et al. Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors. *Neurosci Lett.* 2004;359:99-103.

70. Reubinoff BE, Itsykson P, Turetsky T, et al. Neural progenitors from human embryonic stem cells. *Nat Biotechnol.* 2001;19:1134-1140.

71. Schuldiner M, Eiges R, Eden A, et al. Induced neuronal differentiation of human embryonic stem cells. *Brain Res.* 2001;913:201-205.

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

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

74. Kehat I, Amit M, Gepstein A, Huber I, Itskovitz-Eldor J, Gepstein L. Development of cardiomyocytes from human ES cells. *Methods Enzymol.* 2003;365:461-473.

75. Satin J, Kehat I, Caspi O, et al. Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. *J Physiol.* 2004;559:479-496.

76. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res.* 2002;91:501-508.

77. Gerecht-Nir S, Ziskind A, Cohen S, Itskovitz-Eldor J. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. *Lab Invest.* 2003;83:1811-1820.

78. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA.* 2002;99:4391-4396.

79. Cerdan C, Rouleau A, Bhatia M. VEGF-A165 augments erythropoietic development from human embryonic stem cells. *Blood.* 2004;103:2504-2512.

80. Chadwick K, Wang L, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood.* 2003;102:906-915.

81. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA.* 2001;98:10716-10721.

82. Lu SJ, Li F, Vida L, Honig GR. CD34+CD38- hematopoietic precursors derived from human embryonic stem cells exhibit an embryonic gene expression pattern. *Blood.* 2004;103:4134-4141.

83. Tian X, Kaufman DS. Hematopoietic development of human embryonic stem cells in culture. *Methods Mol Biol.* 2004;290:149-162.

84. Vodyanik MA, Bork JA, Thomson JA, Slukvin II. Human embryonic stem cell-derived CD34+ cells: efficient production in the co-culture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood.* 2004.

85. Wang L, Li L, Shojaei F, et al. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity.* 2004;21:31-41.

86. Zhan X, Dravid G, Ye Z, et al. Functional antigen-presenting leucocytes derived from human embryonic stem cells in vitro. *Lancet.* 2004;364:163-171.

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

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

89. Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. *Differentiation.* 2004;72:230-238.

90. Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant.* 2003;12:1-11.

91. Shirahashi H, Wu J, Yamamoto N, et al. Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. *Cell Transplant.* 2004;13:197-211.

92. Sottile V, Thomson JA, McWhir J. In vitro osteogenic differentiation of human ES cells. *Cloning Stem Cells.* 2003;5:149-155.

93. Calhoun JD, Rao RR, Warrenfeltz S, et al. Transcriptional profiling of initial differentiation events in human embryonic stem cells. *Biochem Biophys Res Commun.* 2004;323:453-464.

94. Conley BJ, Trounson AO, Mollard R. Human embryonic stem cells form embryoid bodies containing visceral endoderm-like derivatives. *Fetal Diagn Ther.* 2004;19:218-223.

95. Dang SM, Gerecht-Nir S, Chen J, Itskovitz-Eldor J, Zandstra PW. Controlled, scalable embryonic stem cell differentiation culture. *Stem Cells.* 2004;22:275-282.

96. Dang SM, Zandstra PW. Scalable production of embryonic stem cell-derived cells. *Methods Mol Biol.* 2004;290:353-364.

97. Gertow K, Wolbank S, Rozell B, et al. Organized development from human embryonic stem cells after injection into immunodeficient mice. *Stem Cells Dev.* 2004;13:421-435.

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

99. Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. *Proc Natl Acad Sci USA.* 2003;100:12741-12746.

2. BONE MARROW (HEMATOPOIETIC) STEM CELLS

by Jos Domen, Amy Wagers** and Irving L. Weissman****

CONTENTS

* Cellerant Therapeutics, 1531 Industrial Road, San Carlos, CA 94070. Current address: Department of Surgery, Arizona Health Sciences Center, 1501 N. Campbell Avenue, P.O. Box 245071, Tucson, AZ 85724-5071, *Email:* jdomen@surgery.arizona.edu

** Section on Developmental and Stem Cell Biology, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215, *Email:* amy.wagers@joslin.harvard.edu

*** Director, Institute for Cancer/Stem Cell Biology and Medicine, Professor of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, *Email:* Irv@Stanford.edu

INTRODUCTION

Blood and the system that forms it, known as the hematopoietic system, consist of many cell types with specialized functions *(see Figure 2.1).* Red blood cells (erythrocytes) carry oxygen to the tissues. Platelets (derived from megakaryocytes) help prevent bleeding. Granulocytes (neutrophils, basophils and eosinophils) and macrophages (collectively known as myeloid cells) fight infections from bacteria, fungi, and other parasites such as nematodes (ubiquitous small worms). Some of these cells are also involved in tissue and bone remodeling and removal of dead cells. B-lymphocytes produce antibodies, while T-lymphocytes can directly kill or isolate by inflammation cells recognized as foreign to the body, including many virus-infected cells and cancer cells. Many blood cells are short-lived and need to be replenished continuously; the average human requires approximately one hundred billion new hematopoietic cells each day. The continued production of these cells depends directly on the presence of Hematopoietic Stem Cells (HSCs), the ultimate, and only, source of all these cells.

HISTORICAL OVERVIEW

The search for stem cells began in the aftermath of the bombings in Hiroshima and Nagasaki in 1945. Those who died over a prolonged period from lower doses of radiation had compromised hematopoietic systems that could not regenerate either sufficient white blood cells to protect against otherwise nonpathogenic infections or enough platelets to clot their blood. Higher doses of radiation also killed the stem cells of the intestinal tract, resulting in more rapid death. Later, it was demonstrated that mice that were given doses of whole body X-irradiation developed the same radiation syndromes; at the minimal lethal dose, the mice died from hematopoietic failure approximately two weeks after radiation exposure.[1] Significantly, however, shielding a single bone or the spleen from radiation prevented this irradiation syndrome. Soon thereafter, using inbred strains of mice, scientists showed that whole-body-irradiated mice could be rescued from otherwise fatal hematopoietic failure by injection of suspensions of cells from blood-forming organs such as the bone marrow.[2] In 1956, three laboratories

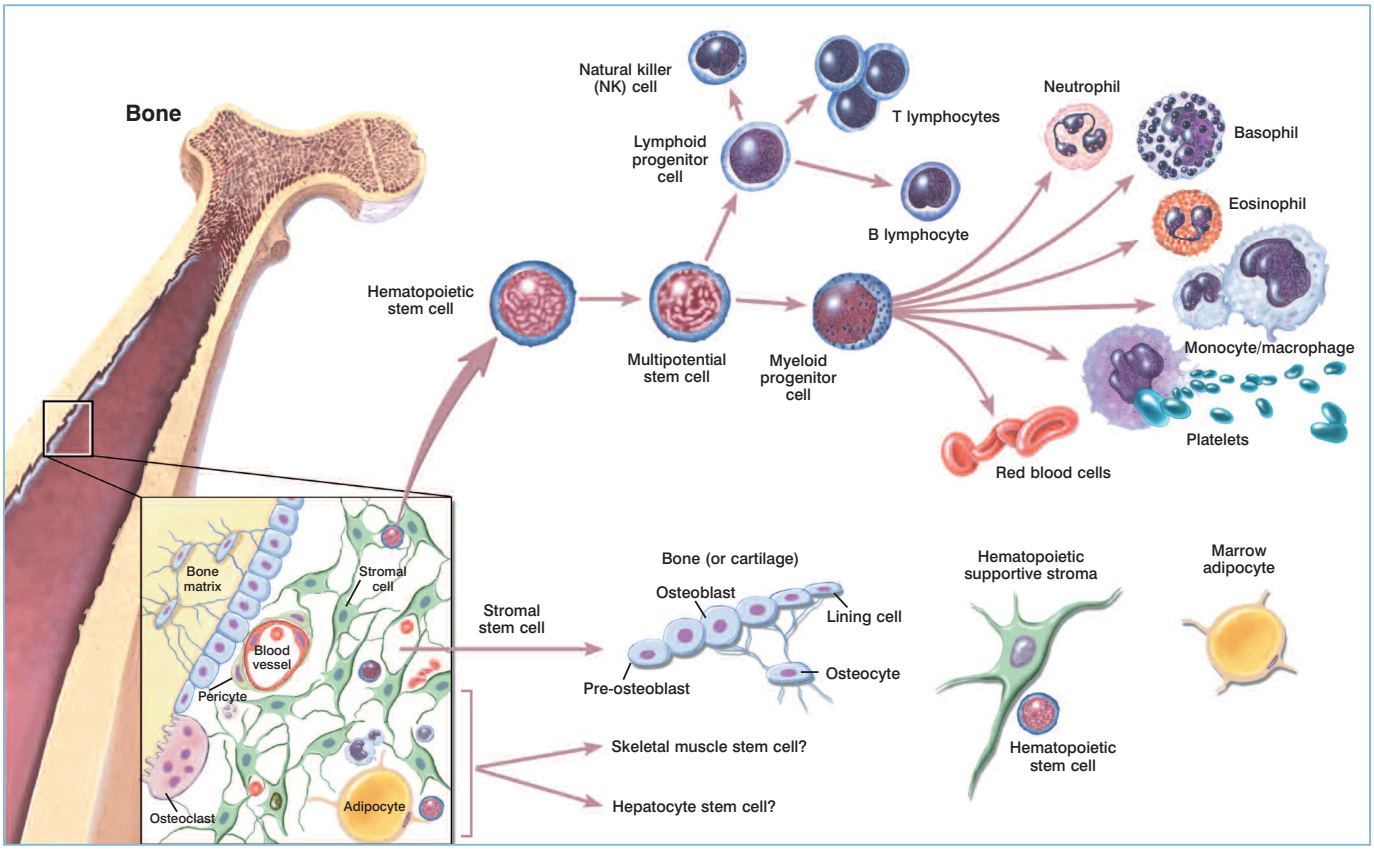


Figure 2.1. Hematopoietic and stromal cell differentiation.

demonstrated that the injected bone marrow cells directly regenerated the blood-forming system, rather than releasing factors that caused the recipients' cells to repair irradiation damage.[3–5] To date, the only known treatment for hematopoietic failure following whole body irradiation is transplantation of bone marrow cells or HSCs to regenerate the blood-forming system in the host organisms.[6,7]

The hematopoietic system is not only destroyed by the lowest doses of lethal X-irradiation (it is the most sensitive of the affected vital organs), but also by chemotherapeutic agents that kill dividing cells. By the 1960s, physicians who sought to treat cancer that had spread (metastasized) beyond the primary cancer site attempted to take advantage of the fact that a large fraction of cancer cells are undergoing cell division at any given point in time. They began using agents (*e.g.,* chemical and X-irradiation) that kill dividing cells to attempt to kill the cancer cells. This required the development of a quantitative assessment of damage to the cancer cells compared that inflicted on normal cells. Till and McCulloch began to assess quantitatively the radiation sensitivity of one normal cell type, the bone marrow cells used in transplantation, as it exists in the body. They found that, at sub-radioprotective doses of bone marrow cells, mice that died 10–15 days after irradiation developed colonies of myeloid and erythroid cells *(see Figure 2.1 for an example)* in their spleens. These colonies correlated directly in number with the number of bone marrow cells originally injected (approximately 1 colony per 7,000 bone marrow cells injected).[8] To test whether these colonies of blood cells derived from single precursor cells, they pre-irradiated the bone marrow donors with low doses of irradiation that would induce unique chromosome breaks in most hematopoietic cells but allow some cells to survive. Surviving cells displayed radiation-induced and repaired chromosomal breaks that marked each clonogenic (colony-initiating) hematopoietic cell.[9] The researchers discovered that all dividing cells within a single spleen colony, which contained different types of blood cells, contained the same unique chromosomal marker. Each colony displayed its own unique chromosomal marker, seen in its dividing cells.[9] Furthermore, when cells from a single spleen colony were re-injected into a second set of lethally-irradiated mice, donor-derived spleen colonies that contained the same unique chromosomal marker were often observed, indicating that these colonies had been regenerated from the same, single cell that had generated the first colony. Rarely, these colonies contained sufficient numbers of regenerative cells both to radioprotect secondary recipients (*e.g.,* to prevent their deaths from radiation-induced blood cell loss) and to give rise to lymphocytes and myeloerythroid cells that bore markers of the donor-injected cells.[10,11] These genetic marking experiments established the fact that cells that can both self-renew and generate most (if not all) of the cell populations in the blood must exist in bone marrow. At the time, such cells were called *pluripotent* HSCs, a term later modified to *multipotent* HSCs.[12,13] However, identifying stem cells in retrospect by analysis of randomly chromosome-marked cells is not the same as being able to isolate pure populations of HSCs for study or clinical use.

Achieving this goal requires markers that uniquely define HSCs. Interestingly, the development of these markers, discussed below, has revealed that most of the early spleen colonies visible 8 to 10 days after injection, as well as many of the later colonies, visible at least 12 days after injection, are actually derived from progenitors rather than from HSCs. Spleen colonies formed by HSCs are relatively rare and tend to be present among the later colonies.[14,15] However, these findings do not detract from Till and McCulloch's seminal experiments to identify HSCs and define these unique cells by their capacities for self-renewal and multilineage differentiation.

THE ISOLATION OF HSCS IN MOUSE AND MAN

While much of the original work was, and continues to be, performed in murine model systems, strides have been made to develop assays to study human HSCs. The development of Fluorescence Activated Cell Sorting (FACS) has been crucial for this field *(see Figure 2.2).* This technique enables the recognition and quantification of small numbers of cells in large mixed populations. More importantly, FACS-based cell sorting allows these rare cells (1 in 2000 to less than 1 in 10,000) to be purified, resulting in preparations of near 100% purity. This capability enables the testing of these cells in various assays.

HSC Assays

Assays have been developed to characterize hematopoietic stem and progenitor cells *in vitro* and *in vivo (Figure 2.3).*[16,17] *In vivo* assays that are used to study

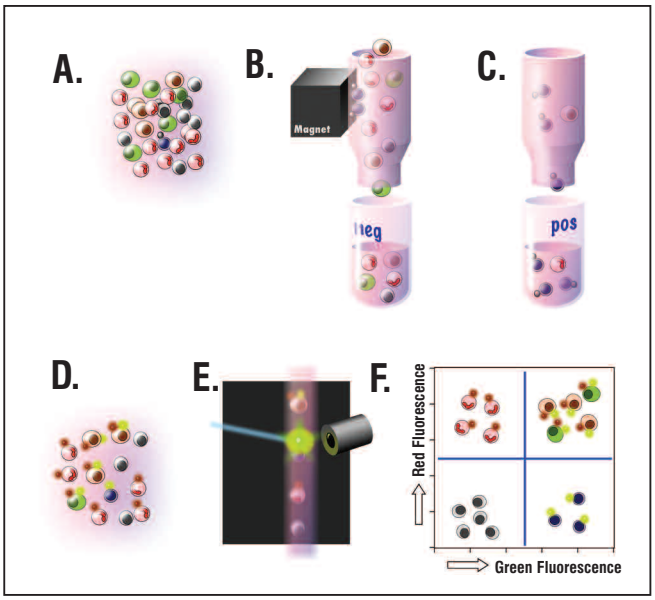


Figure 2.2. Enrichment and purification methods for hematopoietic stem cells. Upper panels illustrate column-based magnetic enrichment. In this method, the cells of interest are labeled with very small iron particles (A). These particles are bound to antibodies that only recognize specific cells. The cell suspension is then passed over a column through a strong magnetic field which retains the cells with the iron particles (B). Other cells flow through and are collected as the depleted negative fraction. The magnet is removed, and the retained cells are collected in a separate tube as the positive or enriched fraction (C). Magnetic enrichment devices exist both as small research instruments and large closed-system clinical instruments. Lower panels illustrate Fluorescence Activated Cell Sorting (FACS). In this setting, the cell mixture is labeled with fluorescent markers that emit light of different colors after being activated by light from a laser. Each of these fluorescent markers is attached to a different monoclonal antibody that recognizes specific sets of cells (D). The cells are then passed one by one in a very tight stream through a laser beam (blue in the figure) in front of detectors (E) that determine which colors fluoresce in response to the laser. The results can be displayed in a FACS-plot (F). FACS-plots (see figures 3 and 4 for examples) typically show fluorescence levels per cell as dots or probability fields. In the example, four groups can be distinguished: Unstained, red-only, green-only, and red-green double labeling. Each of these groups, *e.g.,* green fluorescence-only, can be sorted to very high purity. The actual sorting happens by breaking the stream shown in (E) into tiny droplets, each containing 1 cell, that then can be sorted using electric charges to move the drops. Modern FACS machines use three different lasers (that can activate different set of fluorochromes), to distinguish up to 8 to 12 different fluorescence colors and sort 4 separate populations, all simultaneously.

Magnetic enrichment can process very large samples (billions of cells) in one run, but the resulting cell preparation is enriched for only one parameter (*e.g.,* CD34) and is not pure. Significant levels of contaminants (such as T-cells or tumor cells) remain present. FACS results in very pure cell populations that can be selected for several parameters simultaneously (*e.g.,* Lin^{neg}, $CD34^{pos}$, $CD90^{pos}$), but it is more time consuming (10,000 to 50,000 cells can be sorted per second) and requires expensive instrumentation.

HSCs include Till and McCulloch' s classical spleen colony forming (CFU-S) assay,[8] which measures the ability of HSC (as well as blood-forming progenitor cells) to form large colonies in the spleens of lethally irradiated mice. Its' main advantage (and limitation) is the short-term nature of the assay (now typically 12 days). However, the assays that truly define HSCs are reconstitution assays.[16,18] Mice that have been "preconditioned" by lethal irradiation to accept new HSCs are injected with purified HSCs or mixed populations containing HSCs, which will repopulate the hematopoietic systems of the host mice for the life of the animal. These assays typically use different types of markers to distinguish host and donor-derived cells.

For example, allelic assays distinguish different versions of a particular gene, either by direct analysis of DNA or of the proteins expressed by these alleles. These proteins may be cell-surface proteins that are recognized by specific monoclonal antibodies that can distinguish between the variants (*e.g.,* CD45 in Figure 2.3) or cellular proteins that may be recognized through methods such as gel-based analysis. Other assays take advantage of the fact that male cells can be detected in a female host by detecting the male-cell-specific Y-chromosome by molecular assays (*e.g.,* polymerase chain reaction, or PCR).

Small numbers of HSCs (as few as one cell in mouse experiments) can be assayed using competitive reconstitutions, in which a small amount of host-type bone marrow cells (enough to radioprotect the host and thus ensure survival) is mixed in with the donor-HSC population. To establish long-term reconstitutions in mouse models, the mice are followed for at least 4 months after receiving the HSCs. Serial reconstitution, in which the bone marrow from a previously-irradiated and reconstituted mouse becomes the HSC source for

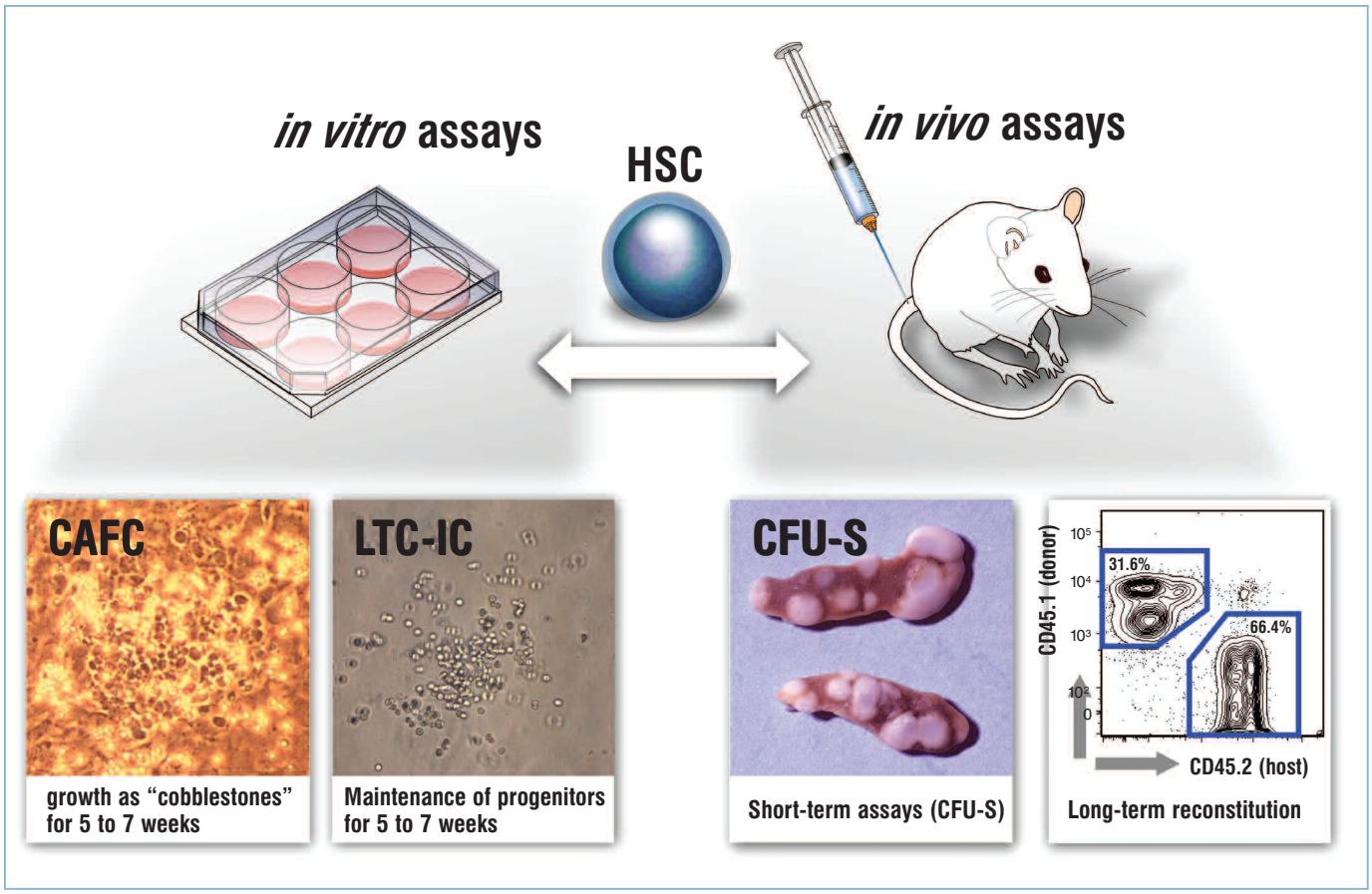


Figure 2.3. Assays used to detect hematopoietic stem cells. The tissue culture assays, which are used frequently to test human cells, include the ability of the cells to be tested to grow as "cobblestones" (the dark cells in the picture) for 5 to 7 weeks in culture. The Long Term Culture-Initiating Cell assay measures whether hematopoietic progenitor cells (capable of forming colonies in secondary assays, as shown in the picture) are still present after 5 to 7 weeks of culture.

In vivo assays in mice include the CFU-S assay, the original stem cell assay discussed in the introduction. The most stringent hematopoietic stem cell assay involves looking for the long-term presence of donor-derived cells in a reconstituted host. The example shows host-donor recognition by antibodies that recognize two different mouse alleles of CD45, a marker present on nearly all blood cells. CD45 is also a good marker for distinguishing human blood cells from mouse blood cells when testing human cells in immunocompromised mice such as NOD/SCID. Other methods such as pcr-markers, chromosomal markers, and enzyme markers can also be used to distinguish host and donor cells.

second irradiated mouse, extends the potential of this assay to test lifespan and expansion limits of HSCs. Unfortunately, the serial transfer assay measures both the lifespan and the transplantability of the stem cells. The transplantability may be altered under various conditions, so this assay is not the *sine qua non* of HSC function. Testing the *in vivo* activity of human cells is obviously more problematic.

Several experimental models have been developed that allow the testing of human cells in mice. These assays employ immunologically-incompetent mice (mutant mice that cannot mount an immune response against foreign cells) such as SCID[19–21] or NOD-SCID mice.[22,23] Reconstitution can be performed in either the presence or absence of human fetal bone or thymus implants to provide a more natural environment in which the human cells can grow in the mice. Recently NOD/SCID/cγ$^{-/-}$ mice have been used as improved recipients for human HSCs, capable of complete reconstitution with human lymphocytes, even in the absence of additional human tissues.[24] Even more promising has been the use of newborn mice with an impaired immune system (Rag-2$^{-/-}$Cγ$^{-/-}$), which results in reproducible production of human B- and T-lymphoid and myeloerythroid cells.[25] These assays

are clearly more stringent, and thus more informative, but also more difficult than the *in vitro* HSC assays discussed below. However, they can only assay a fraction of the lifespan under which the cells would usually have to function. Information on the long-term functioning of cells can only be derived from clinical HSC transplantations.

A number of assays have been developed to recognize HSCs *in vitro* (*e.g.,* in tissue culture). These are especially important when assaying human cells. Since transplantation assays for human cells are limited, cell culture assays often represent the only viable option. *In vitro* assays for HSCs include Long-Term Culture-Initializing Cell (LTC-IC) assays[26–28] and Cobble-stone Area Forming Cell (CAFC) assays.[29] LTC-IC assays are based on the ability of HSCs, but not more mature progenitor cells, to maintain progenitor cells with clonogenic potential over at least a five-week culture period. CAFC assays measure the ability of HSCs to maintain a specific and easily recognizable way of growing under stromal cells for five to seven weeks after the initial plating. Progenitor cells can only grow in culture in this manner for shorter periods of time.

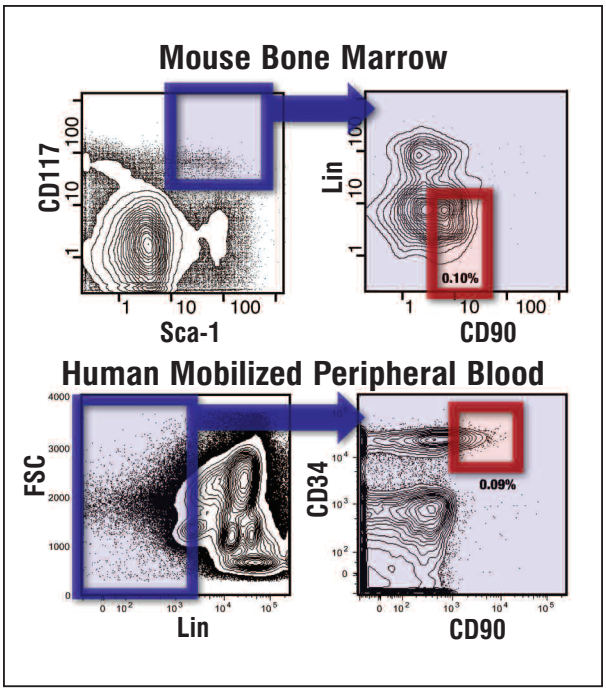


Figure 2.4. Examples of Hematopoietic Stem Cell staining patterns in mouse bone marrow (top) and human mobilized peripheral blood (bottom). The plots on the right show only the cells present in the left blue box. The cells in the right blue box represent HSCs. Stem cells form a rare fraction of the cells present in both cases.

Cell Markers Can Identify HSCs

While initial experiments studied HSC activity in mixed populations, much progress has been made in specifically describing the cells that have HSC activity. A variety of markers have been discovered to help recognize and isolate HSCs. Initial marker efforts focused on cell size, density, and recognition by lectins (carbohydrate-binding proteins derived largely from plants),[30] but more recent efforts have focused mainly on cell surface protein markers, as defined by monoclonal antibodies. For mouse HSCs, these markers include panels of 8 to 14 different monoclonal antibodies that recognize cell surface proteins present on differentiated hematopoietic lineages, such as the red blood cell and macrophage lineages (thus, these markers are collectively referred to as "Lin"),[13,31] as well as the proteins Sca-1,[13,31] CD27,[32] CD34,[33] CD38,[34] CD43,[35] CD90.1 (Thy-1.1),[13,31] CD117 (c-Kit),[36] AA4.1,[37] and MHC class I,[30] and CD150.[38] Human HSCs have been defined with respect to staining for Lin,[39] CD34,[40] CD38,[41] CD43,[35] CD45RO,[42] CD45RA,[42] CD59,[43] CD90,[39] CD109,[44] CD117,[45] CD133,[46,47] CD166,[48] and HLA DR (human).[49,50] In addition, metabolic markers/dyes such as rhodamine123 (which stains mitochondria),[51] Hoechst33342 (which identifies MDR-type drug efflux activity),[52] Pyronin-Y (which stains RNA),[53] and BAAA (indicative of aldehyde dehydrogenase enzyme activity)[54] have been described. While none of these markers recognizes functional stem cell activity, combinations (typically with 3 to 5 different markers, see examples below) allow for the purification of near-homogenous populations of HSCs. The ability to obtain pure preparations of HSCs, albeit in limited numbers, has greatly facilitated the functional and biochemical characterization of these important cells. However, to date there has been limited impact of these discoveries on clinical practice, as highly purified HSCs have only rarely been used to treat patients (discussed below). The undeniable advantages of using purified cells (*e.g.,* the absence of contaminating tumor cells in autologous transplantations) have been offset by practical difficulties and increased purification costs.

Cell Surface Marker Combinations That Define Hematopoietic Stem Cells

HSC assays, when combined with the ability to purify HSCs, have provided increasingly detailed insight into the cells and the early steps involved in the differentiation process. Several marker combinations have been developed that describe murine HSCs, including [CD117^{high}, CD90.1^{low}, Lin$^{neg/low}$, Sca-1^{pos}],[15] [CD90.1^{low}, Lin^{neg}, Sca-1^{pos} Rhodamine123^{low}],[55] [CD34$^{neg/low}$, CD117^{pos}, Sca-1^{pos}, Lin^{neg}],[33] [CD150 ^{pos}, CD48^{neg}, CD244^{neg}],[38] and "side-population" cells using Hoechst-dye.[52] Each of these combinations allows purification of HSCs to near-homogeneity. Figure 2.4 shows an example of an antibody combination that can recognize mouse HSCs. Similar strategies have been developed to purify human HSCs, employing markers such as CD34, CD38, Lin, CD90, CD133 and fluorescent substrates for the enzyme, aldehyde dehydrogenase. The use of highly purified human HSCs has been mainly experimental, and clinical use typically employs enrichment for one marker, usually CD34. CD34 enrichment yields a population of cells enriched for HSC and blood progenitor cells but still contains many other cell types. However, limited trials in which highly FACS-purified CD34^{pos} CD90^{pos} HSCs *(see Figure 2.4)* were used as a source of reconstituting cells have demonstrated that rapid reconstitution of the blood system can reliably be obtained using only HSCs.[56–58]

The purification strategies described above recognize a rare subset of cells. Exact numbers depend on the assay used as well as on the genetic background studied.[16] In mouse bone marrow, 1 in 10,000 cells is a hematopoietic stem cell with the ability to support long-term hematopoiesis following transplantation into a suitable host. When short-term stem cells, which have a limited self-renewal capacity, are included in the estimation, the frequency of stem cells in bone marrow increases to 1 in 1,000 to 1 in 2,000 cells in humans and mice. The numbers present in normal blood are at least ten-fold lower than in marrow.

None of the HSC markers currently used is directly linked to an essential HSC function, and consequently, even within a species, markers can differ depending on genetic alleles,[59] mouse strains,[60] developmental stages,[61] and cell activation stages.[62,63] Despite this, there is a clear correlation in HSC markers between divergent species such as humans and mice. However, unless the ongoing attempts at defining the complete HSC gene expression patterns will yield usable markers that are linked to essential functions for maintaining the "stemness" of the cells,[64,65] functional assays will remain necessary to identify HSCs unequivocally.[16]

Cell Surface Marker Patterns of Hematopoietic Progenitor Cells

More recently, efforts at defining hematopoietic populations by cell surface or other FACS-based markers have been extended to several of the progenitor populations that are derived from HSCs *(see Figure 2.5)*. Progenitors differ from stem cells in that they have a reduced differentiation capacity (they can generate only a subset of the possible lineages) but even more importantly, progenitors lack the ability to self-renew. Thus, they have to be constantly regenerated from the HSC population. However, progenitors do have extensive proliferative potential and can typically generate large numbers of mature cells. Among the progenitors defined in mice and humans are the Common Lymphoid Progenitor (CLP),[66,67] which in adults has the potential to generate all of the lymphoid but not myeloerythroid cells, and a Common Myeloid Progenitor (CMP), which has the potential to generate all of the mature myeloerythroid, but not lymphoid, cells.[68,69] While beyond the scope of this overview, hematopoietic progenitors have clinical potential and will likely see clinical use.[70,71]

HALLMARKS OF HSCS

HSCs have a number of unique properties, the combination of which defines them as such.[16] Among the core properties are the ability to choose between self-renewal (remain a stem cell after cell division) or differentiation (start the path towards becoming a mature hematopoietic cell). In addition, HSCs migrate in regulated fashion and are subject to regulation by apoptosis (programmed cell death). The balance between these activities determines the number of stem cells that are present in the body.

Self-Renewal

One essential feature of HSCs is the ability to self-renew, that is, to make copies with the same or very similar potential. This is an essential property because

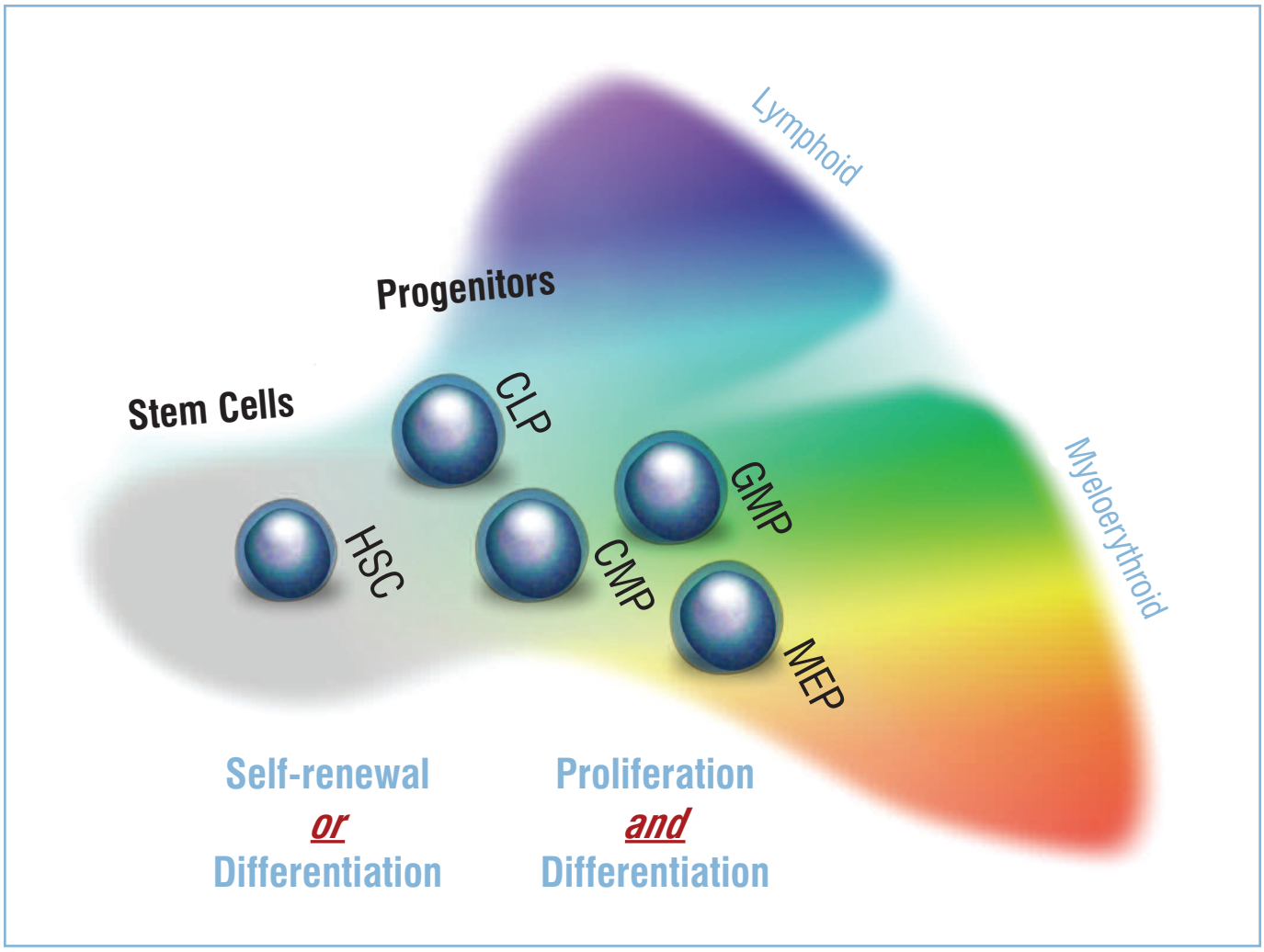


Figure 2.5. Relationship between several of the characterized hematopoietic stem cells and early progenitor cells. Differentiation is indicated by colors; the more intense the color, the more mature the cells. Surface marker distinctions are subtle between these early cell populations, yet they have clearly distinct potentials. Stem cells can choose between self-renewal and differentiation. Progenitors can expand temporarily but always continue to differentiate (other than in certain leukemias). The mature lymphoid (T-cells, B-cells, and Natural Killer cells) and myeloerythroid cells (granulocytes, macrophages, red blood cells, and platelets) that are produced by these stem and progenitor cells are shown in more detail in Figure 2.1.

more differentiated cells, such as hematopoietic progenitors, cannot do this, even though most progenitors can expand significantly during a limited period of time after being generated. However, for continued production of the many (and often short-lived) mature blood cells, the continued presence of stem cells is essential. While it has not been established that adult HSCs can self-renew indefinitely (this would be difficult to prove experimentally), it is clear from serial transplantation experiments that they can produce enough cells to last several (at least four to five) lifetimes in mice. It is still unclear which key signals allow self-renewal. One link that has been noted is telomerase, the enzyme necessary for maintaining telomeres, the DNA regions at the end of chromosomes that protect them from accumulating damage due to DNA replication. Expression of telomerase is associated with self-renewal activity.[72] However, while absence of telomerase reduces the self-renewal capacity of mouse HSCs, forced expression is not sufficient to enable HSCs to be transplanted indefinitely; other barriers must exist.[73,74]

It has proven surprisingly difficult to grow HSCs in culture despite their ability to self-renew. Expansion in culture is routine with many other cells, including

neural stem cells and ES cells. The lack of this capacity for HSCs severely limits their application, because the number of HSCs that can be isolated from mobilized blood, umbilical cord blood, or bone marrow restricts the full application of HSC transplantation in man (whether in the treatment of nuclear radiation exposure or transplantation in the treatment of blood cell cancers or genetic diseases of the blood or blood-forming system). Engraftment periods of 50 days or more were standard when limited numbers of bone marrow or umbilical cord blood cells were used in a transplant setting, reflecting the low level of HSCs found in these native tissues. Attempts to expand HSCs in tissue culture with known stem-cell stimulators, such as the cytokines stem cell factor/steel factor (KitL), thrombopoietin (TPO), interleukins 1, 3, 6, 11, plus or minus the myeloerythroid cytokines GM-CSF, G-CSF, M-CSF, and erythropoietin have never resulted in a significant expansion of HSCs.[16,75] Rather, these compounds induce many HSCs into cell divisions that are always accompanied by cellular differentiation.[76] Yet many experiments demonstrate that the transplantation of a single or a few HSCs into an animal results in a 100,000-fold or greater expansion in the number of HSCs at the steady state while simultaneously generating daughter cells that permitted the regeneration of the full blood-forming system.[77–80] Thus, we do not know the factors necessary to regenerate HSCs by self-renewing cell divisions. By investigating genes transcribed in purified mouse LT-HSCs, investigators have found that these cells contain expressed elements of the Wnt/fzd/beta-catenin signaling pathway, which enables mouse HSCs to undergo self-renewing cell divisions.[81,82] Overexpression of several other proteins, including HoxB4[83-86] and HoxA9[87] has also been reported to achieve this. Other signaling pathways that are under investigation include Notch and Sonic hedgehog.[75] Among the intracellular proteins thought to be essential for maintaining the "stem cell" state are Polycomb group genes, including Bmi-1.[88] Other genes, such as c-*Myc* and JunB have also been shown to play a role in this process.[89,90] Much remains to be discovered, including the identity of the stimuli that govern self-renewal *in vivo,* as well as the composition of the environment (the stem cell "niche") that provides these stimuli.[91] The recent identification of osteoblasts, a cell type known to be involved in bone formation, as a critical component of this environment[92,93] will help to focus this search. For instance, signaling by Angiopoietin-1 on osteoblasts to Tie-2 receptors on HSCs has recently been suggested to regulate stem cell quiescence (the lack of cell division).[94] It is critical to discover which pathways operate in the expansion of human HSCs to take advantage of these pathways to improve hematopoietic transplantation.

Differentiation

Differentiation into progenitors and mature cells that fulfill the functions performed by the hematopoietic system is not a unique HSC property, but, together with the option to self-renew, defines the core function of HSCs. Differentiation is driven and guided by an intricate network of growth factors and cytokines. As discussed earlier, differentiation, rather than self-renewal, seems to be the default outcome for HSCs when stimulated by many of the factors to which they have been shown to respond. It appears that, once they commit to differentiation, HSCs cannot revert to a self-renewing state. Thus, specific signals, provided by specific factors, seem to be needed to maintain HSCs. This strict regulation may reflect the proliferative potential present in HSCs, deregulation of which could easily result in malignant diseases such as leukemia or lymphoma.

Migration

Migration of HSCs occurs at specific times during development (*i.e.,* seeding of fetal liver, spleen and eventually, bone marrow) and under certain conditions (*e.g.,* cytokine-induced mobilization) later in life. The latter has proven clinically useful as a strategy to enhance normal HSC proliferation and migration, and the optimal mobilization regimen for HSCs currently used in the clinic is to treat the stem cell donor with a drug such as cytoxan, which kills most of his or her dividing cells. Normally, only about 8% of LT-HSCs enter the cell cycle per day,[95,96] so HSCs are not significantly affected by a short treatment with cytoxan. However, most of the downstream blood progenitors are actively dividing,[66,68] and their numbers are therefore greatly depleted by this dose, creating a demand for a regenerated blood-forming system. Empirically, cytokines or growth factors such as G-CSF and KitL can increase the number of HSCs in the blood, especially if administered for several days following a cytoxan pulse. The optimized protocol of cytoxan plus G-CSF results in several self-renewing cell divisions for

each resident LT-HSC in mouse bone marrow, expanding the number of HSCs 12- to 15-fold within two to three days.[97] Then, up to one-half of the daughter cells of self-renewing dividing LT-HSCs (estimated to be up to 10^5 per mouse per day[98]) leave the bone marrow, enter the blood, and within minutes engraft other hematopoietic sites, including bone marrow, spleen, and liver.[98] These migrating cells can and do enter empty hematopoietic niches elsewhere in the bone marrow and provide sustained hematopoietic stem cell self-renewal and hematopoiesis.[98,99] It is assumed that this property of mobilization of HSCs is highly conserved in evolution (it has been shown in mouse, dog and humans) and presumably results from contact with natural cell-killing agents in the environment, after which regeneration of hematopoiesis requires restoring empty HSC niches. This means that functional, transplantable HSCs course through every tissue of the body in large numbers every day in normal individuals.

Apoptosis

Apoptosis, or programmed cell death, is a mechanism that results in cells actively self-destructing without causing inflammation. Apoptosis is an essential feature in multicellular organisms, necessary during development and normal maintenance of tissues. Apoptosis can be triggered by specific signals, by cells failing to receive the required signals to avoid apoptosis, and by exposure to infectious agents such as viruses. HSCs are not exempt; apoptosis is one mechanism to regulate their numbers. This was demonstrated in transgenic mouse experiments in which HSC numbers doubled when the apoptosis threshold was increased.[76] This study also showed that HSCs are particularly sensitive and require two signals to avoid undergoing apoptosis.

SOURCES OF HSCS

Bone Marrow and Mobilized Peripheral Blood

The best-known location for HSCs is bone marrow, and bone marrow transplantation has become synonymous with hematopoietic cell transplantation, even though bone marrow itself is increasingly infrequently used as a source due to an invasive harvesting procedure that requires general anesthesia. In adults, under steady-state conditions, the majority of HSCs reside in bone marrow. However, cytokine mobilization can result in the release of large numbers of HSCs into the blood. As a clinical source of HSCs, mobilized peripheral blood (MPB) is now replacing bone marrow, as harvesting peripheral blood is easier for the donors than harvesting bone marrow. As with bone marrow, mobilized peripheral blood contains a mixture of hematopoietic stem and progenitor cells. MPB is normally passed through a device that enriches cells that express CD34, a marker on both stem and progenitor cells. Consequently, the resulting cell preparation that is infused back into patients is not a pure HSC preparation, but a mixture of HSCs, hematopoietic progenitors (the major component), and various contaminants, including T cells and, in the case of autologous grafts from cancer patients, quite possibly tumor cells. It is important to distinguish these kinds of grafts, which are the grafts routinely given, from highly purified HSC preparations, which essentially lack other cell types.

Umbilical Cord Blood

In the late 1980s, umbilical cord blood (UCB) was recognized as an important clinical source of HSCs.[100,101] Blood from the placenta and umbilical cord is a rich source of hematopoietic stem cells, and these cells are typically discarded with the afterbirth. Increasingly, UCB is harvested, frozen, and stored in cord blood banks, as an individual resource (donor-specific source) or as a general resource, directly available when needed. Cord blood has been used successfully to transplant children and (far less frequently) adults. Specific limitations of UCB include the limited number of cells that can be harvested and the delayed immune reconstitution observed following UCB transplant, which leaves patients vulnerable to infections for a longer period of time. Advantages of cord blood include its availability, ease of harvest, and the reduced risk of graft-versus-host-disease (GVHD). In addition, cord blood HSCs have been noted to have a greater proliferative capacity than adult HSCs. Several approaches have been tested to overcome the cell dose issue, including, with some success, pooling of cord blood samples.[101,102] *Ex vivo* expansion in tissue culture, to which cord blood cells are more amenable than adult cells, is another approach under active investigation.[103]

The use of cord blood has opened a controversial treatment strategy — embryo selection to create a related UCB donor.[104] In this procedure, embryos are conceived by *in vitro* fertilization. The embryos are

tested by pre-implantation genetic diagnosis, and embryos with transplantation antigens matching those of the affected sibling are implanted. Cord blood from the resulting newborn is then used to treat this sibling. This approach, successfully pioneered at the University of Minnesota, can in principle be applied to a wide variety of hematopoietic disorders. However, the ethical questions involved argue for clear regulatory guidelines.[105]

Embryonic Stem Cells

Embryonic stem (ES) cells form a potential future source of HSCs. Both mouse and human ES cells have yielded hematopoietic cells in tissue culture, and they do so relatively readily.[106] However, recognizing the actual HSCs in these cultures has proven problematic, which may reflect the variability in HSC markers or the altered reconstitution behavior of these HSCs, which are expected to mimic fetal HSC. This, combined with the potential risks of including undifferentiated cells in an ES-cell-derived graft means that, based on the current science, clinical use of ES cell-derived HSCs remains only a theoretical possibility for now.

HSC PLASTICITY

An ongoing set of investigations has led to claims that HSCs, as well as other stem cells, have the capacity to differentiate into a much wider range of tissues than previously thought possible. It has been claimed that, following reconstitution, bone marrow cells can differentiate not only into blood cells but also muscle cells (both skeletal myocytes and cardiomyocytes),[107–111] brain cells,[112,113] liver cells,[114,115] skin cells, lung cells, kidney cells, intestinal cells,[116] and pancreatic cells.[117] Bone marrow is a complex mixture that contains numerous cell types. In addition to HSCs, at least one other type of stem cell, the mesenchymal stem cell (MSC), is present in bone marrow. MSCs, which have become the subject of increasingly intense investigation, seem to retain a wide range of differentiation capabilities i*n vitro* that is not restricted to mesodermal tissues, but includes tissues normally derived from other embryonic germ layers (*e.g.,* neurons).[118–120] MSCs are discussed in detail in Dr. Catherine Verfaillie's testimony to the President's Council on Bioethics at this website: http://bioethicsprint.bioethics.gov/transcripts/apr02/apr25session2.html and will not be discussed further here. However, similar claims of differentiation into multiple diverse cell types, including muscle,[111] liver,[114] and different types of epithelium[116] have been made in experiments that assayed partially- or fully-purified HSCs. These experiments have spawned the idea that HSCs may not be entirely or irreversibly committed to forming the blood, but under the proper circumstances, HSCs may also function in the regeneration or repair of non-blood tissues. This concept has in turn given rise to the hypothesis that the fate of stem cells is "plastic," or changeable, allowing these cells to adopt alternate fates if needed in response to tissue-derived regenerative signals (a phenomenon sometimes referred to as "trans-differentiation"). This in turn seems to bolster the argument that the full clinical potential of stem cells can be realized by studying only adult stem cells, foregoing research into defining the conditions necessary for the clinical use of the extensive differentiation potential of embryonic stem cells. However, as discussed below, such "transdifferentiation" claims for specialized adult stem cells are controversial, and alternative explanations for these observations remain possible, and, in several cases, have been documented directly.

While a full discussion of this issue is beyond the scope of this overview, several investigators have formulated criteria that must be fulfilled to demonstrate stem cell plasticity.[121,122] These include *(i)* clonal analysis, which requires the transfer and analysis of single, highly-purified cells or individually marked cells and the subsequent demonstration of both "normal" and "plastic" differentiation outcomes, *(ii)* robust levels of "plastic" differentiation outcome, as extremely rare events are difficult to analyze and may be induced by artefact, and *(iii)* demonstration of tissue-specific function of the "transdifferentiated" cell type. Few of the current reports fulfill these criteria, and careful analysis of individually transplanted KTLS HSCs has failed to show significant levels of non-hematopoietic engraftment.[123,124] In addition, several reported trans-differentiation events that employed highly purified HSCs, and in some cases a very strong selection pressure for trans-differentiation, now have been shown to result from fusion of a blood cell with a non-blood cell, rather than from a change in fate of blood stem cells.[125–127] Finally, in the vast majority of cases, reported contributions of adult stem cells to cell types outside their tissue of origin are exceedingly rare, far too rare to be considered therapeutically useful. These findings have raised significant doubts about the

biological importance and immediate clinical utility of adult hematopoietic stem cell plasticity. Instead, these results suggest that normal tissue regeneration relies predominantly on the function of cell type-specific stem or progenitor cells, and that the identification, isolation, and characterization of these cells may be more useful in designing novel approaches to regenerative medicine. Nonetheless, it is possible that a rigorous and concerted effort to identify, purify, and potentially expand the appropriate cell populations responsible for apparent "plasticity" events, characterize the tissue-specific and injury-related signals that recruit, stimulate, or regulate plasticity, and determine the mechanism(s) underlying cell fusion or transdifferentiation, may eventually enhance tissue regeneration via this mechanism to clinically useful levels.

HSC SYSTEMS BIOLOGY

Recent progress in genomic sequencing and genome-wide expression analysis at the RNA and protein levels has greatly increased our ability to study cells such as HSCs as "systems," that is, as combinations of defined components with defined interactions. This goal has yet to be realized fully, as computational biology and system-wide protein biochemistry and proteomics still must catch up with the wealth of data currently generated at the genomic and transcriptional levels. Recent landmark events have included the sequencing of the human and mouse genomes and the development of techniques such as array-based analysis. Several research groups have combined cDNA cloning and sequencing with array-based analysis to begin to define the full transcriptional profile of HSCs from different species and developmental stages and compare these to other stem cells.[64,65,128–131] Many of the data are available in online databases, such as the NIH/NIDDK Stem Cell Genome Anatomy Projects (http://www.scgap.org). While transcriptional profiling is clearly a work in progress, comparisons among various types of stem cells may eventually identify sets of genes that are involved in defining the general "stemness" of a cell, as well as sets of genes that define their exit from the stem cell pool (*e.g.,* the beginning of their path toward becoming mature differentiated cells, also referred to as *commitment*). In addition, these datasets will reveal sets of genes that are associated with specific stem cell populations, such as HSCs and MSCs, and thus define their unique properties. Assembly of these datasets into pathways will greatly help to understand and to predict the responses of HSCs (and other stem cells) to various stimuli.

CLINICAL USE OF HSCS

The clinical use of stem cells holds great promise, although the application of most classes of adult stem cells is either currently untested or is in the earliest phases of clinical testing.[132,133] The only exception is HSCs, which have been used clinically since 1959 and are used increasingly routinely for transplantations, albeit almost exclusively in a non-pure form. By 1995, more than 40,000 transplants were performed annually world-wide.[134,135] Currently the main indications for bone marrow transplantation are either hematopoietic cancers (leukemias and lymphomas), or the use of high-dose chemotherapy for non-hematopoietic malignancies (cancers in other organs). Other indications include diseases that involve genetic or acquired bone marrow failure, such as aplastic anemia, thalassemia sickle cell anemia, and increasingly, autoimmune diseases.

Autologous versus Allogeneic Grafts

Transplantation of bone marrow and HSCs are carried out in two rather different settings, autologous and allogeneic. Autologous transplantations employ a patient's own bone marrow tissue and thus present no tissue incompatibility between the donor and the host. Allogeneic transplantations occur between two individuals who are not genetically identical (with the rare exceptions of transplantations between identical twins, often referred to as syngeneic transplantations). Non-identical individuals differ in their human leukocyte antigens (HLAs), proteins that are expressed by their white blood cells. The immune system uses these HLAs to distinguish between "self" and "non-self." For successful transplantation, allogeneic grafts must match most, if not all, of the six to ten major HLA antigens between host and donor. Even if they do, however, enough differences remain in mostly uncharacterized minor antigens to enable immune cells from the donor and the host to recognize the other as "non-self." This is an important issue, as virtually all HSC transplants are carried out with either non-purified, mixed cell populations (mobilized peripheral blood, cord blood, or bone marrow) or cell populations that have been enriched for HSCs (*e.g.,* by column selection

for CD34+ cells) but have not been fully purified. These mixed population grafts contain sufficient lymphoid cells to mount an immune response against host cells if they are recognized as "non-self." The clinical syndrome that results from this "non-self" response is known as graft-versus-host disease (GVHD).[136]

In contrast, autologous grafts use cells harvested from the patient and offer the advantage of not causing GVHD. The main disadvantage of an autologous graft in the treatment of cancer is the absence of a graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) response, the specific immunological recognition of host tumor cells by donor-immune effector cells present in the transplant. Moreover, the possibility exists for contamination with cancerous or pre-cancerous cells.

Allogeneic grafts also have disadvantages. They are limited by the availability of immunologically-matched donors and the possibility of developing potentially lethal GVHD. The main advantage of allogeneic grafts is the potential for a GVL response, which can be an important contribution to achieving and maintaining complete remission.[137,138]

CD34+-Enriched versus Highly Purified HSC Grafts

Today, most grafts used in the treatment of patients consist of either whole or CD34+-enriched bone marrow or, more likely, mobilized peripheral blood. The use of highly purified hematopoietic stem cells as grafts is rare.[56–58] However, the latter have the advantage of containing no detectable contaminating tumor cells in the case of autologous grafts, therefore not inducing GVHD, or presumably GVL,[139–141] in allogeneic grafts. While they do so less efficiently than lymphocyte-containing cell mixtures, HSCs alone can engraft across full allogeneic barriers (*i.e.,* when transplanted from a donor who is a complete mismatch for both major and minor transplantation antigens).[139–141] The use of donor lymphocyte infusions (DLI) in the context of HSC transplantation allows for the controlled addition of lymphocytes, if necessary, to obtain or maintain high levels of donor cells and/or to induce a potentially curative GVL-response.[142,143] The main problems associated with clinical use of highly purified HSCs are the additional labor and costs[144] involved in obtaining highly purified cells in sufficient quantities.

While the possibilities of GVL and other immune responses to malignancies remain the focus of intense interest, it is also clear that in many cases, less-directed approaches such as chemotherapy or irradiation offer promise. However, while high-dose chemotherapy combined with autologous bone marrow transplantation has been reported to improve outcome (usually measured as the increase in time to progression, or increase in survival time),[145–154] this has not been observed by other researchers and remains controversial.[155–161] The tumor cells present in autologous grafts may be an important limitation in achieving long-term disease-free survival. Only further purification/purging of the grafts, with rigorous separation of HSCs from cancer cells, can overcome this limitation. Initial small scale trials with HSCs purified by flow cytometry suggest that this is both possible and beneficial to the clinical outcome.[56] In summary, purification of HSCs from cancer/lymphoma/leukemia patients offers the only possibility of using these cells post-chemotherapy to regenerate the host with cancer-free grafts. Purification of HSCs in allotransplantation allows transplantation with cells that regenerate the blood-forming system but cannot induce GVHD.

Non-Myeloablative Conditioning

An important recent advance in the clinical use of HSCs is the development of non-myeloablative preconditioning regimens, sometimes referred to as "mini transplants."[162–164] Traditionally, bone marrow or stem cell transplantation has been preceded by a preconditioning regimen consisting of chemotherapeutic agents, often combined with irradiation, that completely destroys host blood and bone marrow tissues (a process called *myeloablation*). This creates "space" for the incoming cells by freeing stem cell niches and prevents an undesired immune response of the host cells against the graft cells, which could result in graft failure. However, myeloablation immunocompromises the patient severely and necessitates a prolonged hospital stay under sterile conditions. Many protocols have been developed that use a more limited and targeted approach to preconditioning. These non-myeloablative preconditioning protocols, which combine excellent engraftment results with the ability to perform hematopoietic cell transplantation on an outpatient basis, have greatly changed the clinical practice of bone marrow transplantation.

Additional Indications

FACS purification of HSCs in mouse and man completely eliminates contaminating T cells, and thus GVHD (which is caused by T-lymphocytes) in allogeneic transplants. Many HSC transplants have been carried out in different combinations of mouse strains. Some of these were matched at the major transplantation antigens but otherwise different (Matched Unrelated Donors or MUD); in others, no match at the major or minor transplantation antigens was expected. To achieve rapid and sustained engraftment, higher doses of HSCs were required in these mismatched allogeneic transplants than in syngeneic transplants.[139–141,165–167] In these experiments, hosts whose immune and blood-forming systems were generated from genetically distinct donors were permanently capable of accepting organ transplants (such as the heart) from either donor or host, but not from mice unrelated to the donor or host. This phenomenon is known as transplant-induced tolerance and was observed whether the organ transplants were given the same day as the HSCs or up to one year later.[139,166] Hematopoietic cell transplant-related complications have limited the clinical application of such tolerance induction for solid organ grafts, but the use of non-myeloablative regimens to prepare the host, as discussed above, should significantly reduce the risk associated with combined HSC and organ transplants. Translation of these findings to human patients should enable a switch from chronic immunosuppression to prevent rejection to protocols wherein a single conditioning dose allows permanent engraftment of both the transplanted blood system and solid organ(s) or other tissue stem cells from the same donor. This should eliminate both GVHD and chronic host transplant immunosuppression, which lead to many complications, including life-threatening opportunistic infections and the development of malignant neoplasms.

We now know that several autoimmune diseases — diseases in which immune cells attack normal body tissues — involve the inheritance of high risk-factor genes.[168] Many of these genes are expressed only in blood cells. Researchers have recently tested whether HSCs could be used in mice with autoimmune disease (*e.g.,* type 1 diabetes) to replace an autoimmune blood system with one that lacks the autoimmune risk genes. The HSC transplants cured mice that were in the process of disease development when non-myeloablative conditioning was used for transplant.[169] It has been observed that transplant-induced tolerance allows co-transplantation of pancreatic islet cells to replace destroyed islets.[170] If these results using non-myeloablative conditioning can be translated to humans, type 1 diabetes and several other auto-immune diseases may be treatable with pure HSC grafts. However, the reader should be cautioned that the translation of treatments from mice to humans is often complicated and time-consuming.

Hematopoietic Stem Cell Banking

Banking is currently a routine procedure for UCB samples. If expansion of fully functional HSCs in tissue culture becomes a reality, HSC transplants may be possible by starting with small collections of HSCs rather than massive numbers acquired through mobilization and apheresis. With such a capability, collections of HSCs from volunteer donors or umbilical cords could be theoretically converted into storable, expandable stem cell banks useful on demand for clinical transplantation and/or for protection against radiation accidents. In mice, successful HSC transplants that regenerate fully normal immune and blood-forming systems can be accomplished when there is only a partial transplantation antigen match. Thus, the establishment of useful human HSC banks may require a match between as few as three out of six transplantation antigens (HLA). This might be accomplished with stem cell banks of as few as 4,000–10,000 independent samples.

LEUKEMIA (AND CANCER) STEM CELLS

Leukemias are proliferative diseases of the hematopoietic system that fail to obey normal regulatory signals. They derive from stem cells or progenitors of the hematopoietic system and almost certainly include several stages of progression. During this progression, genetic and/or epigenetic changes occur, either in the DNA sequence itself (genetic) or other heritable modifications that affect the genome (epigenetic). These (epi)genetic changes alter cells from the normal hematopoietic system into cells capable of robust leukemic growth. There are a variety of leukemias, usually classified by the predominant pathologic cell types and/or the clinical course of the disease. It has been proposed that these are diseases in which self-

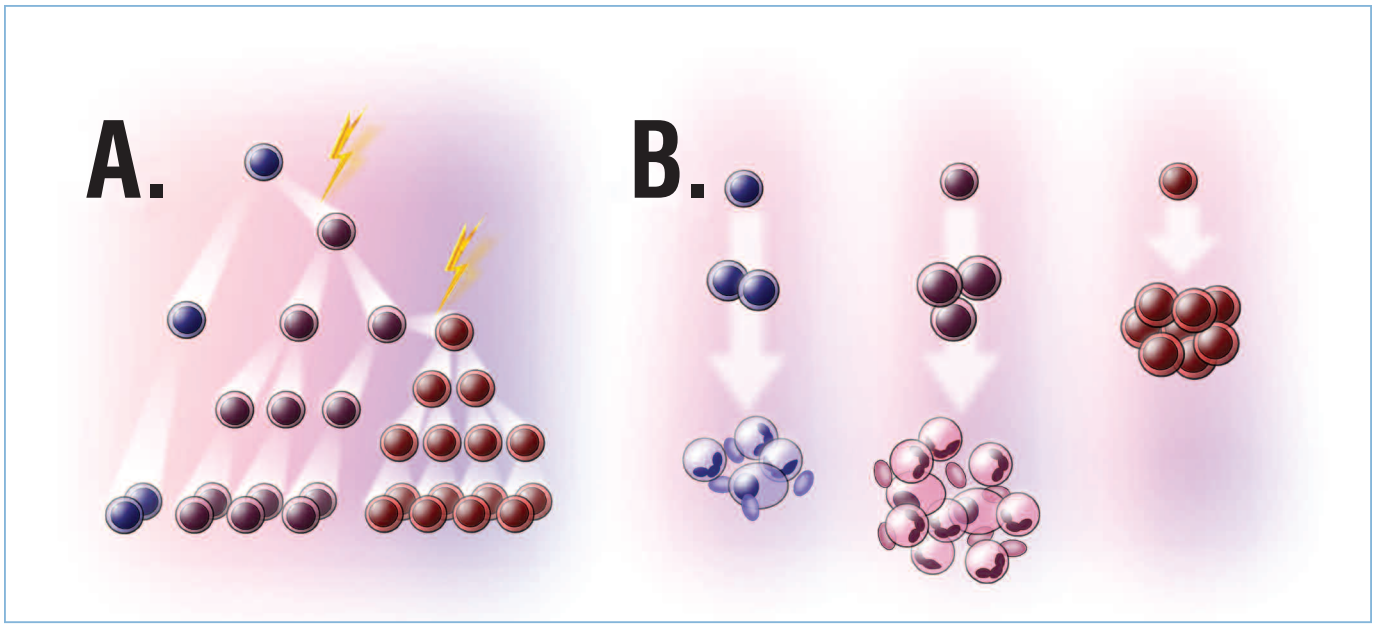


Figure 2.6. Leukemic progression at the hematopoietic stem cell level. Self-renewing HSCs are the cells present long enough to accumulate the many activating events necessary for full transformation into tumorigenic cells. Under normal conditions, half of the offspring of HSC cell divisions would be expected to undergo differentiation, leaving the HSC pool stable in size. (A) (Pre) leukemic progression results in cohorts of HSCs with increasing malignant potential. The cells with the additional event (two events are illustrated, although more would be expected to occur) can outcompete less-transformed cells in the HSC pool if they divide faster (as suggested in the figure) or are more resistant to differentiation or apoptosis (cell death), two major exit routes from the HSC pool. (B) Normal HSCs differentiate into progenitors and mature cells; this is linked with limited proliferation (left). Partially transformed HSCs can still differentiate into progenitors and mature cells, but more cells are produced. Also, the types of mature cells that are produced may be skewed from the normal ratio. Fully transformed cells may be completely blocked in terminal differentiation, and large numbers of primitive blast cells, representing either HSCs or self-renewing, transformed progenitor cells, can be produced. While this sequence of events is true for some leukemias (*e.g.,* AML), not all of the events occur in every leukemia. As with non-transformed cells, most leukemia cells (other than the leukemia stem cells) can retain the potential for (limited) differentiation.

renewing but poorly regulated cells, so-called "leukemia stem cells" (LSCs), are the populations that harbor all the genetic and epigenetic changes that allow leukemic progression.[171–176] While their progeny may be the characteristic cells observed with the leukemia, these progeny cells are not the self-renewing "malignant" cells of the disease. In this view, the events contributing to tumorigenic transformation, such as interrupted or decreased expression of "tumor suppressor" genes, loss of programmed death pathways, evasion of immune cells and macrophage surveillance mechanisms, retention of telomeres, and activation or amplification of self-renewal pathways, occur as single, rare events in the clonal progression to blast-crisis leukemia. As LT HSCs are the only self-renewing cells in the myeloid pathway, it has been proposed that most, if not all, progression events occur at this level of differentiation, creating clonal cohorts of HSCs with increasing malignancy *(see Figure 2.6).* In this disease model, the final event, explosive self-renewal, could occur at the level of HSC or at any of the known progenitors *(see Figures 2.5 and 2.6).* Activation of the β-catenin/lef-tcf signal transduction and transcription pathway has been implicated in leukemic stem cell self-renewal in mouse AML and human CML.[177] In both cases, the granulocyte-macrophage progenitors, not the HSCs or progeny blast cells, are the malignant self-renewing entities. In other models, such as the JunB-deficient tumors in mice and in chronic-phase CML in humans, the leukemic stem cell is the HSC itself.[90,177] However, these HSCs still respond to regulatory signals, thus representing steps in the clonal progression toward blast crisis *(see Figure 2.6).*

Many methods have revealed contributing proto-oncogenes and lost tumor suppressors in myeloid leukemias. Now that LSCs can be isolated, researchers should eventually be able to assess the full sequence of events in HSC clones undergoing leukemic

transformation. For example, early events, such as the AML/ETO translocation in AML or the BCR/ABL translocation in CML can remain present in normal HSCs in patients who are in remission (*e.g.,* without detectable cancer).[177,178] The isolation of LSCs should enable a much more focused attack on these cells, drawing on their known gene expression patterns, the mutant genes they possess, and the proteomic analysis of the pathways altered by the proto-oncogenic events.[173,176,179] Thus, immune therapies for leukemia would become more realistic, and approaches to classify and isolate LSCs in blood could be applied to search for cancer stem cells in other tissues.[180]

SUMMARY

After more than 50 years of research and clinical use, hematopoietic stem cells have become the best-studied stem cells and, more importantly, hematopoietic stem cells have seen widespread clinical use. Yet the study of HSCs remains active and continues to advance very rapidly. Fueled by new basic research and clinical discoveries, HSCs hold promise for such indications as treating autoimmunity, generating tolerance for solid organ transplants, and directing cancer therapy. However, many challenges remain. The availability of (matched) HSCs for all of the potential applications continues to be a major hurdle. Efficient expansion of HSCs in culture remains one of the major research goals. Future developments in genomics and proteomics, as well as in gene therapy, have the potential to widen the horizon for clinical application of hematopoietic stem cells even further.

REFERENCES

1. Jacobsen LO, Marks EK, Gaston EO, Zirkle RE. Effect of spleen protection on mortality following X-irradiation. *J Lab Clin Med.* Vol 34; 1949:1538-1543.
2. Lorenz E, Uphoff ED, Reid TR, Shelton E. Modification of acute irradiation injury in mice and guinea pigs by bone marrow injection. *Radiology.* Vol 58; 1951:863-877.
3. Gengozian N, Urso IS, Congdon CC, Conger AD, Makinodan T. Thymus specificity in lethally irradiated mice treated with rat bone marrow. *Proc Soc Exp Biol Med.* Vol 96; 1957:714-720.
4. Ford CE, Hamerton JL, Barnes DWH, Loutit JF. Cytological identification of radiation-chimaeras. *Nature.* Vol 177; 1956:452-454.
5. Nowell PC, Cole LJ, Habermyer JG, Roan PL. Growth and continued function of rat marrow cells in x-irradiated mice. *Cancer Res.* Vol 16; 1956:258-261.
6. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science.* Vol 287; 2000:1442-1446.
7. Manz MG, Akashi K, Weissman IL. Biology of Hematopoietic Stem and Progenitor Cells. In: Appelbaum FR, ed. *Thomas' Hematopoietic Cell Transplantation.* Third ed. Malden, MA: Blackwell Publishing; 2004.
8. Till J, McCulloch E. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* Vol 14; 1961:213-224.
9. Becker A, McCulloch E, Till J. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature.* Vol 197; 1963:452-454.
10. Siminovitch L, McCulloch E, Till J. The distribution of colony-forming cells among spleen colonies. *J Cell Comp Physiol.* Vol 62; 1963:327-336.
11. Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cell of the lymphoid system. *J Exp Med.* Vol 127; 1963:455-467.
12. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell.* Vol 100; 2000:157-168.
13. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science.* Vol 241; 1988:58-62.
14. Na Nakorn T, Traver D, Weissman IL, Akashi K. Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. *J Clin Invest.* 2002;109:1579-1585.
15. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity.* 1994;1:661-673.
16. Domen J, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol Med Today.* Vol 5; 1999:201-208.
17. Ploemacher RE. Stem cells: characterization and measurement. *Baillieres Clin Haematol.* Vol 10; 1997:429-444.
18. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol.* Vol 11; 1995:35-71.
19. McCune JM, Namikawa H, Kaneshima H, Schultz LD, al e. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science.* Vol 241; 1988:1632-1639.
20. Kamel-Reid S, Dick JE. Engraftment of immune deficient mice with human hematopoietic stem cells. *Science.* Vol 242; 1988:1706-1709.
21. Peault B, Weissman IL, Baum C, McCune JM, Tsukamoto A. Lymphoid reconstitution of the human fetal thymus in SCID mice with CD34+ precursor cells. *J Exp Med.* Vol 174; 1991:1283-1286.

22. Larochelle A, Vormoor J, Hanenberg H, et al. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med.* Vol 2; 1996:1329-1337.

23. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA.* Vol 94; 1997:5320-5325.

24. Hiramatsu H, Nishikomori R, Heike T, et al. Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. *Blood.* Vol 102; 2003:873-880.

25. Traggiai E, Chicha L, Mazzucchelli L, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science.* Vol 304; 2004:104-107.

26. Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol.* Vol 91; 1977:335-344.

27. Whitlock CA, Witte ON. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc Natl Acad Sci USA.* Vol 79; 1982:3608-3612.

28. Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ. Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci USA.* Vol 87; 1990:3584-3588.

29. Ploemacher R, van der Sluijs J, Voerman JSA, Bron NHC. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cell in the mouse. *Blood.* Vol 74; 1989:2755-2763.

30. Bauman JG, de Vries P, Pronk B, Visser JW. Purification of murine hemopoietic stem cells and committed progenitors by fluorescence activated cell sorting using wheat germ agglutinin and monoclonal antibodies. *Acta Histochem Suppl.* Vol 36; 1988:241-253.

31. Uchida N, Weissman IL. Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *J Exp Med.* Vol 175; 1992:175-184.

32. Wiesmann A, Phillips RL, Mojica M, et al. Expression of CD27 on murine hematopoietic stem and progenitor cells. *Immunity.* Vol 12; 2000:193-199.

33. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. Vol 273; 1996:242-245.

34. Randall TD, Lund FE, Howard MC, Weissman IL. Expression of murine CD38 defines a population of long-term reconstituting hematopoietic stem cells. *Blood.* Vol 87; 1996:4057-4067.

35. Moore T, Huang S, Terstappen LW, Bennett M, Kumar V. Expression of CD43 on murine and human pluripotent hematopoietic stem cells. *J Immunol.* Vol 153; 1994:4978-4987.

36. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci USA.* Vol 89; 1992:1502-1506.

37. Jordan CT, McKearn JP, Lemischka IR. Cellular and developmental properties of fetal hematopoietic stem cells. *Cell.* Vol 61; 1990:953-963.

38. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopioetic stem and progenitor cells and reveal enothelial niches for stem cells. *Cell.* 2005;121:1109-1121.

39. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci USA.* Vol 89; 1992:2804-2808.

40. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG- 1a cells. *J Immunol.* Vol 133; 1984:157-165.

41. Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38– progenitor cells. *Blood.* Vol 77; 1991:1218-1227.

42. Lansdorp PM, Sutherland HJ, Eaves CJ. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. *J Exp Med.* Vol 172; 1990:363-366.

43. Hill B, Rozler E, Travis M, et al. High-level expression of a novel epitope of CD59 identifies a subset of CD34+ bone marrow cells highly enriched for pluripotent stem cells. *Exp Hematol.* Vol 24; 1996:936-943.

44. Sutherland DR, Yeo EL, Stewart AK, et al. Identification of CD34+ subsets after glycoprotease selection: engraftment of CD34+Thy-1+Lin– stem cells in fetal sheep. *Exp Hematol.* Vol 24; 1996:795-806.

45. Gunji Y, Nakamura M, Osawa H, et al. Human primitive hematopoietic progenitor cells are more enriched in KITlow cells than in KIThigh cells. *Blood.* Vol 82; 1993:3283-3289.

46. Miraglia S, Godfrey W, Yin AH, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood.* Vol 90; 1997:5013-5021.

47. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood.* Vol 90; 1997:5002-5012.

48. Uchida N, Yang Z, Combs J, et al. The characterization, molecular cloning, and expression of a novel hematopoietic cell antigen from CD34+ human bone marrow cells. *Blood.* Vol 89; 1997:2706-2716.

49. Srour EF, Brandt JE, Leemhuis T, Ballas CB, Hoffman R. Relationship between cytokine-dependent cell cycle progression and MHC class II antigen expression by human CD34+ HLA-DR- bone marrow cells. *J Immunol.* Vol 148; 1992:815-820.

50. Tsukamoto A, Weissman I, Chen B, et al. Phenotypic and functional analysis of hematopoietic stem cells in mouse and man. In: Mertelsman La, ed. *Hematopoietic Stem Cells, Biology and Therapeutic Applications:* Phenotypic and functional analysis of hematopoietic stem cells in mouse and man.; 1995.

51. Spangrude GJ, Johnson GR. Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc Natl Acad Sci USA.* Vol 87; 1990:7433-7437.

52. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med.* Vol 183; 1996:1797-1806.

53. Gothot A, Pyatt R, McMahel J, Rice S, Srour EF. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. *Blood.* Vol 90; 1997:4384-4393.

54. Storms RW, Trujillo AP, Springer JB, et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA.* Vol 96; 1999:9118-9123.

55. Kim M, Cooper DD, Hayes SF, Spangrude GJ. Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood.* Vol 91; 1998:4106-4117.

56. Negrin RS, Atkinson K, Leemhuis T, et al. Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with metastatic breast cancer. *Biol Blood Marrow Transplant.* Vol 6; 2000:262-271.

57. Michallet M, Philip T, Philip I, et al. Transplantation with selected autologous peripheral blood CD34+Thy-1+ hematopoietic stem cells (HSCs) in multiple myeloma: impact of HSC dose on engraftment, safety, and immune reconstitution. *Exp Hematol.* Vol 28; 2000:858-870.

58. Vose JM, Bierman PJ, Lynch JC, et al. Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with recurrent indolent non-Hodgkin's lymphoma. *Biol Blood Marrow Transplant.* Vol 7; 2001:680-687.

59. Spangrude GJ, Brooks DM. Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1- negative subset. *Blood.* Vol 80; 1992:1957-1964.

60. Spangrude GJ, Brooks DM. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. *Blood.* Vol 82; 1993:3327-3332.

61. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci USA.* Vol 92; 1995:10302-10306.

62. Randall TD, Weissman IL. Phenotypic and functional changes induced at the clonal level in hematopoietic stem cells after 5-fluorouracil treatment. *Blood.* Vol 89; 1997:3596-3606.

63. Sato T, Laver JH, Ogawa M. Reversible Expression of CD34 by Murine Hematopoietic Stem Cells. *Blood.* Vol 94; 1999:2548-2554.

64. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science.* Vol 298; 2002:597-600.

65. Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A stem cell molecular signature. *Science.* Vol 298; 2002:601-604.

66. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* Vol 91; 1997:661-672.

67. Galy A, Travis M, Cen D, Chen B. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity.* Vol 3; 1995:459-473.

68. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* Vol 404; 2000:193-197.

69. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci USA.* Vol 99; 2002:11872-11877.

70. Arber C, BitMansour A, Sparer TE, et al. Common lymphoid progenitors rapidly engraft and protect against lethal murine cytomegalovirus infection after hematopoietic stem cell transplantation. *Blood.* Vol 102; 2003:421-428.

71. BitMansour A, Burns SM, Traver D, et al. Myeloid progenitors protect against invasive aspergillosis and Pseudomonas aeruginosa infection following hematopoietic stem cell transplantation. *Blood.* Vol 100; 2002:4660-4667.

72. Greenwood MJ, Lansdorp PM. Telomeres, telomerase, and hematopoietic stem cell biology. *Arch Med Res.* Vol 34; 2003:489-495.

73. Allsopp RC, Morin GB, Horner JW, DePinho R, Harley CB, Weissman IL. Effect of TERT over-expression on the long-term transplantation capacity of hematopoietic stem cells. *Nat Med.* Vol 9; 2003:369-371.

74. Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood.* Vol 102; 2003:517-520.

75. Reya T. Regulation of hematopoietic stem cell self-renewal. *Recent Prog Horm Res.* Vol 58; 2003:283-295.

76. Domen J, Weissman IL. Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med.* Vol 192; 2000:1707-1718.

77. Smith LG, Weissman IL, Heimfeld S. Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proc Natl Acad Sci USA.* Vol 88; 1991:2788-2792.

78. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science.* Vol 297; 2002:2256-2259.

79. Morrison SJ, Wandycz AM, Hemmati HD, Wright DE, Weissman IL. Identification of a lineage of multipotent hematopoietic progenitors. *Development.* Vol 124; 1997:1929-1939.

80. Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr Biol.* Vol 7; 1997:805-808.

81. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature.* Vol 423; 2003:409-414.

82. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature.* Vol 423; 2003:448-452.

83. Sauvageau G, Thorsteinsdottir U, Eaves CJ, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* Vol 9; 1995:1753-1765.

84. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell.* Vol 109; 2002:39-45.

85. Krosl J, Austin P, Beslu N, Kroon E, Humphries RK, Sauvageau G. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat Med.* Vol 9; 2003:1428-1432.

86. Amsellem S, Pflumio F, Bardinet D, et al. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat Med.* Vol 9; 2003:1423-1427.

87. Thorsteinsdottir U, Mamo A, Kroon E, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. *Blood.* Vol 99; 2002:121-129.

88. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. Stem cells and cancer; the polycomb connection. *Cell.* Vol 118; 2004:409-418.

89. Wilson A, Murphy MJ, Oskarsson T, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev.* Vol 18; 2004:2747-2763.

90. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. *Cell.* Vol 119; 2004:431-443.

91. Watt FM, Hogan BL. Out of Eden: stem cells and their niches. *Science.* Vol 287; 2000:1427-1430.

92. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature.* Vol 425; 2003:841-846.

93. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature.* Vol 425; 2003:836-841.

94. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell.* Vol 118; 2004:149-161.

95. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self- renewing hematopoietic stem cells. *Proc Natl Acad Sci USA.* Vol 96; 1999:3120-3125.

96. Bradford GB, Williams B, Rossi R, Bertoncello I. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol.* Vol 25; 1997:445-453.

97. Morrison SJ, Wright DE, Weissman IL. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci USA.* Vol 94; 1997:1908-1913.

98. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. *Science.* Vol 294; 2001:1933-1936.

99. Abkowitz JL, Robinson AE, Kale S, Long MW, Chen J. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. *Blood.* Vol 102; 2003:1249-1253.

100. Barker JN, Wagner JE. Umbilical cord blood transplantation: current practice and future innovations. *Crit Rev Oncol Hematol.* Vol 48; 2003:35-43.

101. Koh LP, Chao NJ. Umbilical cord blood transplantation in adults using myeloablative and nonmyeloablative preparative regimens. *Biol Blood Marrow Transplant.* Vol 10; 2004:1-22.

102. Wagner Je J, Barker J. Umbilical cord blood transplantation: Novel approaches toward improving engraftment. *Biol Blood Marrow Transplant.* Vol 10; 2004:733.

103. Jaroscak J, Goltry K, Smith A, et al. Augmentation of umbilical cord blood (UCB) transplantation with ex vivo-expanded UCB cells: results of a phase 1 trial using the AastromReplicell System. *Blood.* Vol 101; 2003:5061-5067.

104. Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE. Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. *Blood.* Vol 103; 2004:1147-1151.

105. Wolf SM, Kahn JP, Wagner JE. Using preimplantation genetic diagnosis to create a stem cell donor: issues, guidelines & limits. *J Law Med Ethics.* Vol 31; 2003:327-339.

106. Vodyanik MA, Bork JA, Thomson JA, Slukvin, II. Human embryonic stem cell-derived CD34+ cells: efficient production in the co-culture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood;* 2004.

107. Ferrari G, Cusella-De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science.* Vol 279; 1998:1528-1530.

108. Gussoni E, Soneoka Y, Strickland CD, et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature.* Vol 401; 1999:390-394.

109. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* Vol 410; 2001:701-705.

110. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA.* Vol 98; 2001:10344-10349.

111. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest.* Vol 107; 2001:1395-1402.

112. Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science.* Vol 290; 2000:1775-1779.

113. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science.* Vol 290; 2000:1779-1782.

114. Lagasse E, Connors H, Al-Dhalimy M, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med.* Vol 6; 2000:1229-1234.

115. Petersen BE, Bowen WC, Patrene KD, et al. Bone marrow as a potential source of hepatic oval cells. *Science.* Vol 284; 1999:1168-1170.

116. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell.* Vol 105; 2001:369-377.

117. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest.* Vol 111; 2003:843-850.

118. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science.* Vol 276; 1997:71-74.

119. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* Vol 284; 1999:143-147.

120. Verfaillie CM, Schwartz R, Reyes M, Jiang Y. Unexpected potential of adult stem cells. *Ann NY Acad Sci.* Vol 996; 2003:231-234.

121. Lemischka I. A few thoughts about the plasticity of stem cells. *Exp Hematol.* Vol 30; 2002:848-852.

122. Verfaillie CM. Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol.* Vol 12; 2002:502-508.

123. Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell.* Vol 116; 2004:639-648.

124. Sherwood RI, Christensen JL, Conboy IM, et al. Isolation of Adult Mouse Myogenic Progenitors; Functional Heterogeneity of Cells within and Engrafting Skeletal Muscle. *Cell.* Vol 119; 2004:543-554.

125. Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature.* Apr 24 2003;422(6934):897-901.

126. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, et al. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature.* Vol 425; 2003:968-973.

127. Camargo FD, Finegold M, Goodell MA. Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. *J Clin Invest.* Vol 113; 2004:1266-1270.

128. Phillips RL, Ernst RE, Brunk B, et al. The genetic program of hematopoietic stem cells. *Science.* Vol 288; 2000:1635-1640.

129. Terskikh AV, Easterday MC, Li L, et al. From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs. *Proc Natl Acad Sci USA.* Vol 98; 2001:7934-7939.

130. Park IK, He Y, Lin F, et al. Differential gene expression profiling of adult murine hematopoietic stem cells. *Blood.* Vol 99; 2002:488-498.

131. Terskikh AV, Miyamoto T, Chang C, Diatchenko L, Weissman IL. Gene expression analysis of purified hematopoietic stem cells and committed progenitors. *Blood.* Vol 102; 2003:94-101.

132. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol.* Vol 18; 2000:307-316.

133. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthritis Cartilage.* Vol 10; 2002:199-206.

134. Santos GW. Historical background to hematopoietic stem cell transplantation. In: Atkinson K, ed. *Clinical bone marrow and blood stem cell transplantation.* Cambridge, UK: Cambridge University Press; 2000:1-12.

135. Horowitz MM. Uses and growth of hematopoietic cell transplantationj. In: Forman SJ, ed. *Hematopoietic cell transplantation.* Second ed. Malden, MA: Blackwell Science Inc; 1999:12-18.

136. Ferrara JL, Levy R, Chao NJ. Pathophysiologic mechanisms of acute graft-vs.-host disease. *Biol Blood Marrow Transplant.* Vol 5; 1999:347-356.

137. Thomas ED, Blume KG, Forman SJ. Hematopoietic cell transplantation. 2nd ed. Malden, MA: Blackwell Science, Inc.; 1999.

138. Atkinson K. Clinical bone marrow and blood stem cell transplantation. Cambridge, UK: Cambridge University Press; 2000.

139. Gandy KL, Weissman IL. Tolerance of allogeneic heart grafts in mice simultaneously reconstituted with purified allogeneic hematopoietic stem cells. *Transplantation.* Vol 65; 1998:295-304.

140. Shizuru JA, Jerabek L, Edwards CT, Weissman IL. Transplantation of purified hematopoietic stem cells: requirements for overcoming the barriers of allogeneic engraftment. *Biol Blood Marrow Transplant.* Vol 2; 1996:3-14.

141. Wang B, El-Badri NS, Cherry, Good RA. Purified hematopoietic stem cells without facilitating cells can repopulate fully allogeneic recipients across entire major histocompatibility complex transplantation barrier in mice. *Proc Natl Acad Sci USA.* Vol 94; 1997:14632-14636.

142. Slavin S, Morecki S, Weiss L, Or R. Donor lymphocyte infusion: the use of alloreactive and tumor-reactive lymphocytes for immunotherapy of malignant and non-malignant diseases in conjunction with allogeneic stem cell transplantation. *J Hematother Stem Cell Res.* Vol 11; 2002:265-276.

143. Feinstein L, Sandmaier B, Maloney D, et al. Nonmyeloablative hematopoietic cell transplantation. Replacing high-dose cytotoxic therapy by the graft-versus-tumor effect. *Ann NY Acad Sci.* Vol 938; 2001:328-337; discussion 337-329.

144. Goldman JM. Autologous hematopoietic stem cell transplantation for chronic myeloid leukemia. In: Atkinson K, ed. *Clinical bone marrow and blood stem cell transplantation.* Cambridge, UK: Cambridge University Press; 2000:279-286.

145. Attal M, Harousseau JL, Stoppa AM, et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. *N Engl J Med.* Vol 335; 1996:91-97.

146. Haioun C, Lepage E, Gisselbrecht C, et al. Benefit of autologous bone marrow transplantation over sequential chemotherapy in poor-risk aggressive non-Hodgkin's lymphoma: updated results of the prospective study LNH87-2. Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol.* Vol 15; 1997:1131-1137.

147. Gianni AM, Bregni M, Siena S, et al. High-dose chemotherapy and autologous bone marrow transplantation compared with MACOP-B in aggressive B-cell lymphoma. *N Engl J Med.* Vol 336; 1997:1290-1297.

148. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis- retinoic acid. Children's Cancer Group. *N Engl J Med.* Vol 341; 1999:1165-1173.

149. Damon LE, Hu WW, Stockerl-Goldstein KE, et al. High-dose chemotherapy and hematopoietic stem cell rescue for breast cancer: experience in California. *Biol Blood Marrow Transplant.* Vol 6; 2000:496-505.

150. Nieto Y, Champlin RE, Wingard JR, et al. Status of high-dose chemotherapy for breast cancer: a review. *Biol Blood Marrow Transplant.* Vol 6; 2000:476-495.

151. Somlo G, Chow W, Hamasaki V, et al. Tandem-cycle high-dose melphalan and cisplatin with peripheral blood progenitor cell support in patients with breast cancer and other malignancies. *Biol Blood Marrow Transplant.* Vol 7; 2001:284-293.

152. Child JA, Morgan GJ, Davies FE, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. *N Engl J Med.* Vol 348; 2003:1875-1883.

153. Rodenhuis S, Bontenbal M, Beex LV, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for high-risk breast cancer. *N Engl J Med.* Vol 349; 2003:7-16.

154. Monitoring treatment and survival in chronic myeloid leukemia. Italian Cooperative Study Group on Chronic Myeloid Leukemia and Italian Group for Bone Marrow Transplantation. *J Clin Oncol.* Vol 17; 1999:1858-1868.

155. Harousseau JL, Cahn JY, Pignon B, et al. Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. The Groupe Ouest Est Leucemies Aigues Myeloblastiques (GOELAM). *Blood.* Vol 90; 1997:2978-2986.

156. Cassileth PA, Harrington DP, Appelbaum FR, et al. Chemotherapy compared with autologous or allogeneic bone marrow transplantation in the management of acute myeloid leukemia in first remission. *N Engl J Med.* Vol 339; 1998:1649-1656.

157. Stevens RF, Hann IM, Wheatley K, Gray RG. Marked improvements in outcome with chemotherapy alone in paediatric acute myeloid leukemia: results of the United Kingdom Medical Research Council's 10th AML trial. MRC Childhood Leukaemia Working Party. *Br J Haematol.* Vol 101; 1998:130-140.

158. Hortobagyi GN, Buzdar AU, Theriault RL, et al. Randomized trial of high-dose chemotherapy and blood cell autografts for high-risk primary breast carcinoma. *J Natl Cancer Inst.* Vol 92; 2000:225-233.

159. Bashey A, Corringham S, Garrett J, et al. A phase II study of two cycles of high-dose chemotherapy with autologous stem cell support in patients with metastatic breast cancer who meet eligibility criteria for a single cycle. *Bone Marrow Transplant.* Vol 25; 2000:519-524.

160. Stadtmauer EA, O'Neill A, Goldstein LJ, et al. Conventional-dose chemotherapy compared with high-dose chemotherapy plus autologous hematopoietic stem-cell transplantation for metastatic breast cancer. Philadelphia Bone Marrow Transplant Group. *N Engl J Med.* Vol 342; 2000:1069-1076.

161. Tallman MS, Gray R, Robert NJ, et al. Conventional adjuvant chemotherapy with or without high-dose chemotherapy and autologous stem-cell transplantation in high-risk breast cancer. *N Engl J Med.* Vol 349; 2003:17-26.

162. Slavin S. Non-myeloablative stem cell transplantation for induction of host-versus-graft tolerance for adoptive immunotherapy of malignant and nonmalignant diseases and towards transplantation of organ allografts. *Transplant Proc.* Vol 34; 2002:3371-3373.

163. Spitzer TR. The expanding applications of non-myeloablative stem cell transplantation. *Pediatr Transplant.* Vol 7 Suppl 3; 2003:95-100.

164. Maris M, Sandmaier BM, Maloney DG, et al. Non-myeloablative hematopoietic stem cell transplantation. *Transfus Clin Biol.* Vol 8; 2001:231-234.

165. Uchida N, Tsukamoto A, He D, Friera AM, Scollay R, Weissman IL. High doses of purified stem cells cause early hematopoietic recovery in syngeneic and allogeneic hosts. *J Clin Invest.* Vol 101; 1998:961-966.

166. Shizuru JA, Weissman IL, Kernoff R, Masek M, Scheffold YC. Purified hematopoietic stem cell grafts induce tolerance to alloantigens and can mediate positive and negative T cell selection. *Proc Natl Acad Sci USA.* Vol 97; 2000:9555-9560.

167. Gandy KL, Domen J, Aguila H, Weissman IL. *CD8+ TCR+ and CD8+ TCR+ cells in whole bone marrow facilitate the engraftment of hematopoietic stem cells across allogeneic barriers:* Immunity; 1999.

168. McDevitt HO. The role of MHC class II molecules in susceptibility and resistance to autoimmunity. *Curr Opin Immunol.* 1998;10:677-681.

169. Beilhack GF, Landa RR, Masek MA, Shizuru JA. Prevention of type 1 diabetes with major histocompatibility complex-compatible and nonmarrow ablative hematopoietic stem cell transplants. *Diabetes.* 2005;54:1770-1779.

170. Beilhack GF, Scheffold YC, Weissman IL, et al. Purified allogeneic hematopoietic stem cell transplantation blocks diabetes pathogenesis in NOD mice. *Diabetes.* 2003;52:59-68.

171. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* Vol 414; 2001:105-111.

172. Hope KJ, Jin L, Dick JE. Human acute myeloid leukemia stem cells. *Arch Med Res.* Vol 34; 2003:507-514.

173. Guzman ML, Jordan CT. Considerations for targeting malignant stem cells in leukemia. *Cancer Control.* Vol 11; 2004:97-104.

174. Scadden DT. Cancer stem cells refined. *Nat Immunol.* Vol 5; 2004:701-703.

175. Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci USA.* Vol 100 Suppl 1; 2003:11842-11849.

176. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF. Therapeutic implications of cancer stem cells. *Curr Opin Genet Dev.* Vol 14; 2004:43-47.

177. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med.* Vol 351; 2004:657-667.

178. Miyamoto T, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci USA.* Vol 97; 2000:7521-7526.

179. Jones RJ, Matsui WH, Smith BD. Cancer stem cells: are we missing the target? *J Natl Cancer Inst.* Vol 96; 2004:583-585.

180. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene.* Vol 23; 2004:7274-7282.

3. REPAIRING THE NERVOUS SYSTEM WITH STEM CELLS

*by David M. Panchision**

Diseases of the nervous system, including congenital disorders, cancers, and degenerative diseases, affect millions of people of all ages. Congenital disorders occur when the brain or spinal cord does not form correctly during development. Cancers of the nervous system result from the uncontrolled spread of aberrant cells. Degenerative diseases occur when the nervous system loses functioning of nerve cells. Most of the advances in stem cell research have been directed at treating degenerative diseases. While many treatments aim to limit the damage of these diseases, in some cases scientists believe that damage can be reversed by replacing lost cells with new ones derived from cells that can mature into nerve cells, called neural stem cells. Research that uses stem cells to treat nervous system disorders remains an area of great promise and challenge to demonstrate that cell-replacement therapy can restore lost function.

STRATEGIES TO REPAIR THE NERVOUS SYSTEM

The nervous system is a complex organ made up of nerve cells (also called neurons) and glial cells, which surround and support neurons *(see Figure 3.1)*. Neurons send signals that affect numerous functions including thought processes and movement. One type of glial cell, the oligodendrocyte, acts to speed up the signals of neurons that extend over long distances, such as in the spinal cord. The loss of any of these cell types may have catastrophic results on brain function.

Although reports dating back as early as the 1960s pointed towards the possibility that new nerve cells are formed in adult mammalian brains, this knowledge was not applied in the context of curing devastating brain diseases until the 1990s. While earlier medical research focused on limiting damage once it had occurred, in recent years researchers have been working hard to find out if the cells that can give rise to new neurons can be coaxed to restore brain function. New neurons in the adult brain arise from slowly-dividing cells that appear to be the remnants of stem cells that existed during fetal brain development. Since some of these adult cells still retain the ability to generate both neurons and glia, they are referred to as adult neural stem cells.

These findings are exciting because they suggest that the brain may contain a built-in mechanism to repair itself. Unfortunately, these new neurons are only generated in a few sites in the brain and turn into only a few specialized types of nerve cells. Although there are many different neuronal cell types in the brain, we now know that these new neurons can "plug in" correctly to assist brain function.[1] The discovery of these cells has spurred further research into the characteristics of neural stem cells from the fetus and the adult, mostly using rodents and primates as model species. The hope is that these cells may be able to replenish those that are functionally lost in human degenerative diseases such as Parkinson's Disease, Huntington's Disease, and amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease), as well as from brain and spinal cord injuries that result from stroke or trauma.

Scientists are applying these new stem cell discoveries in two ways in their experiments. First, they are using current knowledge of normal brain development to modulate stem cells that are harvested and grown in culture. Researchers can then transplant these cultured cells into the brain of an animal model and allow the brain's own signals to differentiate the stem cells into neurons or glia. Alternatively, the stem cells

* Investigator, Center for Neuroscience Research and Assistant Professor, Dept. of Pediatrics and Pharmacology, George Washington University School of Medicine, Children's Research Institute, Children's National Medical Center, Washington, DC 20010-2970, *Email:* dpanchision@cnmcresearch.org

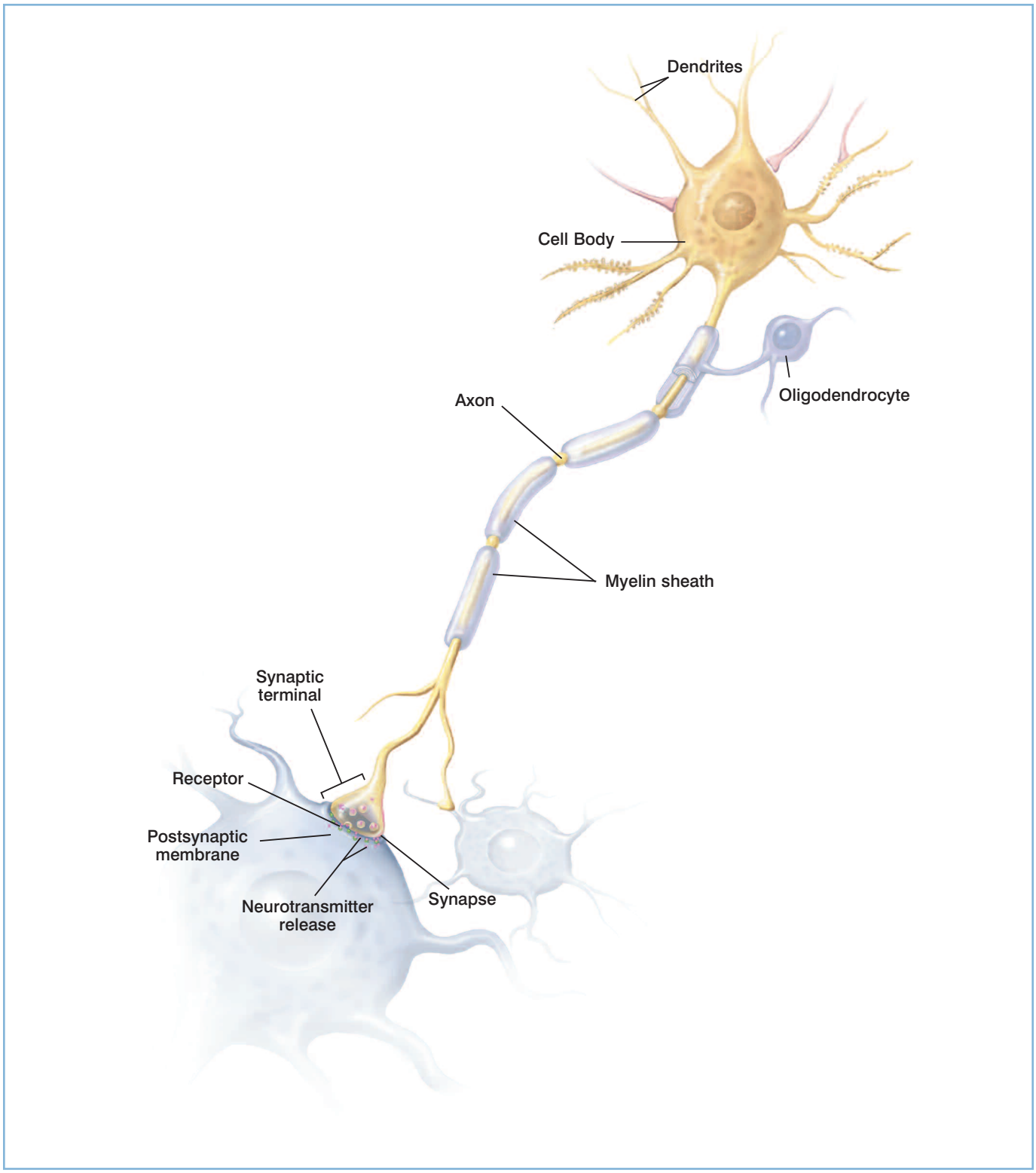


Figure 3.1. The Neuron.
When sufficient neurotransmitters cross synapses and bind receptors on the neuronal cell body and dendrites, the neuron sends an electrical signal down its axon to synaptic terminals, which in turn release neurotransmitters into the synapse that affects the following neuron. The brain neurons that die in Parkinson's Disease release the transmitter dopamine. Oligodendrocytes supply the axon with an insulating myelin sheath.

can be induced to differentiate into neurons and glia while in the culture dish, before being transplanted into the brain. Much progress has been made the last several years with human embryonic stem (ES) cells that can differentiate into all cell types in the body. While ES cells can be maintained in culture for relatively long periods of time without differentiating, they usually must be coaxed through many more steps of differentiation to produce the desired cell types. Recent studies, however, suggest that ES cells may differentiate into neurons in a more straightforward manner than may other cell types.

Second, scientists are identifying growth (trophic) factors that are normally produced and used by the developing and adult brain. They are using these factors to minimize damage to the brain and to activate the patient's own stem cells to repair damage that has occurred. Each of these strategies is being aggressively pursued to identify the most effective treatments for degenerative diseases. Most of these studies have been carried out initially with animal stem cells and recipients to determine their likelihood of success. Still, much more research is necessary to develop stem cell therapies that will be useful for treating brain and spinal cord disease in the same way that hematopoietic stem cell therapies are routinely used for immune system replacement (see Chapter 2).

The majority of stem cell studies of neurological disease have used rats and mice, since these models are convenient to use and are well-characterized biologically. If preliminary studies with rodent stem cells are successful, scientists will attempt to transplant human stem cells into rodents. Studies may then be carried out in primates (*e.g.,* monkeys) to offer insight into how humans might respond to neurological treatment. Human studies are rarely undertaken until these other experiments have shown promising results. While human transplant studies have been carried out for decades in the case of Parkinson's disease, animal research continues to provide improved strategies to generate an abundant supply of transplantable cells.

PARKINSON'S DISEASE — A MAJOR TARGET FOR STEM CELL RESEARCH

The intensive research aiming at curing Parkinson's disease with stem cells is a good example for the various strategies, successful results, and remaining challenges of stem cell-based brain repair. Parkinson's disease is a progressive disorder of motor control that affects roughly 2% of persons 65 years and older. Triggered by the death of neurons in a brain region called the substantia nigra, Parkinson's disease begins with minor tremors that progress to limb and bodily rigidity and difficulty initiating movement. These neurons connect via long axons to another region called the striatum, composed of subregions called the caudate nucleus and the putamen. These neurons that reach from the substantia nigra to the striatum release the chemical transmitter dopamine onto their target neurons in the striatum. One of dopamine's major roles is to regulate the nerves that control body movement. As these cells die, less dopamine is produced, leading to the movement difficulties characteristic of Parkinson's disease. Currently, the causes of death of these neurons are not well understood.

For many years, doctors have treated Parkinson's disease patients with the drug levodopa (L-dopa), which the brain converts into dopamine. Although the drug works well initially, levodopa eventually loses its effectiveness, and side-effects increase. Ultimately, many doctors and patients find themselves fighting a losing battle. For this reason, a huge effort is underway to develop new treatments, including growth factors that help the remaining dopamine neurons survive and transplantation procedures to replace those that have died.

RESEARCH ON FETAL TISSUE TRANSPLANTS IN PARKINSON'S DISEASE

The strategy to use new cells to replace lost ones is not new. Surgeons first attempted to transplant dopamine-releasing cells from a patient's own adrenal glands in the 1980s.[2,3] Although one of these studies reported a dramatic improvement in the patients' conditions, U.S. surgeons were only able to achieve modest and temporary improvement, insufficient to outweigh the risks of such a procedure. As a result, these human studies were not pursued further.

Another strategy was attempted in the 1970s, in which cells derived from fetal tissue from the mouse substantia nigra was transplanted into the adult rat eye and found to develop into mature dopamine neurons.[4] In the 1980s, several groups showed that transplantation of this type of tissue could reverse Parkinson's-like symptoms in rats and monkeys when placed in the damaged areas.The success of the animal studies led to

several human trials beginning in the mid-1980s.[5,6] In some cases, patients showed a lessening of their symptoms. Also, researchers could measure an increase in dopamine neuron function in the striatum of these patients by using a brain-imaging method called positron emission tomography (PET) *(see Figure 3.2).*[7]

The NIH has funded two large and well-controlled clinical trials in the past 15 years in which researchers transplanted tissue from aborted fetuses into the striatum of patients with Parkinson's disease.[7,8] These studies, performed in Colorado and New York, included controls where patients received "sham" surgery (no tissue was implanted), and neither the patients nor the scientists who evaluated their progress knew which patients received the implants. The patients' progress was followed for up to eight years. Unfortunately, both studies showed that the transplants offered little benefit to the patients as a group. While some patients showed improvement, others began to suffer from dyskinesias, jerky involuntary movements that are often side effects of long-term L-dopa treatment. This effect occurred in 15% of the patients in the Colorado study.[7] and more than half of the patients in the New York study.[8] Additionally, the New York study showed evidence that some patients' immune systems were attacking the grafts.

However, promising findings emerged from these studies as well. Younger and milder Parkinson's patients responded relatively well to the grafts, and PET scans of patients showed that some of the transplanted dopamine neurons survived and matured. Additionally, autopsies on three patients who died of unrelated causes, years after the surgeries, indicated the presence of dopamine neurons from the graft. These cells appeared to have matured in the same way as normal dopamine neurons, which suggested that they were acting normally in the brain.

Researchers in Sweden followed the severity of dyskinesia in patients for eleven years after neural transplantation and found that the severity was

Dopamine-Neuron Transplantation

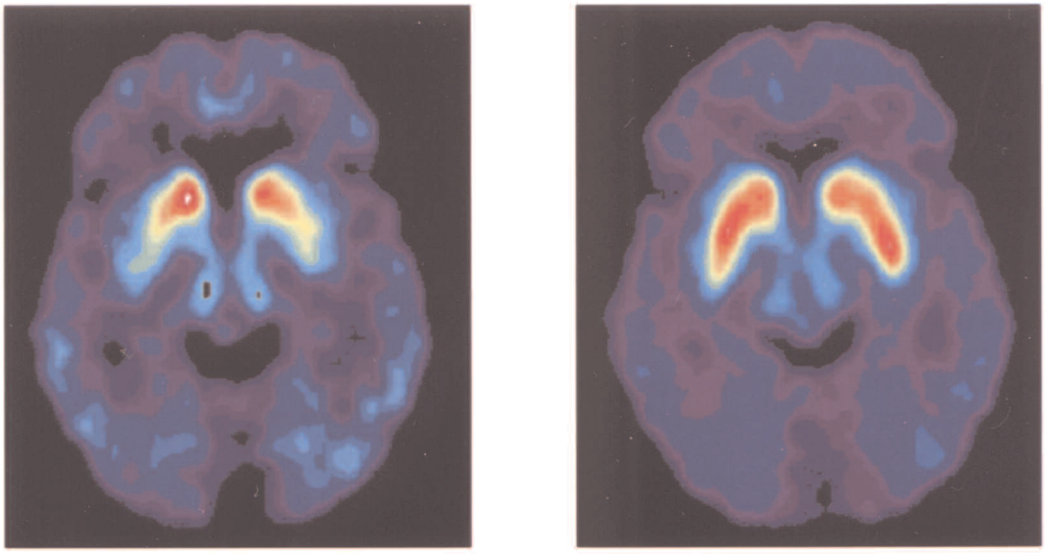


Figure 3.2. Positron Emission Tomography (PET) images from a Parkinson's patient before and after fetal tissue transplantation. The image taken before surgery (left) shows uptake of a radioactive form of dopamine (red) only in the caudate nucleus, indicating that dopamine neurons have degenerated. Twelve months after surgery, an image from the same patient (right) reveals increased dopamine function, especially in the putamen. (Reprinted with permission from N Eng J Med 2001;344 (10) p. 710.)

typically mild or moderate. These results suggested that dyskinesias were due to effects that were distinct from the beneficial effects of the grafts.[9] Dyskinesias may therefore be related to the ways that transplantation disturbs other cells in the brain and so may be minimized by future improvements in therapy. Another study that involved the grafting of cells both into the striatum (the target of dopamine neurons) and the substantia nigra (where dopamine neurons normally reside) of three patients showed no adverse effects and some modest improvement in patient movement.[10] To determine the full extent of therapeutic benefits from such a procedure and confirm the reliability of these results, this study will need to be repeated with a larger patient population that includes the appropriate controls.

The limited success of these studies may reflect variations in the fetal tissue used for transplantation, which is of limited quantity and can not be standardized or well-characterized. The full complement of cells in these fetal tissue samples is not known at present. As a result, the tissue remains the greatest source of uncertainty in patient outcome following transplantation.

STEM CELLS AS A SOURCE OF NEURONS FOR TRANSPLANTATION IN PARKINSON'S DISEASE

The major goal for Parkinson's investigators is to generate a source of cells that can be grown in large supply, maintained indefinitely in the laboratory, and differentiated efficiently into dopamine neurons that work when transplanted into the brain of a Parkinson's patient. Scientists have investigated the behavior of stem cells in culture and the mechanisms that govern dopamine neuron production during development in their attempts to identify optimal culture conditions that allow stem cells to turn into dopamine-producing neurons.

Preliminary studies have been carried out using immature stem cell-like precursors from the rodent ventral midbrain, the region that normally gives rise to these dopamine neurons. In one study these precursors were turned into functional dopamine neurons, which were then grafted into rats previously treated with 6-hydroxy-dopamine (6-OHDA) to kill the dopamine neurons in their substantia nigra and induce Parkinson's-like symptoms. Even though the percentage of surviving dopamine neurons was low following transplantation, it was sufficient to relieve the Parkinson's-like symptoms.[11] Unfortunately, these fetal cells cannot be maintained in culture for very long before they lose the ability to differentiate into dopamine neurons.

Cells with features of neural stem cells have been derived from ES-cells, fetal brain tissue, brain tissue from neurosurgery, and brain tissue that was obtained after a person's death. There is controversy about whether other organ stem cell populations, such as hematopoietic stem cells, either contain or give rise to neural stem cells

Many researchers believe that the more primitive ES cells may be an excellent source of dopamine neurons because ES-cells can be grown indefinitely in a laboratory dish and can differentiate into any cell type, even after long periods in culture. Mouse ES cells injected directly into 6-OHDA-treated rat brains led to relief of Parkinson-like symptoms. Further investigation showed that these ES cells had differentiated into both dopamine and serotonin neurons.[12] This latter type of neuron is generated in an adjacent region of the brain and may complicate the response to transplantation. Since ES cells can generate all cell types in the body, unwanted cell types such as muscle or bone could theoretically also be introduced into the brain. As a result, a great deal of effort is being currently put into finding the right "recipe" for turning ES cells into dopamine neurons — and only this cell type — to treat Parkinson's disease. Researchers strive to learn more about normal brain development to help emulate the natural progression of ES cells toward dopamine neurons in the culture dish.

The recent availability of human ES cells has led to further studies to examine their potential for differentiation into dopamine neurons. Recently, dopamine neurons from human embryonic stem cells have been generated.[13] One research group used a special type of companion cell, along with specific growth factors, to promote the differentiation of the ES cells through several stages into dopamine neurons. These neurons showed many of the characteristic properties of normal dopamine neurons.[13] Furthermore, recent evidence of more direct neuronal differentiation methods from mouse ES cells fuels hope that scientists can refine and streamline the production of transplantable human dopamine neurons.

One method with great therapeutic potential is nuclear transfer. This method fuses the genetic material from one individual donor with a recipient egg cell that has had its nucleus removed. The early embryo that develops from this fusion is a genetic match for the donor. This process is sometimes called "therapeutic cloning" and is regarded by some to be ethically questionable. However, mouse ES cells have been differentiated successfully in this way into dopamine neurons that corrected Parkinsonian symptoms when transplanted into 6-OHDA-treated rats.[14] Similar results have been obtained using parthenogenetic primate stem cells, which are cells that are genetic matches from a female donor with no contribution from a male donor.[15] These approaches may offer the possibility of treating patients with genetically-matched cells, thereby eliminating the possibility of graft rejection.

ACTIVATING THE BRAIN'S OWN STEM CELLS TO REPAIR PARKINSON'S DISEASE

Scientists are also studying the possibility that the brain may be able to repair itself with therapeutic support. This avenue of study is in its early stages but may involve administering drugs that stimulate the birth of new neurons from the brain's own stem cells. The concept is based on research showing that new nerve cells are born in the adult brains of humans. The phenomenon occurs in a brain region called the dentate gyrus of the hippocampus. While it is not yet clear how these new neurons contribute to normal brain function, their presence suggests that stem cells in the adult brain may have the potential to re-wire dysfunctional neuronal circuitry.

The adult brain's capacity for self-repair has been studied by investigating how the adult rat brain responds to transforming growth factor alpha (TGFα), a protein important for early brain development that is expressed in limited quantities in adults.[16] Injection of TGFα into a healthy rat brain causes stem cells to divide for several days before ceasing division. In 6-OHDA-treated (Parkinsonian) rats, however, the cells proliferated and migrated to the damaged areas. Surprisingly, the TGFα-treated rats showed few of the behavioral problems associated with untreated Parkinsonian rats.[16] Additionally, in 2002 and 2003, two research groups isolated small numbers of dividing cells in the substantia nigra of adult rodents.[17,18] These findings suggest that the brain can repair itself, as long as the repair process is triggered sufficiently. It is not clear, though, whether stem cells are responsible for this repair or if the TGFα activates a different repair mechanism.

POSSIBILITIES FOR STEM CELLS IN THE TREATMENT OF OTHER NERVOUS SYSTEM DISORDERS

Many other diseases that affect the nervous system hold the potential for being treated with stem cells. Experimental therapies for chronic diseases of the nervous system, such as Alzheimer's disease, Lou Gehrig's disease, or Huntington's disease, and for acute injuries, such as spinal cord and brain trauma or stoke, are being currently developed and tested. These diverse disorders must be investigated within the contexts of their unique disease processes and treated accordingly with highly adapted cell-based approaches.

Although severe spinal cord injury is an area of intense research, the therapeutic targets are not as clear-cut as in Parkinson's disease. Spinal cord trauma destroys numerous cell types, including the neurons that carry messages between the brain and the rest of the body. In many spinal injuries, the cord is not actually severed, and at least some of the signal-carrying neuronal axons remain intact. However, the surviving axons no longer carry messages because oligodendrocytes, which make the axons' insulating myelin sheath, are lost. Researchers have recently made progress to replenish these lost myelin-producing cells. In one study, scientists cultured human ES cells through several steps to make mixed cultures that contained oligodendrocytes. When they injected these cells into the spinal cords of chemically-demyelinated rats, the treated rats regained limited use of their hind limbs compared with un-grafted rats.[19] Researchers are not certain, however, whether the limited increase in function observed in rats is actually due to the remyelination or to an unidentified trophic effect of the treatment.

Getting neurons to grow new axons through the injury site to reconnect with their targets is even more challenging. While myelin promotes normal neuronal function, it also inhibits the growth of new axons following spinal injury. In a recent study to attempt post-trauma axonal growth, Harper and colleagues

treated ES cells with a combination of factors that are known to promote motor neuron differentiation.[20] The researchers then transplanted these cells into adult rats that had received spinal cord injuries. While many of these cells survived and differentiated into neurons, they did not send out axons unless the researchers also added drugs that interfered with the inhibitory effects of myelin. The growth effect was modest, and the researchers have not yet seen evidence of functional neuron connections. However, their results raise the possibility that signals can be turned on and off in the correct order to allow neurons to reconnect and function properly. Spinal injury researchers emphasize that additional basic and preclinical research must be completed before attempting human trials using stem cell therapies to repair the trauma-damaged nervous system.

Since myelin loss is at the heart of many other degenerative diseases, oligodendrocytes made from ES cells may be useful to treat these conditions as well. For example, scientists recently cultured human ES cells with a combination of growth factors to generate a highly enriched population of myelinating oligodendrocyte precursors.[21,22] The researchers then tested these cells in a genetically-mutated mouse that does not produce myelin properly. When the growth factor-cultured ES cells were transplanted into affected mice, the cells migrated and differentiated into mature oligodendrocytes that made myelin sheaths around neighboring axons. These researchers subsequently showed that these cells matured and improved movement when grafted in rats with spinal cord injury.[23] Improved movement only occurred when grafting was completed soon after injury, suggesting that some post-injury responses may interfere with the grafted cells. However, these results are sufficiently encouraging to plan clinical trials to test whether replacement of myelinating glia can treat spinal cord injury.

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is characterized by a progressive destruction of motor neurons in the spinal cord. Patients with ALS develop increasing muscle weakness over time, which ultimately leads to paralysis and death. The cause of ALS is largely unknown, and there are no effective treatments. Researchers recently have used different sources of stem cells to test in rat models of ALS to test for possible nerve cell-restoring properties. In one study, researchers injected cell clusters made from embryonic germ (EG) cells into the spinal cord fluid of the partially-paralyzed rats.[24] Three months after the injections, many of the treated rats were able to move their hind limbs and walk with difficulty, while the rats that did not receive cell injections remained paralyzed. Moreover, the transplanted cells had migrated throughout the spinal fluid and developed into cells that displayed molecular characteristics of mature motor neurons. However, too few cells matured in this way to account for the recovery, and there was no evidence that the transplanted cells formed functional connections with muscles. The researchers suggest that the transplanted cells may be promoting recovery in some other way, such as by producing trophic factors.

This possibility was addressed in a second study in which scientists grew human fetal CNS stem cells in culture and genetically modified them to produce a trophic factor that promotes the survival of cells that are lost in ALS. When grafted into the spinal cords of the ALS-like rats, these cells secreted the desired growth factor and promoted the survival of the neurons that are normally lost in the ALS-like rats.[25] While promising, these results highlight the need for additional basic research into functional recovery in ALS disease models.

Stroke affects about 750,000 patients per year in the U.S. and is the most common cause of disability in adults. A stroke occurs when blood flow to the brain is disrupted. As a consequence, cells in affected brain regions die from insufficient amounts of oxygen. The treatment of stroke with anti-clotting drugs has dramatically improved the odds of patient recovery. However, in many patients the damage cannot be prevented, and the patient may permanently lose the functions of affected areas of the brain. For these patients, researchers are now considering stem cells as a way to repair the damaged brain regions. This problem is made more challenging because the damage in stroke may be widespread and may affect many cell types and connections.

However, researchers from Sweden recently observed that strokes in rats cause the brain's own stem cells to divide and give rise to new neurons.[26] However, these neurons, which survived only a couple of weeks, are few in number compared to the extent of damage caused. A group from the University of Tokyo added a growth factor, bFGF, into the brains of rats after stroke and showed that the hippocampus was able to generate

large numbers of new neurons.[27] The researchers found evidence that these new neurons were actually making connections with other neurons. These and other results suggest that future stroke treatments may be able to coax the brain's own stem cells to make replacement neurons.

Taking an alternative approach, another group attempted transplantation as a means to treat the loss of brain mass after a severe stroke. By adding stem cells onto a polymer scaffold that they implanted into the stroke-damaged brains of mice, the researchers demonstrated that the seeded stem cells differentiated into neurons and that the polymer scaffold reduced scarring.[28] Two groups transplanted human fetal stem cells in independent studies into the brains of stroke-affected rodents; these stem cells not only survived but migrated to the damaged areas of the brain.[29,30] These studies increase our knowledge of how stem cells are attracted to diseased areas of the brain.

There is also increasing evidence from numerous animal disease models that stem cells are actively drawn to brain damage. Once they reach these damaged areas, they have been shown to exert beneficial effects such as reducing brain inflammation or supporting nerve cells. It is hoped that, once these mechanisms are better understood, this stem cell recruitment can potentially be exploited to mobilize a patient's own stem cells.

Similar lines of research are being considered with other disorders such as Huntington's Disease and certain congenital defects. While much attention has been called to the treatment of Alzheimer's Disease, it is still not clear if stem cells hold the key to its treatment. But despite the fact that much basic work remains and many fundamental questions are yet to be answered, researchers are hopeful that repair for once-incurable nervous system disorders may be amenable to stem cell based therapies.

Considerable progress has been made the last few years in our understanding of stem cell biology and devising sources of cells for transplantation. New methods are also being developed for cell delivery and targeting to affected areas of the body. These advances have fueled optimism that new treatments will come for millions of persons who suffer from neurological disorders. But it is the current task of scientists to bring these methods from the laboratory bench to the clinic in a scientifically sound and ethically acceptable fashion.

REFERENCES

1. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. *Nature.* 2/28/2002 2002;415(6875):1030-1034.

2. Backlund EO, Granberg PO, Hamberger P, et al. Transplantation of adrenal medullary tissue to striatum in parkinsonism. First clinical trials. *J Neurosurg.* 1985;62:169-173.

3. Madrazo I, Drucker-Colin R, Diaz V, Martinez-Mata J, Torres C, Becerril JJ. Open microsurgical autograft of adrenal medulla to the right caudate nucleus in two patients with intractable Parkinson's disease. *N Engl J Med.* 1987;316:831-834.

4. Olson L, Malmfors T. Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and 3H-noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. *Acta Physiol Scand Suppl.* 1970;348:1-112.

5. Madrazo I, Leon V, Torres C, et al. Transplantation of fetal substantia nigra and adrenal medulla to the caudate nucleus in two patients with Parkinson's disease. *N Engl J Med.* 1988;318:51.

6. Lindvall O, Rehncrona S, Brundin P, et al. Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up. *Arch Neurol.* 1989;46:615-631.

7. Freed CR, Greene PE, Breeze RE, et al. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med.* 2001;344:710-719.

8. Olanow CW, Goetz CG, Kordower JH, et al. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann Neurol.* 2003;54:403-414.

9. Hagell P, Piccini P, Bjorklund A, et al. Dyskinesias following neural transplantation in Parkinson's disease. *Nat Neurosci.* 2002;5:627-628.

10. Mendez I, Dagher A, Hong M, et al. Simultaneous intrastriatal and intranigral fetal dopaminergic grafts in patients with Parkinson disease: a pilot study. Report of three cases. *J Neurosurg.* 2002;96:589-596.

11. Studer L, Tabar V, McKay RD. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat Neurosci.* 1998;1:290-295.

12. Bjorklund LM, Sanchez-Pernaute R, Chung S, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinsonian rat model. *Proc Natl Acad Sci USA.* 2002;99:2344-2349.

13. Perrier AL, Tabar V, Barberi T, et al. Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA.* 2004;101:12543-12548.

14. Barberi T, Klivenyi P, Calingasan NY, et al. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol.* 2003;21:1200-1207.

15. Vrana KE, Hipp JD, Goss AM, et al. Nonhuman primate parthenogenetic stem cells. *Proc Natl Acad Sci USA.* 2003;100 Suppl 1:11911-11916.

16. Fallon J, Reid S, Kinyamu R, et al. In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *Proc Natl Acad Sci USA.* 2000;97:14686-14691.

17. Lie DC, Dziewczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci.* 2002;22:6639-6649.

18. Zhao M, Momma S, Delfani K, et al. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci USA.* 2003;100:7925-7930.

19. Liu S, Qu Y, Stewart TJ, et al. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci USA.* 2000;97:6126-6131.

20. Harper JM, Krishnan C, Darman JS, et al. Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. *Proc Natl Acad Sci USA.* 2004;101:7123-7128.

21. Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia.* Feb 2005;49(3):385-396.

22. Brustle O, Jones KN, Learish RD, et al. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science.* 1999;285:754-756.

23. Keirstead HS, Nistor G, Bernal G, et al. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci.* May 11 2005;25(19):4694-4705.

24. Kerr DA, Llado J, Shamblott MJ, et al. Human embryonic germ cell derivatives facilitate motor recovery of rats with diffuse motor neuron injury. *J Neurosci.* 2003;23:5131-5140.

25. Klein SM, Behrstock S, McHugh J, et al. GDNF delivery using human neural progenitor cells in a rat model of ALS. *Hum Gene Ther.* Apr 2005;16(4):509-521.

26. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med.* 2002;8:963-970.

27. Nakatomi H, Kuriu T, Okabe S, et al. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell.* 2002;110:429-441.

28. Park KI, Teng YD, Synder EY. The injured brain interacts reciprocally with neural stem cells supported by scaffolds to reconstitute lost tissue. *Nat Biotechnol.* 2002;20:1111-1117.

29. Kelly S, Bliss TM, Shah AK, et al. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *Proc Natl Acad Sci USA.* 2004;101:11839-11844.

30. Imitola J, Raddassi K, Park KI, et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci USA.* Dec 28 2004;101(52):18117-18122.

[This page intentionally left blank]

4. USE OF GENETICALLY MODIFIED STEM CELLS IN EXPERIMENTAL GENE THERAPIES

*by Thomas P. Zwaka**

INTRODUCTION

Gene therapy is a novel therapeutic branch of modern medicine. Its emergence is a direct consequence of the revolution heralded by the introduction of recombinant DNA methodology in the 1970s. Gene therapy is still highly experimental, but has the potential to become an important treatment regimen. In principle, it allows the transfer of genetic information into patient tissues and organs. Consequently, diseased genes can be eliminated or their normal functions rescued. Furthermore, the procedure allows the addition of new functions to cells, such as the production of immune system mediator proteins that help to combat cancer and other diseases.

Originally, monogenic inherited diseases (those caused by inherited single gene defects), such as cystic fibrosis, were considered primary targets for gene therapy. For instance, in pioneering studies on the correction of adenosine deaminase deficiency, a lymphocyte-associated severe combined immunodeficiency (SCID), was attempted.[1] Although no modulation of immune function was observed, data from this study, together with other early clinical trials, demonstrated the potential feasibility of gene transfer approaches as effective therapeutic strategies. The first successful clinical trials using gene therapy to treat a monogenic disorder involved a different type of SCID, caused by mutation of an X chromosome-linked lymphocyte growth factor receptor.[2]

While the positive therapeutic outcome was celebrated as a breakthrough for gene therapy, a serious drawback subsequently became evident. By February 2005, three children out of seventeen who had been successfully treated for X-linked SCID developed leukemia because the vector inserted near an oncogene (a cancer-causing gene), inadvertently causing it to be inappropriately expressed in the genetically-engineered lymphocyte target cell.[3] On a more positive note, a small number of patients with adenosine deaminase-deficient SCID have been successfully treated by gene therapy without any adverse side effects.[4]

A small number of more recent gene therapy clinical trials, however, are concerned with monogenic disorders. Out of the approximately 1000 recorded clinical trials (January 2005), fewer than 10% target these diseases *(see Figure 4.1)*. The majority of current clinical trials (66% of all trials) focus on polygenic diseases, particularly cancer.

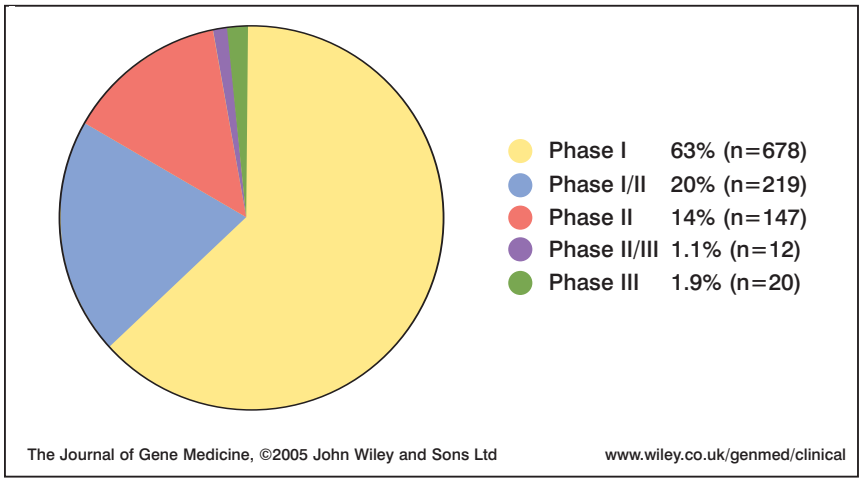


Figure 4.1. Indications Addressed by Gene Therapy Clinical Trials.

Gene therapy relies on similar principles as traditional pharmacologic therapy; specifically, regional specificity for the targeted tissue, specificity of the introduced gene function in relation to disease, and stability and controllability of expression of the introduced gene. To integrate all these aspects into a successful therapy is an exceedingly complex process that requires expertise from many disciplines, including molecular and cell biology, genetics and virology, in addition to bioprocess manufacturing capability and clinical laboratory infrastructure.

* Center for Cell and Gene Therapy & Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, Email: tpzwaka@bcm.tmc.edu

THE TWO PATHS TO GENE THERAPY

Gene therapy can be performed either by direct transfer of genes into the patient or by using living cells as vehicles to transport the genes of interest. Both modes have certain advantages and disadvantages.

Direct gene transfer is particularly attractive because of its relative simplicity. In this scenario, genes are delivered directly into a patient's tissues or bloodstream by packaging into liposomes (spherical vessels composed of the molecules that form the membranes of cells) or other biological microparticles. Alternately, the genes are packaged into genetically-engineered viruses, such as retroviruses or adenoviruses. Because of biosafety concerns, the viruses are typically altered so that they are not toxic or infectious (that is, they are replication incompetent). These basic tools of gene therapists have been extensively optimized over the past 10 years.

However, their biggest strength — simplicity — is simultaneously their biggest weakness. In many cases, direct gene transfer does not allow very sophisticated control over the therapeutic gene. This is because the transferred gene either randomly integrates into the patient's chromosomes or persists unintegrated for a relatively short period of time in the targeted tissue. Additionally, the targeted organ or tissue is not always easily accessible for direct application of the therapeutic gene.

On the other hand, therapeutic genes can be delivered using living cells. This procedure is relatively complex in comparison to direct gene transfer, and can be divided into three major steps. In the first step, cells from the patient or other sources are isolated and propagated in the laboratory. Second, the therapeutic gene is introduced into these cells, applying methods similar to those used in direct gene transfer. Finally, the genetically-modified cells are returned to the patient. The use of cells as gene transfer vehicles has certain advantages. In the laboratory dish *(in vitro),* cells can be manipulated much more precisely than in the body *(in vivo).* Some of the cell types that continue to divide under laboratory conditions may be expanded significantly before reintroduction into the patient. Moreover, some cell types are able to localize to particular regions of the human body, such as hematopoietic (blood-forming) stem cells, which return to the bone marrow. This "homing" phenomenon may be useful for applying the therapeutic gene with regional specificity.

A major disadvantage, however, is the additional biological complexity brought into systems by living cells. Isolation of a specific cell type requires not only extensive knowledge of biological markers, but also insight into the requirements for that cell type to stay alive *in vitro* and continue to divide. Unfortunately, specific biological markers are not known for many cell types, and the majority of normal human cells cannot be maintained for long periods of time *in vitro* without acquiring deleterious mutations.

STEM CELLS AS VEHICLES FOR GENE THERAPY

Stem cells can be classified as embryonic or adult, depending on their tissue of origin. The role of adult stem cells is to sustain an established repertoire of mature cell types in essentially steady-state numbers over the lifetime of the organism. Although adult tissues with a high turnover rate, such as blood, skin, and intestinal epithelium, are maintained by tissue-specific stem cells, the stem cells themselves rarely divide. However, in certain situations, such as during tissue repair after injury or following transplantation, stem cell divisions may become more frequent. The prototypic example of adult stem cells, the hematopoietic stem cell, has already been demonstrated to be of utility in gene therapy.[4,5] Although they are relatively rare in the human body, these cells can be readily isolated from bone marrow or after mobilization into peripheral blood. Specific surface markers allow the identification and enrichment of hematopoietic stem cells from a mixed population of bone marrow or peripheral blood cells.

After *in vitro* manipulation, these cells may be re-transplanted into patients by injection into the bloodstream, where they travel automatically to the place in the bone marrow in which they are functionally active. Hematopoietic stem cells that have been explanted, *in vitro* manipulated, and re-transplanted into the same patient (autologous transplantation) or a different patient (allogeneic transplantation) retain the ability to contribute to all mature blood cell types of the recipient for an extended period of time (when patients' cells are temporarily grown "outside the body" before being returned to them, the *in vitro* process is typically referred to as an *"ex vivo"* approach).

Another adult bone marrow-derived stem cell type with potential use as a vehicle for gene transfer is the mesenchymal stem cell, which has the ability to form cartilage, bone, adipose (fat) tissue, and marrow stroma (the bone marrow microenvironment).[6] Recently, a related stem cell type, the multipotent adult progenitor cell, has been isolated from bone marrow that can differentiate into multiple lineages, including neurons, hepatocytes (liver cells), endothelial cells (such as the cells that form the lining of blood vessels), and other cell types.[7] Other adult stem cells have been identified, such as those in the central nervous system and heart, but these are less well characterized and not as easily accessible.[8]

The traditional method to introduce a therapeutic gene into hematopoietic stem cells from bone marrow or peripheral blood involves the use of a vector derived from a certain class of virus, called a retrovirus. One type of retroviral vector was initially employed to show proof-of-principle that a foreign gene (in that instance the gene was not therapeutic, but was used as a molecular tag to genetically mark the cells) introduced into bone marrow cells may be stably maintained for several months.[9] However, these particular retroviral vectors were only capable of transferring the therapeutic gene into actively dividing cells. Since most adult stem cells divide at a relatively slow rate, efficiency was rather low. Vectors derived from other types of retroviruses (lentiviruses) and adenoviruses have the potential to overcome this limitation, since they also target non-dividing cells.

The major drawback of these methods is that the therapeutic gene frequently integrates more or less randomly into the chromosomes of the target cell. In principle, this is dangerous, because the gene therapy vector can potentially modify the activity of neighboring genes (positively or negatively) in close proximity to the insertion site or even inactivate host genes by integrating into them. These phenomena are referred to as "insertional mutagenesis." In extreme cases, such as in the X-linked SCID gene therapy trials, these mutations contribute to the malignant transformation of the targeted cells, ultimately resulting in cancer.

Another major limitation of using adult stem cells is that it is relatively difficult to maintain the stem cell state during *ex vivo* manipulations. Under current suboptimal conditions, adult stem cells tend to lose their stem cell properties and become more specialized, giving rise to mature cell types through a process termed "differentiation." Recent advances in supportive culture conditions for mouse hematopoietic stem cells may ultimately facilitate more effective use of human hematopoietic stem cells in gene therapy applications.[10,11]

EMBRYONIC STEM CELL: "THE ULTIMATE STEM CELL"

Embryonic stem cells are capable of unlimited self-renewal while maintaining the potential to differentiate into derivatives of all three germ layers. Even after months and years of growth in the laboratory, they retain the ability to form any cell type in the body. These properties reflect their origin from cells of the early embryo at a stage during which the cellular machinery is geared toward the rapid expansion and diversification of cell types.

Murine (mouse) embryonic stem cells were isolated over 20 years ago,[12,13] and paved the way for the isolation of nonhuman primate, and finally human embryonic stem cells.[14] Much of the anticipated potential surrounding human embryonic stem cells is an extrapolation from pioneering experiments in the mouse system. Experiments performed with human embryonic stem cells in the last couple of years indicate that these cells have the potential to make an important impact on medical science, at least in certain fields. In particular, this impact includes: a) differentiation of human embryonic stem cells into various cell types, such as neurons, cardiac, vascular, hematopoietic, pancreatic, hepatic, and placental cells, b) the derivation of new cell lines under alternative conditions, c) and the establishment of protocols that allow the genetic modification of these cells.

THE POTENTIAL OF HUMAN EMBRYONIC STEM CELLS FOR GENE THERAPY

Following derivation, human embryonic stem cells are easily accessible for controlled and specific genetic manipulation. When this facility is combined with their rapid growth, remarkable stability, and ability to mature *in vitro* into multiple cell types of the body, human embryonic stem cells are attractive potential tools for gene therapy. Two possible scenarios whereby human embryonic stem cells may benefit the gene therapy field are discussed below.

First, human embryonic stem cells could be genetically manipulated to introduce the therapeutic gene. This

gene may either be active or awaiting later activation, once the modified embryonic stem cell has differentiated into the desired cell type. Recently published reports establish the feasibility of such an approach.[15] Skin cells from an immunodeficient mouse were used to generate cellular therapy that partially restored immune function in the mouse. In these experiments, embryonic stem cells were generated from an immunodeficient mouse by nuclear transfer technology. The nucleus of an egg cell was replaced with that from a skin cell of an adult mouse with the genetic immunodeficiency. The egg was developed to the blastula stage at which embryonic stem cells were derived. The genetic defect was corrected by a genetic modification strategy designated "gene targeting." These "cured" embryonic stem cells were differentiated into hematopoietic "stem" cells and transplanted into immunodeficient mice. Interestingly, the immune function in these animals was partially restored. In principle, this approach may be employed for treating human patients with immunodeficiency or other diseases that may be corrected by cell transplantation.

However, significant advances must first be made. The levels of immune system reconstitution observed in the mice were quite modest (<1% of normal), while the methodology employed to achieve hematopoietic engraftment is not clinically feasible. This methodology involved using a more severely immunodeficient mouse as a recipient (which also had the murine equivalent of the human X-linked SCID mutation) and genetically engineering the hematopoietic engrafting cells with a potential oncogene prior to transplantation.

Embryonic stem cells may additionally be indirectly beneficial for cellular gene therapy. Since these cells can be differentiated *in vitro* into many cell types, including presumably tissue-specific stem cells, they may provide a constant *in vitro* source of cellular material. Such "adult" stem cells derived from embryonic stem cells may thus be utilized to optimize protocols for propagation and genetic manipulation techniques.[16] To acquire optimal cellular material from clinical samples in larger quantities for experimental and optimization purposes is usually rather difficult since access to these samples is limited.

GENETIC MANIPULATION OF STEM CELLS

The therapeutic gene needs to be introduced into the cell type used for therapy. Genes may be introduced into cells by transfection or transduction. Transfection utilizes chemical or physical methods to introduce new genes into cells. Usually, small molecules, such as liposomes, as well as other cationic-lipid based particles are employed to facilitate the entry of DNA encoding the gene of interest into the cells. Brief electric shocks are additionally used to facilitate DNA entry into living cells. All of these techniques have been applied to various stem cells, including human embryonic stem cells. However, the destiny of the introduced DNA is relatively poorly controlled using these procedures. In most cells, the DNA disappears after days or weeks, and in rare cases, integrates randomly into host chromosomal DNA. *In vitro* drug selection strategies allow the isolation and expansion of cells that are stably transfected, as long as they significantly express the newly introduced gene.

Transduction utilizes viral vectors for DNA transfer. Viruses, by nature, introduce DNA or RNA into cells very efficiently. Engineered viruses can be used to introduce almost any genetic information into cells. However, there are usually limitations in the size of the introduced gene. Additionally, some viruses (particularly retroviruses) only infect dividing cells effectively, whereas others (lentiviruses) do not require actively dividing cells. In most cases, the genetic information carried by the viral vector is stably integrated into the host cell genome (the total complement of chromosomes in the cell).

An important parameter that must be carefully monitored is the random integration into the host genome, since this process can induce mutations that lead to malignant transformation or serious gene dysfunction. However, several copies of the therapeutic gene may also be integrated into the genome, helping to bypass positional effects and gene silencing. Positional effects are caused by certain areas within the genome and directly influence the activity of the introduced gene. Gene silencing refers to the phenomenon whereby over time, most artificially introduced active genes are turned off by the host cell, a mechanism that is not currently well understood. In these cases, integration of several copies may help to achieve stable gene expression, since a subset of the introduced genes may integrate into favorable sites. In the past, gene silencing and positional effects were a particular problem in mouse hematopoietic stem cells.[17] These problems led to the optimization of retroviral and lentiviral vector systems by the addition of

genetic control elements (referred to as chromatin domain insulators and scaffold/matrix attachment regions) into the vectors, resulting in more robust expression in differentiating cell systems, including human embryonic stem cells.[18]

In some gene transfer systems, the foreign transgene does not integrate at a high rate and remains separate from the host genomic DNA, a status denoted "episomal". Specific proteins stabilizing these episomal DNA molecules have been identified as well as viruses (adenovirus) that persist stably for some time in an episomal condition. Recently, episomal systems have been applied to embryonic stem cells.[19]

An elegant way to circumvent positional effects and gene silencing is to introduce the gene of interest specifically into a defined region of the genome by the gene targeting technique referred to previously.[20] The gene targeting technique takes advantage of a cellular DNA repair process known as homologous recombination.[21] Homologous recombination provides a precise mechanism for defined modifications of genomes in living cells, and has been used extensively with mouse embryonic stem cells to investigate gene function and create mouse models of human diseases. Recombinant DNA is altered in vitro, and the therapeutic gene is introduced into a copy of the genomic DNA that is targeted during this process. Next, recombinant DNA is introduced by transfection into the cell, where it recombines with the homologous part of the cell genome. This in turn results in the replacement of normal genomic DNA with recombinant DNA containing genetic modifications.

Homologous recombination is a very rare event in cells, and thus a powerful selection strategy is necessary to identify the cells in which it occurs. Usually, the introduced construct has an additional gene coding for antibiotic resistance (referred to as a selectable marker), allowing cells that have incorporated the recombinant DNA to be positively selected in culture. However, antibiotic resistance only reveals that the cells have taken up recombinant DNA and incorporated it somewhere in the genome. To select for cells in which homologous recombination has occurred, the end of the recombination construct often includes the thymidine kinase gene from the herpes simplex virus. Cells that randomly incorporate recombinant DNA usually retain the entire DNA construct, including the herpes virus thymidine kinase gene. In cells that display homologous recombination between the recombinant construct and cellular DNA, an exchange of homologous DNA sequences is involved, and the non-homologous thymidine kinase gene at the end of the construct is eliminated. Cells expressing the thymidine kinase gene are killed by the antiviral drug ganciclovir in a process known as negative selection. Therefore, those cells undergoing homologous recombination are unique in that they are resistant to both the antibiotic and ganciclovir, allowing effective selection with these drugs *(see Figure 4.2).*

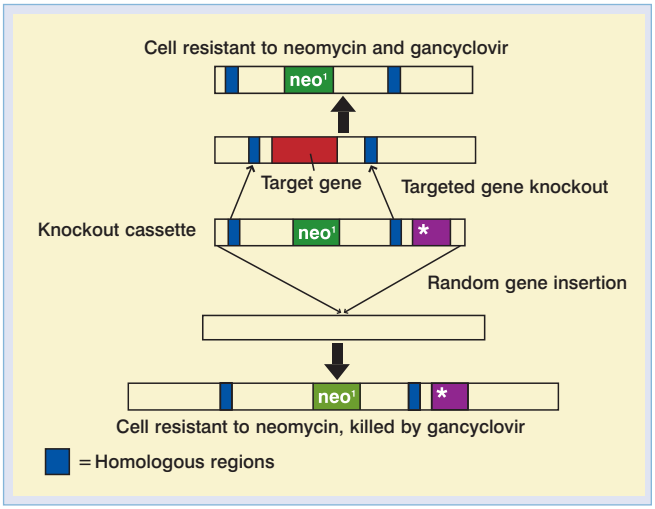


Figure 4.2. Gene targeting by homologous recombination.

Gene targeting by homologous recombination has recently been applied to human embryonic stem cells.[22] This is important for studying gene functions *in vitro* for lineage selection and marking. For therapeutic applications in transplantation medicine, the controlled modification of specific genes should be useful for purifying specific embryonic stem cell-derived, differentiated cell types from a mixed population, altering the antigenicity of embryonic stem cell derivatives, and adding defined markers that allow the identification of transplanted cells. Additionally, since the therapeutic gene can now be introduced into defined regions of the human genome, better controlled expression of the therapeutic gene should be possible. This also significantly reduces the risk of insertional mutagenesis.

FUTURE CHALLENGES FOR STEM CELL-BASED GENE THERAPY

Despite promising scientific results with genetically modified stem cells, some major problems remain to be overcome. The more specific and extensive the

genetic modification, the longer the stem cells have to remain *in vitro*. Although human embryonic stem cells in the culture dish remain remarkably stable, the cells may accumulate genetic and epigenetic changes that might harm the patient (epigenetic changes regulate gene activity without altering the genetic blueprint of the cell). Indeed, sporadic chromosomal abnormalities in human embryonic stem cell culture have been reported, and these may occur more frequently when the cells are passaged as bulk populations. This observation reinforces the necessity to optimize culture conditions further, to explore new human embryonic stem cell lines, and to monitor the existing cell lines.[23,24] Additionally undifferentiated embryonic stem cells have the potential to form a type of cancer called a teratocarcinoma. Safety precautions are therefore necessary, and currently, protocols are being developed to allow the complete depletion of any remaining undifferentiated embryonic stem cells.[25] This may be achieved by rigorous purification of embryonic stem cell derivatives or introducing suicide genes that can be externally controlled.

Another issue is the patient's immune system response. Transgenic genes, as well as vectors introducing these genes (such as those derived from viruses), potentially trigger immune system responses. If stem cells are not autologous, they eventually cause immuno-rejection of the transplanted cell type. Strategies to circumvent these problems, such as the expression of immune system-modulating genes by stem cells, creation of chimeric, immunotolerable bone marrow or suppression

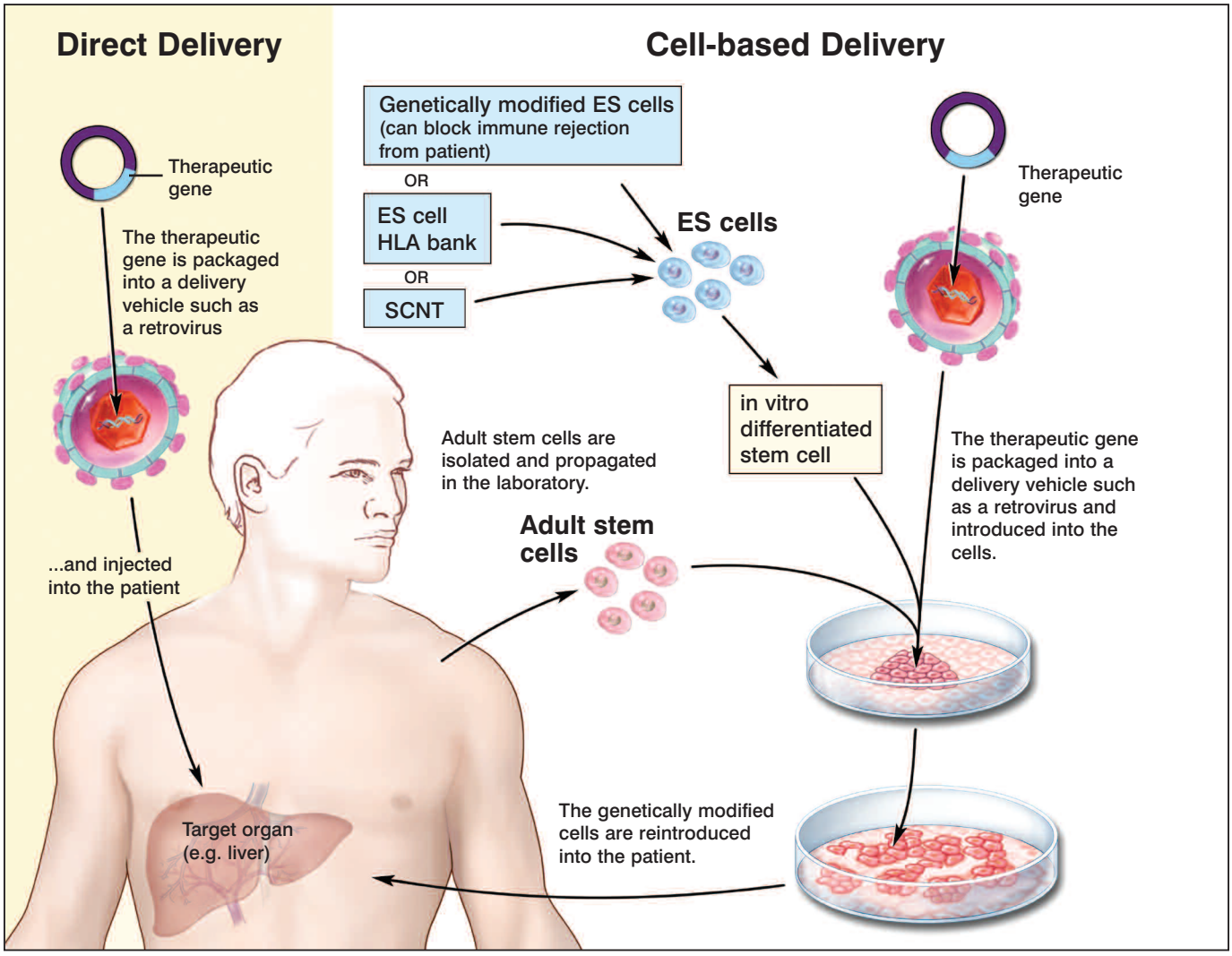


Figure 4.3. Strategies for Delivering Therapeutic Transgenes into Patients.

of HLA genes have been suggested.[25] In this context, nuclear transfer technology has been recently extended to human embryonic stem cells.[26]* Notably, immune-matched human embryonic stem cells have now been established from patients, including an individual with an immunodeficiency disease, congenital hypogammaglobulinemia.[27]* Strategies that combine gene targeting with embryonic stem cell-based therapy are thus potential novel therapeutic options.

The addition of human embryonic stem cells to the experimental gene therapy arsenal offers great promise in overcoming many of the existing problems of cellular based gene therapy that have been encountered in clinic trials *(see Figure 4.3)*. Further research is essential to determine the full potential of both adult and embryonic stem cells in this exciting new field.

REFERENCES

1. Mullen CA, Snitzer K, Culver KW, Morgan RA, Anderson WF, Blaese RM. Molecular analysis of T lymphocyte-directed gene therapy for adenosine deaminase deficiency: long-term expression in vivo of genes introduced with a retroviral vector. *Hum Gene Ther.* 1996;7:1123-1129.
2. Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med.* Apr 18 2002;346(16):1185-1193.
3. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med.* Jan 16 2003;348(3):255-256.
4. Aiuti A, Slavin S, Aker M, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science.* 2002;296:2410-2413.
5. Hacein-Bey-Abina S, Le Diest F, Carlier F, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med.* 2002;346:1185-1193.
6. Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: molecular control of expansion and differentiation. *Exp Cell Res.* 2005;306:330-335.
7. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* Jul 4 2002;418(6893):41-49.
8. Stocum DL. Stem cells in CNS and cardiac regeneration. *Adv Biochem Eng Biotechnol.* 2005;93:135-159.
9. Brenner MK, Rill DR, Holladay MS, et al. Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet.* Nov 6 1993;342(8880):1134-1137.
10. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature.* May 22 2003;423(6938):409-414.
11. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature.* May 22 2003;423(6938):448-452.
12. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* Jul 9 1981;292(5819):154-156.
13. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA.* Dec 1981;78(12):7634-7638.
14. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* Nov 6 1998;282(5391):1145-1147.
15. Rideout WM, 3rd, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell.* Apr 5 2002;109(1):17-27.
16. Barberi T, Willis LM, Socci ND, Studer L. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med.* 2005;2:e161.
17. Challita PM, Kohn DB. Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc Natl Acad Sci USA.* Mar 29 1994;91(7):2567-2571.
18. 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(1):111-117.
19. Aubert J, Dunstan H, Chambers I, Smith A. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat Biotechnol.* Dec 2002;20(12):1240-1245.
20. Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell.* Apr 5 2002;109(1):29-37.
21. Smithies O. Forty years with homologous recombination. *Nat Med.* Oct 2001;7(10):1083-1086.
22. Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. *Nat Biotechnol.* Mar 2003;21(3):319-321.

* **Editor's note:** Both papers referenced in 26 and 27 were later retracted. See Science 20 January 2006: Vol. 311. no. 5759, p. 335.

23. Draper JS, Smith K, Gokhale P, et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol.* Jan 2004;22(1):53-54.

24. Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med.* Mar 25 2004;350(13):1353-1356.

25. Gerecht-Nir S, Itskovitz-Eldor J. Cell therapy using human embryonic stem cells. *Transpl Immunol.* Apr 2004;12(3-4):203-209.

26. Hwang WS, Ryu YJ, Park JH, et al. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science.* Mar 12 2004;303(5664):1669-1674.

27. Hwang WS, Roh SI, Lee BC, et al. Patient-specific embryonic stem cells derived from human SCNT blastocysts. *Science.* 2005;308:1777-1783.

5. INTELLECTUAL PROPERTY OF HUMAN PLURIPOTENT STEM CELLS

*by Mark L. Rohrbaugh**

This report will provide an update in the area of intellectual property issues related to human pluripotent stem cells, and specifically, to human embryonic stem cells (hESCs). As anticipated, the patent landscape with respect to stem cells continues to become more complex in the United States, with new patents issued in various areas involving differentiated or modified cells and methods to differentiate cells. In Europe, some patent claims that involve unmodified hESCs currently stand rejected, although their ultimate outcomes are undetermined, as several parties have appealed the rejections they have received.

THE UNITED STATES PATENT LANDSCAPE

Since Thomson and colleagues were issued a patent on March 13, 2001 that specifically claimed hESCs,[1] a number of patents have issued in the U.S. involving claims to methods of using, maintaining, or inducing differentiation of hESCs or to the modified or differentiated cells themselves. According to data provided by the United States Patent and Trademark Office (USPTO) on October 22, 2004, nearly 300 patents had been issued with claims to embryonic stem (ES) cells or processes, of which approximately 38 encompass human products or processes. Approximately 700 pending patent applications had been published with claims to ES cells or processes, of which approximately 200 encompass human products or processes. Approximately 150 published patient applications encompass "totipotent" ES cells or processes. These patents claim various cell types that would be used in regenerative medicine (as described below) or auxiliary technologies, such as conditioned medium for cell growth, that support the use of hESCs.[2]

Among the patents issued more recently, one stands out in particular — a patent issued to Geron with broad claims to cells grown feeder-free.[3] One broad claim from this patent states, "A cellular composition comprising undifferentiated primate primordial stem (pPS) cells proliferating on an extracellular matrix, wherein the composition is free of feeder cells." Another recites, "A cell population consisting essentially of primate embryonic stem (ES) cells proliferating in culture on an extracellular matrix in a manner such that at least 50% of the proliferating ES cells are undifferentiated." The term "primordial" as used in the application refers to pluripotent or totipotent cells such as embryonic germ cells and ES cells. The claims cover cells that have been weaned from feeder cells as well as those that were derived *de novo* in feeder-free cultures. This patented technology, along with the original Thomson hESC technology, will likely be necessary in the use of many anticipated therapeutic applications of hESCs.

Other patents have issued to methods of inducing differentiation and to partially or fully differentiated cells. Such patents include the University of Utah's patent claiming neuroepithelial stem cells and Geron's patent claiming "directed differentiation of human pluripotent stem cells to cells of the hepatocyte lineage."[4] The Thomson patent will dominate such technologies to the extent that they utilize hESCs as starting or intermediate materials. However, technologies exist that do not require the use of the Thomson patent claims because they rely on lineage-specific stem cells obtained from sources other than hESCs. One such technology patented by Snyder *et al.* is a "pluripotent and self-renewing neural stem cell of human origin" isolated from embryonic neural tissue.[5] Another patent claim is directed to a method of obtaining a "substantially homogeneous population of pluripotent brain stem cells" from brain tissue rather than from hESCs.[6]

* Director, National Institutes of Health Office of Technology Transfer, Bethesda, MD 20892, *Email:* rohrbaugh@nih.gov

Scientists and physicians envision therapeutic uses of stem cells that are genetically modified in some manner to enhance their utility. For example, a pluripotent stem cell could be modified with a gene construct that enhances the ability to remove trace undifferentiated hESCs from an otherwise differentiated population of cells. This construct might include a gene encoding an enzyme that converts a pro-drug to a toxic drug linked to a promoter that is active only in undifferentiated hESCs. After isolating a differentiated population of cells modified in this manner, the pro-drug could be added to the culture, where it would be converted to a toxin in any residual undifferentiated cells.[7] The depletion of undifferentiated cells from a population of differentiated cells prior to implantation into patients reduces the risk that "contaminating" undifferentiated cells would form tumors.

THE EUROPEAN PATENT LANDSCAPE

In Europe, the first patents claiming unmodified stem cells have been denied based on a European Patent Convention (EPC) rule that excludes inventions involving the use of human embryos for industrial or commercial purposes. These denials include that of James Thomson of the Wisconsin Alumni Research Foundation (WARF).[8–10] While it does not appear that unmodified human embryonic stem cell patents will issue in Europe, the door has not yet been closed, as these decisions are currently being appealed.[11]

In arriving at the decision to deny the WARF application, the Examining Division maintained that the EPC rule against patenting embryos did not apply to downstream products from embryos as long as those products did not necessitate the use of a human embryo. Because the WARF technology necessitates use of a human embryo, it could not be patented. Commentators opposed to this decision view the rule more narrowly, arguing that the limits of ethical acceptability as defined by the rule should not be so broad as to include claims that involve starting materials that are already embryonic cells or cell mixtures. Such reasoning would limit the exclusion to claims that include a preliminary step of producing freshly disaggregated cells by destroying a human embryo, but not necessarily to isolated human embryonic stem cells *per se,* which are available through legal importation in many European countries.[10]

FACILITATING ACCESS TO STEM CELLS

Several new model agreements have been approved by NIH for use in distributing hESCs under Infrastructure Grants. These include model material transfer agreements (MTAs) from MizMedi Hospital, Seoul, Korea; Technion-Israel Institute of Technology, Haifa, Israel; and Cellartis, AB, Göteborg, Sweden (for details, see http://stemcells.nih.gov/research/registry/eligibilityCriteria.asp). The terms are similar to the previous model agreements that the NIH has entered into or approved for use with NIH-funded hESC distribution.

CONCLUSIONS

To date, two patents, one from WARF and one from Geron, dominate most of the anticipated commercial uses of hESCs in the U.S. Europe has taken a different course by not currently permitting the patenting of unmodified hESCs. In both North America and Europe, it is likely that more patents will continue to issue on other types of pluripotent stem cells, tissue-specific stem cells, methods that use these cells, and materials and methods associated with their propagation. More stem cells are now available for broad distribution with U.S. Federal funding under terms that permit reasonably unrestricted use in non-profit research.

While many scientists have received hESCs for non-profit research, fewer have been able to reach agreements with providers for collaborative research that directly benefits the commercial sector. In these instances, the research is high-risk and often does not result in new intellectual property, yet the industrial collaborator seeks an agreement in advance that includes the right to license new inventions, particularly new uses of the materials, should they occur. The industrial collaborator usually must negotiate an agreement and pay a fee in advance to patent holders and owners of the cell lines. This can be a high hurdle for small companies that have limited funds and for large companies that do not have a strong interest in the field but want to protect their investment in proprietary materials while providing them to non-profit researchers. Finally, WiCell, recipient of the NIH contract for the National Stem Cell Bank, must reach agreements with owners of patents and proprietary cell lines to facilitate the distribution of the cells through the Bank while protecting the interests of all parties.

The NIH experience with agreements to transfer proprietary materials from companies to government researchers suggests that only a small fraction of these collaborations lead to new inventions, yet they result in important scientific publications that advance biomedical research. Hopefully, patent owners, cell providers, and researchers will work together to facilitate these public-private partnerships.

REFERENCES

1. Thomson JA, inventor; Wisconsin Alumni Research Foundation, assignee. Primate embryonic stem cells. US patent 6,200,806, 2001.
2. Xu C, Gold JD, inventors; Geron Corporation, assignee. Conditioned media for propagating human pluripotent stem cells, 2003.
3. Bodnar AG, Chiu C-P, Gold JD, Inokuma M, Murai JT, West MD, inventors; Geron Corporation, assignee. Methods and materials for the growth of primate-derived primordial stem cells in feeder-free culture. US patent 6,800,480, 2004.
4. Rambhatla L, Carpenter MK, inventors; Geron Corporation, assignee. Hepatocyte lineage cells derived from pluripotent stem cells. US patent 6,458,589, 2002.
5. Snyder EY, Wolfe JH, Kim SU, inventors; The Children's Medical Center Corporation, University of British Columbia, University of Pennsylvania,, assignee. Engraftable human neural stem cells. US patent 6,680,198, 2004.
6. Steindler DA, Laywell ED, Kukekou VG, Thomas LB, inventors; University of Tennessee Research Foundation, assignee. Isolated mammalian neural stem cells. US patent 6,638,763, 2003.
7. Gold JD, Lebkowski JS, inventors; Geron Corporation, assignee. Methods for providing differentiated stem cells. US patent 6,576,464, 2003.
8. Decision to refuse European Patent application #96 903 521.1-2401, Primate embryonic stem cells, applicant: Wisconsin Alumni Research Foundation. *Examining Division;* 2004.
9. Webber P. Embryonic cell patenting. *Biosci Law Rev.* 2004;3:87-98.
10. Germinario C. The value of life. *Patent World.* 2004(June):87-98.
11. Gallagher K. Battles loom over basic patent on stem cells. *Milwaukee J Sentinel.* October 13, 2004.

[This page intentionally left blank]

6. MENDING A BROKEN HEART: STEM CELLS AND CARDIAC REPAIR

Charles A. Goldthwaite, Jr., Ph.D.

HEART FAILURE: THE DISEASE AND ITS CAUSES

Cardiovascular disease (CVD), which includes hypertension, coronary heart disease (CHD), stroke, and congestive heart failure (CHF), has ranked as the number one cause of death in the United States every year since 1900 except 1918, when the nation struggled with an influenza epidemic.[1] In 2002, CVD claimed roughly as many lives as cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, influenza, and pneumonia combined. According to data from the 1999-2002 National Health and Nutrition Examination Survey (NHANES), CVD caused approximately 1.4 million deaths (38.0 percent of all deaths) in the U.S. in 2002. Nearly 2600 Americans die of CVD each day, roughly one death every 34 seconds. Moreover, within a year of diagnosis, one in five patients with CHF will die. CVD also creates a growing economic burden; the total health care cost of CVD in 2005 was estimated at $393.5 billion dollars.

Given the aging of the U.S. population and the relatively dramatic recent increases in the prevalence of cardiovascular risk factors such as obesity and type 2 diabetes,[2,3] CVD will continue to be a significant health concern well into the 21st century. However, improvements in the acute treatment of heart attacks and an increasing arsenal of drugs have facilitated survival. In the U.S. alone, an estimated 7.1 million people have survived a heart attack, while 4.9 million live with CHF.[1] These trends suggest an unmet need for therapies to regenerate or repair damaged cardiac tissue.

Ischemic heart failure occurs when cardiac tissue is deprived of oxygen. When the ischemic insult is severe enough to cause the loss of critical amounts of cardiac muscle cells (cardiomyocytes), this loss initiates a cascade of detrimental events, including formation of a non-contractile scar, ventricular wall thinning, an overload of blood flow and pressure, ventricular remodeling (the overstretching of viable cardiac cells to sustain cardiac output), heart failure, and eventual death.[4] Restoring damaged heart muscle tissue, through repair or regeneration, therefore represents a fundamental mechanistic strategy to treat heart failure. However, endogenous repair mechanisms, including the proliferation of cardiomyocytes under conditions of severe blood vessel stress or vessel formation and tissue generation via the migration of bone-marrow-derived stem cells to the site of damage, are in themselves insufficient to restore lost heart muscle tissue (myocardium) or cardiac function.[5] Current pharmacologic interventions for heart disease, including beta-blockers, diuretics, and angiotensin-converting enzyme (ACE) inhibitors, and surgical treatment options, such as changing the shape of the left ventricle and implanting assistive devices such as pacemakers or defibrillators, do not restore function to damaged tissue. Moreover, while implantation of mechanical ventricular assist devices can provide long-term improvement in heart function, complications such as infection and blood clots remain problematic.[6] Although heart transplantation offers a viable option to replace damaged myocardium in selected individuals, organ availability and transplant rejection complications limit the widespread practical use of this approach.

The difficulty in regenerating damaged myocardial tissue has led researchers to explore the application of embryonic and adult-derived stem cells for cardiac repair. A number of stem cell types, including embryonic stem (ES) cells, cardiac stem cells that naturally reside within the heart, myoblasts (muscle stem cells), adult bone marrow-derived cells, mesenchymal cells (bone marrow-derived cells that give rise to tissues such as muscle, bone, tendons, ligaments, and adipose tissue), endothelial progenitor cells (cells that give rise to the endothelium, the interior lining of blood vessels), and umbilical cord blood cells, have been investigated to varying extents as possible sources for regenerating

damaged myocardium. All have been tested in mouse or rat models, and some have been tested in large animal models such as pigs. Preliminary clinical data for many of these cell types have also been gathered in selected patient populations.

However, clinical trials to date using stem cells to repair damaged cardiac tissue vary in terms of the condition being treated, the method of cell delivery, and the primary outcome measured by the study, thus hampering direct comparisons between trials.[7] Some patients who have received stem cells for myocardial repair have reduced cardiac blood flow (myocardial ischemia), while others have more pronounced congestive heart failure and still others are recovering from heart attacks. In some cases, the patient's underlying condition influences the way that the stem cells are delivered to his/her heart (see the section, "Methods of Cell Delivery" for details). Even among patients undergoing comparable procedures, the clinical study design can affect the reporting of results. Some studies have focused on safety issues and adverse effects of the transplantation procedures; others have assessed improvements in ventricular function or the delivery of arterial blood. Furthermore, no published trial has directly compared two or more stem cell types, and the transplanted cells may be autologous (i.e., derived from the person on whom they are used) or allogeneic (i.e., originating from another person) in origin. Finally, most of these trials use unlabeled cells, making it difficult for investigators to follow the cells' course through the body after transplantation (see the section "Considerations for Using These Stem Cells in the Clinical Setting" at the end of this article for more details).

Despite the relative infancy of this field, initial results from the application of stem cells to restore cardiac function have been promising. This article will review the research supporting each of the aforementioned cell types as potential source materials for myocardial regeneration and will conclude with a discussion of general issues that relate to their clinical application.

MECHANISMS OF ACTION

In 2001, Menasche, *et.al.* described the successful implantation of autologous skeletal myoblasts (cells that divide to repair and/or increase the size of voluntary muscles) into the post-infarction scar of a patient with severe ischemic heart failure who was undergoing coronary artery bypass surgery.[8] Following the procedure, the researchers used imaging techniques to observe the heart's muscular wall and to assess its ability to beat. When they examined patients 5 months after treatment, they concluded that treated hearts pumped blood more efficiently and seemed to demonstrate improved tissue health. This case study suggested that stem cells may represent a viable resource for treating ischemic heart failure, spawning several dozen clinical studies of stem cell therapy for cardiac repair (see Boyle, *et.al.*[7] for a complete list) and inspiring the development of Phase I and Phase II clinical trials. These trials have revealed the complexity of using stem cells for cardiac repair, and considerations for using stem cells in the clinical setting are discussed in a subsequent section of this report.

The mechanism by which stem cells promote cardiac repair remains controversial, and it is likely that the cells regenerate myocardium through several pathways. Initially, scientists believed that transplanted cells differentiated into cardiac cells, blood vessels, or other cells damaged by CVD.[9-11] However, this model has been recently supplanted by the idea that transplanted stem cells release growth factors and other molecules that promote blood vessel formation (angiogenesis) or stimulate "resident" cardiac stem cells to repair damage.[12-14] Additional mechanisms for stem-cell mediated heart repair, including strengthening of the post-infarct scar[15] and the fusion of donor cells with host cardiomyocytes,[16] have also been proposed.

METHODS OF CELL DELIVERY

Regardless of which mechanism(s) will ultimately prove to be the most significant in stem-cell mediated cardiac repair, cells must be successfully delivered to the site of injury to maximize the restored function. In preliminary clinical studies, researchers have used several approaches to deliver stem cells. Common approaches include intravenous injection and direct infusion into the coronary arteries. These methods can be used in patients whose blood flow has been restored to their hearts after a heart attack, provided that they do not have additional cardiac dysfunction that results in total occlusion or poor arterial flow.[12, 17] Of these two methods, intracoronary infusion offers the advantage of directed local delivery, thereby increasing the number of cells that reach the target tissue relative to the number that will home to the heart once they

have been placed in the circulation. However, these strategies may be of limited benefit to those who have poor circulation, and stem cells are often injected directly into the ventricular wall of these patients. This endomyocardial injection may be carried out either via a catheter or during open-heart surgery.[18]

To determine the ideal site to inject stem cells, doctors use mapping or direct visualization to identify the locations of scars and viable cardiac tissue. Despite improvements in delivery efficiency, however, the success of these methods remains limited by the death of the transplanted cells; as many as 90% of transplanted cells die shortly after implantation as a result of physical stress, myocardial inflammation, and myocardial hypoxia.[4] Timing of delivery may slow the rate of deterioration of tissue function, although this issue remains a hurdle for therapeutic approaches.

TYPES OF STEM CELLS INVESTIGATED TO REGENERATE DAMAGED MYOCARDIAL TISSUE

Embryonic and adult stem cells have been investigated to regenerate damaged myocardial tissue in animal models and in a limited number of clinical studies. A brief review of work to date and specific considerations for the application of various cell types will be discussed in the following sections.

Embryonic Stem (ES) Cells

Because ES cells are pluripotent, they can potentially give rise to the variety of cell types that are instrumental in regenerating damaged myocardium, including cardiomyocytes, endothelial cells, and smooth muscle cells. To this end, mouse and human ES cells have been shown to differentiate spontaneously to form endothelial and smooth muscle cells *in vitro*[19] and *in vivo,*[20,21] and human ES cells differentiate into myocytes with the structural and functional properties of cardiomyocytes.[22-24] Moreover, ES cells that were transplanted into ischemically-injured myocardium in rats differentiated into normal myocardial cells that remained viable for up to four months,[25] suggesting that these cells may be candidates for regenerative therapy in humans.

However, several key hurdles must be overcome before human ES cells can be used for clinical applications. Foremost, ethical issues related to embryo access currently limit the avenues of investigation. In addition, human ES cells must go through rigorous testing and purification procedures before the cells can be used as sources to regenerate tissue. First, researchers must verify that their putative ES cells are pluripotent. To prove that they have established a human ES cell line, researchers inject the cells into immunocompromised mice; i.e., mice that have a dysfunctional immune system. Because the injected cells cannot be destroyed by the mouse's immune system, they survive and proliferate. Under these conditions, pluripotent cells will form a teratoma, a multi-layered, benign tumor that contains cells derived from all three embryonic germ layers. Teratoma formation indicates that the stem cells have the capacity to give rise to all cell types in the body.

The pluripotency of ES cells can complicate their clinical application. While undifferentiated ES cells may possibly serve as sources of specific cell populations used in myocardial repair, it is essential that tight quality control be maintained with respect to the differentiated cells. Any differentiated cells that would be used to regenerate heart tissue must be purified before transplantation can be considered. If injected regenerative cells are accidentally contaminated with undifferentiated ES cells, a tumor could possibly form as a result of the cell transplant.[4] However, purification methodologies continue to improve; one recent report describes a method to identify and select cardiomyocytes during human ES cell differentiation that may make these cells a viable option in the future.[26]

This concern illustrates the scientific challenges that accompany the use of all human stem cells, whether derived from embryonic or adult tissues. Predictable control of cell proliferation and differentiation requires additional basic research on the molecular and genetic signals that regulate cell division and specialization. Furthermore, long-term cell stability must be well understood before human ES-derived cells can be used in regenerative medicine. The propensity for genetic mutation in the human ES cells must be determined, and the survival of differentiated, ES-derived cells following transplantation must be assessed. Furthermore, once cells have been transplanted, undesirable interactions between the host tissue and the injected cells must be minimized. Cells or tissues derived from ES cells that are currently available for use in humans are not tissue-matched to patients and thus would require immunosuppression to limit immune rejection.[18]

Skeletal Myoblasts

While skeletal myoblasts (SMs) are committed progenitors of skeletal muscle cells, their autologous origin, high proliferative potential, commitment to a myogenic lineage, and resistance to ischemia promoted their use as the first stem cell type to be explored extensively for cardiac application. Studies in rats and humans have demonstrated that these cells can repopulate scar tissue and improve left ventricular function following transplantation.[27] However, SM-derived cardiomyocytes do not function in complete concert with native myocardium. The expression of two key proteins involved in electromechanical cell integration, N-cadherin and connexin 43, are downregulated *in vivo,*[28] and the engrafted cells develop a contractile activity phenotype that appears to be unaffected by neighboring cardiomyocytes.[29]

To date, the safety and feasibility of transplanting SM cells have been explored in a series of small studies enrolling a collective total of nearly 100 patients. Most of these procedures were carried out during open-heart surgery, although a couple of studies have investigated direct myocardial injection and transcoronary administration. Sustained ventricular tachycardia, a life-threatening arrhythmia and unexpected side-effect, occurred in early implantation studies, possibly resulting from the lack of electrical coupling between SM-derived cardiomyocytes and native tissue.[30,31] Changes in pre-implantation protocols have minimized the occurrence of arrhythmias in conjunction with the use of SM cells, and Phase II studies of skeletal myoblast therapy are presently underway.

Human Adult Bone-Marrow Derived Cells

In 2001, Jackson, *et.al.* demonstrated that cardiomyocytes and endothelial cells could be regenerated in a mouse heart attack model through the introduction of adult mouse bone marrow-derived stem cells.[9] That same year, Orlic and colleagues showed that direct injection of mouse bone marrow-derived cells into the damaged ventricular wall following an induced heart attack led to the formation of new cardiomyocytes, vascular endothelium, and smooth muscle cells.[11] Nine days after transplanting the stem cells, the newly-formed myocardium occupied nearly 70 percent of the damaged portion of the ventricle, and survival rates were greater in mice that received these cells than in those that did not. While several subsequent studies have questioned whether these cells actually differentiate into cardiomyocytes,[32,33] the evidence to support their ability to prevent remodeling has been demonstrated in many laboratories.[7]

Based on these findings, researchers have investigated the potential of human adult bone marrow as a source of stem cells for cardiac repair. Adult bone marrow contains several stem cell populations, including hematopoietic stem cells (which differentiate into all of the cellular components of blood), endothelial progenitor cells, and mesenchymal stem cells; successful application of these cells usually necessitates isolating a particular cell type on the basis of its' unique cell-surface receptors. In the past three years, the transplantation of bone marrow mononuclear cells (BMMNCs), a mixed population of blood and cells that includes stem and progenitor cells, has been explored in more patients and clinical studies of cardiac repair than any other type of stem cell.7

The results from clinical studies of BMMNC transplantation have been promising but mixed. However, it should be noted that these studies have been conducted under a variety of conditions, thereby hampering direct comparison. The cells have been delivered via open-heart surgery and endomyocardial and intracoronary catheterization. Several studies, including the Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) and the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trials, have shown that intracoronary infusion of BMMNCs following a heart attack significantly improves the left ventricular (LV) ejection fraction, or the volume of blood pumped out of the left ventricle with each heartbeat.[34-36] However, other studies have indicated either no improvement in LV ejection fraction upon treatment[37] or an increased LV ejection fraction in the control group.[38] An early study that used endomyocardial injection to enhance targeted delivery indicated a significant improvement in overall LV function.[39] Discrepancies such as these may reflect differences in cell preparation protocols or baseline patient statistics. As larger trials are developed, these issues can be explored more systematically.

Mesenchymal (Bone Marrow Stromal) Cells

Mesenchymal stem cells (MSCs) are precursors of non-hematopoietic tissues (e.g., muscle, bone, tendons, ligaments, adipose tissue, and fibroblasts) that are obtained relatively easily from autologous bone marrow. They remain multipotent following expan-

sion *in vitro,* exhibit relatively low immunogenicity, and can be frozen easily. While these properties make the cells amenable to preparation and delivery protocols, scientists can also culture them under special conditions to differentiate them into cells that resemble cardiac myocytes. This property enables their application to cardiac regeneration. MSCs differentiate into endothelial cells when cultured with vascular endothelial growth factor[40] and cardiomyogenic (CMG) cells when treated with the DNA-demethylating agent, 5-azacytidine.41 More important, however, is the observation that MSCs can differentiate into cardiomyocytes and endothelial cells *in vivo* when transplanted to the heart following myocardial infarct (MI) or non-injury in pig, mouse, or rat models.[42-45] Additionally, the ability of MSCs to restore functionality may be enhanced by the simultaneous transplantation of other stem cell types.[43]

Several animal model studies have shown that treatment with MSCs significantly increases myocardial function and capillary formation.[5,41] One advantage of using these cells in human studies is their low immunogenicity; allogeneic MSCs injected into infarcted myocardium in a pig model regenerated myocardium and reduced infarct size without evidence of rejection.[46] A randomized clinical trial implanting MSCs after MI has demonstrated significant improvement in global and regional LV function,[47] and clinical trials are currently underway to investigate the application of allogeneic and autologous MSCs for acute MI and myocardial ischemia, respectively.

Resident Cardiac Stem Cells

Recent evidence suggests that the heart contains a small population of endogenous stem cells that most likely facilitate minor repair and turnover-mediated cell replacement.[7] These cells have been isolated and characterized in mouse, rat, and human tissues.[48,49] The cells can be harvested in limited quantity from human endomyocardial biopsy specimens[50] and can be injected into the site of infarction to promote cardiomyocyte formation and improvements in systolic function.[49] Separation and expansion *ex vivo* over a period of weeks are necessary to obtain sufficient quantities of these cells for experimental purposes. However, their potential as a convenient resource for autologous stem cell therapy has led the National Heart, Lung, and Blood Institute to fund forthcoming clinical trials that will explore the use of cardiac stem cells for myocardial regeneration.

Endothelial Progenitor Cells

The endothelium is a layer of specialized cells that lines the interior surface of all blood vessels (including the heart). This layer provides an interface between circulating blood and the vessel wall. Endothelial progenitor cells (EPCs) are bone marrow-derived stem cells that are recruited into the peripheral blood in response to tissue ischemia.[4] EPCs are precursor cells that express some cell-surface markers characteristic of mature endothelium and some of hematopoietic cells.[19,51-53] EPCs home in on ischemic areas, where they differentiate into new blood vessels; following a heart attack, intravenously injected EPCs home to the damaged region within 48 hours.[12] The new vascularization induced by these cells prevents cardiomyocyte apoptosis (programmed cell death) and LV remodeling, thereby preserving ventricular function.[13] However, no change has been observed in non-infarcted regions upon EPC administration. Clinical trials are currently underway to assess EPC therapy for growing new blood vessels and regenerating myocardium.

Other Cells: Umbilical Cord Blood Stem Cells, Fibroblasts, and Peripheral Blood CD34$^+$ Cells

Several other cell populations, including umbilical cord blood (UCB) stem cells, fibroblasts (cells that synthesize the extracellular matrix of connective tissues), and peripheral blood CD34$^+$ cells, have potential therapeutic uses for regenerating cardiac tissue. Although these cell types have not been investigated in clinical trials of heart disease, preliminary studies in animal models indicate several potential applications in humans.

Umbilical cord blood contains enriched populations of hematopoietic stem cells and mesencyhmal precursor cells relative to the quantities present in adult blood or bone marrow.[54,55] When injected intravenously into the tail vein in a mouse model of MI, human mononuclear UCB cells formed new blood vessels in the infarcted heart.[56] A human DNA assay was used to determine the migration pattern of the cells after injection; although they homed only to injured areas within the heart, they were also detected in the marrow, spleen, and liver. When injected directly into the infarcted area in a rat model of MI, human mononuclear UCB cells improved ventricular function.[57] Staining for CD34 and other markers found on the cell surface of hematopoietic stem cells indicated that some of the cells survived in the myocardium. Results similar to these have been

observed following the injection of human unrestricted somatic stem cells from UCB into a pig MI model.[58]

Adult peripheral blood $CD34^+$ cells offer the advantage of being obtained relatively easily from autologous sources.[59] Although some studies using a mouse model of MI claim that these cells can transdifferentiate into cardiomyocytes, endothelial cells, and smooth muscle cells at the site of tissue injury,[60] this conclusion is highly contested. Recent studies that involve the direct injection of blood-borne or bone marrow-derived hematopoietic stem cells into the infarcted region of a mouse model of MI found no evidence of myocardial regeneration following injection of either cell type.[33] Instead, these hematopoietic stem cells followed traditional differentiation patterns into blood cells within the microenvironment of the injured heart. Whether these cells will ultimately find application in myocardial regeneration remains to be determined.

Autologous fibroblasts offer a different strategy to combat myocardial damage by replacing scar tissue with a more elastic, muscle-like tissue and inhibiting host matrix degradation.[4] The cells may be manipulated to express muscle-specific transcription factors that promote their differentiation into myotubes such as those derived from skeletal myoblasts.[61] One month after these cells were implanted into the post-infarction scar in a rat model of MI, they occupied a large portion of the scar but were not functionally integrated.[61] Although the effects on ventricular function were not evaluated in this study, authors noted that modified autologous fibroblasts may ultimately prove useful in elderly patients who have a limited population of autologous skeletal myoblasts or bone marrow stem cells.

CONSIDERATIONS FOR USING THESE STEM CELLS IN THE CLINICAL SETTING

As these examples indicate, many types of stem cells have been applied to regenerate damaged myocardium. In select applications, stem cells have demonstrated sufficient promise to warrant further exploration in large-scale, controlled clinical trials. However, the current breadth of application of these cells has made it difficult to compare and contextualize the results generated by the various trials. Most studies published to date have enrolled fewer than 25 patients, and the studies vary in terms of cell types and preparations used, methods of delivery, patient populations, and trial outcomes. However, the mixed results that have been observed in these studies do not necessarily argue against using stem cells for cardiac repair. Rather, preliminary results illuminate the many gaps in understanding of the mechanisms by which these cells regenerate myocardial tissue and argue for improved characterization of cell preparations and delivery methods to support clinical applications.

Future clinical trials that use stem cells for myocardial repair must address two concerns that accompany the delivery of these cells: 1) safety and 2) tracking the cells to their ultimate destination(s). Although stem cells appear to be relatively safe in the majority of recipients to date, an increased frequency of non-sustained ventricular tachycardia, an arrhythmia, has been reported in conjunction with the use of skeletal myoblasts.[30,62-64] While this proarrhythmic effect occurs relatively early after cell delivery and does not appear to be permanent, its presence highlights the need for careful safety monitoring when these cells are used. Additionally, animal models have demonstrated that stem cells rapidly diffuse from the heart to other organs (e.g., lungs, kidneys, liver, spleen) within a few hours of transplantation,[65,66] an effect observed regardless of whether the cells are injected locally into the myocardium. This migration may or may not cause side-effects in patients; however, it remains a concern related to the delivery of stem cells in humans. (*Note:* Techniques to label stem cells for tracking purposes and to assess their safety are discussed in more detail in other articles in this publication).

In addition to safety and tracking, several logistical issues must also be addressed before stem cells can be used routinely in the clinic. While cell tracking methodologies allow researchers to determine migration patterns, the stem cells must target their desired destination(s) and be retained there for a sufficient amount of time to achieve benefit. To facilitate targeting and enable clinical use, stem cells must be delivered easily and efficiently to their sites of application. Finally, the ease by which the cells can be obtained and the cost of cell preparation will also influence their transition to the clinic.

CONCLUSIONS

The evidence to date suggests that stem cells hold promise as a therapy to regenerate damaged myocardium. Given the worldwide prevalence of

cardiac dysfunction and the limited availability of tissue for cardiac transplantation, stem cells could ultimately fulfill a large-scale unmet clinical need and improve the quality of life for millions of people with CVD. However, the use of these cells in this setting is currently in its infancy — much remains to be learned about the mechanisms by which stem cells repair and regenerate myocardium, the optimal cell types and modes of their delivery, and the safety issues that will accompany their use. As the results of large-scale clinical trials become available, researchers will begin to identify ways to standardize and optimize the use of these cells, thereby providing clinicians with powerful tools to mend a broken heart.

REFERENCES

1. American Heart Association. *Heart Disease and Stroke Statistics–2005.* Dallas: American Heart Association; 2005.
2. Mokdad AH, Ford ES, Bowman BA, et al. Prevalence of obesity, diabetes, and obesity-related health-risk factors, 2001. *JAMA.* 2003;289:76-79.
3. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. *JAMA.* 2002;288:1723-1727.
4. Rosenstrauch D, Poglajen G, Zidar N, Gregoric ID. Stem cell therapy for ischemic heart failure. *Tex Heart Ist J.* 2005;32:339-347.
5. Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med.* 2003;9:1195-1201.
6. Lietz K, Long JW, Kfoury AG, et al. Outcomes of left ventricular assist device implantation as destination therapy in the post-REMATCH era: implications for patient selection. *Circulation.* 2007;116:497-505.
7. Boyle AJ, Schulman SP, Hare JM. Is stem cell therapy ready for patients? Stem cell therapy for cardiac repair. *Circulation.* 2006;114:339-352.
8. Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. *Lancet.* 2001;357:279-280.
9. Jackson KA, Majka SM, Wang H, et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest.* 2001;107:1395-1402.
10. Condorelli G, Borello U, De Angelis L, et al. Cardiomyocytes induce endothelial cells to transdifferentiate into cardiac muscle: implications for myocardium regeneration. *Proc Natl Acad Sci USA.* 2001;98:10733-10738.
11. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001; 410:701-705.
12. Kocher AA, Schuster MD, Szaboles MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow derived angioblasts prevents cardiomyocyte apoptosis, reduces remodelling and improves cardiac function. *Nat Med.* 2001;7:430-436.
13. Schuster MD, Kocher AA, Seki T, et al. Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration. Am J Physiol Heart Circ Physiol. 2004;287:H525-H532.
14. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med.* 2005;11:367-368.
15. Fujii T, Yau TM, Weisel RD, et al. Cell transplantation to prevent heart failure: a comparison of cell types. *Ann Thorac Surg.* 2003;76:2062-2070.
16. Nygren JM, Jovinge S, Breitbach M, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at low frequency through cell fusion, but not transdifferentiation. *Nat Med.* 2004;10:494-501.
17. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation.* 2002;106:1913-1918.
18. Oettgen P. Cardiac stem cell therapy: need for optimization of efficacy and safety monitoring. *Circulation.* 2006;114:353-358.
19. Vittet D, Prandidni MH, Berthier R, et al. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood.* 1996;88:3424-3431.
20. Marchetti S, Gimond C, Iljin K, et al. Endothelial cells genetically selected from differentiating mouse embryonic stem cells incorporate at sites of neovascularization in vivo. *J Cell Sci.* 2002;115:2075-2085.
21. Yamashita J, Itoh H, Hirashima M, et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature.* 2000;408:92-96.
22. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest.* 2001;108:407-414.
23. Kehat I, Gepstein A, Spira A, Itskovitz-Eldor J, Gepstein L. High-resolution electrophysiological assessment of human embryonic stem cell-derived cardiomyocytes: a novel in vitro model for the study of conduction. *Circ Res.* 2002;91:659-661.
24. Westfall MV, Pasyk KA, Yule DI, Samuelson LC, Metzger JM. Ultrastructure and cell-cell coupling of cardiac myocytes differentiating in embryonic stem cell cultures. *Cell Motil Cytoskel.* 1998;36:43-54.
25. Min JY, Yang Y, Converso KL, et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Appl Physiol.* 2002;92:288-296.

26. Huber I, Itzhaki I, Caspi O, et al. Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. *FASEB J.* 2007;21:2551-2563.

27. Dowell JD, Rubart M, Pasumarthi KB, Soonpaa MH, Field LJ. Myocyte and myogenic stem cell transplantation in the heart. *Cardiovasc Res.* 2003;58:336-350.

28. Reinecke H, MacDonald GH, Hauschka SD, Murry CE. Electromechanical coupling between skeletal and cardiac muscle. Implications for infarct repair. *J Cell Biol.* 2000;149:731-740.

29. Leobon B, Garcin I, Menasche P, Vilquin JT, Audinat E, Charpak S. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. *Proc Natl Acad Sci USA.* 2003;100:7808-7811.

30. Menasche P, Hagege AA, Vilquin J-T, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol.* 2003;41:1078-1083.

31. Siminiak T, Kalawski R, Fiszer D, et al. Autologous skeletal myoblast transplantation for the treatment of post-infarction myocardial injury: phase I clinical study with 12 months of follow-up. *Am Heart J.* 2004;148:531-537.

32. Murry CE, Soonpaa MH, Reinecke H, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature.* 2004;428:664-668.

33. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature.* 2004;428:668-673.

34. Assmus B, Schachinger V, Teupe C, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation.* 2002;106:3009-3017.

35. Schachinger V, Assmus B, Britten MB, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol.* 2004;44:1690.

36. Wollert KC, Meyer GP, Lotz J, et al. Intra-coronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet.* 2004;364:141-148.

37. Janssens S, Dubois C, Bogaert J, et al. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised, controlled trial. *Lancet.* 2005;367:113-121.

38. Cleland JG, Freemantle N, Coletta AP, Clark AL. Clinical trials update from the American Heart Association: REPAIR-AMI, ASTAMI, JELIS, MEGA, REVIVE-II, SURVIVE, and PROACTIVE. *Eur J Heart Fail.* 2006;8:105-110.

39. Perin EC, Dohmann HFR, Borojevic R, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation.* 2003;107:2294-2302.

40. MacKenzie TC, Flake AW. Human mesenchymal stem cells: insights from a surrogate in vivo assay system. *Cells Tissues Organs.* 2002;171:90-95.

41. Davani S, Marandin A, Mersin N, et al. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation.* 2003;108(suppl II):II-253-II-258.

42. Makino S, Fukuda K, Miyoshi S, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest.* 1999;103:697-705.

43. Min J-Y, Sullivan MF, Yang Y, et al. Significant improvement of heart function by cotransplantation of human mesenchymal stem cells and fetal cardiomyocytes in postinfarcted pigs. *Ann Thorac Surg.* 2002;74:1568-1575.

44. Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg.* 2002;73:1919-1926.

45. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.* 2002;105:93-98.

46. Amado LC, Saliaris AP, Schuleri KH, et al. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci USA.* 2005;102:11474-11479.

47. Chen S-I, Fang W-W, Ye F, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. *Am J Cardiol.* 2004;94:92-95.

48. Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003;114:763-776.

49. Messina E, De Angelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res.* 2004;95:911-921.

50. Smith RR, Barile L, Cho HC, et al. Unique phenotype of cardiospheres derived from human endomyocardial biopsies. *Circulation.* 2005;112(suppl II):II-334.

51. Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Dourain NM. Ligand-dependent development of the endothelial and hematopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci USA.* 1997; 94:5141-5146.

52. Sato TN, Quin Y, Kozak CA, Audus KL. Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA.* 1993;90:9355-9358.

53. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell.* 1996;87:1171-1180.

54. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol.* 2000;109:235-242.

55. Mayani H, Lansdorp PM. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. *Stem Cells.* 1998;16:153-165.

56. Ma N, Stamm C, Kaminski A, et al. Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. *Cardiovasc Res.* 2005;66:45-54.

57. Hirata Y, Sata M, Motomura N, et al. Human umbilical cord blood cells improve cardiac function after myocardial infarction. *Biochem Biophys Res Commun.* 2005; 327:609-614.

58. Kim B-O, Tian H, Prasongsukarn K, et al. Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation.* 2005; 112 (suppl I):I-96-I-104.

59. Korbling M, Katz RL, Khanna A, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med.* 2002;346:738-746.

60. Yeh ET, Zhang S, Wu HD, Korbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood $CD34^+$-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. *Circulation.* 2003;108:2070-2073.

61. Etzion S, Barbash IM, Feinberg MS, et al. Cellular cardiomyoplasty of cardiac fibroblasts by adenoviral delivery of MyoD ex vivo: an unlimited source of cells for myocardial repair. *Circulation.* 2002;106(12 Suppl 1):I125-I130.

62. Smits PC, van Genus RJ, Poldermans D, et al. Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. *J Am Coll Cardiol.* 2003;42:2063-2069.

63. Dib N, McCarthy P, Campbell A, et al. Feasibility and safety of autologous myoblast transplantation in patients with ischemic cardiomyopathy. *Cell Transplant.* 2005;14:11-19.

64. Pagani FD, DerSimonian H, Zawadzka A, et al. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans: histological analysis of cell survival and differentiation. *J Am Coll Cardiol.* 2003;41:879-888.

65. Dow J, Simkhovich BZ, Kedes L, Kloner RA. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. *Cardiovasc Res.* 2005;67:301-307.

66. Muller-Ehmsen J, Whittaker P, Kloner RA, et al. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. *J Mol Cell Cardiol.* 2002;34:107-116.

7. ARE STEM CELLS THE NEXT FRONTIER FOR DIABETES TREATMENT?

Charles A. Goldthwaite, Jr., Ph.D.

Diabetes is a devastating disease that affects millions of people worldwide. The major forms of the disease are type 1 and type 2 diabetes. In type 1 diabetes, the body's immune system aberrantly destroys the insulin-producing beta cells (β-cells) of the pancreas. Type 2 diabetes, the more common form, is characterized both by insulin resistance, a condition in which various tissues in the body no longer respond properly to insulin action, and by subsequent progressive decline in β-cell function to the point that the cells can no longer produce enough additional insulin to overcome the insulin resistance. Researchers are actively exploring cell replacement therapy as a potential strategy to treat type 1 diabetes, because patients with this disease have lost all or nearly all β-cell function. However, if a safe and cost-effective means for replenishing β-cells were developed, such a treatment strategy could also be useful for the larger population with type 2 diabetes. One of the major challenges of cell replacement therapy is the current insufficient supply of β-cells from human organ donors. This article focuses on stem cells as potential sources for deriving new β-cells.

DIABETES: A CRITICAL HEALTH ISSUE FOR THE 21ST CENTURY

According to the International Diabetes Federation, diabetes currently affects 7% of the world's population — nearly 250 million individuals worldwide.[1] This total is expected to rise to 380 million by 2025 as a result of aging populations, changing lifestyles, and a recent worldwide increase in obesity. Although projections for increases in diabetes prevalence suggest that the greatest percentage gains will occur in Asia and South America,[2,3] all nations will experience a rising disease burden.

According to the National Diabetes Fact Sheet, which was compiled using information from the Centers for Disease Control and Prevention and other Federal and non-Federal organizations, 20.8 million U.S. children and adults have diabetes (6.2 million of whom are currently undiagnosed).[4] An estimated 54 million Americans have "pre-diabetes", a condition defined by blood glucose levels that are above normal but not sufficiently high to be diagnosed as diabetes. In 2005, 1.5 million new cases of diabetes were diagnosed in Americans aged 20 years or older.[4] If present trends continue, 1 in 3 Americans (1 in 2 minorities) born in 2000 will develop diabetes in their lifetimes.[5]

Diabetes is currently the sixth leading cause of death in the U.S.[4] It is associated with numerous health complications, including increased risk for heart disease, stroke, kidney disease, blindness, and amputations. In 2007, the total annual economic cost of diabetes was estimated to be $174 billion dollars.[6] Direct medical expenditures account for the vast majority of this total ($116 billion), although lost productivity and other indirect costs approached nearly $58 billion. The American Diabetes Association estimates that one out of every 10 health care dollars currently spent in the U.S. is used for diabetes and its complications.[6]

While diabetes can be managed, at present it cannot be cured. As a result, it is a lifelong and often disabling disease that can severely impact the quality of life of those who are afflicted. Based on several recent discoveries, however, researchers have begun to ask if a new treatment approach is on the horizon — can stem cells that are derived from adult or embryonic tissues generate new pancreatic β-cells to replace those that have failed or been destroyed? Cell replacement therapy is one of many research avenues being pursued as a potential treatment strategy for type 1 diabetes. The strategy may also have implications for ameliorating type 2 diabetes. One of the key obstacles to advancing such therapy is the current inadequate supply of cadaveric donor pancreata as a source of cells for transplantation. Additionally, it is not currently possible

to induce a patient's own cells to regenerate new β-cells within the body. Thus, researchers are actively investigating potential sources of new beta cells, including different types of stem cells. This article will focus on the various types of stem cells that are candidates for use in pancreatic regeneration and will discuss the challenges of using such cells as therapy for diabetes.

DEFINING DIABETES

Diabetes results from the body's inability to regulate the concentration of sugar (glucose) in the blood. Blood glucose concentration is modulated by insulin, a hormone produced by pancreatic β-cells and released into the bloodstream to maintain homeostasis. In healthy individuals, β-cells counteract sharp increases in blood glucose, such as those caused by a meal, by releasing an initial "spike" of insulin within a few minutes of the glucose challenge. This acute release is then followed by a more sustained release that may last for several hours, depending on the persistence of the elevated blood glucose concentration. The insulin release gradually tapers as the body's steady-state glucose concentration is reestablished. While postprandial insulin release is stimulated by factors other than blood glucose, the blood sugar concentration is the major driver. When the β-cells fail to produce enough insulin to meet regulatory needs, however, the blood glucose concentration rises. This elevated concentration imposes a metabolic burden on numerous body systems, dramatically increasing the risk of premature cardiovascular disease, stroke, and kidney failure. Moreover, the risk for certain diabetes-related complications increases even at blood glucose concentrations below the threshold for diagnosing diabetes.

At present, there is no cure for diabetes. β-cell failure is progressive[7]; once the condition is manifest, full function usually cannot be restored. Those with type 1 diabetes require daily insulin administration to survive. Persons with type 2 diabetes must control their elevated blood glucose levels through various means, including diet and exercise, oral antihyperglycemic (blood glucose-lowering) drugs, and/or daily insulin shots. Most people who live with type 2 diabetes for a period of time will eventually require insulin to survive.

As noted earlier, there are different forms of diabetes. Type 1 diabetes results when a person's immune system mistakenly attacks and destroys the β-cells. This type of diabetes was once referred to as "juvenile-onset diabetes," because it usually begins in childhood. Type 1 diabetes accounts for 5–10% of diabetes cases, and people with type 1 diabetes depend on daily insulin administration to survive.

By contrast, type 2 diabetes is a metabolic disorder that results from a decline in β-cell function combined with insulin resistance, or the inability to use insulin effectively in peripheral tissues such as the liver, muscles, and fat.[8] Onset is associated with genetic factors and with obesity, and type 2 diabetes disproportionately affects certain minority groups.[3] Unlike type 1 diabetes, type 2 is largely preventable. Numerous studies have suggested that the environmental and behavioral factors that promote obesity (e.g., a sedentary lifestyle, a high-calorie diet) have profoundly influenced the recent rise in the prevalence of type 2 diabetes.[9] This trend suggests that type 2 diabetes will continue to be a major health care issue.

THE CASE FOR STEM CELLS

There is great interest in developing strategies to expand the population of functional β-cells. Possible ways to achieve this include physically replacing the β-cell mass via transplantation, increasing β-cell replication, decreasing β-cell death, and deriving new β-cells from appropriate progenitor cells.[10] In 1990, physicians at the Washington University Medical Center in St. Louis reported the first successful transplant of donor-supplied pancreatic islet tissue (which includes β-cells; *see below*) in humans with type 1 diabetes.[11] By the end of the decade, many other transplants had been reported using various protocols, including the widely-known "Edmonton protocol" (named for the islet transplantation researchers at the University of Alberta in Edmonton).[12-14] This protocol involves isolating islets from the cadaveric pancreatic tissue of multiple donors and infusing them into the recipient's portal vein. However, the lack of available appropriate donor tissue and the strenuous regimen of immunosuppressive drugs necessary to keep the body from rejecting the transplanted tissue limit the widespread use of this approach. Moreover, the isolation process for islets damages the transplantable tissue; as such, 2–3 donors are required to obtain the minimal β-cell mass sufficient for transplantation into a single recipient.[13] While these strategies continue to be improved, islet

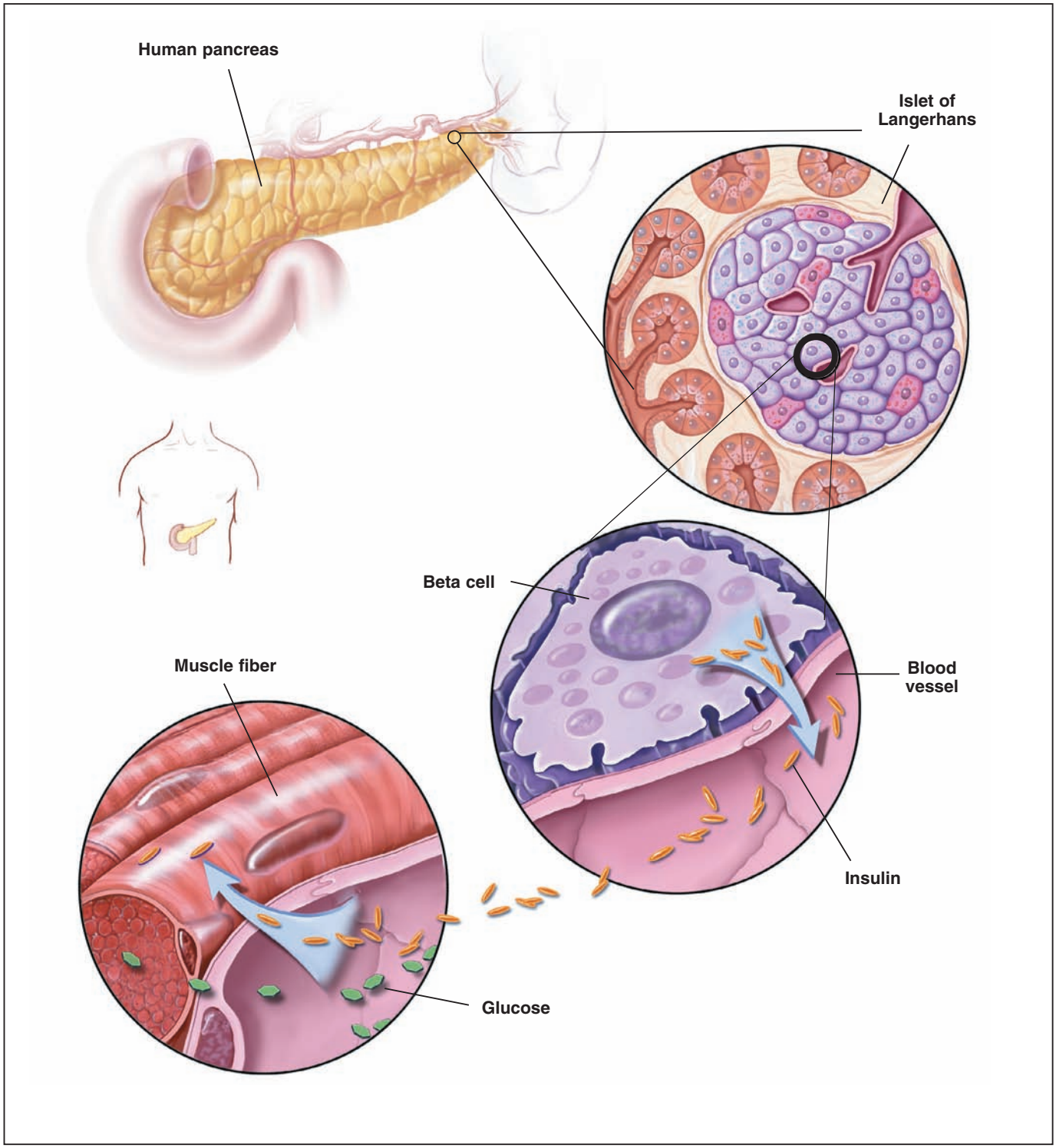


Figure 7.1. Insulin Production in the Human Pancreas.
The pancreas is located in the abdomen, adjacent to the duodenum (the first portion of the small intestine). A cross-section of the pancreas shows the islet of Langerhans which is the functional unit of the endocrine pancreas. Encircled is the beta cell that synthesizes and secretes insulin. Beta cells are located adjacent to blood vessels and can easily respond to changes in blood glucose concentration by adjusting insulin production. Insulin facilitates uptake of glucose, the main fuel source, into cells of tissues such as muscle.

function declines relatively rapidly post-transplant. For example, a long-term follow-up study of Edmonton transplant patients indicated that less than 10% of recipients remained insulin-independent five years after transplant.[15]

These challenges have led researchers to explore the use of stem cells a possible therapeutic option. Type 1 diabetes is an appropriate candidate disease for stem cell therapy, as the causative damage is localized to a particular cell type. In theory, stem cells that can differentiate into β-cells in response to molecular signals in the local pancreatic environment could be introduced into the body, where they would migrate to the damaged tissue and differentiate as necessary to maintain the appropriate β-cell mass. Alternately, methods could be developed to coax stem cells grown in the laboratory to differentiate into insulin-producing β-cells. Once isolated from other cells, these differentiated cells could be transplanted into a patient. As such, stem cell therapy would directly benefit persons with type 1 diabetes by replenishing β-cells that are destroyed by autoimmune processes, although it would still be necessary to mitigate the autoimmune destruction of β-cells. The strategy would also benefit those with type 2 diabetes to a lesser extent by replacing failing β-cells, although the insulin resistance in peripheral tissues would remain present. As discussed in the following sections, however, debate continues about potential source(s) of pancreatic stem cells.

SEARCHING FOR THE "PANCREATIC STEM CELL"

The pancreas is a complex organ made up of many cell types. The majority of its mass is comprised of exocrine tissue, which contains acinar cells that secrete pancreatic enzymes into the intestine to aid in food digestion. Dispersed throughout this tissue are thousands of islets of Langerhans, clusters of endocrine cells that produce and secrete hormones into the blood to maintain homeostasis. The insulin-producing β-cell is one type of endocrine cell in the islet; other types include alpha cells (α-cells), which produce glucagon, gamma cells (γ-cells), which produce pancreatic polypeptide, and delta cells (δ-cells), which produce somatostatin.

Each of these cell types arises from a precursor cell type during the process of development. Therefore, the key step for using stem cells to treat diabetes is to identify the precursor cell(s) that ultimately give rise to the β-cell. However, generating these cells is more complex than simply isolating a hypothetical "pancreatic stem cell." Experiments have indicated that embryonic and adult stem cells can serve as sources of insulin-secreting cells,[16] leading researchers to explore several avenues through which stem cells could feasibly be used to regenerate β-cells. However, many challenges must be addressed before a particular cell type will become established for this approach.

The human body has inherent mechanisms to repair damaged tissue, and these mechanisms remain active throughout life. Thus, there is reason to speculate that the adult pancreas may be aided by some type of regenerative system that replaces worn-out cells and repairs damaged tissue in response to injury. Such a system could theoretically be supported by precursor or stem cells, located in the endocrine pancreas or elsewhere, which could be coaxed to differentiate in response to select molecular or chemical stimuli. But do these cells exist? If so, how can they be recognized, isolated, and cultured for therapeutic use? How quickly could they produce sufficient numbers of β-cells to offset damage caused by diabetes processes? Alternately, what if cells that have the capability to regenerate β-cells exist in the body but are committed to differentiate into some other cell type? Could embryonic stem (ES) cell lines, which have the potential to develop into cells from all lineages, then be derived *in vitro* and be directed to differentiate into β-cells? These questions will be explored in the following sections, which review the types of candidate stem cells for diabetes.

ARE ADULT PANCREATIC STEM CELLS PRESENT IN THE PANCREAS?

Whether β-cell progenitors are present in the adult pancreas is a controversial topic in diabetes research. Several recent studies in rodents have indicated that the adult pancreas contains some type of endocrine progenitor cells that can differentiate toward β-cells.[16] However, researchers have not reached consensus about the origin of the bona fide pancreatic stem cell (if it exists) or the mechanism(s) by which β-cells are regenerated.[17] For example, a pivotal study by Dor and colleagues used genetic lineage tracing in adult mice to determine how stem cells contribute to the development of β-cells.[18] Their analysis indicated that new β-cells arise from pre-existing ones, rather

than from pluripotent stem cells, in adult mice. As such, the authors noted that β-cells can proliferate *in vivo,* thereby "cast[ing] doubt on the idea that adult stem cells have a significant role in beta-cell replenishment." Soon after this report was published, Seaberg and coworkers reported the identification of multipotent precursor cells from the adult mouse pancreas.[19] These novel cells proliferated in vitro to form colonies that could differentiate into pancreatic α-, β-, and δ-cells as well as exocrine cells, neurons, and glial cells. Moreover, the beta-like cells demonstrated glucose-dependent insulin release, suggesting possible therapeutic application to diabetes. Several subsequent studies have also reported the existence of pancreatic stem/precursor cells *in vitro* or *in vivo.*[20-22] One recent report suggests that such cells exist in the pancreatic ductal lining and can be activated autonomously in response to injury, increasing the β-cell mass through differentiation and proliferation.[23]

The study of pancreatic regeneration continues to evolve, and many claims have been made regarding cells believed to be involved in the process. In the last decade, reports have described various putative pancreatic stem cells embedded in the pancreatic islets,[24,25] pancreatic ducts,[23,26] among the exocrine acinar cells,[20,21] and in unspecified pancreatic locales[19,27] in rodent models, as well as from human adult pancreatic cell lines,[28] islet tissue,[29] and non-islet tissues discarded after islets have been removed for transplantation.[30-32] These cells are identified by the presence of one or more cell-surface proteins, or markers, known to be associated with a particular stem cell lineage. However, these studies illustrate several challenges shared by all researchers who seek to identify the "pancreatic stem cell". First, all potential stem cell candidates identified to date are relatively rare; for instance, the precursor cells identified by Seaberg are present at the rate of 1 cell per 3,000–9,000 pancreatic cells.[19] Because there are so few of these putative stem cells, they can be difficult to identify. Additionally, the choice of marker can select for certain stem cell populations while possibly excluding others. Interestingly, the progenitor cells identified in the Seaberg study lacked some known β-cell markers such as HNF3β, yet they were able to generate β-cells. Thus, a hypothetical experiment that used only HNF3β as a marker for β-cell differentiation would likely not identify this stem cell population. Moreover, techniques used to study the pancreatic tissue, such as the genetic lineage technique of Dor, *et.al.* could possibly interfere with the generation of new β-cells from stem or precursor cells.[33]

As such, the possibility remains that β-cells could be regenerated by differentiation of endogenous stem cells, by proliferation of existing β-cells, or a combination of the two mechanisms.

Further research to elucidate conditions under which β-cells can proliferate may help to develop new therapeutic approaches. For example, several advances have recently been made from studies of pregnancy and pregnancy-related diabetes (gestational diabetes) in mice. During pregnancy, pancreatic islet cells normally expand in number to meet increased metabolic demands. Researchers have found that the protein HNF4-alpha helps increase β-cell mass, and that pregnancy-related decreases in levels of another protein, menin, also enable β-cell proliferation.[34,35] Insights may also arise from research on another organ, the liver. Unlike the pancreas, the liver has an inherently high capacity for regeneration. New strategies for inducing pancreatic islet cell growth may emerge from knowledge of how liver cells develop from progenitor cells during early development such that the resulting adult organ retains substantial regenerative capacity.[36] In another research avenue, scientists are exploring whether it may be possible to redirect adult pancreatic cells in the body to change from their original cell type into β-cells.

OTHER POTENTIAL SOURCES OF STEM CELLS DERIVED FROM ADULT CELLS

Furthermore, various reports have also described putative stem cells in the liver, spleen, central nervous system, and bone marrow that can differentiate into insulin-producing cells.[17] While it is possible that such pathways may exist, these results are currently under debate within the research community. In another research avenue, scientists recently reported that differentiated cells, including adult human skin cells, can be genetically "reprogrammed" to revert to a pluripotent state, resembling that of embryonic stem (ES) cells.[37] The researchers refer to these cells as induced pluripotent stem (iPS) cells. Their method involved introducing a defined set of genes into the differentiated cells. This approach may facilitate the establishment of human iPS cell lines from patients with specific diseases that could be used as research tools. This technique, or variations of it, may also one day allow patient-specific stem cells to be generated

for use in stem cell-based therapies. However, the genes used for reprogramming were introduced into the cells using a virus-based method, which could have adverse clinical effects. If, however, safe alternate methods based on this research can be developed for reprogramming cells, then iPS cells may lead to novel, personalized therapies.

CAN EMBRYONIC STEM CELLS BE USED?

The challenges associated with identifying and isolating adult "pancreatic stem cells" has led some researchers to explore the use of ES cells as a source of insulin-producing cells. Several factors make ES cells attractive for this application.[33] First, given the complexity of pancreatic tissue, identified β-cell precursors would likely be difficult to isolate from the adult pancreas. If isolated, the cells would then need to be replicated *ex vivo* while keeping them directed toward a β-cell lineage. Second, protocols to grow and expand mature β-cells in culture have met with technical challenges. ES cells, which are pluripotent cell lines (they can give rise to all cell types of the embryo) that can be induced to develop into various lineages based on culture conditions, may therefore represent a future option for β-cell regeneration.

To date, several human ES cells lines have been successfully derived.[38-40] While these cell lines serve as resources for exploring the mechanisms of development, their potential use in a clinical setting is limited by several factors, most notably ethical concerns and the risk of teratoma development. (For a more detailed discussion of the scientific challenges associated with clinical application of ES cells, see Chapter 6, "Mending a Broken Heart: Stem Cells and Cardiac Repair," p.59). In addition, researchers are only beginning to unlock the myriad factors that come into play as a once-pluripotent cell differentiates into a unipotent cell, one that can contribute to only one mature cell type.[41] For example, several recent reports indicate that mouse[42] and human[43] ES cells can be successfully differentiated into endodermal cells, the precursors of pancreatic cells. In addition, insulin-producing cells have been derived from mouse[44,45] and human[46] ES cells.

However, it should be noted that directed differentiation of ES cells toward the β-cell has not been reported. Beta cells appear relatively late during embryonic development, suggesting that their presence involves the temporal control of a considerable number of genes. Moreover, the creation of patient-specific, stem cell-derived β-cells for transplantation requires genetic matching to lessen the immune response. Generating immune-matched tissues requires the therapeutic cloning of human ES cells, which has not been accomplished to date. A fraudulent claim to the contrary in 2005 by South Korean researcher Woo Suk Hwang[47] ignited international controversy within the scientific community[48] and illustrated the scientific and ethical challenges of using ES cells as a source of transplant tissue. Despite current gaps in knowledge, researchers recognize the potential of ES cells as sources of specialized cells such as the β-cell, and the study of ES cells provides insight into the processes that govern differentiation and specialization.

CLINICAL CHALLENGES

Clearly, using stem cells to treat diabetes will require additional knowledge, both in the laboratory and in the clinic. This section will suggest several envisioned approaches for stem-cell derived diabetes therapies and discuss key considerations that must be addressed for their successful application.

Contingent upon the development of appropriate protocols, stem cells could theoretically be used to treat diabetes through two approaches.[49] Both strategies would require the isolation and *in vitro* expansion of a homogenous population of β-cell precursor cells from appropriate donor tissue. Once a population of these cells has been generated, they could either 1) be induced to differentiate into insulin-producing cells *in vitro* and then be transplanted into the diabetic patient's liver, or 2) be injected into the circulation along with stem cell stimulators, with the hope that the cells will "home in" to the injured islets and differentiate into a permanent self-renewing β-cell population.

Because type 1 diabetes is an autoimmune disease, controlling the autoimmune response is critical to the success of any potential stem cell-based therapy. Type 1 diabetes is characterized by the action of β-cell-specific, autoreactive T-cells. Even if the regenerative properties of the pancreas remain functional, the continued presence of these T-cells effectively counteracts any endogenous repair and would likely decimate populations of newly-regenerated or transplanted insulin-producing cells. However, the autoimmune response has been successfully averted in non-obese diabetic mice either by using anti-T-cell antibodies to

eliminate the majority of the autoreactive cells[50] or by transplanting bone marrow from a diabetes-resistant donor (with a sublethal dose of irradiation) into the diabetic animal.[51-53] Both strategies appear to enable the replenishment of insulin-secreting cells and the eventual restoration of normal blood glucose levels, although the process requires weeks to months and may necessitate additional therapy. Other strategies being explored include altering the immune tolerance through the use of monoclonal antibodies,[54] proteins,[55] and oligonucleotides.[56]

Other clinical challenges, including safety, tracking of the stem cells, delivery of the cells to the targeted tissue within a clinically relevant time frame (for transplanted cells), identification of ways to promote long-term survival and functioning of regenerated β-cells, ease of obtaining the cells, and cost, parallel those encountered with all applications of stem cell-based regenerative therapy. These issues must be addressed once the "pancreatic stem cell" population has been identified conclusively. Given current debate on this issue, the routine clinical application of stem-cell based regenerative therapy for the treatment of diabetes remains a future goal, albeit one with great potential.

As an additional source of information, an extensive discussion of research challenges and strategies for achieving the goal of cell replacement therapy for Type 1 diabetes is presented in *Advances and Emerging Opportunities in Type 1 Diabetes Research: A Strategic Plan,* available on the NIH web site at http://www2.niddk.nih.gov/AboutNIDDK/ResearchAndPlanning/Type1Diabetes/.

CONCLUSIONS

The results discussed in this article demonstrate the many challenges that must be addressed before stem cells can be used to regenerate islet tissue in persons with diabetes. Debate continues on the identification of the "pancreatic stem cell," and at present it is difficult to ascertain which cell type has the greatest potential for diabetes therapy. Moreover, modulating the autoimmune response in type 1 diabetes remains a significant challenge regardless of the type of cell that is transplanted, and it will also be important to address the insulin resistance in type 2 diabetes, as well as factors that contribute to obesity. However, diabetes is a disease with a major deficiency in the functioning of one type of cell, and there is potential of stem cells to treat type 1 diabetes and to improve the quality of life for those with type 2 diabetes. As researchers learn more about the mechanisms that govern stem cell programming, differentiation, and renewal, their ability to identify, isolate, and culture candidate stem cells will continue to improve. While stem cells can be currently considered a frontier for diabetes therapy, they may one day become its basis.

REFERENCES

1. International Diabetes Federation. Facts & figures: diabetes prevalence. http://www.idf.org/home/index.cfm?node=264. Accessed September 15, 2007.
2. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care.* 1998;21:1414-1431.
3. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature.* 2001;414:782-787.
4. Centers for Disease Control and Prevention. National diabetes fact sheet: general information and national estimates on diabetes in the United States, 2005. http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2005.pdf. Accessed April 24, 2008.
5. Narayan KM, Boyle JP, Thompson TJ, Sorensen SW, Williamson DF. Lifetime risk for diabetes mellitus in the United States. *JAMA.* 2003;290:1884-1890.
6. American Diabetes Association. Economic costs of diabetes in the U.S. In 2007. *Diabetes Care.* 2008;31:596-615.
7. UK Prospective Diabetes Study Group. Overview of 6 years' therapy of type II diabetes: a progressive disease (UKPDS 16). *Diabetes.* 1995;44:1249-1258.
8. Ferranninni E. Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. *Endocr Rev.* 1998;19:477-490.
9. Hill JO, Peters JC. Environmental contributions to the obesity epidemic. *Science.* 1998;280:1371-1374.
10. Lipsett M, Finegood DT. Beta-cell neogenesis during prolonged hyperglycemia in rats. *Diabetes.* 2001;51:1834-1841.
11. Scharp DW, Lacy PE, Santiago JV, et al. Insulin independence after islet transplantation into type I diabetic patient. *Diabetes.* 1990(39):515-518.
12. Ryan EA, Lakey JR, Paty BW, et al. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes.* 2002;51:2148-2157.
13. Ryan EA, Lakey JR, Rajotte RV, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes.* 2001;50:710-719.

14. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* 2000;343:230-238.

15. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54:2060-2069.

16. Soria B, Bedoya FJ, Martin F. Gastrointestinal stem cells: I. Pancreatic stem cells. *Am J Physiol Gastrointest Liver Physiol.* 2005;289:G177-G180.

17. Zulewski H. Stem cells with potential to generate insulin-producing cells in man. *Swiss Med Wkly.* 2006;136:647-654.

18. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature.* 2004;429:41-46.

19. Seaberg RM, Smukler SR, Kieffer TJ, et al. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol.* 2004;22:1115-1124.

20. Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L. In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. *Diabetologia.* 2005;48:49-57.

21. Minami K, Okuno M, Miyawaki K, et al. Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. *Proc Natl Acad Sci USA.* 2005;102:15116-15121.

22. Lee CS, De Leon DD, Kaestner KH, Stoffers DA. Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. *Diabetes.* 2006;55:269-272.

23. Xu X, D'Hoker J, Stangé G, et al. Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell.* 2008;132:197-207.

24. Guz Y, Nasir I, Teitelman G. Regeneration of pancreatic beta cells from intra-islet precursor cells in an experimental model of diabetes. *Endocrinology.* 2001;142:4956-4968.

25. Zulewski H, Abraham EJ, Gerlach MJ, et al. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes.* 2001;50:521-533.

26. Oshima Y, Suzuki A, Kawashimo K, Ishikawa M, Ohkohchi N, Taniguchi H. Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. *Gastroenterology.* 2007;132:720-732.

27. Raimya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med.* 2000;6:278-282.

28. Tsang WG, Zheng T, Wang Y, et al. Generation of functional islet-like clusters after monolayer culture and intracapsular aggregation of adult human pancreatic islet tissue. *Transplantation.* 2007;83:685-693.

29. Abraham EJ, Leech CA, Lin JC, Zulewski H, Habener JF. Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells. *Endocrinology.* 2002;143:3152-3161.

30. Todorov I, Nair I, Ferreri K, et al. Multipotent progenitor cells isolated from human adult pancreatic tissue. *Transplant Proc.* 2005;37:3420-3421.

31. Todorov I, Omori K, Pascual M, et al. Generation of human islets through expansion and differentiation of non-islet pancreatic cells discarded (pancreatic discard) after islet isolation. *Pancreas.* 2006;32:130-138.

32. Bonner-Weir S, Taneja M, Weir GC, et al. In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci USA.* 2000;97:7999-8004.

33. Trucco M. Regeneration of the pancreatic beta cell. *J Clin Invest.* 2005;115:5-12.

34. Gupta RK, Gao N, Gorski RK, et al. Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. *Genes Dev.* 2007;21:756-769.

35. Karnik SK, Chen H, McLean GW, et al. Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. *Science.* 2007;318:806-809.

36. Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. *Nat Rev Genet.* 2008;9:329-340.

37. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature.* 2008;451:141-146.

38. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282:1145-1147.

39. Shamblott MJ, Axelman J, Wang S, et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA.* 1998;95:13726-13731.

40. Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med.* 2004;350:1353-1356.

41. Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell.* 2004;116:639-648.

42. Yasunaga M, Tada S, Torikai-Nishikawa S, et al. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotechnol.* 2005;23:1542-1550.

43. D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol.* 2005;23:1534-1541.

44. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science.* 2001;292:1389-1394.

45. Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes.* 2000;49:157-162.

46. Seguev H, Fishman B, Ziskind A, Shulman M, Itskovitz-Eldor J. Differentiation of human stem cells into insulin-producing cell clusters. *Stem Cells.* 2004;22:265-274.

47. Hwang WS, Roh SI, Lee BC, et al. Patient-specific embryonic stem cells derived from human SCNT blastocysts. *Science.* 2005;308:1777-1783.

48. Chong AS, Normile D. Stem cells: how young Korean researchers helped unearth a scandal… *Science.* 2006;311:22-25.

49. Lechner A, Habener JF. Stem/progenitor cells derived from adult tissues: potential for the treatment of diabetes mellitus. *Am J Physiol Endocrinol Metab.* 2003;284:E259-E266.

50. Ogawa N, List JF, Habener JF, Maki T. Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. *Diabetes.* 2004;53:1700-1705.

51. Ikehara S, Ohtsuki H, Good RA, et al. Prevention of type 1 diabetes in nonobese diabetic mice by allogenic bone marrow transplantation. *Proc Natl Acad Sci USA.* 1985;82:7743-7747.

52. Li H, Kaufman CL, Boggs SS, Johnson PC, Patrene KD, Ildstad ST. Mixed allogeneic chimerism induced by a sublethal approach prevents autoimmune diabetes and reverses insulitis in nonobese diabetic (NOD) mice. *J Immunol.* 1996;156:380-388.

53. Zorina TD, Subbotin VM, Bertera S, et al. Distinct characteristics and features of allogeneic chimerism in the NOD mouse model of autoimmune diabetes. *Cell Transplant.* 2002;11:113-123.

54. Chatenoud L, Primo J, Bach J-F. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol.* 1997;158:2947-2954.

55. Zheng XX, Sanchez-Fueyo A, Sho M, Domenig C, Sayegh MH, Strom TB. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity.* 2003;19:503-514.

56. Machen J, Harnaha J, Lakomy R, Styche A, Trucco M, Giannoukakis N. Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells. *J Immunol.* 2004;173:4331-4341.

8. ALTERNATE METHODS FOR PREPARING PLURIPOTENT STEM CELLS

James F. Battey, Jr., MD, PhD; Laura K. Cole, PhD; and Charles A. Goldthwaite, Jr., PhD

THE CLINICAL APPLICATION OF PLURIPOTENT CELLS: THE PROMISE AND THE CHALLENGES

Stem cells are distinguished from other cells by two characteristics: (1) they can divide to produce copies of themselves (self-renewal) under appropriate conditions and (2) they are pluripotent, or able to differentiate into any of the three germ layers: the endoderm (which forms the lungs, gastrointestinal tract, and interior lining of the stomach), mesoderm (which forms the bones, muscles, blood, and urogenital tract), and ectoderm (which forms the epidermal tissues and nervous system). Pluripotent cells, which can differentiate into any mature cell type, are distinct from multipotent cells (such as hematopoietic, or blood-forming, cells) that can differ into a limited number of mature cell types. Because of their pluripotency and capacity for self-renewal, stem cells hold great potential to renew tissues that have been damaged by conditions such as type 1 diabetes, Parkinson's disease, heart attacks, and spinal cord injury. Although techniques to transplant multipotent or pluripotent cells are being developed for many specific applications, some procedures are sufficiently mature to be established options for care. For example, human hematopoietic cells from the umbilical cord and bone marrow are currently being used to treat patients with disorders that require replacement of cells made by the bone marrow, including Fanconi's anemia and chemotherapy-induced bone marrow failure after cancer treatment.

However, differentiation is influenced by numerous factors, and investigators are just beginning to understand the fundamental properties of human pluripotent cells. Researchers are gradually learning how to direct these cells to differentiate into specialized cell types and to use them for research, drug discovery, and transplantation therapy (see Figure 8.1). However, before stem cell derivatives are suitable for clinical application, scientists require a more complete understanding of the molecular mechanisms that drive pluripotent cells into differentiated cells. Scientists will need to pilot experimental transplantation therapies in animal model systems to assess the safety and long-term stable functioning of transplanted cells. In particular, they must be certain that any transplanted cells do not continue to self-renew in an unregulated fashion after transplantation, which may result in a teratoma, or stem

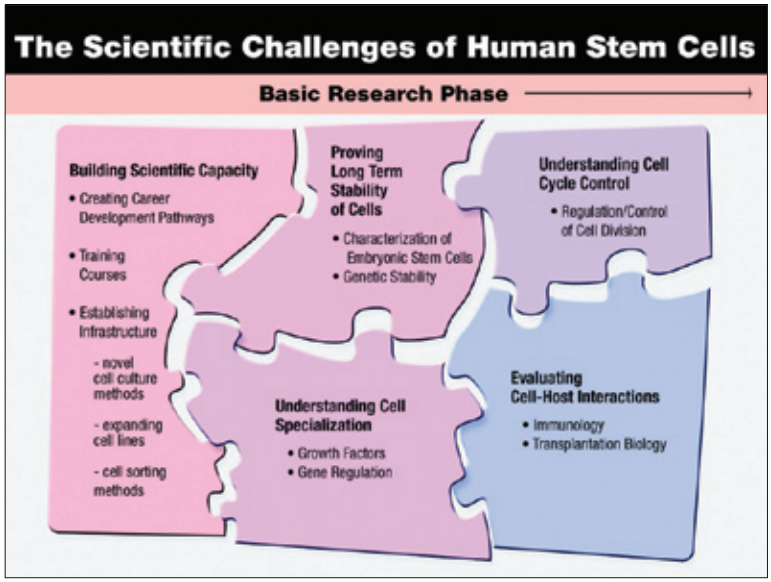


Figure 8.1. The Scientific Challenge of Human Stem Cells
The state of the science currently lies in the development of fundamental knowledge of the properties of human pluripotent cells. The scientific capacity needs to be built, an understanding of the molecular mechanisms that drive cell specialization needs to be advanced, the nature and regulation of interaction between host and transplanted cells needs to be explored and understood, cell division needs to be understood and regulated, and the long-term stability of the function in transplanted cells needs to be established.

cell tumor. In addition, scientists must ascertain that cells transplanted into a patient are not recognized as foreign by the patient's immune system and rejected.

Stem cells derived from an early-stage human blastocyst (an embryo fertilized in vitro and grown approximately five days in culture) have the capacity to renew indefinitely, and can theoretically provide an unlimited supply of cells. It is also possible to derive stem cells from non-embryonic tissues, including amniotic fluid, placenta, umbilical cord, brain, gut, bone marrow, and liver. These stem cells are sometimes called "adult" stem cells, and they are typically rare in the tissue of origin. For example, blood-forming (hematopoietic) stem cell experts estimate that only 1 in 2000 to fewer than 1 in 10,000 cells found in the bone marrow is actually a stem cell.[1] Because so-called "adult" stem cells include cells from the placenta and other early stages of development, they are more correctly termed "non-embryonic stem cells." Non-embryonic stem cells are more limited in their capacity to self renew in the laboratory, making it more difficult to generate a large number of stem cells for a specific experimental or therapeutic application. Under normal conditions, non-embryonic stem cells serve as a repair pool for the body, so they typically differentiate only into the cell types found in the organ of origin. Moreover, there is little compelling evidence for trans-differentiation, whereby a stem cell from one organ differentiates into a mature cell type of a different organ. New discoveries may overcome these limitations of stem cells derived from non-embryonic sources, and research directed toward this goal is currently underway in a number of laboratories.

THE ROLE OF CULTURED CELLS IN UNDERSTANDING THE DIFFERENTIATION PROCESS

Cultures of human pluripotent, self-renewing cells enable researchers to understand the molecular mechanisms that regulate differentiation (see Figure 8.2), including epigenetic changes (traits that may be inherited that do not arise from changes in the DNA sequence) in the chromatin structure, developmental changes in gene expression, exposure to growth factors, and interactions between adjacent cells. Understanding these basic mechanisms may enable future scientists to mobilize and differentiate endogenous populations of pluripotent cells to replace a cell type ravaged by injury or disease. Alternatively, scientists may some day be able to coax human pluripotent cells grown in the laboratory to become a specific type of specialized cell, which physicians could subsequently transplant into a patient to replace cells damaged by these same disease processes.

Scientists are gradually learning to direct the differentiation of pluripotent cell cultures into a specific type of cell, which can then be used as cellular models of human disease for drug discovery or toxicity studies. While it is not possible to predict the myriad ways that a basic understanding of stem cell differentiation may lead to new approaches for treating patients with cellular degenerative diseases, some avenues can be theorized. For example, in the case of Huntington's disease, a fatal neurodegenerative disorder, one could imagine that pluripotent cells derived from an embryo that carries Huntington's disease and differentiated into neurons in culture could be used to test drugs to delay or prevent degeneration.

Despite the incredible growth in knowledge that has occurred in stem cell research within the last couple of decades, investigators are just beginning to unravel the process of differentiation. Human pluripotent cell lines are an essential tool to understand this process and to facilitate the ultimate use of these cells in the clinic. To provide background on this fundamental topic, this article reviews the various potential sources and approaches that have been used to generate human pluripotent and multipotent cell lines, both of embryonic and non-embryonic origin.

ESTABLISHING HUMAN PLURIPOTENT STEM CELL LINES FROM EMBRYONIC OR FETAL TISSUES

Currently, at least six embryonic sources have been used to establish human pluripotent stem cell lines. All approaches involve isolation of viable cells during an early phase of development, followed by growth of these cells in appropriate culture medium. The various sources of these initial cell populations are discussed in brief below. It should be noted that the manipulation and use of embryonic tissues has raised a number of ethical issues.[2,3] This article focuses on the scientific and technical issues associated with creating pluripotent cells, with the understanding that some of these techniques are currently subject to debates that extend beyond discussions of their scientific merits.

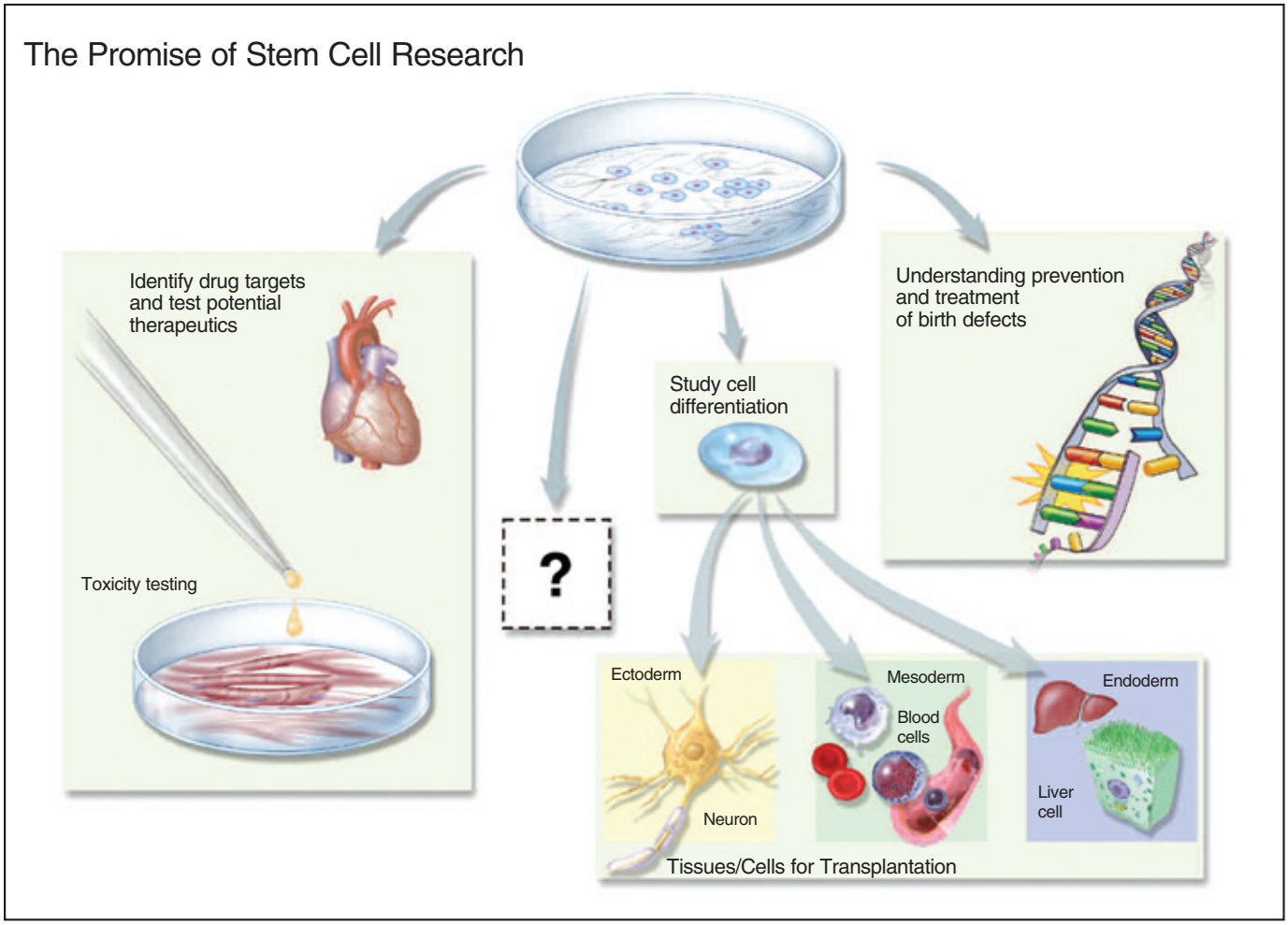


Figure 8.2. The Promise of Stem Cell Research
Stem cell research provides a useful tool for unraveling the molecular mechanisms that determine the differentiation fate of a pluripotent cell and for understanding the gene expression properties and epigenetic modifications essential to maintain the pluripotent state. In the future, this knowledge may be used to generate cells for transplantation therapies, whereby a specific cell population compromised by disease is replaced with new, functional cells. Differentiated derivatives of human pluripotent cells may also prove to be useful as models for understanding the biology of disease and developing new drugs, particularly when there is no animal model for the disease being studied. The greatest promise of stem cell research may lie in an area not yet imagined.

Traditional Human Embryonic Stem Cell (hESC) Line Generation

Drawing upon twenty years of communal expertise with mouse ES cells[4] and on human inner cell mass culture conditions developed by Ariff Bongso and colleagues[5], James Thomson and colleagues at the University of Wisconsin generated the first hESC lines in 1998 using tissue from embryos fertilized *in vitro*.[6] This method uses embryos generated for *in vitro* fertilization (IVF) that are no longer needed for reproductive purposes. During IVF, medical professionals usually produce more embryos than a couple attempting to start a family may need. Spare embryos are typically stored in a freezer to support possible future attempts for additional children if desired. It is estimated that there are approximately 400,000 such spare embryos worldwide.[7] If these embryos are never used by the couple, they either remain in storage or are discarded as medical waste. Alternatively, these embryos can potentially be used to generate a hESC line.

To generate a hESC line, scientists begin with a donated blastocyst-stage embryo, at approximately five days after IVF (see Figure 8.3a). The blastocyst consists of approximately 150–200 cells that form a hollow sphere of cells, the outer layer of which is called the trophectoderm. During normal development, the trophoblast becomes the placenta and umbilical cord. At one pole of this hollow sphere, 30–50 cells form a

cluster that is called the inner cell mass (ICM), which would give rise to the developing fetus. ICM cells are pluripotent, possessing the capacity to become any of the several hundred specialized cell types found in a developed human, with the exception of the placenta and umbilical cord.

Scientists remove the ICM from the donated blastocyst and place these cells into a specialized culture medium. In approximately one in five attempts, a hESC line begins to grow. Stem cells grown in such a manner can then be directed to differentiate into various lineages, including neural precursor cells,[8] cardiomyocytes,[9] and hematopoietic (blood forming) precursor cells.[10]

However, hESC lines are extremely difficult to grow in culture; the cells require highly specialized growth media that contain essential ingredients that are difficult to standardize. Yet the culture conditions are critical to maintain the cells' self-renewing and pluripotent properties. Culture requires the support of mouse or human cells, either directly as a "feeder" cell layer[6,11,12] or indirectly as a source of conditioned medium in feeder-free culture systems.[13] The feeder cells secrete important nutrients and otherwise support stem cell growth, but are treated so they cannot divide. Although the complete role of these feeder cells is not known, they promote stem cell growth, including detoxifying the culture medium and secreting proteins that participate in cell growth.[14] hESC lines used to produce human cells for transplantation therapies may need to be propagated on a human feeder cell layer to reduce the risk of contamination by murine viruses or other proteins that may cause rejection. Thus, hESC lines often grow only under highly specific culture conditions, and the identification of ideal growth conditions presents a challenge regardless of the source of the hESCs.

Furthermore, human ES cell cultures must be expanded using an exacting protocol to avoid cell death and to control spontaneous differentiation. Since a limited number of laboratories in the United States are growing these cells, there is a shortage of people well-versed in the art and science of successful hESC culture. In the short term, challenges of working with these cells include developing robust culture conditions and protocols, understanding the molecular mechanisms that direct differentiation into specific cell types, and developing the infrastructure to advance this scientific opportunity. Once these challenges have been met, scientists will need to conduct transplantation studies in animal models (rodent and non-human primates) to demonstrate safety, effectiveness, and long-term benefit before stem cell therapies may enter clinical trials.

hESC Lines from Human Primordial Germ Cells

A second method for generating human pluripotent stem cell lines was published in 1998 by John Gearhart and coworkers at The Johns Hopkins Medical School.[15] These researchers isolated specialized cells known as primordial germ cells (PGCs) from a 5–7-week-old embryo and placed these cells into culture (see Figure 8.3b). PGCs are destined to become either oocytes or sperm cells, depending on the sex of the developing embryo. The resulting cell lines are called embryonic germ cell lines, and they share many properties with ES cells. As with ES cells, however, PGCs present challenges with sustained growth in culture.[16,17] Spontaneous differentiation, which hinders the isolation of pure clonal lines, is a particular issue. Therefore, the clinical application of these cells requires a more complete understanding of their derivation and maintenance *in vitro.*

hESC Lines from Dead Embryos

Embryos that stop dividing after being fertilized *in vitro* are not preferentially selected for implantation in a woman undergoing fertility treatment. These embryos are typically either frozen for future use or discarded as medical waste. In 2006, scientists at the University of Newcastle, United Kingdom, generated hESC lines from IVF embryos that had stopped dividing.[18] These scientists used similar methods as described under "Traditional hESC Line Generation" except that their source material was so-called "dead" IVF embryos (see Figure 8.3c). The human stem cells created using this technique behaved like pluripotent stem cells, including producing proteins critical for "stemness" and being able to produce cells from all three germ layers. It has been proposed that an IVF embryo can be considered dead when it ceases to divide.[19] If one accepts this definition, such an embryo that "dies" from natural causes presumably cannot develop into a human being, thereby providing a source to derive human ES cells without destroying a living embryo.

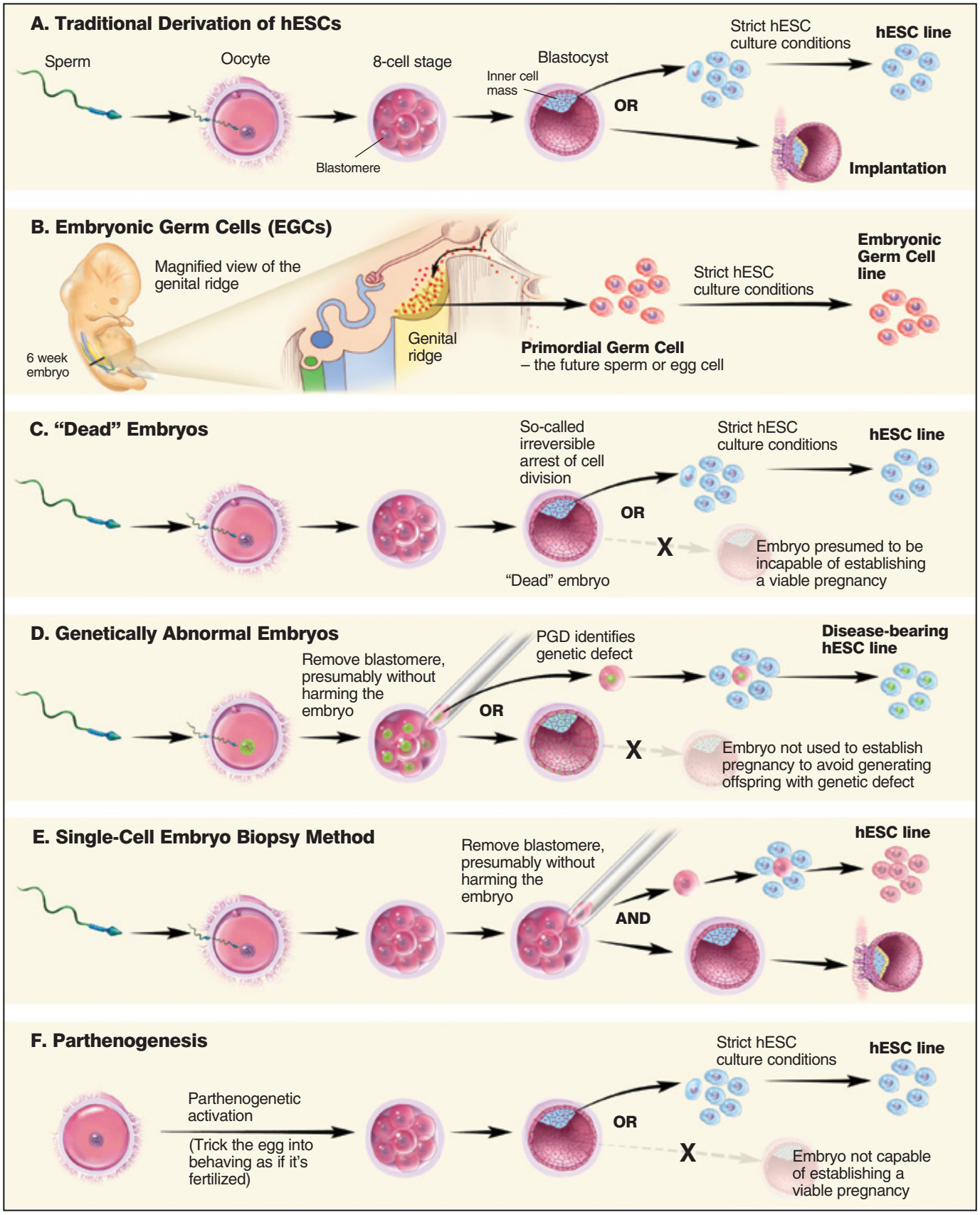


Figure 8.3. Alternative Methods for Preparing Pluripotent Stem Cells

hESC Lines from Genetically Abnormal Embryos

Couples who have learned that they carry a genetic disorder sometimes use pre-implantation genetic diagnosis (PGD) and IVF to have a child that does not carry the disorder. PGD requires scientists to remove one cell from a very early IVF human embryo and test it for diseases known to be carried by the hopeful couple. Normally, embryos identified with genetic disorders are discarded as medical waste. However, Dr.Yuri Verlinsky

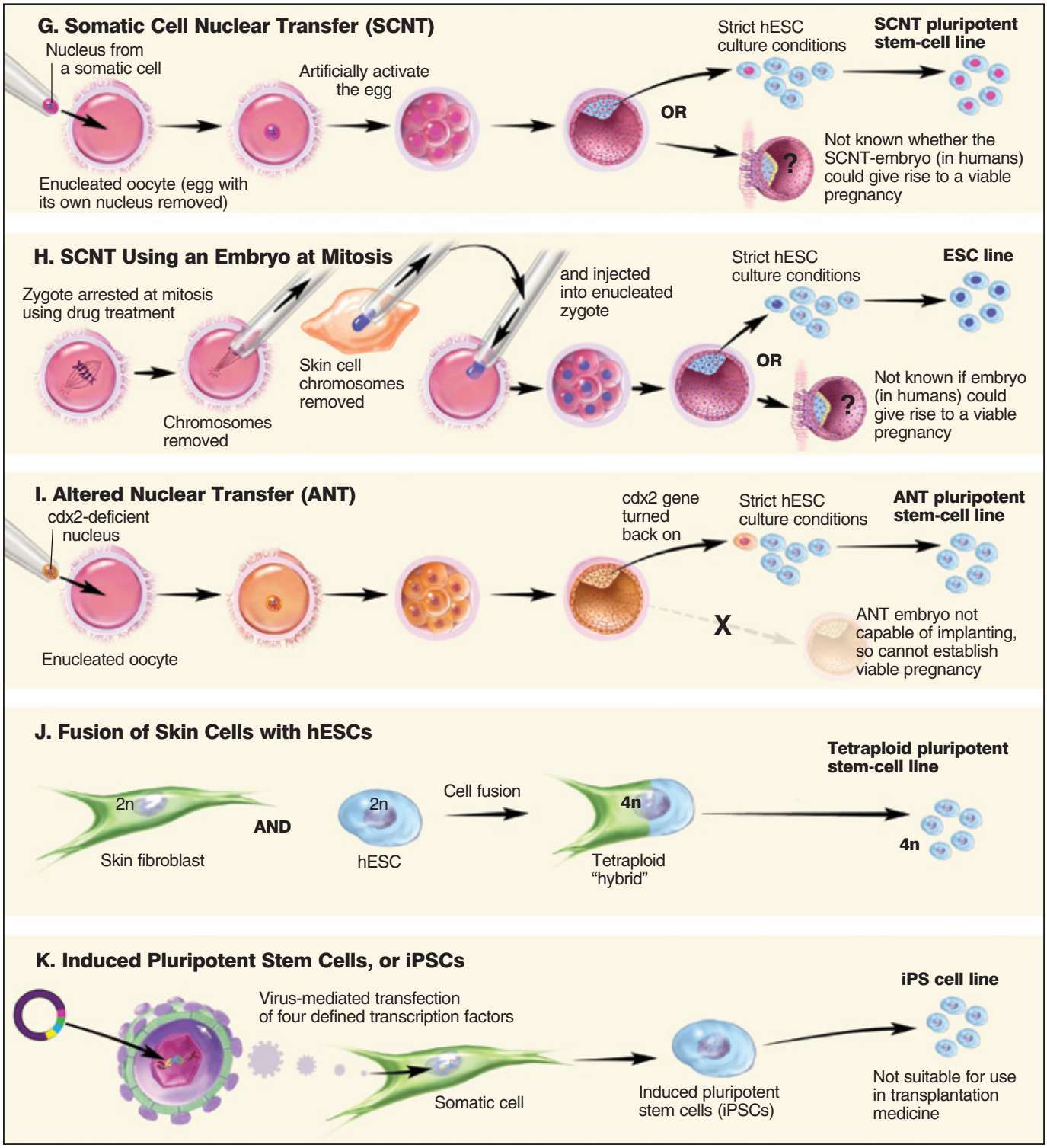


Figure 8.3. Alternative Methods for Preparing Pluripotent Stem Cells

and colleagues have capitalized on these embryos as a way to further our understanding of the diseases they carry (see Figure 8.3d) by deriving hESC lines from them.[20] These stem cell lines can then be used to help scientists understand genetically-based disorders such as muscular dystrophy, Huntington's disease, thalessemia, Fanconi's anemia, Marfan syndrome, adrenoleukodystrophy, and neurofibromatosis.

hESC Lines from Single Cell Embryo Biopsy

In 2006, Dr. Robert Lanza and colleagues demonstrated that it is possible to remove a single cell from a pre-implantation mouse embryo and generate a mouse ES cell line.[21] This work was based upon their experience with cleavage-stage mouse embryos. Later that same year, Dr. Lanza's laboratory reported that it had successfully established hESC lines (see Figure 8.3e) from single cells taken from pre-implantation human embryos.[22] The human stem cells created using this technique behaved like pluripotent stem cells, including making proteins critical for "stemness" and producing cells from all three germ layers. Proponents of this technique suggest that since it requires only one embryonic cell, the remaining cells may yet be implanted in the womb and develop into a human being. Therefore, scientists could potentially derive human embryonic stem cells without having to destroy an embryo. However, ethical considerations make it uncertain whether scientists will ever test if the cells remaining after removal of a single cell can develop into a human being, at least in embryos that are not at risk for carrying a genetic disorder. Moreover, it is unclear whether the single cell used to generate a pluripotent stem cell line has the capacity to become a human being.

hESC Lines Created via Parthenogenesis

Parthenogenesis is the creation of an embryo without fertilizing the egg with a sperm, thus omitting the sperm's genetic contributions. To achieve this feat, scientists "trick" the egg into believing it is fertilized, so that it will begin to divide and form a blastocyst (see Figure 8.3f). In 2007, Dr. E.S. Revazova and colleagues reported that they successfully used parthenogenesis to derive hESCs.[23] These stem cell lines, derived and grown using a human feeder cell layer, retained the genetic information of the egg donor and demonstrated characteristics of pluripotency. This technique may lead to the ability to generate tissue-matched cells for transplantation to treat women who are willing to provide their own egg cells.[24] It also offers an alternate method for deriving tissue-matched hESCs that does not require destruction of a fertilized embryo.

HUMAN STEM CELL LINES WHOSE POTENCY IS CURRENTLY BEING DETERMINED: AMNIOTIC FLUID STEM CELLS

Amniotic fluid surrounding the developing fetus contains cells shed by the fetus and is regularly collected from pregnant women during amniocentesis. In 2003, researchers identified a subset of cells in amniotic fluid that express Oct-4, a marker for pluripotent human stem cells that is expressed in ES cells and embryonic germ cells.[25] Since then, investigators have shown that amniotic fluid stem cells can differentiate into cells of all three embryonic germ layers and that these cells do not form tumors *in vivo*.[26,27]

For example, Anthony Atala and colleagues at the Wake Forest University have recently generated non-embryonic stem cell lines from cells found in human and rat amniotic fluid.[27] They named these cells amniotic fluid-derived stem cells (AFS). Experiments demonstrate that AFS can produce cells that originate from each of the three embryonic germ layers, and the self-renewing cells maintained the normal number of chromosomes after a prolonged period in culture. However, undifferentiated AFS did not produce all of the proteins expected of pluripotent cells, and they were not capable of forming a teratoma. The scientists developed *in vitro* conditions that enabled AFS to produce nerve cells, liver cells, and bone-forming cells. AFS-derived human nerve cells could make proteins typical of specialized nerve cells and were able to integrate into a mouse brain and survive for at least two months. Cultured AFS-derived human liver cells secreted urea and made proteins characteristic of normal human liver cells. Cultured AFS-derived human bone cells made proteins expected of human bone cells and formed bone in mice when seeded onto scaffolds and implanted under the mouse's skin. Although scientists do not yet know how many different cell types AFS can generate, AFS may one day allow researchers to establish a bank of cells for transplantation into humans.

STRATEGIES TO "REPROGRAM" NON-PLURIPOTENT CELLS TO BECOME PLURIPOTENT CELLS

An alternative to searching for an existing population of stem cells is to create a new one from a population of non-pluripotent cells. This strategy, which may or may not involve the creation of an embryo, is known as "reprogramming." This section will summarize reprogramming approaches, including several recent breakthroughs in the field.

Reprogramming through Somatic Cell Nuclear Transfer (SCNT)

In SCNT (see Figure 8.3g), human oocytes (eggs) are collected from a volunteer donor who has taken drugs that stimulate the production of more than one oocyte during the menstrual cycle. Scientists then remove the nucleus from the donated oocyte and replace it with the nucleus from a somatic cell, a differentiated adult cell from elsewhere in the body. The oocyte with the newly-transferred nucleus is then stimulated to develop. The oocyte may develop only if the transplanted nucleus is returned to the pluripotent state by factors present in the oocyte cytoplasm. This alteration in the state of the mature nucleus is called nuclear reprogramming. When development progresses to the blastocyst stage, the ICM is removed and placed into culture in an attempt to establish a pluripotent stem cell line. To date, the technique has been successfully demonstrated in two primates: macaque monkeys[28] and humans.[29]

However, successful SCNT creates an embryo-like entity, thereby raising the ethical issues that confront the use of spare IVF embryos. However, pluripotent cell lines created by embryos generated by SCNT offer several advantages over ES cells. First, the nuclear genes of such a pluripotent cell line will be identical to the genes in the donor nucleus. If the nucleus comes from a cell that carries a mutation underlying a human genetic disease such as Huntington's disease, then all cells derived from the pluripotent cell line will carry this mutation. In this case, the SCNT procedure would enable the development of cellular models of human genetic disease that can inform our understanding of the biology of disease and facilitate development of drugs to slow or halt disease progression. Alternatively, if the cell providing the donor nucleus comes from a specific patient, all cells derived from the resulting pluripotent cell line will be genetically matched to the patient with respect to the nuclear genome. If these cells were used in transplantation therapy, the likelihood that the patient's immune system would recognize the transplanted cells as foreign and initiate tissue rejection would be reduced. However, because mitochondria also contain DNA, the donor oocyte will be the source of the mitochondrial genome, which is likely to carry mitochondrial gene differences from the patient which may still lead to tissue rejection.

A technique reported in 2007 by Dr. Kevin Eggan and colleagues at Harvard University may expand scientists' options when trying to "reprogram" an adult cell's DNA[30]. Previously, successful SCNT relied upon the use of an unfertilized egg. Now, the Harvard scientists have demonstrated that by using a drug to stop cell division in a fertilized mouse egg (zygote) during mitosis, they can successfully reprogram an adult mouse skin cell by taking advantage of the "reprogramming factors" that are active in the zygote at mitosis. They removed the chromosomes from the single-celled zygote's nucleus and replaced them with the adult donor cell's chromosomes (see Figure 8.3h). The active reprogramming factors present in the zygote turned genes on and off in the adult donor chromosomes, to make them behave like the chromosomes of a normally fertilized zygote. After the zygote was stimulated to divide, the cloned mouse embryo developed to the blastocyst stage, and the scientists were able to harvest embryonic stem cells from the resulting blastocyst. When the scientists applied their new method to abnormal mouse zygotes, they succeeded at reprogramming adult mouse skin cells and harvesting stem cells. If this technique can be repeated with abnormal human zygotes created in excess after IVF procedures, scientists could use them for research instead of discarding them as medical waste.

Reprogramming Through Altered Nuclear Transfer (ANT)

Altered nuclear transfer is a variation on standard SCNT that proposes to create patient-specific stem cells without destroying an embryo. In ANT, scientists turn off a gene needed for implantation in the uterus *(Cdx2)* in the patient cell nucleus before it is transferred into the donor egg (see Figure 8.3i). In 2006, Dr. Rudolph

Jaenisch and colleagues at MIT demonstrated that ANT can be carried out in mice.[31] Mouse ANT entities whose *Cdx2* gene is switched off are unable to implant in the uterus and do not survive to birth. Although ANT has been used to create viable stem cell lines capable of producing almost all cell types, the authors point out that this technique must still be tested with monkey and human embryos. Moreover, the manipulation needed to control *Cdx2* expression introduces another logistical hurdle that may complicate the use of ANT to derive embryonic stem cells. Proponents of ANT, such as William Hurlbut of the Stanford University Medical Center, suggest that the entity created by ANT is not a true embryo because it cannot implant in the uterus.[32,33] However, the technique is highly controversial, and its ethical implications remain a source of current debate.[3,32]

Reprogramming Through Cell Fusion

In 2005, Kevin Eggan and colleagues at Harvard University reported that they had fused cultured adult human skin cells with hESCs (see Figure 8.3j).[36] The resulting "hybrid" cells featured many characteristics of hESCs, including a similar manner of growth and division and the manufacture of proteins typically produced by hESCs. Some factor(s) within the hESCs enabled them to "reprogram" the adult skin cells to behave as hESCs. However, these cells raised a significant technical barrier to clinical use. Because fused cells are tetraploid (they contain four copies of the cellular DNA rather than the normal two copies), scientists would need to develop a method to remove the extra DNA without eliminating their hESC-like properties. The fusion method serves as a useful model system for studying how stem cells "reprogram" adult cells to have properties of pluripotent cells. However, if the reprogramming technique could be carried out without the fusion strategy, a powerful avenue for creating patient-specific stem cells without using human eggs could be developed.

Induced Pluripotent Stem Cells (iPSCs): Reprogramming Adult Somatic Cells to Become Pluripotent Stem Cells

In 2007, two independent research groups published manuscripts that described successful genetic reprogramming of human adult somatic cells into pluripotent human stem cells.[34,35] Although some technical limitations remain, this strategy suggests a promising new avenue for generating pluripotent cell lines that can inform drug development, models of disease, and ultimately, transplantation medicine. These experiments, which are discussed below, were breakthroughs because they used adult somatic cells to create pluripotent stem cells that featured hallmarks of ES cells.

In 2006, Shinya Yamanaka and colleagues at Kyoto University reported that they could use a retroviral expression vector to introduce four important stem cell factors into adult mouse cells and reprogram them to behave like ES cells (see Figure 8.3k).[37] They called the reprogrammed cells "iPSCs," for induced pluripotent stem cells. However, iPSCs produced using the original technique failed to produce sperm and egg cells when injected into an early mouse blastocyst and did not make certain critical DNA changes. These researchers then modified the technique to select for iPSCs that can produce sperm and eggs,[38] results that have since been reproduced by Rudolph Jaenisch and colleagues at the Massachusetts Institute of Technology (MIT).[39] In addition, the MIT scientists determined that iPSCs DNA is modified in a manner similar to ES cells, and important stem cell genes are expressed at similar levels. They also demonstrated that iPSCs injected into an early mouse blastocyst can produce all cell types within the developing embryo, and such embryos can complete gestation and are born alive.

Once these research advances were made in mice, they suggested that similar techniques might be used to reprogram adult human cells. In 2007, Yamanaka and coworkers reported that introducing the same four genetic factors that reprogrammed the mouse cells into adult human dermal fibroblasts reprogrammed the cells into human iPSCs.[35] These iPSCs were similar to human ES cells in numerous ways, including morphology, proliferative capacity, expression of cell surface antigens, and gene expression. Moreover, the cells could differentiate into cell types from the three embryonic germ layers both *in vitro* and in teratoma assays. Concurrent with the Yamanaka report, James Thomson and coworkers at the University of Wisconsin published a separate manuscript that detailed the creation of human iPSCs through somatic cell reprogramming using four genetic factors (two of which were in common with the Yamanaka report).[34] The cells generated by the Thomson group met all

defining criteria for ES cells, with the exception that they were not derived from embryos.

These breakthroughs have spurred interest in the field of iPSCs research. In early 2008, investigators at the Massachusetts General Hospital[40] and the University of California, Los Angeles[41] reported generating reprogrammed cells. As scientists explore the mechanisms that govern reprogramming, it is anticipated that more reports will be forthcoming in this emerging area. Although these reprogramming methods require the use of a virus, non-viral strategies may also be possible in the future. In any case, these approaches have created powerful new tools to enable the "dedifferentation" of cells that scientists had previously believed to be terminally differentiated.[42,43]

Although further study is warranted to determine if iPS and ES cells differ in clinically significant ways, these breakthrough reports suggest that reprogramming is a promising strategy for future clinical applications. Induced pluripotent cells offer the obvious advantage that they are not derived from embryonic tissues, thereby circumventing the ethical issues that surround use of these materials. Successful reprogramming of adult somatic cells could also lead to the development of stem cell lines from patients who suffer from genetically-based diseases, such as Huntington's Disease, spinal muscular atrophy, muscular dystrophy, and thalessemia. These lines would be invaluable research tools to understand the mechanisms of these diseases and to test potential drug treatments. Additionally, reprogrammed cells could potentially be used to repair damaged tissues; patient-specific cell lines could greatly reduce the concerns of immune rejection that are prevalent with many transplantation strategies.

However, several technical hurdles must be overcome before iPSCs can be used in humans. For example, in preliminary experiments with mice, the virus used to introduce the stem cell factors sometimes caused cancers.[37] The viral vectors used in these experiments will have to be selected carefully and tested fully to verify that they do not integrate into the genome, thereby harboring the potential to introduce genetic mutations at their site of insertion. This represents a significant concern that must be addressed before the technique can lead to useful treatments for humans. However, this strategy identifies a method for creating pluripotent stem cells that, together with studies of other types of pluripotent stem cells, will help researchers learn how to reprogram cells to repair damaged tissues in the human body.

OTHER SOURCES OF PLURIPOTENT AND/OR MULTIPOTENT CELLS

Stem cell research is a rapidly evolving field, and researchers continue to isolate new pluripotent cells and create additional cell lines. This section briefly reviews other sources of pluripotent cells and the implications that their discovery may have on future research.

Epiblast Cells. While rodent and human ES cells are pluripotent, they maintain their respective pluripotencies through different molecular signaling pathways. It is not known why these differences exist. Recently, several research groups have reported the generation of stable, pluripotent cell lines from mouse and rat epiblast, a tissue of the post-implantation embryo that ultimately generates the embryo proper.[44,45] These cells are distinct from mouse ES cells in terms of the signals that control their differentiation. However, the cells share patterns of gene expression and signaling responses with human ES cells. The establishment of epiblast cell lines can therefore provide insight into the distinctions between pluripotent cells from different species and illuminate ways that pluripotent cells pursue distinct fates during early development.

Existing Adult Stem Cells. As has been discussed in other chapters, numerous types of precursor cells have been isolated in adult tissues.[46] Although these cells tend to be relatively rare and are dispersed throughout the tissues, they hold great potential for clinical application and tissue engineering. For example, tissues created using stem cells harvested from an adult patient could theoretically be used clinically in that patient without engendering an immune response. Moreover, the use of adult stem cells avoids the ethical concerns associated with the use of ES cells. In addition, adult-derived stem cells do not spontaneously differentiate as do ES cells, thus eliminating the formation of teratomas often seen with implantation of ES cells. The potential of adult stem cells for regenerative medicine is great; it is likely that these various cells will find clinical application in the upcoming decades.

CONCLUSION: PLURIPOTENT CELL LINES ARE TOOLS FOR FUTURE RESEARCH

Although the recent advances in reprogramming of adult somatic cells has generated a wave of interest in the scientific community, these cell lines will not likely replace hESC lines as tools for research and discovery. Rather, both categories of cells will find unique uses in the study of stem cell biology and the development and evaluation of therapeutic strategies. Pluripotent cells offer a number of potential clinical applications, especially for diseases with a genetic basis. However, researchers are just beginning to unlock the many factors that govern the cells' growth and differentiation. As scientists make strides toward understanding how these cells can be manipulated, additional applications, approaches, and techniques will likely emerge. As such, pluripotent cells will play a pivotal role in future research into the biology of development and the treatment of disease.

REFERENCES

1. Domen J, Wagers A, Weissman IL. Bone marrow (hematopoietic) stem cells. *Regenerative Medicine* [http://stemcells.nih.gov/info/scireport/2006report. Accessed February 22, 2008.
2. Rao M, Condic ML. Alternative sources of pluripotent stem cells: scientific solutions to an ethical dilemma. *Stem Cells Dev.* 2008;17:1-10.
3. The President's Council on Bioethics. White paper: alternative sources of human pluripotent stem cells. http://www.bioethics.gov/reports/white_paper/index.html Accessed February 26, 2008.
4. Downing GJ, Battey JF Jr. Technical assessment of the first 20 years of research using mouse embryonic stem cell lines. *Stem Cells.* 2004;22:1168-1180.
5. Bongso A, Chui-Yee F, Soon-Chye N, Ratnam S. Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 1994; 9: 2110-2117.
6. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998;282:1145-1147.
7. Hoffman DI, Zellman GL, Fair CC, et al. Cryopreserved embryos in the United States and their availability for research. *Fertil Steril.* 2003;79:1063-1069.
8. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol.* 2001;19:1129-1133.
9. He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res.* 2003;93:32-39.
10. Wang L, Li L, Menendez P, Cerdan C, Bhatia M. Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. *Blood.* 2005;105:4598-4603.
11. 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.* 2002;20:933-936.
12. Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells.* 2003;21:546-556.
13. Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol.* 2001;19:971-974.
14. Lim JWE, Bodnar A. Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. *Proteomics.* 2002;2:1187-1203.
15. Shamblott MJ, Axelman J, Wang S, et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA.* 1998;95:13726-13731.
16. Turnpenny L, Brickwood S, Spalluto CM, et al. Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. *Stem Cells.* 2003;21:598-609.
17. Aflatoonian B, Moore H. Human primordial germ cells and embryonic germ cells, and their use in cell therapy. *Curr Opin Biotechnol.* 2005;16:530-535.
18. Zhang X, Stojkovic P, Przyborski S, et al. Derivation of human embryonic stem cells from developing and arrested embryos. *Stem Cells.* 2006;24:2669-2676.
19. Landry DW, Zucker HA. Embryonic death and the creation of human embryonic stem cells. *J Clin Invest.* 2004;114:1184-1186.
20. Verlinsky Y, Strelchenko N, Kukharenko V, et al. Human embryonic stem cell lines with genetic disorders. *Reprod Biomed Online.* 2005;10:105-110.
21. Chung Y, Klimanskaya I, Becker S, et al. Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres. *Nature.* 2006;439:216-219.
22. Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R. Human embryonic stem cell lines derived from single blastomeres. *Nature.* 2006;444:481-485.
23. Revazova ES, Turovets NA, Kochetkova OD, et al. Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells.* 2007;9:432-449.

24. Revazova ES, Turovets NA, Kochetkova OD, et al. HLA homozygous stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells.* 2007;Dec 19:epub ahead of print.

25. Prusa AR, Marton E, Rosner M, Bernaschek G, M H. Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? *Hum Reprod.* 2003;18:1489-1493.

26. Siegel N, Rosner M, Hanneder M, Valli A, Hengstschlager M. Stem cells in amniotic fluid as new tools to study human genetic diseases. *Stem Cell Rev.* 2007;3:256-264.

27. De Coppi P, Bartsch G Jr, Siddiqui MM, et al. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol.* 2007;25:100-106.

28. Byrne JA, Pedersen DA, Clepper LL, et al. Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature.* 2007;450:497-502.

29. French A, Adams C, Anderson L, Kitchen J, Hughes M, Wood S. Development of human cloned blastocysts following somatic cell nuclear transfer (SCNT) with adult fibroblasts. *Stem Cells.* 2008;Epub ahead of print.

30. Egli, D, Rosains, J, Birkhoff, G, Eggan, K. Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature.* 2007; 447: 679-685.

31. Meissner A, Jaenisch R. Generation of nuclear transfer-derived pluripotent ES cells from cloned *Cdx2*-deficient blastocysts. *Nature.* 2006;439:212-215.

32. Hurlbut WB. Ethics and embryonic stem cell research: altered nuclear transfer as a way forward. *BioDrugs.* 2007;21:79-83.

33. Hurlbut WB. Altered nuclear transfer: a way forward for embryonic stem cell research. *Stem Cell Rev.* 2005;1:293-300.

34. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318:1917-1920.

35. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131:1-12.

36. Cowan CA, Atienza J, Melton DA, Eggan K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science.* 2005;309:1369-1373.

37. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126:663-676.

38. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature.* 2007;448:313-317.

38. Wernig M, Meissner A, Foreman R, et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature.* 2007;448:318-324.

40. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature.* 2008;451:141-146.

41. Lowry WE, Richter L, Yachechko R, et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci USA.* 2008; Epub ahead of print.

42. Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell.* 2008;132:567-582.

43. Collas P. Dedifferentiation of cells: new approaches. *Cytotherapy.* 2007;9:236-244.

44. Tesar PJ, Chenoweth JG, Brook FA, et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature.* 2007;448:196-199.

45. Brons IGM, Smither LE, Trotter MWB, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature.* 2007;448:191-195.

46. Young HE, Duplaa C, Katz R, et al. Adult-derived stem cells and their potential for use in tissue repair and molecular medicine. *J Cell Mol Med.* 2005;9:753-769.

9. ARE STEM CELLS INVOLVED IN CANCER?

Charles A. Goldthwaite, Jr., Ph.D.

CANCER: IMPACT AND CHALLENGES

Data from 2007 suggest that approximately 1.4 million men and women in the U.S. population should be diagnosed with cancer, and approximately 566,000 American adults should die from cancer in 2008.[1] Based on prevalence rates from 2003–2005, it has been estimated that 40% of men and women born today will develop cancer in their lifetimes.[1] Data collected between 1996 and 2004 indicate that the overall 5-year survival rate for cancers from all sites, relative to the expected survival from a comparable set of people without cancer, is 65.3%.[1] However, survival and recurrence rates following diagnosis vary greatly as a function of cancer type and the stage of development at diagnosis. For example, in 2000, the relative survival rate five years following diagnosis of melanoma (skin cancer) was greater than 90%; that of cancers of the brain and nervous system was 35%. Once a cancer has metastasized (or spread to secondary sites via the blood or lymph system), however, the survival rate usually declines dramatically. For example, when melanoma is diagnosed at the localized stage, 99% of people will survive more than five years, compared to 65% of those diagnosed with melanoma that has metastasized regionally and 15% of those whose melanoma has spread to distant sites.[2]

The term "cancer" describes a group of diseases that are characterized by uncontrolled cellular growth, cellular invasion into adjacent tissues, and the potential to metastasize if not treated at a sufficiently early stage. These cellular aberrations arise from accumulated genetic modifications, either via changes in the underlying genetic sequence or from epigenetic alterations (e.g., modifications to gene activation- or DNA-related proteins that do not affect the genetic sequence itself).[3,4] Cancers may form tumors in solid organs, such as the lung, brain, or liver, or be present as malignancies in tissues such as the blood or lymph. Tumors and other structures that result from aberrant cell growth, contain heterogeneous cell populations with diverse biological characteristics and potentials. As such, a researcher sequencing all of the genes from tumor specimens of two individuals diagnosed with the same type of lung cancer will identify some consistencies along with many differences. In fact, cancerous tissues are sufficiently heterogeneous that the researcher will likely identify differences in the genetic profiles between several tissue samples from the same specimen. While some groupings of genes allow scientists to classify organ- or tissue-specific cancers into subcategories that may ultimately inform treatment and provide predictive information, the remarkable complexity of cancer biology continues to confound treatment efforts.

Once a cancer has been diagnosed, treatments vary according to cancer type and severity. Surgery, radiation therapy, and systemic treatments such as chemotherapy or hormonal therapy represent traditional approaches designed to remove or kill rapidly-dividing cancer cells. These methods have limitations in clinical use. For example, cancer surgeons may be unable to remove all of the tumor tissue due to its location or extent of spreading. Radiation and chemotherapy, on the other hand, are non-specific strategies – while targeting rapidly-dividing cells, these treatments often destroy healthy tissue as well. Recently, several agents that target specific proteins implicated in cancer-associated molecular pathways have been developed for clinical use. These include trastuzumab, a monoclonal antibody that targets the protein HER2 in breast cancer,[5] gefitinib and erlotnib, which target epidermal growth factor receptor (EGFR) in lung cancer,[6] imatinib, which targets the BCR-ABL tyrosine kinase in chronic myelogenous leukemia,[7] the monoclonal antibodies bevacizumab, which targets vascular endothelial growth factor in colorectal and lung cancer,[8] and cetuximab and panitumumab, which target EGFR in colorectal cancer.[8] These agents have shown that a targeted approach can be successful,

although they are effective only in patients who feature select subclasses of these respective cancers.

All of these treatments are most successful when a cancer is localized; most fail in the metastatic setting.[9] The key to treatment therefore lies in the ability to retard or halt the processes of aberrant cell division common to all cancers. To address this challenge, researchers have sought to understand how tumor cells override the signals for cell division that restrain other cells and how cancer cells can successfully create a neoplasm in a new tissue microenvironment. Based on what is known about tumoral heterogeneity and the replication process, cells that initiate tumor formation must somehow accomplish two feats: 1) generate numerous daughter cells without dying and 2) differentiate into a variety of cell types. Given that stem cells renew themselves indefinitely and have the potential to differentiate into various cell types, researchers have begun to gather evidence to support a "cancer stem cell" (CSC) hypothesis, an evolving theory that explains tumor formation as the outcome of consecutive genetic changes in a small population of stem cell-like, tumor-forming cells.[9–11] This article will discuss the CSC hypothesis and its supporting evidence and provide some perspectives on how CSCs could impact the development of future cancer therapy.

DEFINING THE "CANCER STEM CELL"

A consensus panel convened by the American Association of Cancer Research has defined a CSC as "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor."[12] It should be noted that this definition does not indicate the source of these cells – these tumor-forming cells could hypothetically originate from stem, progenitor, or differentiated cells.[13] As such, the terms "tumor-initiating cell" or "cancer-initiating cell" are sometimes used instead of "cancer stem cell" to avoid confusion. Tumors originate from the transformation of normal cells through the accumulation of genetic modifications, but it has not been established unequivocally that stem cells are the origin of all CSCs. The CSC hypothesis therefore does not imply that cancer is always caused by stem cells or that the potential application of stem cells to treat conditions such as heart disease or diabetes as discussed in other chapters of this guide will result in tumor formation. Rather, tumor-initiating cells possess stem-like characteristics to a degree sufficient to warrant the comparison with stem cells; the observed experimental and clinical behaviors of metastatic cancer cells are highly reminiscent of the classical properties of stem cells.[9]

THE CSC HYPOTHESIS AND THE SEARCH FOR CSCs

The CSC hypothesis suggests that the malignancies associated with cancer originate from a small population of stem-like, tumor-initiating cells. Although cancer researchers first isolated CSCs in 1994,[14] the concept dates to the mid-19th century. In 1855, German pathologist Rudolf Virchow proposed that cancers arise from the activation of dormant, embryonic-like cells present in mature tissue.[15] Virchow argued that cancer does not simply appear spontaneously; rather, cancerous cells, like their non-cancerous counterparts, must originate from other living cells. One hundred and fifty years after Virchow's observation, Lapidot and colleagues provided the first solid evidence to support the CSC hypothesis when they used cell-surface protein markers to identify a relatively rare population of stem-like cells in acute myeloid leukemia (AML).[14] Present in the peripheral blood of persons with leukemia at approximately 1:250,000 cells, these cells could initiate human AML when transplanted into mice with compromised immune systems. Subsequent analysis of populations of leukemia-initiating cells from various AML subtypes indicated that the cells were relatively immature in terms of differentiation.[16] In other words, the cells were "stem-like" – more closely related to primitive blood-forming (hematopoietic) stem cells than to more mature, committed blood cells.

The identification of leukemia-inducing cells has fostered an intense effort to isolate and characterize CSCs in solid tumors. Stem cell-like populations have since been characterized using cell-surface protein markers in tumors of the breast,[17] colon,[18] brain,[19] pancreas,[20,21] and prostate.[22,23] However, identifying markers that unequivocally characterize a population of CSCs remains challenging, even when there is evidence that putative CSCs exist in a given solid tumor type. For example, in hepatocellular carcinoma, cellular analysis suggests the presence of stem-like cells.[24] Definitive markers have yet to be identified to characterize these putative CSCs, although several potential candidates

have been proposed recently.[25,26] In other cancers in which CSCs have yet to be identified, researchers are beginning to link established stem-cell markers with malignant cancer cells. For instance, the proteins Nanog, nucleostemin, and musashi1, which are highly expressed in embryonic stem cells and are critical to maintaining those cells' pluripotency, are also highly expressed in malignant cervical epithelial cells.[27] While this finding does not indicate the existence of cervical cancer CSCs, it suggests that these proteins may play roles in cervical carcinogenesis and progression.

DO CSCs ARISE FROM STEM CELLS?

Given the similarities between tumor-initiating cells and stem cells, researchers have sought to determine whether CSCs arise from stem cells, progenitor cells, or differentiated cells present in adult tissue. Of course, different malignancies may present different answers to this question. The issue is currently under debate,[9,12] and this section will review several theories about the cellular precursors of cancer cells (see Fig. 9.1).

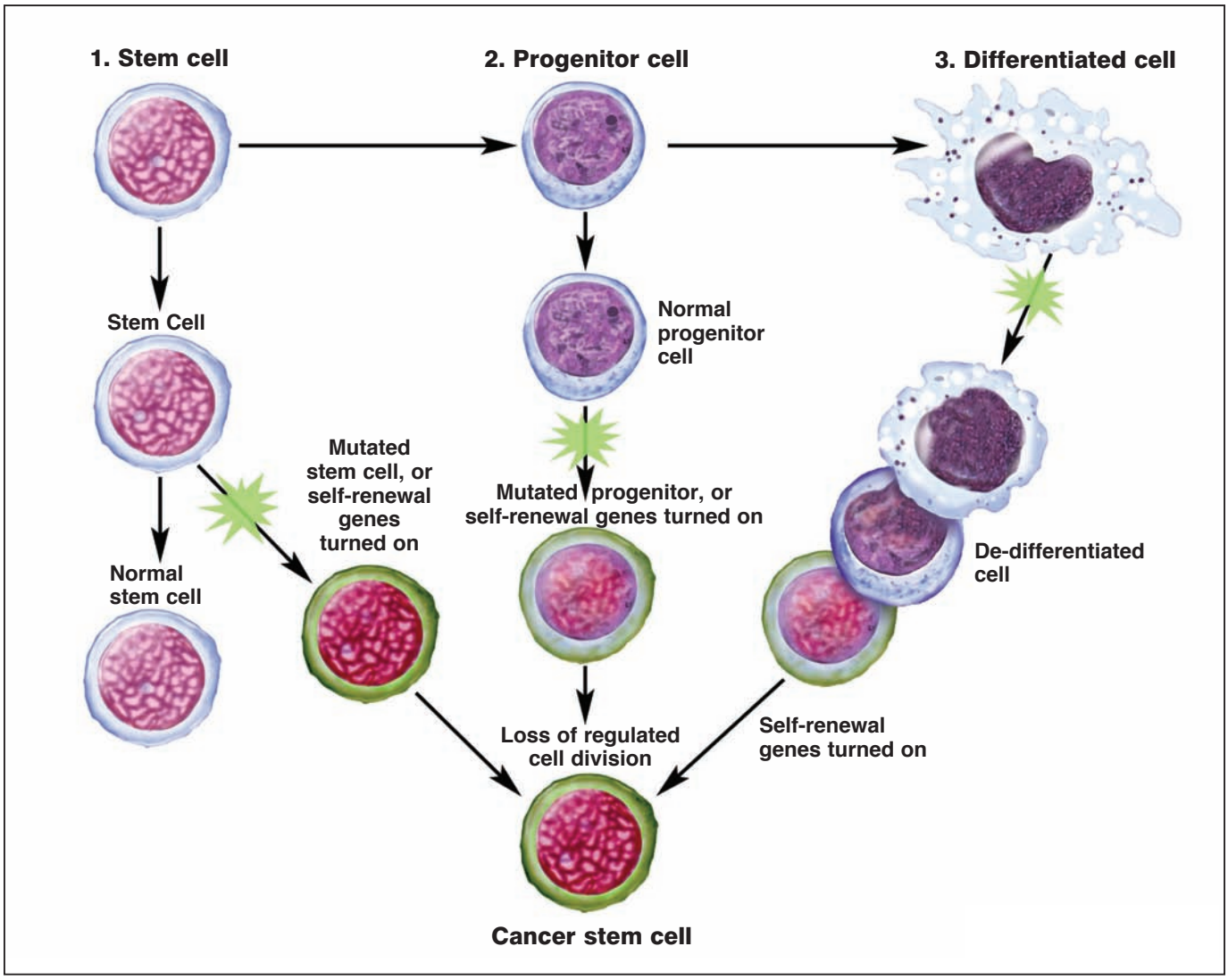


Figure 9.1. How Do Cancer Stem Cells Arise?
The molecular pathways that maintain "stem-ness" in stem cells are also active in numerous cancers. This similarity has led scientists to propose that cancers may arise when some event produces a mutation in a stem cell, robbing it of the ability to regulate cell division. This figure illustrates 3 hypotheses of how a cancer stem cell may arise: (1) A stem cell undergoes a mutation, (2) A progenitor cell undergoes two or more mutations, or (3) A fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all 3 scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division.

Hypothesis #1: Cancer Cells Arise from Stem Cells. Stem cells are distinguished from other cells by two characteristics: (1) they can divide to produce copies of themselves, or self-renew, under appropriate conditions and (2) they are pluripotent, or able to differentiate into most, if not all, mature cell types. If CSCs arise from normal stem cells present in the adult tissue, de-differentiation would not be necessary for tumor formation. In this scenario, cancer cells could simply utilize the existing stem-cell regulatory pathways to promote their self-renewal. The ability to self-renew gives stem cells long lifespans relative to those of mature, differentiated cells.[30] It has therefore been hypothesized that the limited lifespan of a mature cell makes it less likely to live long enough to undergo the multiple mutations necessary for tumor formation and metastasis.

Several characteristics of the leukemia-initiating cells support the stem-cell origin hypothesis. Recently, the CSCs associated with AML have been shown to comprise distinct, hierarchically-arranged classes (similar to those observed with hematopoietic stem cells) that dictate distinct fates.[31] To investigate whether these CSCs derive from hematopoietic stem cells, researchers have used a technique known as serial dilution to determine the CSCs' ability to self-renew. Serial dilution involves transplanting cells (usually hematopoietic stem cells, but in this case, CSCs) into a mouse during a bone-marrow transplant. Prior to the transplant, this "primary recipient" mouse's natural supply of hematopoietic stem cells is ablated. If the transplant is successful and if the cells undergo substantial self-renewal, the primary recipient can then become a successful donor for a subsequent, or serial, transplant. Following cell division within primary recipients, a subset of the AML-associated CSCs divided only rarely and underwent self-renewal instead of committing to a lineage. This heterogeneity in self-renewal potential supports the hypothesis that these CSCs derive from normal hematopoietic stem cells.[31] It should be noted, however, that the leukemia-inducing cells are the longest-studied of the known CSCs; the identification and characterization of other CSCs will allow researchers to understand more about the origin of these unique cells.

Hypothesis #2: Cancer Cells Arise from Progenitor Cells. The differentiation pathway from a stem cell to a differentiated cell usually involves one or more intermediate cell types. These intermediate cells, which are more abundant in adult tissue than are stem cells, are called progenitor or precursor cells. They are partly-differentiated cells present in fetal and adult tissues that usually divide to produce mature cells. However, they retain a partial capacity for self-renewal. This property, when considered with their abundance relative to stem cells in adult tissue, has led some researchers to postulate that progenitor cells could be a source of CSCs.[32,33]

Hypothesis #3: Cancer Cells Arise from Differentiated Cells. Some researchers have suggested that cancer cells could arise from mature, differentiated cells that somehow de-differentiate to become more stem cell-like. In this scenario, the requisite oncogenic (cancer-causing) genetic mutations would need to drive the de-differentiation process as well as the subsequent self-renewal of the proliferating cells. This model leaves open the possibility that a relatively large population of cells in the tissue could have tumorigenic potential; a small subset of these would actually initiate the tumor. Specific mechanisms to select which cells would de-differentiate have not been proposed. However, if a tissue contains a sufficient population of differentiated cells, the laws of probability indicate that a small portion of them could, in principle, undergo the sequence of events necessary for de-differentiation. Moreover, this sequence may contain surprisingly few steps; researchers have recently demonstrated that human adult somatic cells can be genetically "re-programmed" into pluripotent human stem cells by applying only four stem-cell factors (see the chapter, "Alternate Methods for Preparing Pluripotent Stem Cells" for detailed discussion of inducing pluripotent stem cells).[28,29]

HOW CANCER STEM CELLS COULD SUPPORT METASTASIS

Metastasis is a complex, multi-step process that involves a specific sequence of events; namely, cancer cells must escape from the original tumor, migrate through the blood or lymph to a new site, adhere to the new site, move from the circulation into the local tissue, form micrometastases, develop a blood supply, and grow to form macroscopic and clinically relevant metastases.[9,34,35] Perhaps not surprisingly, metastasis is highly inefficient.[9] It has been estimated that less than 2% of solitary cells that successfully migrate to a new site are able to initiate growth once there.[34,36,37] Moreover, less than 1% of cells that initiate growth

at the secondary site are able to maintain this growth sufficiently to become macroscopic metastases.[36] These observations suggest that a small, and most likely specialized, subset of cancer cells drives the spread of disease to distant organs.

Some researchers have proposed that these unique cells may be CSCs.[9,30,32,33,38] In this hypothesis, metastatic inefficiency may reflect the relative rarity of CSCs combined with the varying compatibilities of these cells with destination microenvironments. Researchers have demonstrated that stem cells and metastatic cancer cells share several properties that are essential to the metastatic process, including the requirement of a specific microenvironment (or "niche") to support growth and provide protection, the use of specific cellular pathways for migration, enhanced resistance to cell death, and an increased capacity for drug resistance.[9] There is emerging, albeit limited, evidence that these properties may also hold for CSCs.[9] Metastatic sites for a given cancer type could therefore represent those tissues that provide or promote the development of a compatible CSC niche, from which CSCs could expand through normal or dysregulated cellular signaling. Moreover, normal stem cells tend to be quiescent unless activated to divide.[39] If the CSC hypothesis holds true, then undifferentiated, dormant CSCs would be relatively resistant to chemotherapeutic agents, which act mainly on dividing cells.[10] As such, this subpopulation could form the kernel of cells responsible for metastasis and cancer recurrence following treatment and remission.

HOW THE CSC MODEL COULD AFFECT CANCER THERAPY

As noted previously, most contemporary cancer treatments have limited selectivity – systemic therapies and surgeries remove or damage normal tissue in addition to tumor tissue. These methods must therefore be employed judiciously to limit adverse effects associated with treatment. Moreover, these approaches are often only temporarily effective; cancers that appear to be successfully eliminated immediately following treatment may recur at a later time and often do so at a new site. Agents that target molecules implicated in cancer pathways have illustrated the power of a selective approach, and many researchers and drug developers are shifting toward this paradigm. If the CSC hypothesis proves to be correct, then a strategy designed to target CSCs selectively could potentially stop the "seeds" of the tumor before they have a chance to germinate and spread.

The CSC hypothesis accounts for observed patterns of cancer recurrence and metastasis following an apparently successful therapeutic intervention. In clinical practice, however, some cancers prove quite aggressive, resisting chemotherapy or radiation even when administered at relatively early stages of tumor progression. These tumors therefore have an increased likelihood of metastasizing, confounding further treatment strategies while compromising the cancer patient's quality of life. The presence of CSC in some malignancies may account for some of these metastases. So why do some tumors succumb to therapy, while others resist it? Some scientists have suggested that the tumor aggressiveness may correlate with the proportion of CSCs within a corresponding tumor.[40–42] In this scenario, less aggressive cancers contain fewer CSCs and a greater proportion of therapy-sensitive non-CSCs.[9]

There is also some evidence to suggest that CSCs may be able to selectively resist many current cancer therapies, although this property has yet to be proven in the clinic.[9] For example, normal stem cells and metastatic cancer cells over-express several common, known drug-resistance genes.[43] As a result, breast cancer CSCs express increased levels of several membrane proteins implicated in resistance to chemotherapy.[17] These cells have also been shown to express intercellular signaling molecules such as Hedgehog and Bmi-1,[44] which are essential for promoting self-renewal and proliferation of several types of stem cells.[45] Moreover, experiments in cell lines from breast cancer[46] and glioma[40] have shown that CSCs (as identified by cell-surface markers) are more resistant to radiotherapy than their non-CSC counterparts. In the face of radiation, the CSCs appear to survive preferentially, repair their damaged DNA more efficiently, and begin the process of self-renewal.

These discoveries have led researchers to propose several avenues for treating cancer by targeting molecules involved in CSC renewal and proliferation pathways. Potential strategies include interfering with molecular pathways that increase drug resistance, targeting proteins that may sensitize CSCs to radiation, or restraining the CSCs' self-renewal capacity by modifying their cell differentiation capabilities.[9] In each case, successful development of a therapy

would require additional basic and clinical research. Researchers must characterize the CSCs associated with a given tumor type, identify relevant molecules to target, develop effective agents, and test the agents in pre-clinical models, such as animals or cell lines. However, by targeting fundamental CSC cellular signaling processes, it is possible that a given treatment could be effective against multiple tumor types.

CONCLUSION

Cancer represents a major health challenge for the 21st century. Governed by an intricate, complex interplay of molecular signals, cancers often resist systemic treatments. Yet the uncontrolled cellular growth that characterizes cancers may paradoxically hold the key to understanding the spread of disease. It has long been postulated that tumors form and proliferate from the actions of a small population of unique cells. The observation that metastatic cancer cells exhibit experimental and clinical behaviors highly reminiscent of the classical properties of stem cells has led researchers to search for and to characterize "cancer stem cells" believed to be implicated in the cancer process.

The discovery of CSCs in some tumor types has ushered in a new era of cancer research. Cancer stem cell science is an emerging field that will ultimately impact researchers' understanding of cancer processes and may identify new therapeutic strategies. However, much remains to be learned about these unique cells, which as of yet have not been identified in all tumor types. At present, evidence continues to mount to support a CSC Hypothesis – that cancers are perpetuated by a small population of tumor-initiating cells that exhibit numerous stem cell-like properties. Whether or not the Hypothesis ultimately proves true in all cases, understanding the similarities between cancer cells and stem cells will illuminate many molecular pathways that are triggered in carcinogenesis. Thus, the question, "Are stem cells involved in cancer?" has no simple answer. However, the characterization of CSCs will likely play a role in the development of novel targeted therapies designed to eradicate the most dangerous tumor cells, that may be resistant to current chemotherapy regimens, thereby providing researchers and clinicians with additional targets to alleviate the burden of cancer.

REFERENCES

1. National Cancer Institute. Surveillance Epidemiology and End Results: SEER stat fact sheets. http://seer.cancer.gov/statfacts/html/all.html#ref04. Accessed February 15, 2009.
2. American Cancer Society. Cancer Facts & Figures 2008. http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf. Accessed February 15, 2009.
3. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Rev Genet.* 2006; 7:21–33.
4. Jones PA, Baylin SB. The epigenomics of cancer. *Cell.* 2007;128:683–692.
5. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med.* 2001;344:783–792.
6. Silvestri GA, Rivera MP. Targeted therapy for the treatment of advanced non-small cell lung cancer: a review of the epidermal growth factor receptor antagonists. *Chest.* 2005;128:3975–3984.
7. Sherbenou DW, Druker BJ. Applying the discovery of the Philadelphia chromosome. *J Clin Invest.* 2007; 117:2067–2074.
8. Hedge SR, Sun W, Lynch JP. Systemic and targeted therapy for advanced colon cancer. *Expert Rev Gastroenterol Hepatol.* 2008;2:135–149.
9. Croker AK, Allan AL. Cancer stem cells: implications for the progression and treatment of metastatic disease. *J Cell Mol Med.* 2008;12:374–390.
10. Gil J, Stembalska A, Pesz KA, Sasiadek MM. Cancer stem cells: the theory and perspectives in cancer therapy. *J App Genet.* 2008;49:193–199.
11. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414:105–111.
12. Clarke MF, Dick JE, Dirks PB, et al. Cancer Stem Cells – Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Res.* 2006; 66:9339–9344.
13. Rapp UR, Ceteci F, Schreck R. Oncogene-induced plasticity and cancer stem cells. *Cell Cycle.* 2008;7:45–51.
14. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* 1994;367:645–648.
15. Huntly BJP, Gilliland DG. Leukemia stem cells and the evolution of cancer stem cells. *Nat Rev Cancer.* 2005;5:311–321.
16. Bonnet D, Dick JE. Human myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3:730–737.

17. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA.* 2003;100:3983–3988.

18. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature.* 2007;445:106–110.

19. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumor initiating cells. *Nature.* 2004; 432:396–401.

20. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007;67:1030–1037.

21. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cancer Stem Cell.* 2007;1:313–323.

22. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland MJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* 2005;65:10946–10951.

23. Patrawala L, Calhoun T, Schneider-Broussard R, et al. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene.* 2006;25:1696–1708.

24. Sell S, Leffert HL. Liver cancer stem cells. *J Clin Oncol.* 2008;26:2800–2805.

25. Yang ZF, Ho DW, Ng MN, et al. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell.* 2008;13:153–166.

26. Yang ZF, Ngai P, Ho DW, et al. Identification of local and circulating cancer stem cells in human liver cancer. *Hepatology.* 2008;47:919–928.

27. Ye F, Zhou C, Cheng Q, Shen J, Chen H. Stem-cell abundant proteins Nanog, Nucleostemin and Musashi1 are highly expressed in malignant cervical epithelial cells. *BMC Cancer.* 2008;8:108.

28. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318:1917–1920.

29. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131:1–12.

30. Allan AL, Vantyghem SA, Tuck AB, Chambers AF. Tumor dormancy and cancer stem cells: implications for the biology and treatment of breast cancer metastasis. *Breast Dis.* 2007;26:87–98.

31. Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol.* 2004;5:738–743.

32. Li F, Tiede B, Massague J, Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. *Cell Res.* 2007;17:3–14.

33. Kucia M, Ratajczak MZ. Stem cells as a two-edged sword – from regeneration to tumor formation. *J Physiol Pharmacol.* 2006;57:5–16.

34. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* 2002;2:563–572.

35. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer.* 2004;4:448–456.

36. Luzzi KJ, MacDonald IC, Schmidt EE, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol.* 1998;153:865–873.

37. Weiss L. Metastatic inefficiency. *Adv Cancer Res.* 1990;54:159–211.

38. Vaidya JS. An alternative model of cancer cell growth and metastasis. *Int J Surg.* 2007;5:73–75.

39. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer.* 2003; 3:895-902.

40. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006;444:756–760.

41. Diehn M, Clarke MF. Cancer stem cells and radiotherapy: new insights into tumor radioresistance. *J Natl Cancer Inst.* 2006;98:1755–1757.

42. Smalley M, Ashworth A. Stem cells and breast cancer: a field in transit. *Nat Rev Cancer.* 2003;3:832–844.

43. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer.* 2005;5:275–284.

44. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and *Bmi-1* regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* 2006;66:6063–6071.

45. Park I-K, Morrison SJ, Clarke MF. *Bmi1,* stem cells, and senescence regulation. *J Clin Invest.* 2004;113:175–179.

46. Phillips TM, McBride WH, Pajonk F. The response of $CD24^{-/low}/CD44^{+}$ breast cancer-initiating cells to radiation. *J Natl Cancer Inst.* 2006;98:1777–1785.

10. THE PROMISE OF INDUCED PLURIPOTENT STEM CELLS (iPSCs)

Charles A. Goldthwaite, Jr., Ph.D.

In 2006, researchers at Kyoto University in Japan identified conditions that would allow specialized adult cells to be genetically "reprogrammed" to assume a stem cell-like state. These adult cells, called induced pluripotent stem cells (iPSCs), were reprogrammed to an embryonic stem cell-like state by introducing genes important for maintaining the essential properties of embryonic stem cells (ESCs). Since this initial discovery, researchers have rapidly improved the techniques to generate iPSCs, creating a powerful new way to "de-differentiate" cells whose developmental fates had been previously assumed to be determined.

Although much additional research is needed, investigators are beginning to focus on the potential utility of iPSCs as a tool for drug development, modeling of disease, and transplantation medicine. The idea that a patient's tissues could provide him/her a copious, immune-matched supply of pluripotent cells has captured the imagination of researchers and clinicians worldwide. Furthermore, ethical issues associated with the production of ESCs do not apply to iPSCs, which offer a non-controversial strategy to generate patient-specific stem cell lines. As an introduction to this exciting new field of stem cell research, this chapter will review the characteristics of iPSCs, the technical challenges that must be overcome before this strategy can be deployed, and the cells' potential applications to regenerative medicine.

"REPROGRAMMING" CELLS: ACHIEVING PLURIPOTENCY

As noted in other chapters, stem cells represent a precious commodity. Although present in embryonic and adult tissues, practical considerations such as obtaining embryonic tissues and isolating relatively rare cell types have limited the large-scale production of populations of pure stem cells (see the Chapter, "Alternate Methods for Preparing Pluripotent Stem Cells" for details). As such, the logistical challenges of isolating, culturing, purifying, and differentiating stem cell lines that are extracted from tissues have led researchers to explore options for "creating" pluripotent cells using existing non-pluripotent cells. Coaxing abundant, readily available differentiated cells to pluripotency would in principle eliminate the search for rare cells while providing the opportunity to culture clinically useful quantities of stem-like cells.

One strategy to accomplish this goal is nuclear reprogramming, a technique that involves experimentally inducing a stable change in the nucleus of a mature cell that can then be maintained and replicated as the cell divides through mitosis. These changes are most frequently associated with the reacquisition of a pluripotent state, thereby endowing the cell with developmental potential. The strategy has historically been carried out using techniques such as somatic cell nuclear transfer (SCNT),[1,2] altered nuclear transfer (ANT),[3,4] and methods to fuse somatic cells with ESCs [5,6] (see "Alternate Methods for Preparing Pluripotent Stem Cells" for details of these approaches). From a clinical perspective, these methods feature several drawbacks, such as the creation of an embryo or the development of hybrid cells that are not viable to treat disease. However, in 2006, these efforts informed the development of nuclear reprogramming *in vitro,* the breakthrough method that creates iPSCs.

This approach involves taking mature "somatic" cells from an adult and introducing the genes that encode critical transcription factor proteins, which themselves regulate the function of other genes important for early steps in embryonic development (See Fig. 10.1). In the initial 2006 study, it was reported that only four transcription factors *(Oct4, Sox2, Klf4,* and *c-Myc)* were required to reprogram mouse fibroblasts (cells found in the skin and other connective tissue) to an embryonic stem cell–like state by forcing them to express genes important for maintaining the defining

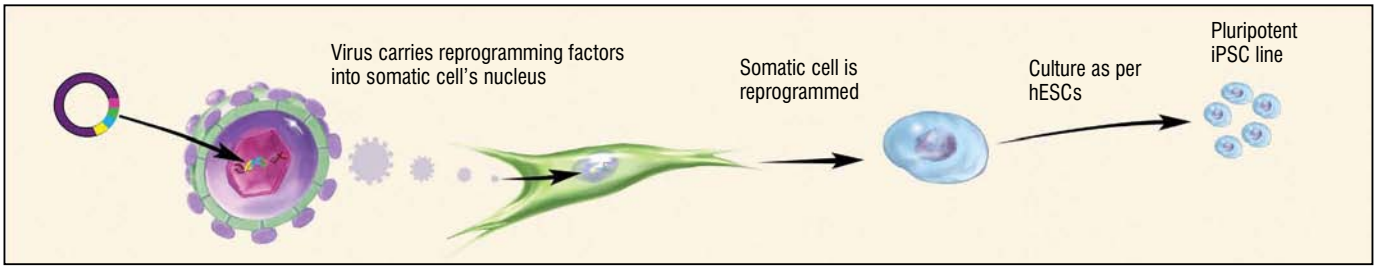


Figure 10.1. Generating Induced Pluripotent Stem Cells (iPSCs)

properties of ESCs.[7] These factors were chosen because they were known to be involved in the maintenance of pluripotency, which is the capability to generate all other cell types of the body. The newly-created iPSCs were found to be highly similar to ESCs and could be established after several weeks in culture.[7,8] In 2007, two different research groups reached a new milestone by deriving iPSCs from human cells, using either the original four genes[9] or a different combination containing *Oct4, Sox2, Nanog,* and *Lin28*.[10] Since then, researchers have reported generating iPSCs from somatic tissues of the monkey[11] and rat.[12,13]

However, these original methods of reprogramming are inefficient, yielding iPSCs in less than 1% of the starting adult cells.[14,15] The type of adult cell used also affects efficiency; fibroblasts require more time for factor expression and have lower efficiency of reprogramming than do human keratinocytes, mouse liver and stomach cells, or mouse neural stem cells.[14–19]

Several approaches have been investigated to improve reprogramming efficiency and decrease potentially detrimental side effects of the reprogramming process. Since the retroviruses used to deliver the four transcription factors in the earliest studies can potentially cause mutagenesis (see below), researchers have investigated whether all four factors are absolutely necessary. In particular, the gene *c-Myc* is known to promote tumor growth in some cases, which would negatively affect iPSC usefulness in transplantation therapies. To this end, researchers tested a three-factor approach that uses the orphan nuclear receptor *Esrrb* with *Oct4* and *Sox2,* and were able to convert mouse embryonic fibroblasts to iPSCs.[20] This achievement corroborates other reports that *c-Myc* is dispensable for direct reprogramming of mouse fibroblasts.[21] Subsequent studies have further reduced the number of genes required for reprogramming,[22–26] and researchers continue to identify chemicals that can either substitute for or enhance the efficiency of transcription factors in this process.[27] These breakthroughs continue to inform and to simplify the reprogramming process, thereby advancing the field toward the generation of patient-specific stem cells for clinical application. However, as the next section will discuss, the method by which transcription factors are delivered to the somatic cells is critical to their potential use in the clinic.

CURRENT CHALLENGES IN iPSC RESEARCH

Reprogramming poses several challenges for researchers who hope to apply it to regenerative medicine. To deliver the desired transcription factors, the DNA that encodes their production must be introduced and integrated into the genome of the somatic cells. Early efforts to generate iPSCs accomplished this goal using retroviral vectors. A retrovirus is an RNA virus that uses an enzyme, reverse transcriptase, to replicate in a host cell and subsequently produce DNA from its RNA genome. This DNA incorporates into the host's genome, allowing the virus to replicate as part of the host cell's DNA. However, the forced expression of these genes cannot be controlled fully, leading to unpredictable effects.[28] While other types of integrating viruses, such as lentiviruses, can increase the efficiency of reprogramming,[16] the expression of viral transgenes remains a critical clinical issue. Given the dual needs of reducing the drawbacks of viral integration and maximizing reprogramming efficiency, researchers are exploring a number of strategies to reprogram cells in the absence of integrating viral vectors[27–30] or to use potentially more efficient integrative approaches.[31,32]

Before reprogramming can be considered for use as a clinical tool, the efficiency of the process must improve substantially. Although researchers have

begun to identify the myriad molecular pathways that are implicated in reprogramming somatic cells,[15] much more basic research will be required to identify the full spectrum of events that enable this process. Simply adding transcription factors to a population of differentiated cells does not guarantee reprogramming – the low efficiency of reprogramming *in vitro* suggests that additional rare events are necessary to generate iPSCs, and the efficiency of reprogramming decreases even further with fibroblasts that have been cultured for long time periods.[33] Furthermore, the differentiation stage of the starting cell appears to impact directly the reprogramming efficiency; mouse hematopoietic stem and progenitor cells give rise to iPSCs up to 300 times more efficiently than do their terminally-differentiated B- and T-cell counterparts.[34] As this field continues to develop, researchers are exploring the reprogramming of stem or adult progenitor cells from mice[24,25,34,35] and humans[23,26] as one strategy to increase efficiency compared to that observed with mature cells.

As these discussions suggest, clinical application of iPSCs will require safe and highly efficient generation of stem cells. As scientists increase their understanding of the molecular mechanisms that underlie reprogramming, they will be able to identify the cell types and conditions that most effectively enable the process and use this information to design tools for widespread use. Clinical application of these cells will require methods to reprogram cells while minimizing DNA alterations. To this end, researchers have found ways to introduce combinations of factors in a single viral "cassette" into a known genetic location.[36] Evolving tools such as these will enable researchers to induce programming more safely, thereby informing basic iPSC research and moving this technology closer to clinical application.

ARE iPSCs TRULY EQUIVALENT TO ESCs?

ESCs and iPSCs are created using different strategies and conditions, leading researchers to ask whether the cell types are truly equivalent. To assess this issue, investigators have begun extensive comparisons to determine pluripotency, gene expression, and function of differentiated cell derivatives. Ultimately, the two cell types exhibit some differences, yet they are remarkably similar in many key aspects that could impact their application to regenerative medicine. Future experiments will determine the clinical significance (if any) of the observed differences between the cell types.

Other than their derivation from adult tissues, iPSCs meet the defining criteria for ESCs. Mouse and human iPSCs demonstrate important characteristics of pluripotent stem cells, including expressing stem cell markers, forming tumors containing cell types from all three primitive embryonic layers, and displaying the capacity to contribute to many different tissues when injected into mouse embryos at a very early stage of development. Initially, it was unclear that iPSCs were truly pluripotent, as early iPSC lines contributed to mouse embryonic development but failed to produce live-born progeny as do ESCs. In late 2009, however, several research groups reported mouse iPSC lines that are capable of producing live births,[37,38] noting that the cells maintain a pluripotent potential that is "very close to" that of ESCs.[38] Therefore, iPSCs appear to be truly pluripotent, although they are less efficient than ESCs with respect to differentiating into all cell types.[38] In addition, the two cell types appear to have similar defense mechanisms to thwart the production of DNA-damaging reactive oxygen species, thereby conferring the cells with comparable capabilities to maintain genomic integrity.[39]

Undifferentiated iPSCs appear molecularly indistinguishable from ESCs. However, comparative genomic analyses reveal differences between the two cell types. For example, hundreds of genes are differentially expressed in ESCs and iPSCs,[40] and there appear to be subtle but detectable differences in epigenetic methylation between the two cell types.[41,42] Genomic differences are to be expected; it has been reported that gene-expression profiles of iPSCs and ESCs from the same species differ no more than observed variability among individual ESC lines.[43] It should be noted that the functional implications of these findings are presently unknown, and observed differences may ultimately prove functionally inconsequential.[44]

Recently, some of the researchers who first generated human iPSCs compared the ability of iPSCs and human ESCs to differentiate into neural cells (e.g., neurons and glia).[45] Their results demonstrated that both cell types follow the same steps and time course during differentiation. However, although human ESCs differentiate into neural cells with a similar efficiency regardless of the cell line used, iPSC-derived neural cells demonstrate lower efficiency and greater variability when differentiating into neural cells. These observations occurred regardless of which of several iPSC-generation protocols were used to reprogram

the original cell to the pluripotent state. Experimental evidence suggests that individual iPSC lines may be "epigenetically unique" and predisposed to generate cells of a particular lineage. However, the authors believe that improvements to the culturing techniques may be able to overcome the variability and inefficiency described in this report.

These findings underpin the importance of understanding the inherent variability among discrete cell populations, whether they are iPSCs or ESCs. Characterizing the variability among iPSC lines will be crucial to apply the cells clinically. Indeed, the factors that make each iPSC line unique may also delay the cells' widespread use, as differences among the cell lines will affect comparisons and potentially influence their clinical behavior. For example, successfully modeling disease requires being able to identify the cellular differences between patients and controls that lead to dysfunction. These differences must be framed in the context of the biologic variability inherent in a given patient population. If iPSC lines are to be used to model disease or screen candidate drugs, then variability among lines must be minimized and characterized fully so that researchers can understand how their observed results match to the biology of the disease being studied. As such, standardized assays and methods will become increasingly important for the clinical application of iPSCs, and controls must be developed that account for variability among the iPSCs and their derivatives.

Additionally, researchers must understand the factors that initiate reprogramming towards pluripotency in different cell types. A recent report has identified one factor that initiates reprogramming in human fibroblasts,[46] setting the groundwork for developing predictive models to identify those cells that will become iPSCs. An iPSC may carry a genetic "memory" of the cell type that it once was, and this "memory" will likely influence its ability to be reprogrammed. Understanding how this memory varies among different cell types and tissues will be necessary to reprogram successfully.

POTENTIAL MEDICAL APPLICATIONS OF IPSCs

iPSCs have the potential to become multipurpose research and clinical tools to understand and model diseases, develop and screen candidate drugs, and deliver cell-replacement therapy to support regenerative medicine. This section will explore the possibilities and the challenges that accompany these medical applications, with the caveat that some uses are more immediate than others. For example, researchers currently use stem cells to test/screen drugs or as study material to identify molecules or genes implicated in regeneration. Conducting experiments or testing candidate drugs on human cells grown in culture enables researchers to understand fundamental principles and relationships that will ultimately inform the use of stem cells as a source of tissue for transplantation. Therefore, using iPSCs in cell-replacement therapies is a future application of these cells, albeit one that has tremendous clinical potential. The following discussion will highlight recent efforts toward this goal while recognizing the challenges that must be overcome for these cells to reach the clinic.

Reprogramming technology offers the potential to treat many diseases, including Alzheimer's disease, Parkinson's disease, cardiovascular disease, diabetes, and amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease). In theory, easily-accessible cell types (such as skin fibroblasts) could be biopsied from a patient and reprogrammed, effectively recapitulating the patient's disease in a culture dish. Such cells could then serve as the basis for autologous cell replacement therapy. Because the source cells originate within the patient, immune rejection of the differentiated derivatives would be minimized. As a result, the need for immunosuppressive drugs to accompany the cell transplant would be lessened and perhaps eliminated altogether. In addition, the reprogrammed cells could be directed to produce the cell types that are compromised or destroyed by the disease in question. A recent experiment has demonstrated the proof of principle in this regard,[47] as iPSCs derived from a patient with ALS were directed to differentiate into motor neurons, which are the cells that are destroyed in the disease.

Although much additional basic research will be required before iPSCs can be applied in the clinic, these cells represent multi-purpose tools for medical research. Using the techniques described in this article, researchers are now generating myriad disease-specific iPSCs. For example, dermal fibroblasts and bone marrow-derived mesenchymal cells have been used to establish iPSCs from patients with a variety of diseases, including ALS, adenosine deaminase deficiency-related

severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophies, Parkinson's disease, Huntington's disease, type 1 diabetes mellitus, Down syndrome/trisomy 21, and spinal muscular atrophy.[47–49] iPSCs created from patients diagnosed with a specific genetically-inherited disease can then be used to model disease pathology. For example, iPSCs created from skin fibroblasts taken from a child with spinal muscular atrophy were used to generate motor neurons that showed selective deficits compared to those derived from the child's unaffected mother.[48] As iPSCs illuminate the development of normal and disease-specific pathologic tissues, it is expected that discoveries made using these cells will inform future drug development or other therapeutic interventions.

One particularly appealing aspect of iPSCs is that, in theory, they can be directed to differentiate into a specified lineage that will support treatment or tissue regeneration. Thus, somatic cells from a patient with cardiovascular disease could be used to generate iPSCs that could then be directed to give rise to functional adult cardiac muscle cells (cardiomyocytes) that replace diseased heart tissue, and so forth. Yet while iPSCs have great potential as sources of adult mature cells, much remains to be learned about the processes by which these cells differentiate. For example, iPSCs created from human[50] and murine fibroblasts[51–53] can give rise to functional cardiomyocytes that display hallmark cardiac action potentials. However, the maturation process into cardiomyocytes is impaired when iPSCs are used – cardiac development of iPSCs is delayed compared to that seen with cardiomyocytes derived from ESCs or fetal tissue. Furthermore, variation exists in the expression of genetic markers in the iPSC-derived cardiac cells as compared to that seen in ESC-derived cardiomyocytes. Therefore, iPSC-derived cardiomyocytes demonstrate normal commitment but impaired maturation, and it is unclear whether observed defects are due to technical (e.g., incomplete reprogramming of iPSCs) or biological barriers (e.g., functional impairment due to genetic factors). Thus, before these cells can be used for therapy, it will be critical to distinguish between iPSC-specific and disease-specific phenotypes.

However, it must be noted that this emerging field is continually evolving; additional basic iPSC research will be required in parallel with the development of disease models. Although the reprogramming technology that creates iPSCs is currently imperfect, these cells will likely impact future therapy, and "imperfect" cells can illuminate many areas related to regenerative medicine. However, iPSC-derived cells that will be used for therapy will require extensive characterization relative to what is sufficient to support disease modeling studies. To this end, researchers have begun to use imaging techniques to observe cells that are undergoing reprogramming to distinguish true iPSCs from partially-reprogrammed cells.[54] The potential for tumor formation must also be addressed fully before any iPSC derivatives can be considered for applied cell therapy. Furthermore, in proposed autologous therapy applications, somatic DNA mutations (e.g., non-inherited mutations that have accumulated during the person's lifetime) retained in the iPSCs and their derivatives could potentially impact downstream cellular function or promote tumor formation (an issue that may possibly be circumvented by creating iPSCs from a "youthful" cell source such as umbilical cord blood).[55] Whether these issues will prove consequential when weighed against the cells' therapeutic potential remains to be determined. While the promise of iPSCs is great, the current levels of understanding of the cells' biology, variability, and utility must also increase greatly before iPSCs become standard tools for regenerative medicine.

CONCLUSION

Since their discovery four years ago, induced pluripotent stem cells have captured the imagination of researchers and clinicians seeking to develop patient-specific therapies. Reprogramming adult tissues to embryonic-like states has countless prospective applications to regenerative medicine, drug development, and basic research on stem cells and developmental processes. To this point, a PubMed search conducted in April 2010 using the term "induced pluripotent stem cells" (which was coined in 2006) returned more than 1400 publications, indicating a highly active and rapidly-developing research field.

However, many technical and basic science issues remain before the promise offered by iPSC technology can be realized fully. For putative regenerative medicine applications, patient safety is the foremost consideration. Standardized methods must be developed to characterize iPSCs and their derivatives. Furthermore, reprogramming has demonstrated a proof-of-principle, yet the process is currently too inefficient for routine clinical application. Thus,

unraveling the molecular mechanisms that govern reprogramming is a critical first step toward standardizing protocols. A grasp on the molecular underpinnings of the process will shed light on the differences between iPSCs and ESCs (and determine whether these differences are clinically significant). Moreover, as researchers delve more deeply into this field, the effects of donor cell populations can be compared to support a given application; i.e., do muscle-derived iPSCs produce more muscle than skin-derived cells? Based on the exciting developments in this area to date, induced pluripotent stem cells will likely support future therapeutic interventions, either directly or as research tools to establish novel models for degenerative disease that will inform drug development. While much remains to be learned in the field of iPSC research, the development of reprogramming techniques represents a breakthrough that will ultimately open many new avenues of research and therapy.

REFERENCES

1. French AJ, Adams CA, Anderson LS, Kitchen JR, Hughes MR, Wood SH. Development of human cloned blastocysts following somatic cell nuclear transfer (SCNT) with adult fibroblasts. *Stem Cells.* 2008;26:485-483.
2. Byrne JA, Pedersen DA, Clepper LL, et al. Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature.* 2007;450:497-502.
3. Hurlbut WB. Ethics and embryonic stem cell research: altered nuclear transfer as a way forward. *BioDrugs.* 2007;21:79-83.
4. Meissner A, Jaenisch R. Generation of nuclear transfer-derived pluripotent ES cells from cloned Cdx2-deficient blastocysts. *Nature.* 2006;439:212-215.
5. Cantz T, Bleidissel M, Stehling M, Schöler HR. In vitro differentiation of reprogrammed murine somatic cells into hepatic precursor cells. *Biol Chem.* 2008;389:889-896.
6. Cowan CA, Atienza J, Melton DA, Eggan K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science.* 2005;309:1369-1373.
7. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126:663-676.
8. Maherali N, Sridharan R, Xie W, et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell.* 2007;1:55-70.
9. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131:1-12.
10. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* 2007;318:1917-1920.
11. Liu H, Zhu F, Yong J, et al. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell.* 2008;3:587-590.
12. Liao J, Cui C, Chen S, et al. Generation of induced pluripotent stem cell lines from adult rat cells. *Cell Stem Cell.* 2009;4:11-15.
13. Li W, Wei W, Zhu S, et al. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell.* 2009;4:16-19.
14. Brambrink T, Foreman R, Welstead GG, et al. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell.* 2008;2:151-159.
15. Stadtfeld M, Maherali N, Breault DT, Hochedlinger K. Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. *Cell Stem Cell.* 2008;2:230-240.
16. Maherali N, Ahfeldt T, Rigamonti A, Utikal J, Cowan C, Hochedlinger K. A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell.* 2008;3:340-345.
17. Aasen T, Raya A, Barrero MJ, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol.* 2008;26:1276-1284.
18. Schöler HR. Commentary: Highlight on stem cell research. *Biol Chem.* 2008;389:789.
19. Aoi T, Yae K, Nakagawa M, et al. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science.* 2008;321:699-702.
20. Feng B, Jiang J, Kraus P, et al. Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nat Cell Biol.* 2009;11:197-203.
21. Wernig M, Meissner A, Cassady JP, Jaenisch R. *c-Myc* is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell.* 2008;2:10-12.
22. Huangfu D, Osafune K, Maehr R, et al. Induction of pluripotent stem cells from primary human fibroblasts with only *Oct4* and *Sox2. Nat Biotechnol.* 2008;26:1269-1275.
23. Hester ME, Song S, Miranda CJ, Eagle A, Schwartz PH, Kaspar BK. Two factor reprogramming of human neural stem cells into pluripotency. *PLoS One.* 2009;4:e7044.
24. Kim JB, Zaehres H, Wu G, et al. Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature.* 2008;454:646-650.
25. Kim JB, Sebastiano V, Wu G, et al. Oct4-induced pluripotency in adult neural stem cells. *Cell.* 2009;136:411-419.
26. Kim JB, Greber B, Arauzo Bravo MJ, et al. Direct reprogramming of human neural stem cells by *Oct4. Nature.* 2009;461:649-653.

27. Feng B, Ng JH, Heng JC, Ng HH. Molecules that promote or enhance reprogramming of somatic cells to induced pluripotent stem cells. *Cell Stem Cell.* 2009;4:301-312.

28. Stadtfeld M, Hochedlinger K. Without a trace? PiggyBac-ing to pluripotency. *Nat Methods.* 2009;6:329-330.

29. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science.* 2008;322:945-949.

30. Page RL, Ambady S, Holmes WF, et al. Induction of stem cell gene expression in adult human fibroblasts without transgenes. *Cloning Stem Cells.* 2009;11:1-10.

31. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science.* 2008;322:949-953.

32. Yusa K, Rad R, Takeda J, Bradley A. Generation of transgene-free induced pluripotent mouse stem cells by the *piggyBac* transposon. *Nat Methods.* 2009;6:363-369.

33. Utikal J, Polo JM, Stadtfeld M, et al. Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature.* 2009;460:1145-1148.

34. Eminli S, Foudi A, Stadtfeld M, et al. Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. *Nat Genet.* 2009;41:968-976.

35. Ko K, Tapia N, Wu G, et al. Induction of pluripotency in adult unipotent germline stem cells. *Cell Stem Cell.* 2009;5:87-96.

36. Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells.* 2009;27:543-549.

37. Kang L, Wang J, Zhang Y, Kou Z, Gao S. iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell.* 2009;5:135-138.

38. Zhao XY, Li W, Lv Z, et al. iPS cells produce viable mice through tetraploid complementation. *Nature.* 2009;461:86-90.

39. Armstrong L, Tilgner K, Saretzki G, et al. Human induced pluripotent stem cell lines show similar stress defence mechanisms and mitochondrial regulation to human embryonic stem cells. *Stem Cells.* 2010;Jan 13 [Epub ahead of print].

40. Chin MH, Mason MJ, Xie W, et al. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell.* 2009;5:111-123.

41. Deng J, Shoemaker R, Xie B, et al. Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat Biotechnol.* 2009;27:353-360.

42. Doi A, Park I-H, Wen B, et al. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet.* 2009;41:1350-1353.

43. Mikkelsen TS, Hanna J, Zhang X, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature.* 2008;454:794.

44. Colman A, Dressen O. Induced pluripotent stem cells and the stability of the differentiated state. E*MBO Reports.* 2009;10:714-721.

45. Hu BY, Weick JP, Yu J, et al. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc Natl Acad Sci USA.* 2010;107:4335-4340.

46. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM. Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature.* 2010;463:1042-1048.

47. Dimos JT, Rodolfa KT, Niakan KK, et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science.* 2008;321:1218-1221.

48. Ebert AD, Yu J, Rose FF Jr, et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature.* 2009;457:277-280.

49. Park I-H, Arora N, Huo H, et al. Disease-specific induced pluripotent stem (iPS) cells. *Cell.* 2008;134:877-886.

50. Zhang J, Wilson GF, Soerens AG, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res.* 2009;104:e30-e41.

51. Mauritz C, Schwanke K, Reppel M, et al. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation.* 2008;118:507-517.

52. Kuzmenkin A, Liang H, Xu G, et al. Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells in vitro. *FASEB J.* 2009;23:4168-4180.

53. Pfannkuche K, Liang H, Hannes T, et al. Cardiac myocytes derived from murine reprogrammed fibroblasts: intact hormonal regulation, cardiac ion channel expression and development of contractility. Cell Physiol Biochem. 2009;24:73-86.

54. Chan EM, Ratanasirintrawoot S, Park I-H, et al. Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. Nat Biotechnol. 2009;27:1033-1037.

55. Haase A, Olmer R, Schwanke K, et al. Generation of induced pluripotent stem cells from human cord blood. Cell Stem Cell. 2009;5:434-441.