3. THE HUMAN EMBRYONIC STEM CELL AND THE HUMAN EMBRYONIC GERM CELL

A new era in stem cell biology began in 1998 with the derivation of cells from human blastocysts and fetal tissue with the unique ability of differentiating into cells of all tissues in the body, i.e., the cells are pluripotent. Since then, several research teams have characterized many of the molecular characteristics of these cells and improved the methods for culturing them. In addition, scientists are just beginning to direct the differentiation of the human pluripotent stem cells and to identify the functional capabilities of the resulting specialized cells. Although in its earliest phases, research with these cells is proving to be important to developing innovative cell replacement strategies to rebuild tissues and restore critical functions of the diseased or damaged human body.

OVERVIEW

In 1998, James Thomson and his colleagues reported methods for deriving and maintaining human embryonic stem (ES) cells from the inner cell mass of human blastocysts that were produced through *in vitro* fertilization (IVF) and donated for research purposes [46]. At the same time, another group, led by John Gearhart, reported the derivation of cells that they identified as embryonic germ (EG) cells. The cells were cultured from primordial germ cells obtained from the gonadal ridge and mesenchyma of 5- to 9-week fetal tissue that resulted from elective abortions [41].

The two research teams developed their methods for culturing human ES and EG cells by drawing on a host of animal studies, some of which date back almost 40 years: derivations of pluripotent mouse ES cells from blastocysts [13, 15], reports of the derivation of EG cells [27, 36], experiments with stem cells derived from mouse teratocarcinomas [24] and human embryonal carcinomas and teratocarcinomas [4, 17, 24], the derivation and culture of ES cells from the blastocysts of rhesus monkeys [46] and marmosets [47], and methods used by IVF clinics to prepare human embryos for transplanting into the uterus to produce a live birth [11, 49].

TIMELINE OF HUMAN EMBRYONIC STEM CELL RESEARCH

- **1878:** First reported attempts to fertilize mammalian eggs outside the body [49].
- **1959:** First report of animals (rabbits) produced through IVF in the United States [49].
- **1960s:** Studies of teratocarcinomas in the testes of several inbred strains of mice indicates they originated from embryonic germ cells. The work establishes embryonal carcinoma (EC) cells as a kind of stem cell [17, 24]. For a more detailed discussion of human embryonal carcinoma cells, see Appendix C.
- **1968:** Edwards and Bavister fertilize the first human egg *in vitro* [49].
- **1970s:** EC cells injected into mouse blastocysts produce chimeric mice. Cultured SC cells are explored as models of embryonic development, although their complement of chromosomes is abnormal [25].
- **1978:** Louise Brown, the first IVF baby, is born in England [49].
- **1980:** Australia's first IVF baby, Candace Reed, is born in Melbourne [49].
- **1981:** Evans and Kaufman, and Martin derive mouse embryonic stem (ES) cells from the inner cell mass of blastocysts. They establish culture conditions for growing pluripotent mouse ES cells *in vitro*. The ES cells yield cell lines with normal, diploid karyotyes and generate derivatives of all three primary germ layers as well as primordial

germ cells. Injecting the ES cells into mice induces the formation of teratomas [15, 26]. The first IVF baby, Elizabeth Carr, is born in the United States [49].

- **1984-88:** Andrews et al., develop pluripotent, genetically identical (clonal) cells called embryonal carcinoma (EC) cells from Tera-2, a cell line of human testicular teratocarcinoma [5]. Cloned human teratoma cells exposed to retinoic acid differentiate into neuron-like cells and other cell types [3, 44].
- **1989:** Pera et al., derive a clonal line of human embryonal carcinoma cells, which yields tissues from all three primary germ layers. The cells are aneuploid (fewer or greater than the normal number of chromosomes in the cell) and their potential to differentiate spontaneously *in vitro* is typically limited. The behavior of human EC cell clones differs from that of mouse ES or EC cells [33].
- **1994:** Human blastocysts created for reproductive purposes using IVF and donated by patients for research, are generated from the 2-pronuclear stage. The inner cell mass of the blastocyst is maintained in culture and generates aggregates with trophoblast-like cells at the periphery and ES-like cells in the center. The cells retain a complete set of chromosomes (normal karyotype); most cultures retain a stem cell-like morphology, although some inner cell mass clumps differentiate into fibroblasts. The cultures are maintained for two passages [6, 7].
- **1995-96:** Non-human primate ES cells are derived and maintained *in vitro*, first from the inner cell mass of rhesus monkeys [46], and then from marmosets [47]. The primate ES cells are diploid and have normal karyotypes. They are pluripotent and differentiate into cells types derived from all three primary germ layers. The primate ES cells resemble human EC cells and indicate that it should be possible to derive and maintain human ES cells *in vitro*.
- **1998:** Thomson et al., derive human ES cells from the inner cell mass of normal human blastocysts donated by couples undergoing treatment for infertility. The cells are cultured through many passages, retain their normal karyotypes, maintain high levels of telomerase activity, and express a panel of markers typical of human EC cells non-human primate ES cells. Several (non-clonal) cell lines are established that form teratomas when injected into immune-deficient mice. The teratomas include cell types derived from all three primary germ layers, demonstrating the pluripotency of human ES cells [48]. Gearhart and colleagues derive human embryonic germ (EG) cells from the gonadal ridge and mesenchyma of 5- to 9-week fetal tissue that resulted from elective abortions. They grow EG cells *in vitro* for approximately 20 passages, and the cells maintain normal karyotypes. The cells spontaneously form aggregates that differentiate spontaneously, and ultimately contain derivatives of all three primary germ layers. Other indications of their pluripotency include the expression of a panel of markers typical of mouse ES and EG cells. The EG cells do not form teratomas when injected into immune-deficient mice [41].
- **2000:** Scientists in Singapore and Australia led by Pera, Trounson, and Bongso derive human ES cells from the inner cell mass of blastocysts donated by couples undergoing treatment for infertility. The ES cells proliferate for extended periods *in vitro*, maintain normal karyotypes, differentiate spontaneously into somatic cell lineages derived from all three primary germ layers, and form teratomas when injected into immune-deficient mice.
- **2001:** As human ES cell lines are shared and new lines are derived, more research groups report methods to direct the differentiation of the cells *in vitro*. Many of the methods are aimed at generating human tissues for transplantation purposes, including pancreatic islet cells, neurons that release dopamine, and cardiac muscle cells.

DERIVATION OF HUMAN EMBRYONIC STEM CELLS

The first documentation of the isolation of embryonic stem cells from human blastocysts was in 1994 [7]. Since then, techniques for deriving and culturing human ES cells have been refined [38, 48]. The ability to isolate human ES cells from blastocysts and grow them in culture seems to depend in large part on the integrity and condition of the blastocyst from which the cells are derived. In general, blastocysts with a

large and distinct inner cell mass tend to yield ES cultures most efficiently [11] (see Figure 3.1. Human Blastocyst Showing Inner Cell Mass and Trophectoderm).

Timeline for the Development of a Human

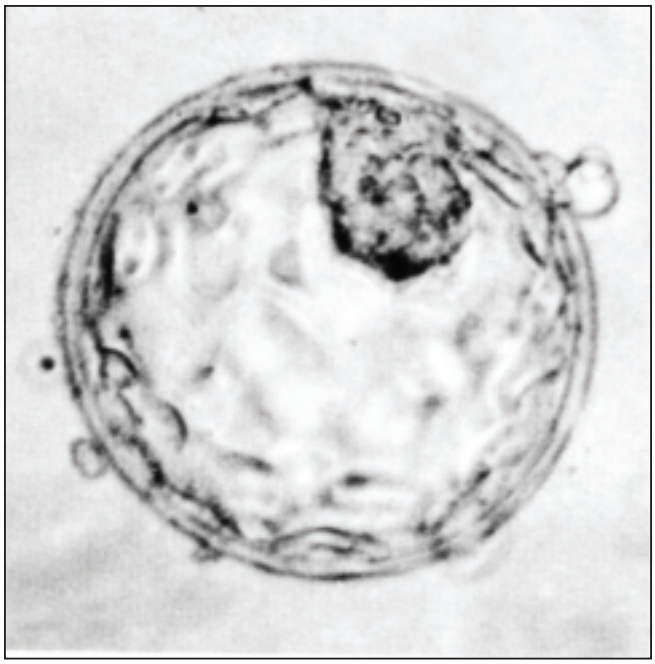

Photo Credit: Mt. J. Conaghan

Figure 3.1. Human Blastocyst Showing Inner Cell Mass and Trophectoderm.

Blastocyst *In Vitro*

After a human oocyte is fertilized *in vitro* by a sperm cell, the following events occur according to a fairly predictable timeline [9, 12, 16]. At 18 to 24 hours after *in vitro* fertilization of the oocyte is considered day 1. By day 2 (24 to 25 hours), the zygote (fertilized egg) undergoes the first cleavage to produce a 2-cell embryo. By day 3 (72 hours), the embryo reaches the 8-cell stage called a morula. It is at this stage that the genome of the embryo begins to control its own development. This means that any maternal influences—due to the presence of mRNA and proteins in the oocyte cytoplasm—are significantly reduced. By day 4, the cells of the embryo adhere tightly to each other in a process known as compaction and by day 5, the cavity of the blastocyst is completed. The inner cell mass begins to separate from the outer cells, which become the trophectoderm that surrounds the blastocyst. This represents the first observable sign of cell differentiation in the embryo. (For a more detailed discussion, see Appendix A. Early Development.)

Many IVF clinics now transfer day-5 embryos to the uterus for optimal implantation, a stage of development that more closely parallels the stage at which a blastocyst would implant in the wall of the uterus *in vivo*. This represents a change—and a greatly improved implantation rate—from earlier IVF procedures in which a 2-cell embryo was used for implantation.

Day-5 blastocysts are used to derive ES cell cultures. A normal day-5 human embryo *in vitro* consists of 200 to 250 cells. Most of the cells comprise the trophectoderm. For deriving ES cell cultures, the trophectoderm is removed, either by microsurgery or immunosurgery (in which antibodies against the trophectoderm help break it down, thus freeing the inner cell mass). At this stage, the inner cell mass is composed of only 30 to 34 cells [10].

The *in vitro* conditions for growing a human embryo to the blastocyst stage vary among IVF clinics and are reviewed elsewhere [6, 8, 14, 16, 18, 21, 39, 49, 50]. However, once the inner cell mass is obtained from either mouse or human blastocysts, the techniques for growing ES cells are similar. (For a detailed discussion see Appendix C. Human Embryonic Stem Cells and Human Embryonic Germ Cells.)

DERIVATION OF HUMAN EMBRYONIC GERM CELLS

As stated earlier, human embryonic germ (EG) cells share many of the characteristics of human ES cells, but differ in significant ways. Human EG cells are derived from the primordial germ cells, which occur in a specific part of the embryo/fetus called the gonadal ridge, and which normally develop into mature gametes (eggs and sperm). Gearhart and his collaborators devised methods for growing pluripotent cells derived from human EG cells. The process requires the generation of embryoid bodies from EG cells, which consists of an unpredictable mix of partially differentiated cell types [19]. The embryoid body-derived cells resulting from this process have high proliferative capacity and gene expression patterns that are representative of multiple cell lineages. This suggests that the embryoid body-derived cells are progenitor or precursor cells for

a variety of differentiated cell types [19]. (For a more detailed description of the derivation of EG cells, see Appendix C. Human Embryonic Stem Cells and Human Embryonic Germ Cells.)

PLURIPOTENCY OF HUMAN EMBRYONIC STEM CELLS AND EMBRYONIC GERM CELLS

As stated earlier, a truly pluripotent stem cell is a cell that is capable of self-renewal and of differentiating into most all of the cells of the body, including cells of all three germ layers. Human ES and EG cells *in vitro* are capable of long-term self-renewal, while retaining a normal karyotype [1, 38, 41, 42, 48]. Human ES cells can proliferate for two years through 300 population doublings [29] or even 450 population doublings [30]. Cultures derived from embryoid bodies generated by human embryonic germ cells have less capacity for proliferation. Most will proliferate for 40 population doublings; the maximum reported is 70 to 80 population doublings [42].

To date, several laboratories have demonstrated that human ES cells *in vitro* are pluripotent; they can produce cell types derived from all three embryonic germ layers [1, 20, 38, 40].

Currently, the only test of the *in vivo* pluripotency of human ES cells is to inject them into immune-deficient mice where they generate differentiated cells that are derived from all three germ layers. These include gut epithelium (which, in the embryo, is derived from endoderm); smooth and striated muscle (derived from mesoderm); and neural epithelium, and stratified squamous epithelium (derived from ectoderm) [20, 38, 48].

However, two aspects of *in vivo* pluripotency typically used in animals have not been met by human ES cells: evidence that cells have the capacity to be injected into a human embryo and form an organism made up of cells from two genetic lineages; and evidence that they have the ability to generate germ cells, the precursors to eggs and sperm in a developing organism. These are theoretical considerations, however, because such tests using human ES cells have not been conducted. In any case, these two demonstrations of human ES cell pluripotency are not likely to be critical for potential therapeutic uses of the cells—in transplants or drug development, for example [43].

COMPARISONS BETWEEN HUMAN EMBRYONIC STEM CELLS AND EMBRYONIC GERM CELLS

The ES cells derived from human blastocysts by Thomson and his colleagues, and from human EG cells derived by Gearhart and his collaborators, are similar in many respects. In both cases, the cells replicate for an extended period of time, show no chromosomal abnormalities, generate both XX (female) and XY (male) cultures, and express a set of markers regarded as characteristic of pluripotent cells. When the culture conditions are adjusted to permit differentiation (see below for details), both ES and EG cells spontaneously differentiate into derivatives of all three primary germ layers—endoderm, mesoderm, and ectoderm (see Table 3.1. Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells).

However, the ES cells derived from human blastocysts and EG cells differ not only in the tissue sources from which they are derived, they also vary with respect to their growth characteristics *in vitro*, and their behavior *in vivo* [34]. In addition, human ES cells have been propagated for approximately two years *in vitro*, for several hundred population doublings [1], whereas human embryoid body-derived cells from cultures of embryonic germ cells have been maintained for only 70 to 80 population doublings [42]. Also, human ES cells will generate teratomas containing differentiated cell types, if injected into immunocompromised mice colonies, while human EG cells will not [20, 37, 38, 41, 48].

Several research groups are trying to grow human ES cells without feeder layers of mouse embryo fibroblasts (MEF), which are labor-intensive to generate. At a recent meeting, scientists from the Geron Corporation reported that they have grown human ES cell without feeder layers, in medium conditioned by MEFs and supplemented with basic FGF [51].

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS AND EMBRYONIC GERM CELLS *IN VITRO*

Currently, a major goal for embryonic stem cell research is to control the differentiation of human ES and EG cell lines into specific kinds of cells—an

Table 3.1. Comparison of Mouse, Monkey, and Human Pluripotent Stem Cells

Marker Name	Mouse EC/ ES/EG cells	Monkey ES cells	Human ES cells	Human EG cells	Human EC cells
SSEA-1	+	–	–	+	–
SSEA-3	–	+	+	+	+
SEA-4	–	+	+	+	+
TRA-1-60	–	+	+	+	+
TRA-1-81	–	+	+	+	+
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	+	Unknown	+
Telomerase activity	+ ES, EC	Unknown	+	Unknown	+
Feeder-cell dependent	ES, EG, some EC	Yes	Yes	Yes	Some; relatively low clonal efficiency
Factors which aid in stem cell self-renewal	LIF and other factors that act through gp130 receptor and can substitute for feeder layer	Co-culture with feeder cells; other promoting factors have not been identified	Feeder cells + serum; feeder layer + serum-free medium + bFGF	LIF, bFGF, forskolin	Unknown; low proliferative capacity
Growth characteristics *in vitro*	Form tight, rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs	Form flat, loose aggregates; can form EBs	Form rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs
Teratoma formation *in vivo*	+	+	+	–	+
Chimera formation	+	Unknown	+	–	+

KEY

ES cell = Embryonic stem cell
EG cell = Embryonic germ cell
EC cell = Embryonal carcinoma cell
SSEA = Stage-specific embryonic antigen
TRA = Tumor rejection antigen-1
LIF = Leukemia inhibitory factor
bFGF = Basic fibroblast growth factor
EB = Embryoid bodies

objective that must be met if the cells are to be used as the basis for therapeutic transplantation, testing drugs, or screening potential toxins. The techniques now being tested to direct human ES cell differentiation are borrowed directly from techniques used to direct the differentiation of mouse ES cells *in vitro*. For more discussion on directed differentiation of human ES and EG cells see Appendix C.

POTENTIAL USES OF HUMAN EMBRYONIC STEM CELLS

Many uses have been proposed for human embryonic stem cells. The most-often discussed is their potential use in transplant therapy—i.e., to replace or restore tissue that has been damaged by disease or injury (see also Chapters 5-9).

Using Human Embryonic Stem Cells for Therapeutic Transplants

Diseases that might be treated by transplanting human ES-derived cells include Parkinson's disease, diabetes, traumatic spinal cord injury, Purkinje cell degeneration, Duchenne's muscular dystrophy, heart failure, and osteogenesis imperfecta. However, treatments for any of these diseases require that human ES cells be directed to differentiate into specific cell types prior to transplant. The research is occurring in several laboratories, but is limited because so few laboratories have access to human ES cells. Thus, at

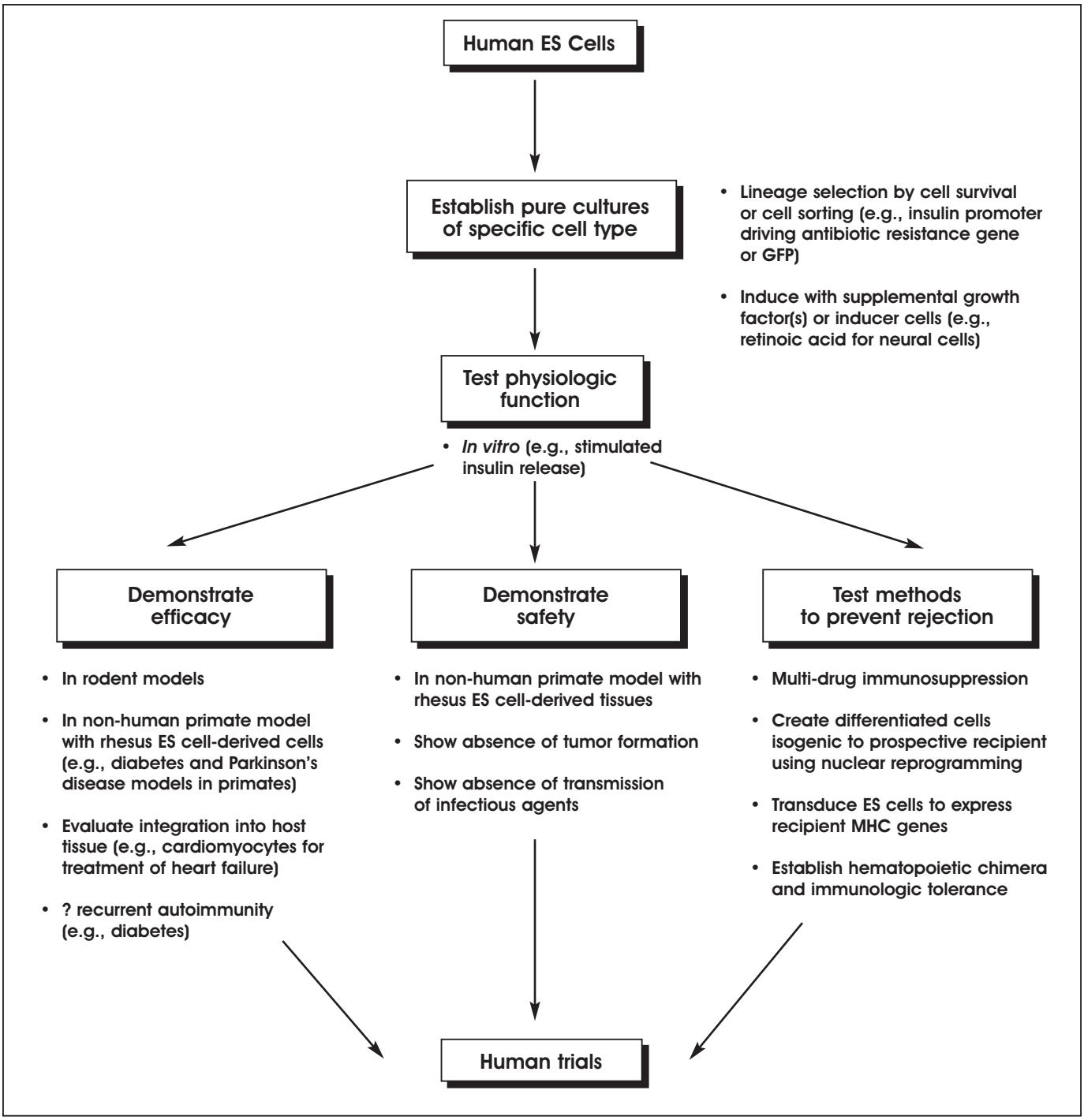

Figure 3.2. Major Goals in the Development of Transplantation Therapies from Human ES Cell Lines. (Reproduced with permission from Stem Cells, 2001)

this stage, any therapies based on the use of human ES cells are still hypothetical and highly experimental [22, 29, 31] (see Figure 3.2. Major Goals in the Development of Transplantation Therapies from Human ES Cell Lines).

One of the current advantages of using ES cells as compared to adult stem cells is that ES cells have an unlimited ability to proliferate *in vitro*, and are more likely to be able to generate a broad range of cell types through directed differentiation. Ultimately, it will also be necessary to both identify the optimal stage(s) of differentiation for transplant, and demonstrate that the transplanted ES-derived cells can survive, integrate, and function in the recipient.

The potential disadvantages of the use of human ES cells for transplant therapy include the propensity of undifferentiated ES cells to induce the formation of tumors (teratomas), which are typically benign. Because it is the undifferentiated cells—rather than their differentiated progeny—that have been shown to induce teratomas, tumor formation might be avoided by devising methods for removing any undifferentiated ES cells prior to transplant. Also, it should be possible to devise a fail-safe mechanism—i.e., to insert into transplanted ES-derived cells suicide genes that can trigger the death of the cells should they become tumorigenic.

Human ES derived cells would also be advantageous for transplantation purposes if they did not trigger immune rejection. The immunological status of human ES cells has not been studied in detail, and it is not known how immunogenic ES-derived cells might be. In general, the immunogenicity of a cell depends on its expression of Class I major histocompatability antigens (MHC), which allow the body to distinguish its own cells from foreign tissue, and on the presence of cells that can bind to foreign antigens and "present" them to the immune system.

The potential immunological rejection of human ES-derived cells might be avoided by genetically engineering the ES cells to express the MHC antigens of the transplant recipient, or by using nuclear transfer technology to generate ES cells that are genetically identical to the person who receives the transplant. It has been suggested that this could be accomplished by using somatic cell nuclear transfer technology (so-called therapeutic cloning) in which the nucleus is removed from one of the transplant patient's cells, such as a skin cell, and injecting the nucleus into an oocyte. The oocyte, thus "fertilized," could be cultured *in vitro* to the blastocyst stage. ES cells could subsequently be derived from its inner cell mass, and directed to differentiate into the desired cell type. The result would be differentiated (or partly differentiated) ES-derived cells that match exactly the immunological profile of the person who donated the somatic cell nucleus, and who is also the intended recipient of the transplant—a labor intensive, but truly customized therapy [29].

Other Potential Uses of Human Embryonic Stem Cells

Many potential uses of human ES cells have been proposed that do not involve transplantation. For example, human ES cells could be used to study early events in human development. Still-unexplained events in early human development can result in congenital birth defects and placental abnormalities that lead to spontaneous abortion. By studying human ES cells *in vitro*, it may be possible to identify the genetic, molecular, and cellular events that lead to these problems and identify methods for preventing them [22, 35, 45].

Such cells could also be used to explore the effects of chromosomal abnormalities in early development. This might include the ability to monitor the development of early childhood tumors, many of which are embryonic in origin [32].

Human ES cells could also be used to test candidate therapeutic drugs. Currently, before candidate drugs are tested in human volunteers, they are subjected to a barrage of preclinical tests. These include drug screening in animal models—*in vitro* tests using cells derived from mice or rats, for example, or *in vivo* tests that involve giving the drug to an animal to assess its safety. Although animal model testing is a mainstay of pharmaceutical research, it cannot always predict the effects that a candidate drug may have on human cells. For this reason, cultures of human cells are often employed in preclinical tests. These human cell lines have usually been maintained *in vitro* for long periods and as such often have different characteristics than do *in vivo* cells. These differences can make it difficult to predict the action of a drug in vivo based on the response of human cell lines in vitro. Therefore, if human ES cells can be directed to differentiate into specific cell types that are important

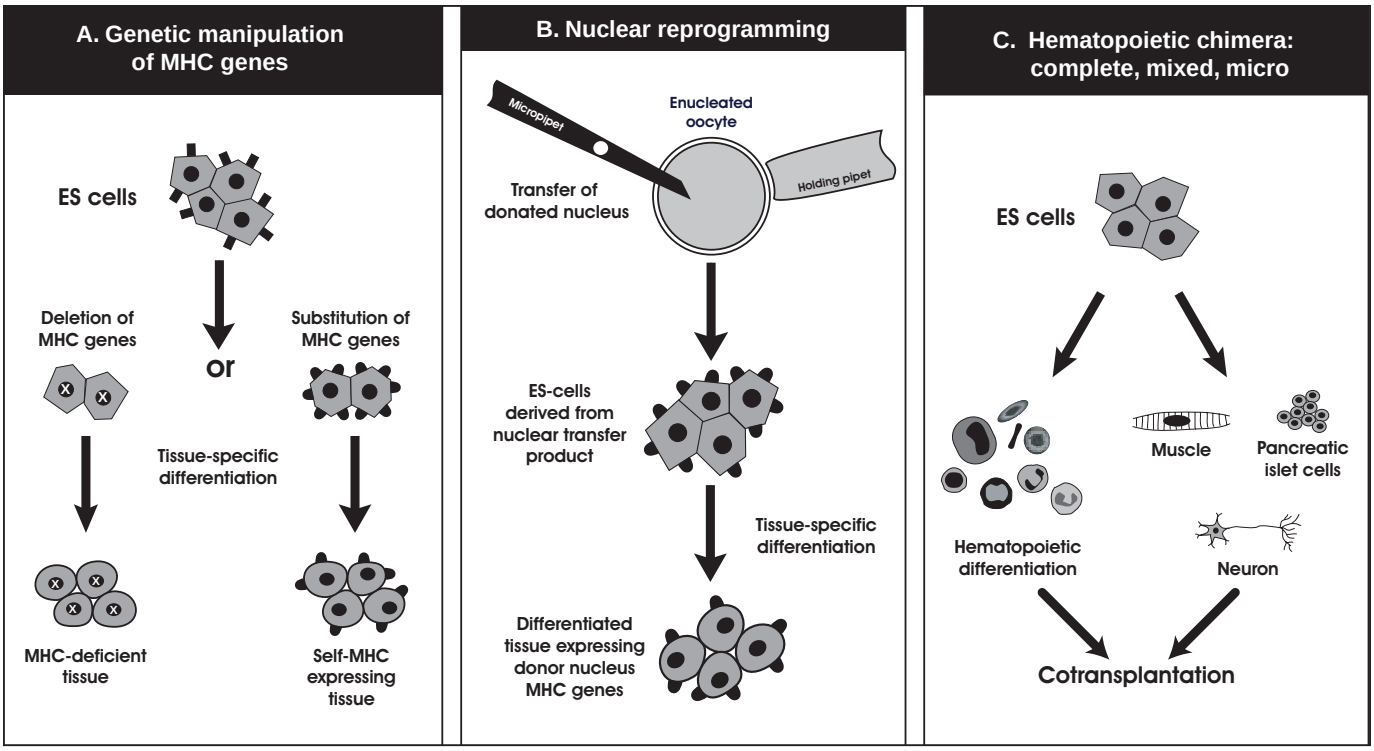

Figure 3.3. Genetic Manipulation of Human Embryonic Stem Cells. (Reproduced with permission from Stem Cells, 2001)

for drug screening, the ES-derived cells may be more likely to mimic the *in vivo* response of the cells/tissues to the drug(s) being tested and so offer safer, and potentially cheaper, models for drug screening.

Human ES cells could be employed to screen potential toxins. The reasons for using human ES cells to screen potential toxins closely resemble those for using human ES-derived cells to test drugs (above). Toxins often have different effects on different animal species, which makes it critical to have the best possible *in vitro* models for evaluating their effects on human cells.

Finally, human ES cells could be used to develop new methods for genetic engineering (see Figure 3.3. Genetic Manipulation of Human Embryonic Stem Cells). Currently, the genetic complement of mouse ES cells *in vitro* can be modified easily by techniques such as homologous recombination. This is a method for replacing or adding genes, which requires that a DNA molecule be artificially introduced into the genome and then expressed. Using this method, genes to direct differentiation to a specific cell type or genes that express a desired protein product might be introduced into the ES cell line. Ultimately, if such techniques could be developed using human ES cells, it may be possible to devise better methods for gene therapy [35] (see Chapter 10. Assessing Human Stem Cell Safety).

SUMMARY

What Do We Know About Human Embryonic Stem Cells?

Since 1998, research teams have refined the techniques for growing human ES cells *in vitro* [1, 20, 38]. Collectively, the studies indicate that it is now possible to grow human ES cells for more than a year in serum-free medium on feeder layers. The cells have normal karyotype and are pluripotent; they generate teratomas that contain differentiated cell types derived from all three primary germ layers. The long-term cultures of human ES cells have active telomerase and maintain relatively long telomeres, another marker of proliferating cells.

Overall, the pluripotent cells that can be generated *in vitro* from human ES cells and human EG cells are apparently not equivalent in their potential to proliferate or differentiate. (ES cells are derived from the inner cell mass of the preimplantation blastocyst, approximately 5 days post-fertilization, whereas human EG cells are derived from fetal primordial

germ cells, 5 to 10 weeks post-fertilization.) ES cells can proliferate for up to 300 population doublings, while cells derived from embryoid bodies that are generated from embryonic germ cells (fetal tissue) double a maximum of 70 to 80 times *in vitro*.

ES cells appear to have a broader ability to differentiate. Both kinds of cells spontaneously generate neural precursor-type cells (widely regarded as a default pathway for differentiation), and both generate cells that resemble cardiac myocytes [19, 45]. However, human ES and EG cells *in vitro* will spontaneously generate embryoid bodies that consist of cell types from all three primary germ layers [1, 20, 38, 42].

What Do We Need To Know About Human Embryonic Stem Cells?

Scientists are just beginning to understand the biology of human embryonic stem cells, and many key questions remain unanswered or only partly answered. For example, in order to refine and improve ES cell culture systems, it is important that scientists identify the mechanisms that allow human ES cells *in vitro* to proliferate without differentiating [29]. Once the mechanisms that regulate human ES proliferation are known, it will likely be possible to apply this knowledge to the long-standing challenge of improving the *in vitro* self-renewal capabilities of adult stem cells.

It will also be important to determine whether the genetic imprinting status of human ES cells plays any significant role in maintaining the cells, directing their differentiation, or determining their suitability for transplant. One of the effects of growing mouse blastocysts in culture is a change in the methylation of specific genes that control embryonic growth and development [23]. Do similar changes in gene imprinting patterns occur in human ES cells (or blastocysts)? If so, what is their effect on *in vitro* development and on any differentiated cell types that may be derived from cultured ES cells?

Efforts will need to be made to determine whether cultures of human ES cells that appear to be homogeneous and undifferentiated are, in fact, homogeneous and undifferentiated. Is it possible that human ES cells *in vitro* cycle in and out of partially differentiated states? And if that occurs, how will it affect attempts to direct their differentiation or maintain the cells in a proliferating state [28]?

Scientists will need to identify which signal transduction pathways must be activated to induce human ES cell differentiation along a particular pathway. This includes understanding ligand-receptor interaction and the intracellular components of the signaling system, as well as identifying the genes that are activated or inactivated during differentiation of specific cell types [29].

Identifying intermediate stages of human ES cell differentiation will also be important. As human ES cells differentiate *in vitro*, do they form distinct precursor or progenitor cells that can be identified and isolated? If ES cells do form such intermediate cell types, can the latter be maintained and expanded? Would such precursor or progenitor cells be useful for therapeutic transplantation [19]?

Finally, scientists will need to determine what differentiation stages of human ES-derived cells are optimal for other practical applications. For example, what differentiation stages of ES-derived cells would be best for screening drugs or toxins, or for delivering potentially therapeutic drugs?

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