CHAPTER 2: HUMAN STEM CELL RESEARCH AND THE POTENTIAL FOR CLINICAL APPLICATION [1]

INTRODUCTION [2]

Stem cells are unique and essential cells in higher order animals. They serve to renew tissue throughout life as they have the capability to continually reproduce themselves. Although there are many kinds of stem cells there is a hierarchy of types. Some stem cells are more committed, or differentiated, than are others. While the term "stem cell" is commonly used to refer to any cells within the adult organism that provide tissue renewal (e.g., hematopoietic stem cells, a type of cell found in the blood), the most fundamental stem cells are those found in the early-stage embryo (Van Blerkom, 1994). These embryonic stem cells, unlike more differentiated stem cells or other cell types, have the special ability to develop into nearly any cell type of the body, a type of biological plasticity called "*pluripotency*." In comparison, most other cell types have differentiated, that is, undergone a sequence of changes to a point at which they stop and remain specialized, for example, as heart, muscle, brain, or blood cells (Weiss et al., 1996).

Because stem cells have the ability to proliferate and renew themselves over the lifetime of the organism—while retaining some or all of their multilineage potential—scientists have long recognized the plausibility of using such cells to generate a large number of specialized cells or tissue through amplification, a possibility that could allow the generation of new cells in response to injury or disease (Weiss et al., 1996).

In late 1998 three separate reports brought to the forefront the scientific prospects of human pluripotent stem cell research. The first two reports, published by two independent teams of scientists, describe the first successful isolation of human pluripotent stem cells and their culture in the laboratory. The research of both teams was supported by private funds from Geron Corp., a biotechnology company in Menlo Park, CA. Dr. John Gearhart and his colleagues derived human pluripotent stem cells from primordial gonadal tissue, which was obtained from a non-living fetus (Gearhart, 1998). Dr. James Thomson and his co-workers derived pluripotent

stem cells from the blastocyst stage of an early embryo—the embryos used were donated by couples who were receiving infertility treatment (Thomson, 1998). The pluripotent stem cells derived by each of these means appear to be very similar in structure, function, and potential, but it will take more research to verify fully this claim (Varmus testimony, December 2, 1998). The third report, which describes work funded by Advanced Cell Technology of Worcester, Massachusetts, appeared in the November 12, 1998 *New York Times*, and has not yet been published or fully verified. In this report, the company claims to have made human cells revert to the primordial, totipotent state by fusing them with cow eggs and creating a hybrid embryo from which human-like stem cells appear to have been isolated (Wade, 1998).

The methodologies used by these investigators for deriving human pluripotent stem cells are not really new; pluripotent stem cells have been derived from mice since the early 1980s and, since then, from non-human primates and other animals. The isolation and culturing of human pluripotent stem cells, however, opens certain avenues of important research for the first time. At the most basic level, the isolation of these cells allows scientists to focus on how stem cells differentiate into specific types of cells, with the goal of identifying the genetic and environmental signals that direct the specialization of a stem cell to develop into specific cell types. Such research might, for example, have implications for the discovery of new ways to prevent and treat birth defects and cancer. This research would build on investigations conducted over the last decade, in which animal models have been used to determine whether pluripotent stem cells can be used to re-establish tissue in an adult organism (Hall and Watt, 1989; Corn et al., 1991; Hollands, 1991; Diukman and Golbus, 1992). Through processes we are only beginning to understand, primitive stem cells can be stimulated to become specialized, so that they are precursors to any one of many different cell types such as muscle cells, skin cells, nerve cells, liver cells.

Box A: Early Development of the Human Embryo (will include drawings)

Fertilization occurs in the oviduct. The union of an oocyte (egg) and sperm results in the formation of the zygote. Three days after fertilization occurs the zygote will have moved through the fallopian tube into the cavity of the uterus. The delayed transport of the zygote through the

fallopian tube allows several stages of division (cleavage) to occur before the zygote enters the uterus. About 36 hours after fertilization, the zygote begins to cleave into two cells called blastomeres.

At about 60 hours after fertilization, the 2 blastomeres divide again to form 4 blastomeres. At 3 days post fertilization, the 4 blastomeres divide to form 8 cells. Each blastomere becomes smaller with each subsequent division. In this early stage of development, all of the blastomeres are of equal size. These cells are unspecialized and have the capacity to differentiate into any of the cell types of the embryo as well as the essential extra-embryonic membranes and tissue. Because each of these blastomeres has the potential, within an appropriate environment, to develop into a human, one or more of them can be removed without affecting the ability of the other blastomeres to develop into a fetus. In fact, if an embryo separates in half at this early stage of development while all of the cells are still totipotent, identical twins—two genetically identical individuals—develop.[1]

When the cell division reaches about 16 cells, the zygote is called a morula. It leaves the fallopian tube and enters the uterine cavity 3 to 4 days after fertilization. After reaching the uterus, the developing zygote usually remains in the uterine cavity an additional 4 to 5 days before it implants in the endometrium (uterine wall), which means that implantation ordinarily occurs on the 7^{th} or 8^{th} day following fertilization.

Cell division continues, creating a cavity known as a blastocele in the center of the morula. With the appearance of the cavity in the center, the entire structure is now called a blastocyst. This first specialization event occurs just before the zygote attaches to the uterus, around 6 to 7 days after fertilization, when there are approximately 100 cells. This specialization

[1] Blastocyst division or induced twinning is performed by taking a single embryo at the blastocyst stage and mechanically dividing it into two so that each part receives an approximately equal number of trophoblast and ICM cells. Each blastocyst is then transferred to the uterus, so that, at most, one embryo gives rise to identical twins. At the 1993 annual meeting of the American Fertility Society, Robert Stillman and Jerry Hall reported "cloning" of human embryos by artificially separating (i.e., twinning) genetically abnormal (triploid) embryos at the 2-cell stage, followed by culture of the individual daughter cells *in vitro* to the 32-cell stage within an artificial zona pellucida (Van Blerkom, 1994). The basic findings of this study were that daughter blastomeres could be separated at the 2-cell stage by micromanipulation, and because the resulting twin embryos progressed through the preimplantation stages, developmental fate at the cellular level was not fixed in the very early human embryo (i.e., the cells were totipotent). These investigators suggested that the production of multiple genetically identical embryos could increase the chances for pregnancy through IVF by permitting more embryos to be returned to the infertile patient. *[Need to find out if this technique is ever used in humans, despite ethical concerns.]*

involves the formation of an outer layer of trophoblast cells, which will give rise to part of the placenta, surrounding a group of about 20 to 30 inner cells (the inner cell mass or ICM) that remain undifferentiated. At this stage, these cells can no longer give rise to all of the cells necessary to form an entire organism, and therefore are not capable of developing into an entire human being. In general, as cells further differentiate they lose the capacity to enter developmental pathways previously open to them.

As the blastocyst attaches to the uterus, the outer layer of blastomere cells secretes an enzyme, which erodes the epithelial uterine lining and creates an implantation site for the blastocyst. Once implantation has taken place, the trophoblast and sub-lying cells proliferate rapidly, forming the placenta and the various membranes that surround and nourish the developing embryonic cells and fetus.

In the week following implantation, the inner cells of the blastocyst give rise to more of the placental tissue and eventually to a small disc of cells from which the fetus will develop. By 14 days the embryonic disc is about 0.5 mm in diameter and consists of about 2,000 cells. It is at this time that the first stage of organized development, known as gastrulation, is initiated, leading over the next few days to the first appearance of differentiated tissues of the body, including primitive neural cells. Gastrulation is the process by which the bilaminar (two-layered) embryonic disc is converted into a trilaminar (three-layered) embryonic disc and its onset at day 14 *in vivo* is marked by the appearance of the primitive streak. This is a region in which cells move from one layer to another in an organized way.

During the third week, the embryo grows to 2.3 mm long, and the foundations of most of the major organ systems begin to form. At the beginning of the third month the embryo becomes a fetus. During the 3rd to 9th month, the organ systems and tissues of the fetus continue to develop.

STEM CELL TYPES [2]

Stem cells can be derived from several sources. Cells derived from teratocarcinomas, malignant embryonic tumors, are call *embryonal carcinoma cells*, cells derived from the inner

cell mass of preimplantation embryos are called *embryonic stem cells*, and cells that are derived from primordial germ cells of fetuses are called *embryonic germ cells*. Stem cells can also be derived through nuclear transfer of a cell into an enucleated oocyte. This may include transfers of embryonic, fetal or somatic cells to an oocyte of the same or a different species. In addition, stem cells can be found in the adult organism, for example, in bone marrow, skin, and intestine. These stem cells serve to replenish terminally differentiated cells in tissues where cells are most susceptible to injury, disease, or natural cell death. As described below, these various stem cells are thought to possess differing levels of potency, or potential to differentiate into various cell types. Although not totipotent, the most potent of these stem cell types are thought to be the embryonic stem cells. Slightly more differentiated and possibly altered in important ways from embryonic stem cells are embryonic germ cells, which arise from primordial germ cells in the developing fetus (Hogan, testimony, Feb. 1999). Stem cells found in the adult organism have been shown to be multipotent, that is in some instances capable of differentiating into selected cell types.

Embryonic Stem Cells [3]

In mammalian embryonic development, cell division gives rise to differentiated progeny that eventually comprise the mature animal. As cells become committed to a particular lineage, or cell type, a progressive decrease in developmental potential occurs. Early in embryonic development (until about 16 cells), each cell of the early cleavage-stage embryo is totipotent and has the developmental potential to contribute to any embryonic or extraembryonic cell type (Winkel and Pedersen, 1988). However, by the blastocyst stage, cells of the trophectoderm are irreversibly committed to forming the placenta and other trophectoderm lineages (Winkel and Pedersen, 1988). By six to seven days post fertilization, the inner cell mass has divided to form two layers, which give rise to the embryo proper, and extra-embryonic tissues (Gardner, 1982).

Although the cells of the inner cell mass are precursors to all adult tissues, they lose their totipotency— becoming pluripotent—and only proliferate and replace themselves in the intact embryo for a limited time before they become committed to specific lineages (Thomson and

Marshall, 1998). **Embryonic stem cells** are derived from the pluripotent cells of the inner cell mass. Once they are placed in the appropriate culture (laboratory growth) conditions, embryonic stem cells seem to be capable of unlimited, undifferentiated proliferation *in vitro*, and maintain the potential to contribute to all adult cell types (Evans and Kaufman, 1981; Martin, 1981).

While these embryonic cells are "stem cells," they differ substantially from the stem cells found within the fully developed, adult organism (see below). Most importantly, embryonic stem cells are highly proliferative, both in the embryo as well as in culture, while some stem cells of the adult can be almost totally quiescent, and may be more difficult to maintain and expand in culture (Van Blerkom, 1994). Therefore, it appears that if stem cells were someday to be used for the treatment of disease, there would be greater advantage in using embryonic stem cells. These later issues, however, remain to be fully resolved.

Embryonic Stem Cells Derived via Nuclear Transfer[4]

It may also be possible to make human pluripotent stem cells by using somatic cell nuclear transfer, the technique used to produce the sheep Dolly. Although there has been no scientific publication of this to date, presumably any cell from the human body (except the egg or sperm cell) could be stimulated to return to highly immature, pluripotent and possibly totipotent state by fusing them with an enucleated egg to form an embryo. Thus, an embryo produced via somatic cell nuclear transfer—fusing the nucleus from an embryo, fetus or somatic cell to an egg from which the genetic material has been removed—could be a source of stem cells. There is no reason to believe that stem cells derived in this way would be different from those derived through in vitro fertilization. One could imagine the prospect of nuclear transfer from an adult cell to generate an early embryo and thence an embryonic stem cell line for an individual patient, which would be ideally tissue-matched for later transplant purposes (NBAC, 1997).

Embryonic Germ Cells [3]

Primordial germ cells are the embryonic precursors of the sperm and ova of the adult animal (Donovan, 1998). The establishment of the germ line in the embryo involves the

separation of primordial germ cells from the somatic cells, proliferation of primordial germ cells, migration of these cells to the gonad, and finally their differentiation to gametes (Donovan, 1994). Primordial germ cells are the only cells in the body that can give rise to successive generations, while the somatic cells that form the body of the animal are destined for death once they start to differentiate (Matsui, 1998).

In vivo and *in vitro*, primordial germ cells can give rise to pluripotent stem cells that are capable of giving rise to cells of multiple lineages (Donovan, 1998). *In vivo*, primordial germ cells give rise to embryonal carcinoma cells. Such cells are the pluripotent stem cells of teratomas, which are benign tumors containing derivatives of the three primary germ layers: endoderm, ectoderm and mesoderm (Donovan, 1998). *In vitro*, primordial germ cells can be converted into pluripotent stem cells called **embryonic germ cells**.

Embryonic germ cells form embryoid bodies in culture, give rise to teratomas when introduced into histocompatible animals, and form germline chimeras when introduced into a host blastocyst (Donovan, 1998). The derivation of embryonic germ cells directly from primordial germ cells represents a mechanism to study some aspects of primordial germ cell development such as imprinting and pluripotency (Donovan, 1994).

Stem Cells Found in the Adult Organism [3]

In the adult mammal, cell division occurs in order to maintain a constant number of terminally differentiated cells in tissues where cells are most susceptible to injury, disease, or natural cell death. Cells with a high turnover rate are replaced by a highly regulated process of proliferation, differentiation, and apoptosis (programmed cell death) from undifferentiated stem cells (Thomson and Marshall, 1998). The best known example of a stem cell is the hematopoietic stem cell found in bone marrow, which is responsible for the production of all the terminally differentiated cell types of the blood (Iscove, 1990). Other examples include the skin epithelium and the epithelium of the small intestine (Hall and Watt, 1989). In the human small intestine, for example, approximately 10^{11} cells are shed and must be replaced daily (Potten and Loeffler,

1990). These tissues contain subpopulations of stem cells that are a source for the replacement of the relatively short-lived, terminally differentiated cells (Weiss et al., 1996).

Until the successful cloning of Dolly the sheep, it was believed that most somatic cell specialization involved irreversible genetic changes (Wilmut, 1997). With Dolly it was shown that even somatic cells conserve genomic totipotentiality. Preliminary studies of stem cells obtained from various systems of the adult organism suggest that in some cases (i.e., stem cells) the reactivation of dormant genetic programs may not require nuclear transfer or experimental modification of the genome. This particular class of stem cells might prove to be versatile to a point, that is able to differentiate along several, but probably not all, cell lineages in response to an appropriate pattern of stimulation. Thus, such cells might more appropriately be called **multipotent**.

Neuronal Stem Cells [4]

It has been recognized for a number of years that transplantation of fresh fetal tissue into the diseased adult brain was a promising therapy for neurodegenerative disorders, and has recently been shown to be effective in younger patients with Parkinsons disease (reported by Curt Freed to American Academy of Neurology, April 21, 1999). However, by developing techniques to culture primary fetal neural cells before transplantation, some of the problems of using fresh tissue may be eliminated, such as the necessity of timing the surgery to the availability of fresh fetal tissue, the need to quickly screen for infectious diseases, and the limiting amount of donor fetal tissue available (Cattaneo and McKay,). In addition, it may be possible to direct cultured cells to develop along certain lineages or to express specific genes before they are transplanted, so that, for example, dopamine-producing cells could be selectively grown to treat Parkinson's disease (Cattaneo and McKay,).

The embryonic nervous system arises from the ectoderm. The first cell type to differentiate from the uncommitted, pluripotent precursor cells are neurons followed by astrocytes and then oligodendrocytes (Frederiksen and McKay, 1998). Recently, Angelo Vescovi, a neurobiologist at the National Neurological Institute Carlo Besta in Milan, Italy, and his colleagues report that neural stem cells, which give rise to the three main types of brain cells, can

also become blood cells when transplanted into mice whose own blood-forming tissue, the bone marrow, has been mostly destroyed (Bjornson, 1999). The study did not explain what caused the neural cells to turn into blood cells, but the investigators speculate that the neural cells might be responding to the same signals that normally stimulate the few remaining blood stem cells to reproduce and mature after irradiation wipes out most of the bone marrow (Strauss, 1999). Although this research is preliminary and has not yet been conducted using human cells, it opens the possibility of using neural stem cell transplants to treat human blood cell disorders such as aplastic anemia and severe combined immunodeficiency—an appealing idea, as bone marrow stem cells do not replenish themselves well in lab cultures. The problem of access to such cells in humans, however, remains problematic, as such cells have to be obtained from the brain, an invasive and risky procedure.

Mesenchymal Stem Cells [4]

Human mesenchymal stem cells, which are present in adult marrow, are thought to be multipotent cells, in that they can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma (Pittenger, 1999). In a recent experiment, cells that have the characteristics of human mesenchymal stem cells were isolated from marrow aspirates of volunteer donors. Individual stem cells were identified that, when expanded to colonies, retained their multilineage potential. It is speculated that these particular adult stem cells could be induced to differentiate exclusively into the adipocytic, chondrocytic, or osteocytic lineages.

These experiments showed that the isolated expanded human mesenchymal stem cells in culture would differentiate, in a controlled manner, to multiple, but limited lineages. The specific environmental cues to initiate the proliferation and differentiation of these cells *in vivo* are not understood (Pittenger, 1999). The ability to isolate, expand the culture, and direct the differentiation of such cells *in vitro* to particular lineages, however, provides the opportunity to study events associated with cell commitment and differentiation. The human mesenchymal stem cells isolated by Pittenger and colleagues appear to have the ability to proliferate extensively, and

maintain the ability to differentiate into certain cell types *in vitro*. Their cultivation and selective differentiation should provide further understanding of this important progenitor of multiple tissue types and the potential of new therapeutic approaches for the restoration of damaged or diseased tissue (Pittenger, 1999).

ANIMAL MODELS [2]

The first embryonic stem cells were isolated from rabbits (Cole et al., 1965a, b). However, embryonic stem cells derived from mouse embryos have become the model system for the study of these cells (Evans and Kaufman, 1981; Martin, 1981). More recently, pluripotent stem cells have been reported for rat, hamster, mink, sheep, pig, cow, common marmoset, rhesus monkey, and humans (reviewed in Pedersen, 1994; See Table 1). The derivation and research uses of pluripotent stem cells from mice, cows, pigs, and primates are described below.

Table 1. Stem Cells Isolated from Mammals

Species	References
Mouse	Evans and Kaufman, 1981 Martin, 1981
Rat	Iannaccone et al., 1994
Hamster	Doetschman et al., 1988
Mink	Sukoyan et al., 1992 Sukoyan et al., 1993
Rabbit	Cole et al., 1965a, b Moreadith and Graves, 1992 Giles et al., 1993 Graves and Moreadith, 1993
Sheep	Handyside et al., 1987 Piedrahita et al., 1990 Notarianni et al., 1991
Pig	Piedrahita et al., 1988 Evans et al., 1990 Notarianni et al., 1990 Piedrahita et al., 1990 Hochereau-de Reiviers and Perreau, 1993 Talbot et al., 1993 Wheeler 1994 Shim et al., 1997
Cow	Evans et al., 1990 Saito et al., 1992 Strelchenko and Stice, 1994 Cibelli et al., 1998
Common Marmosett	Thomson et al., 1996
Rhesus Monkey	Thomson et al., 1995
Human	Bongso et al., 1994 Shamblott et al., 1988 Thomson et al., 1998

Mouse Embryonic Stem Cells [3]

Embryonic stem cells were first isolated from mouse blastocysts in 1981 (Evans and Kaufman, 1981; Martin, 1981). Blastocysts were place in culture and allowed to attach to the culture dish so that trophoblast cells spread out, while the undifferentiated inner cells (the inner

cell mass) continued to grow as a tight, but unorganized, cluster. Before the inner cell mass developed into the equivalent of the embryonic disc, it was drawn up into a fine pipette, dissociated into single cells, and dispersed into another dish with a rich culture medium. Under these circumstances, the dissociated cells continued to grow rapidly and almost indefinitely.

Mouse embryonic stem cells cannot become organized into an embryo by themselves or implant into the uterus if placed there. However, if the cells are injected back into a new blastocyst, they can intermingle with the host inner cell mass to make a chimera and take part in normal development, eventually contributing to all the tissues of the adult mouse, including nerve, blood, skin, bone, and germ cells (reviewed in Robertson and Bradley, 1986). This indicates that mouse embryonic stem cells have not lost the capacity to give rise to specialized tissues, but they will not do so unless placed in the right environment.

The ability of mouse embryonic stem cells to enter the germ line in chimeras allows the introduction of specific genetic changes into the mouse genome and offers a direct approach to understanding gene function in the intact animal (Rossant et al., 1993). Using the technique of homologous recombination in which a gene is either modified or disabled ("knocked out"), mouse embryonic stem cells can be derived that contain specific gene alterations. These genetically altered cells can then be used to form chimeras with normal embryos.

Mouse embryonic stem cells have also been extremely useful as *in vitro* models of the early differentiation events that take place during the development of mammalian embryos (reviewed in Pedersen, 1994). For example:

- When mouse embryonic stem cells were allowed to differentiate in culture, beating heart cells formed spontaneously, providing a model for cardiac-specific gene expression, and the development of cardiac muscle and blood vessels (Robbins et al., 1990; Wobus et al., 1991; Chen and Kosco, 1993; Doetschman et al., 1993; Miller-Hance et al., 1993; Muthuchamy et al., 1993).

- Blood formation will occur spontaneously in embryonic stem cell-derived embryoid bodies, and can be augmented by modifying the culture conditions (reviewed in Snodgrass et al., 1992). Therefore, hematopoietic stem cells have been studied extensively to determine the conditions for differentiation, survival, and proliferation of blood cells.

- Several studies have highlighted the importance of growth and differentiation factors in the regulation of mammalian development. For example, the maintenance of mouse embryonic stem cells in an undifferentiated state was found to require the presence of leukemia inhibitory factor (LIF), a differentiation-inhibiting factor (reviewed in Fry, 1992). Other studies have found several growth and differentiation factors to be important in embryonic stem cell development, including activins, colony-stimulating factor, erythopoietin, basic fibroblast growth factor, insulin-like growth factor 2, interleukins, parathryoid hormone-related peptide, platelet-derived growth factor, steel factor, and transforming growth factor β (reviewed in Pedersen, 1994).

- In mid-gestation embryos and the adult mouse, only one parental allele of imprinted genes is expressed. However, studies have suggested that there is limited relaxation of imprinting in embryonic stem cells so that both maternal and paternal alleles are expressed (reviewed in Pedersen, 1994).

By understanding the mechanisms responsible for growth and differentiation in embryonic development, it may then be possible to attempt to regulate the differentiation of embryonic stem cells along specific pathways. The knowledge gained from these types of studies could someday lead to the effective treatment of certain important human diseases.

Historically, because of its well-defined genetics, short gestational times, and large litters, the mouse has been one of the primary models for the study of mammalian embryonic development. However, there are several differences between early mouse development and early human development, including differences in:

- the timing of embryonic genome expression (Braude et al., 1988);

- the formation, structure, and function of the fetal membranes and placenta (Lucken, 1975, 1978; Benirschke and Kaufmann, 1990); and
- formation of an egg cylinder in the mouse as opposed to an embryonic disc in humans (O'Rahilly and Muller, 1987; Kaufmann, 1992).

Therefore, new models that would directly allow the study of human embryonic development are crucial for our understanding early human development.

Bovine Embryonic Stem Cells [3]

The first bovine embryonic stem cells were reported by Saito et al. (Saito et al., 1992). More recently, transgenic bovine embryonic stem cells were derived by using nuclear transfer of fetal fibroblasts to enucleated bovine oocytes (Cibelli et al., 1998). This technique involved introducing a marker gene into bovine fibroblasts from a 55-day old fetus and then fusing the transgenic fibroblasts to enucleated oocytes to produce blastocyst-stage nuclear transplant embryos (Cibelli et al., 1998). When reintroduced into preimplantation embryos, these transgenic embryonic stem cells differentiated into derivatives from the three embryonic germ layers, ectoderm, mesoderm, and endoderm (Cibelli et al., 1998). Bovine embryonic stem cells have the potential for generating tissues and organs for use in xenotransplantation to treat human diseases.

Primate Embryonic Stem Cells [3]

Primate embryonic stem cells have been derived from both the rhesus monkey (Thomson et al., 1995) and the common marmoset (Thomson et al., 1996). When allowed to grow to confluence and pile up, both marmoset and rhesus embryonic stem cells spontaneously differentiate into more complex structures, including cardiac muscle, neurons, endoderm, trophoblast, and numerous unidentified cell types (Thomson and Marshall, 1998).

Essential characteristics of primate embryonic stem cells include: 1) derivation from the preimplantation or peri-implantation embryo; 2) prolonged undifferentiated proliferation; and 3) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged maintenance in culture (Thomson and Marshall, 1998). In addition, while mouse

embryonic stem cells rarely contribute to trophoblast in chimeras (Beddington and Robertson, 1989), primate embryonic stem cells contribute to derivatives of trophoblast, endoderm, and all three germ layers, suggesting that they more closely resemble early totipotent embryonic cells (Thomson and Marshall, 1998). Furthermore, some primate embryonic stem cell lines have maintained a normal karyotype through undifferentiated culture for at least two years, sustained a stable developmental potential throughout this culture period, and maintained the potential to form trophoblast *in vitro* (Thomson et al., 1995, 1996).

Although there is some variation between species, nonhuman primate embryonic stem cell lines provide an accurate *in vitro* model for understanding the differentiation of human tissues (Thomson and Marshall, 1998). In addition, primate embryonic stem cells provide a powerful model for understanding human development and disease. Furthermore, because of the similarities between embryonic stem cells from humans and primates, primate embryonic stem cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of embryonic stem cell-based therapies (Thomson, et al., 1995).

HUMAN MODELS [2]

Human Embryonic Stem Cell Lines Derived from Blastocysts [3]

The first successful isolation of cells from the human inner cell mass of blastocysts and their culture *in vitro* for at least two passages was reported by Bongso and colleagues in 1994 (Bongso et al., 1994). Starting with 21 spare embryos donated by 9 patients in an IVF program[2], this group isolated cells with typical stem cell characteristics from 17 5-day-old blastocyts (Bongso et al., 1994). These cell were like embryonic stem cells—small and round with high nuclear to cytoplasmic ratios, stained positively for alkaline phosphatase (a biochemical marker for stem cells), and maintained a normal diploid karyotype—however, after the second subculture, the cells differentiated into fibroblasts or died (Bongso et al., 1994).

[2] Consent to carry out this study was approved by the hospital ethical committee based on the guidelines on Assisted Reproductive Technology of the Ministry of Health, Singapore that experimentation of human embryos up to day 14 of embryonic growth may be allowed (Bongso et al., 1994).

In later work, James A. Thomson, an embryologist at the University of Wisconsin, and his colleagues, were able to isolate human embryonic stem cell lines and grow them continuously in culture for at least five to six months, a major technical achievement. This renewable tissue culture source of human cells, capable of differentiating into a wide variety of cell types, is believed to have broad applications in basic research and transplantation therapies (Gearhart, 1998).

In Thomson's work human embryonic stem cells were isolated from embryos that were originally produced by *in vitro* fertilization (IVF) for clinical purposes (see Box B). The embryos were donated by individuals after providing informed consent and receiving approval by an appropriately constituted Institutional Review Board (Thomson et al., 1998). Thirty-six embryos were cultured for approximately five days. The inner cell mass was isolated from fourteen of the 20 blastocysts that developed, and five embryonic stem cell lines, originating from 5 separate embryos, were derived (Thomson et al., 1998). The technique used to derive these human embryonic stem cells was essentially the same as that used to isolate nonhuman primate embryonic stem cell lines (Thomson et al., 1995).

The human embryonic stem cell lines had normal karyotypes (two male and three female), and were grown in culture continuously for at least five to six months (Thomson et al., 1998). The cell lines expressed high levels of telomerase activity, which is indicative of a high replicative life-span and immortality in human cell lines (Thomson et al., 1998). In addition, the cell lines expressed cell surface markers that are also found on nonhuman primate embryonic stem cells (Thomson et al., 1998). Most importantly, the cell lines maintained the potential to form derivatives of all three embryonic germ layers, endoderm, mesoderm, and ectoderm (Thomson et al., 1998).

It is believed that research with human embryonic stem cells could offer insights into developmental events that cannot be studied directly in the intact human embryo, but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss.

Such cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans. Screens based on the *in vitro* differentiation of human embryonic stem cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds (Thomson, 1998).

Box B: *In vitro* Fertilization [to be updated]

The procedure of *in vitro* fertilization of human oocytes is now widely available in many countries throughout the world and in the United States. It was originally developed for the treatment of infertility due to blocked fallopian tubes, but has been extended to assist patients with premature depletion of oocytes, recurrent failure of embryos to implant, and low production of functional sperm. More recently, the technique has been used in conjunction with preimplantation genetic diagnosis to enable fertile couples at risk for transmitting severe or fatal inherited diseases to have healthy children.

Although details of the IVF procedures vary among centers, the basic principles are as follows: oocyte donors are treated with a regimen of hormones over several days designed to stimulate the final maturation of several follicles within the ovary. This is known as hyperstimulation, and carries with it a low risk (less than 1 in 100) of an adverse reaction. Following completion of the hormone treatment mature follicles are detected by sonography and an average of around 10 are collected by transvaginal aspiration under sedation. The oocytes are then inseminated and cultured in sterile fluid for about two days. When they have reached the 4- to 8-cell stage, between 3 and 6 embryos are transferred to the uterus, and untransferred embryos are usually frozen, if they are developing normally. Non-viable embryos are discarded.

The efficiency of the IVF procedure is low, with approximately [5 to 10] percent of fertilized eggs give rise to live born children, depending on factors such as age of the recipient and the reason for infertility. In comparison, approximately 30 percent of normally (in vivo) conceived human embryos result in a successful pregnancy. [need recent refs.]

Unused embryos can be cryopreserved and stored indefinitely. Issues concerning the disposition of unused embryos remaining after infertility treatments are addressed more fully in chapter 4 of this report.

SOURCES: Report of the NIH Human Embryo Research Panel, 1994; *Assisted Reproductive Technologies: Analysis and Recommendations for Public Policy*, The New York State Task Force on Life and the Law, 1998.

Human Pluripotent Stem Cells from Fetal Primordial Germ Cells [3]

Primordial germ cells have also been found to give rise to cells with characteristics of embryonic stem cells, and have been designated *embryonic germ cells* to distinguish their tissue of origin (Gearhart, 1998). A 1998 report from John D. Gearhart of the Johns Hopkins University School of Medicine in Baltimore and his colleagues describes the establishment of human embryonic germ cell lines from human primordial germ cells (Shamblott, 1998). The human embryonic germ cells were isolated from the developing gonads of 5- to 9-week old fetuses that were obtained as a result of elective abortion, using an IRB-approved protocol (Shamblott, 1998). These human embryonic germ cell lines have morphological, immunohistochemical, and karyotypic features consistent with those of previously described pluripotent stem cells and have a demonstrated ability to differentiate *in vitro* into derivatives of the three embryonic germ layers (Shamblott, 1998).

Fusion of Human Somatic Cells with Cow Eggs to Create Hybrid Embryonic Cells [3]

Advanced Cell Technology of Worcester, Mass. announced November 11, 1998, that its scientists had for the first time made human cells revert to the primordial, totipotent state and fusing them with cow eggs to create a hybrid embryo (Wade, 1998). This work with human cells was performed in 1996 by Jose Cibelli. Using 52 of his own cells, some of them white blood cells and others scraped from the inside of his cheek, Cibelli used a pulse of electricity to fuse each cell with a cow egg from which the nucleus containing the DNA had first been removed (PCT/U397/12919, 1997). Out of these 52 attempts, only one embryo, derived from a cheek epithelial cell, developed into a blastocyst. Approximately 12 days after the fusion of cheek cell and cow egg, there were sufficient cells to allow harvesting of the inner cell mass to produce cells resembling human embryonic stem cells (PCT/U397/12919, 1997). The scientists observed that the hybrid cell quickly became more human-like as the human nucleus took control and displaced cow proteins with human proteins. However, it is difficult to judge the validity of this work since it is extremely preliminary and has not been submitted for peer review or publication in a scientific journal.

The stated purpose of these experiments was to create an embryo solely for the purpose of establishing an embryonic stem cell line that could potentially be used to treat any disease caused by loss or malfunction of cells, such as Parkinson's disease, diabetes, and heart disease. The researchers emphasized that they had no intention of transferring the resulting hybrid embryos to a uterus, something they considered to be unethical and unsafe (Wade, 1998).

POTENTIAL MEDICAL APPLICATIONS OF STEM CELL RESEARCH [2]

Gaining the ability to elucidate the mechanisms that control cell differentiation is, at the most elemental level, the promise of stem cell research. This knowledge will facilitate the efficient, directed differentiation of pluripotent stem cells to specific cell types. The standardized production of large, purified populations of, for example, euploid human cells such as cardiomyocytes and neurons could provide a potentially limitless source of cells for drug discovery and transplantation therapies (Thomson et al., 1998). Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types and the replacement of those cells could offer lifelong treatment.

Substantial advances in basic developmental biology are required before one could direct pluripotent stem cells to lineages of human clinical importance. However, progress has already been made in the *in vitro* differentiation of mouse embryonic stem cells to neurons, hematopoietic cells, and cardiac muscle (Deacon, 1998; Brustle, 1997; Shamblott, 1998). Pluripotent stem cells could be put to use in targeting neurodegenerative disorders, diabetes, spinal cord injury, and hematopoietic repopulation.

Use of Stem Cells in Transplantation [3]

One of the major causes of organ transplantation and graft failure is immune rejection. A likely application of stem cell research is in the area of transplantation. Although much research remains to be done, stem cells derived via somatic cell nuclear transfer offer the possibility that therapies could be developed from the patients own cells, that is, a patient's somatic cell could be fused with an enucleated ova, developed to the blastocyst stage, at which point stem cells could be derived for the development of cell-based therapy, essentially an autologous transfer.

Alternatively, other techniques could be used to generate pluripotent stem cells that would be immunologically compatible for transplantation purposes, such as:

1) banking of multiple cell lines representing a spectrum of major histocompatibility complex (MHC) alleles to serve as a source for MHC matching;
2) creation of universal donor lines, in which the MHC genes could be genetically altered so rejection would not occur, an approach that has been tried with moderate success in the mouse;
3) customization of embryonic stem cells through transgenesis and gene targeting so that a potential recipient's MHC genes are introduced into embryonic stem cells through homologous recombination; and
4) production of embryonic stem cell lines containing the genome of the prospective recipient (Gearhart, 1998).

Autologous transplants would obviate the need for immunosuppressive agents in transplantation and the ensuing susceptibility to other diseases. In addition to eliminating the need for immunosuppressive drugs, this would address problems ranging from the supply of donor organs to the difficulty of finding matches between donors and recipients. Research on embryonic stem cells could lead to cures for diseases that require treatment through transplantation, including autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and type-I diabetes.

A near-term prospect for the application of pluripotent stem cell research is in the treatment of type-I diabetes (Varmus testimony, JDF testimony). Such treatment would involve the transplantation of pancreatic islet cells or beta cells produced from autologous embryonic stem cells.

Studies of Human Reproduction and Developmental Biology [3]

Research using human embryonic stem cells could offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss (Thomson et

al., 1998). Embryonic stem cells provide large quantities of homogeneous material that can be used for biochemical analysis of the patterns of gene expression and the molecular mechanisms of embryonic differentiation. Work in the mouse has shown that pluripotential stem cells, primarily embryonic stem cells, are essential to studies of embryogenesis, gene function, and development in the mouse (Thomson, 1998). Gene targeting within embryonic stem cells has enabled whole-animal studies of gene function and the production of mouse models of human genetic diseases and abnormalities.

Cancer Therapy [3]

Pluripotent stem cells may be used to reduce the tissue toxicity brought on by cancer therapy. Already bone marrow stem cells, representing a more committed stem cell, are used to treat patients after high-dose chemotherapy. However, the recovered blood cells appear limited in their ability to recognize abnormal cells such as cancer cells. It is possible that injections of pluripotent stem cells would revive the complete immune response to patients undergoing bone marrow transplantation, an important possibility since current approaches aim to manipulate the immune system after high dose chemotherapy, so that it specifically recognizes cancer cells.

Diseases of the Nervous System [3]

Some believe that in no area of medicine are the potential benefits of stem cell research greater than in diseases of the nervous system (cite Senate testimonies). The most obvious reason is that so many of these diseases result from the loss of nerve cells, and mature nerve cells cannot divide to replace those that are lost. For example, in Parkinson's disease, nerve cells that make the chemical dopamine die; in Alzheimer's disease, it is the cells that make acetylcholine that die; in amyotrophic lateral sclerosis the motor nerve cells that activate muscles die. In stroke, brain trauma, and spinal cord injury many types of cells are lost.

Preliminary results from fetal tissue transplantation trials for Parkinson's disease argue that supplying new cells to a structure as intricate as the brain can slow or stop disease progression (Freed, 1999). Yet the difficulty of obtaining enough cells of the right type, that is, dopamine producing nerve cells—limits success. In 1999 scientists developed methods in animal

models to isolate neural stem cells and coax them to proliferate for several generations in cell culture, and then, on cue, to specialize into mature dopamine nerve cells. When these cells were implanted into the brains of rodents with experimental Parkinson's disease, the animals showed improvements in their movement control (get refs from NINDS). A large supply of "dopamine competent" stem cells, such as pluripotent cell lines, could remove the barrier of limited amounts of tissue. Other diseases that might benefit from a similar approach are Tay-Sachs disease, spinal cord injury, epilepsy, and stroke.

Toxicity and Drug Testing [3]

Human stem cell research offers promise for use in testing the beneficial and toxic effects of biologicals, chemicals and drugs in the most relevant species for clinical validity, humans. Such studies could lead to fewer, less-costly, better-designed human clinical trials yielding more specific diagnostic procedures and more effective systemic therapies. Beyond the drug development screening of pharmacological agents for toxicity and/or efficacy, human pluripotent stem cell research could define new research approaches for clarifying the complex association of environmental agents with human disease processes (NIEHS, 1999). It also makes possible a new means of conducting detailed investigations of the underlying mechanisms of the effects of environmental toxicant or mixtures of toxicants, including their subtle effects on the developing embryonic and fetal development tissue systems.

Diseases of the Bone and Cartilage [3]

Because stem cells constitute a self-renewing population of cells, they can be cultured to generate greater numbers of bone or cartilage cells than could be obtained from a tissue sample. If a self-renewing population of pluripotent stem cells can be established in a transplant recipient, it could effect long-term correction of many diseases and degenerative conditions in which bone or cartilage cells are deficient in numbers or defective in function. This could be done either by transplanting the stem cells from a healthy donor to a recipient, or by genetically modifying a person=s own stem cells and returning them to the marrow. Such an approach holds promise for the treatment of genetic disorders of bone and cartilage, such as osteogenesis imperfecta and the various chondrodysplasias. In a somewhat different potential application, stem cells could

perhaps be stimulated in culture to develop into either bone or cartilage-producing cells. These cells could then be introduced into the damaged areas of joint cartilage in cases of osteoarthritis, or into large gaps in bone that can arise from fractures or surgery. This sort of repair would have a number of advantages over the current practice of tissue grafting (NIAMS, 1999).

Blood Disorders [3]

The epsilon globin gene is expressed only in embryonic red blood cells. This gene recently has been shown to block the sickling of the sickle cell hemoglobin (ref.). Research involving embryonic stem cells could help answer questions about how to turn on the epsilon globin gene in adult blood cells and thereby halt the disease process. Stem cell research may also help produce transplantable cells that would not contain the sickle cell mutation.

Transplantable Organs [3]

Several researchers are investigating ways to isolate adult stem cells and create transplantable organs that may be used to treat a multitude of diseases that do not rely on the use of embryonic or fetal tissue. For example, recent developments indicate that it may soon be possible to create transplantable organs that may be able to overcome such problems as the limited supply of organs and tissue rejection.

For example, using tissue engineering methods, researchers have successfully grown bladders in the laboratory, implanted them into dogs, and shown them to be functional (Oberpenning et al., 1999). To create the bladders, small biopsies of tissue were taken from canine bladders which were teased apart to isolate the urothelial tissue and muscle tissue which were then grown separately in culture (Tanne, 1999). The tissue was then applied to a mold of biodegradable material with the urothelial tissue on the inside and the muscle tissue on the outside (Tanne, 1999). The new organs were transplanted within five weeks (Tanne, 1999).

Dogs that received the tissue engineered organs regained 95 percent of their original bladder capacity, were continent, and voided normally. When the new organs were examined 11 months later, they were completely covered with urothelial and muscle tissue, and had both nerve

and blood vessel growth. Dogs who did not undergo reconstructive procedures or only received implants of the biodegradable molds did not regain normal bladder function (Oberpenning et al., 1999).

This accomplishment marks the first time a mammalian organ has been grown in a laboratory. The ability to create new organs would be extremely useful to treat babies with congenital malformations of organs and people who have lost organs due to trauma or disease (Tanne, 1999).

SUMMARY [2]

Currently, pluripotential stem cells can be derived from the early-stage embryo or from the primordial germ cells of fetal tissue. These cells, present in the early stages of embryo and fetal development, can generate all of the human cell types and are capable of self-renewal. A renewable, tissue culture source of human cells capable of differentiating into a wide variety of cell types would have broad applications in basic research and transplantation therapies. A major step in realizing this goal was taken in 1998 with the demonstration that human embryonic stem cells can be grown in culture. These stem cells have been derived in culture from two embryonic tissues: inner cell masses of blastocysts (those cells within the conceptus that form the embryo proper) and primordial germ cells. The clinical potential for human embryonic stem cells is vast. Such cells will be important for in vitro studies of normal human embryogenesis, abnormal development (through the generation of cell lines with targeted gene alterations and engineered chromosomes), human gene discovery, and drug and teratogen testing and as a renewable source of cells for tissue transplantation, cell replacement, and gene therapies.

GLOSSARY **[being developed]**

blastocyst - a stage in the development of a mammalian embryo which follows the morula. It consists of an outer layer of trophoblast to which is attached an inner cell mass.

blastomere - 1) cleavage cell; 2) one of the cells into which the egg divides after its fertilization; 3) one of the cells resulting from the cleavage of a fertilized ovum.

cloning - the production of a precise genetic copy of a molecule (including DNA), cell, tissue, plant, or animal.

differentiation –

diploid - denoting to state of a cell containing two haploid sets derived from the father and the mother respectively; the normal chromosome complement of somatic cells (in humans, 46 chromosomes)

ectoderm -

embryo - 1) the beginning of any organism in the early stages of development; 2) stage in prenatal development of a mammal between the ovum and the fetus; 3) in humans, stage of development between the 2nd and 8th weeks inclusive. [Definition from: Taber's Cyclopedic Medical Dictionary. Edition 14. Clayton L. Thomas, ed. F.A. Davis Company, Philadelphia. 1981.]

embryonic stem cell –

embryonic germ cell –

endoderm -

fibroblast – a cell present in connective tissue, capable of forming collagen fibers.

gamete - 1) any germ cell, whether ovum or spermatozoon; 2) a mature male or female reproductive cell.

germ cells - gametes or the cells that give rise directly to gametes.

inner cell mass -

karyotype – the chromosome characteristics of an individual cell or of a cell line, usually presented a systematic array of metaphase chromosomes from a photograph of a single cell nucleus arranged in pairs in descending order of size and according to the position of the centromere.

mesoderm – the middle of the three primary germ layers of the embryo; the origin of all connective tissues, all body musculature, blood, cardiovascular and lymphatic systems, most of the urogenital system, and the lining of the pericardial, pleural, and peritoneal cavities.

morula - 1) the mass of blastomeres resulting from the early cleavage divisions of the zygote; 2) solid mass of cells resembling a mulberry, resulting from cleavage of an ovum.

oocyte - 1) a diploid cell that will undergo meiosis (a type of cell division of germ cells) to form an egg; 2) immature ovum.

ovum - 1) female sex cell; 2) female reproductive or germ cell.

pluripotent - cells, present in the early stages of embryo development, that can generate all of the cell types in a fetus and in the adult and are capable of self-renewal. Pluripotent cells are not capable of developing into an entire organism.

somatic cells - [from *soma* - the body] 1) cells of which in mammals and flowering plants normally have 2 sets of chromosomes, one derived from each parent; 2) all cells of an organism with the exception of the germ cells.

stem cell - cells that have the ability to divide without limit and to give rise to specialized cells

totipotent - means the potential is total; having unlimited capacity. Totipotent cells have the capacity to differentiate into the embryo and into extraembryonic membranes and tissues. Totipotent cells contribute to every cell type of the adult organism.

trophoblast - The outermost layer of the developing blastocyst of a mammal. It differentiates into two layers, the cytotrophoblast and syntrophoblast, the latter coming into intimate relationship with the uterine endometrium with which it establishes nutrient relationships.

zygote - 1) the cell resulting from the fusion of two gametes in sexual reproduction; 2) a fertilized egg (ovum); 3) the diploid cell resulting from the union of a sperm and ovum.

References

1. Benirschke, K., and Kaufmann, P. 1990. Pathology of the Human Placenta. Springer-Verlag, New York.

2. Biology. 1979. Third Edition. Helena Curtis. Worth Publishers, Inc., New York.

3. Christopher R. R. Bjornson, C.R.R., Rietze, R.L., Reynolds, B.A., Magli, M.C., and A.L. Vescovi "Turning Brain into Blood: A Hematopoietic Fate Adopted by Adult Neural Stem Cells in Vivo," *Science* 1999 January 22; 283: 534-537.

4. Bongso, A., Fong, C. -Y., Ng, S. -C., and Ratman, S. 1994. Isolation and culture of inner cell mass cells from human blastocysts. *Human Reproduction* 9(11): 2110-2117.

5. Braude, P., Bolton, B., and Moore, S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332**:** 459-461.

6. Brustle, O., *et al., Proc. Natl. Acad. Sci. U.S.A.* 94, 14809 (1997)

7. Cattaneo, E., and McKay, R. Identifying and manipulating neuronal stem cells. *Trends in Neurosciences*

8. Chen, U., and Kosco, M. 1993. Differentiation of mouse embryonic stem cells *in vitro*. III. Morphological evaluation of tissues developed after implantation of differentiated mouse embryoid bodies. *Dev. Dynamics* 197**:** 217-226.

9. Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, K . J., Jerry, J., Blackwell, C., Ponce de Leon, F. A., and Robl, J. M. 1998. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nature Biotechnology* 16**:** 642-646.

10. Cole, R. J., Edwards, R. G., and Paul, J. 1965a. Cytodifferentiation in cell colonies and cell strains derived from cleaving ova and blastocysts of the rabbit. *Exp. Cell Res.* 37: 501-504.

11. Cole, R. J., Edwards, R. G., and Paul, J. 1965b. Cytodifferentiation and embryogenesis in cell colonies and tissue cultures derived from ova and blastocysts of the rabbit. *Dev. Biol.* 13**:** 385-407.

12. Corn, B., Reed, M., Dishong, S., Yunsheng, L., and Wagner, T. 1991. Culture and successful transplantation of embryonic yolk-sac cells. *Clin. Biotech.* **3:** 15.

13. Deacon, T., Dinsmore, J., Costantini, L.C., Ratliff, J., Isacson, O. xxxxx, *Exp. Neurol.* 149**,** 28 (1998)

14. Diukman, R., and Golbus, M. 1992. In utero stem cell therapy. *J. Reprod. Med.* 37: 515-520.

15. Doetschman, T., Williams, P., and Maeda, N. 1988. Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev. Biol.* 127**:** 224-227.

16. Doetschman, T., Shull, M., Kier, A., and Coffin, J. 1993. Embryonic stem cell model systems for vascular morphogenesis and cardiac disorders. *Hypertension* 22: 618-629.

17. Donovan, P. J. 1994. Growth factor regulation of mouse primordial germ cell development. *Current Topics in Developmental Biology* 29: 189-225.

18. Donovan, P. J. 1998. The germ cell - the mother of all stem cells. *Int. J. Dev. Biol.* 42: 1043-1050.

19. Evans, M. F., and Kaufman, M. H. 1981. Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292: 154-156.

20. Evans, M. J., Notarianni, E., Laurie, S., and Moor, R. M. 1990. Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. *Theriogenology* 33: 125-128.

21. Frederiksen, K., and McKay, R. D. G. 1988. Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. *J. Neurosci.* 8: 1144-1151.

22. Fry, R. 1992. The effect of leukemia inhibitory factor (LIF) on embryogenesis. *Reprod. Fertil. Dev.* 4: 449-458.

23. Gardner, R. L. 1982. Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. Exp. Morphol.* 68**:** 175-198.

24. Geahart, J. 1998. New potential for human embryonic stem cells. *Science* 282: 1061-1062.

25. Giles, J. R., Yang, X., Mark, W., and Foote, R. H. 1993. Pluripotency of cultured rabbit inner cell mass cells detected by izozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae. *Mol. Reprod. Dev.* 36: 130-138.

26. Graves, K. H., and Moreadith, R. W. 1993. Derivatin and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. *Mol. Reprod. Dev.* 36(4): 424-433.

27. Hall, P. A., and Watt, F. M. 1989. Stem cells: The generation and maintenance of cellular diversity. *Development* 106: 619-633.

28. Hochereau-de Reviers, M., and Perreau, C. 1993. *In vitro* culture of embryonic disc cells from porcine blastocysts. *Reprod. Nutr. Dev.* 33: 475-483.

29. Hole, N., Graham, G.J., Menzel, U., and J. D. Ansell, *Blood* 88, 1266 (1996)

30. Hollands, P. 1991. Embryonic stem cell grafting: The therapy of the future? *Hum. Reprod.* 6: 79-84.

31. Human Embryo Research Panel. 1994. Report of the Human Embryo Research Panel, National Institutes of Health.

32. Iannaccone, P., Taborn, G., Garton, R., Caplice, M., and Brenin, D. 1994. Pluripotent embryonic stem cells from the rat are capable of producing chimeras. *Dev. Biol.* 163: 288-292.

33. Iscove, N. 1990. Haematopoiesis. Searching for stem cells. *Nature* 347: 126-127.

34. Johnson, W. H., Loskutoff, N. M., Plante, Y., and Betteridge, K. J. 1995. Production of four identical calves by the separation of blastomeres from an in vitro derived four-cell embryo. *Veterinary Record* 137: 15-16.

35. Kaufmann, M. H. 1992. The Atlas of Mouse Development. Academic Press, London.

36. Luckett, W. P. 1975. The develpment of primordial and definitive amniotic cavities in early rhesus monkey and human embryos. *Am. J. Anat*. 144: 149-168.

37. Luckett, W. P. 1978. Origin and differentiation of the yolk sac extraembryonic mesoderm in presomite human and rhesus monkey embryos. *Am. J. Anat*. 152: 59-98.

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

39. Matsui, Y. 1998. Developmental fates of mouse germ cell line. *Int. J. Dev. Biol.* 42: 1037-1042.

40. Miller-Hance, W., LaCorbiere, M., Fuller, S., Evans, S., Lyons, G., Schmidt, C., Robbins, J., and Chien, K. 1993. *In vitro* chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *J. Biol. Chem.* 268: 25244-25252.

41. Moreadith, R., and Graves, K. 1992. Derivation of pluripotential embryonic stem cells from the rabbit. *Trans. Assoc. Am. Physicians* 105: 197-203.

42. Muthuchamy, M., Pajak, L., Howles, P., Doetschman, T., and Wieczorek, D. 1993. Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. *Mol. Cell Biol.* 13: 3311-3323.

43. [NABER. 1995. Report on Human Cloning Through Embryo Splitting: An Amber Light. NABER: National Advisory Board on Ethics in Reproduction Report 1(1): 3,6]

44. Notarianni, E., Laurie, S., Moor, R. M., and Evans, M. J. 1990. Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *J. Reprod. Fertil.* (Suppl.) 40: 51-56.

45. Oberpenning, F., Meng, J., Yoo, J. J., and Atala, A. 1999. De novo reconstruction of a functional mammalian urinary bladder by tissue engineering. *Nature Biotechnology* 17: 149-155.

46. O'Rahilly, R. and Muller, F. 1987. Developmental Stages in Human Embryos. Carnegie Instution of Washington, Washington.

47. PCT/U397/12919. 1997. Embryonic or Stem-Like Cell Lines Produced by Cross Species Nuclear Transplantation. *International Application Published Under the Patent Cooperation Treaty* WO 98/07/841.

48. Pedersen, R. A. 1994. Studies of *in vitro* differentiation with embryonic stem cells. *Reproduction, Fertility, and Development* 6: 543-552.

49. Piedrahita, J. A., Anderson, G. B., Martin, G. R., Bondurant, R. H., and Pashen, R. L. 1988. Isolation of embryonic stem cell-like colonies from porcine embryos. *Theriogenology* 29: 286.

50. Piedrahita, J. A., Anderson, G. B., and Bondurant, R. H. 1990. On the isolation of embryonic stem cells: Comparative behavior of murine, porcine and ovine embryos. *Theriogenology* 34: 879-891.

51. Plante, Y., Schmutz, S. M., and Lang, K. D. M. 1992. *Theriogenology* 38: 897.

52. Potten, C. S., and Loeffler, M. 1990. Stem cells: Attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 110: 1001-1020.

53. Robbins, J., Gulick, J., Sanchez, A., Howles, P., and Doetschman, T. 1990. Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development *in vitro*. *J. Biol. Chem.* 265: 11905-11909.

54. Robertson, E., and Bradley, A. 1986. Productin of permanent cell lines from early embryos and their use in studying developmental problems. In: Experimental Approaches to Mammalian Embryonic Development, eds. J. Rossant and R. A. Pedersen, Cambridge University Press, Cambridge, pp. 475-508.

55. Saito, S., Strelchenko, N. S., and Niemann, H. 1992. Bovine embryonic stem cell-like cell lines cultured over several passages. *Roux's Arch. Dev. Biol.* 201: 134-141.

56. Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Higgins, G. R., and Gearhart, J. D. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. USA* 95: 13726-13731.

57. Shim, H., Gutierrez-Adan, A., Chen, L., BonDurant, R., Behboodi, E., and Anderson, G. 1997. *Biol. Reprod*. 57: 1089-1095.

58. Snodgrass, H., Schmitt, R., and Bruyns, E. 1992. Embryonic stem cells and *in vitro* hematopoiesis. *J.Cell. Biochem.* 49**:** 225-230.

59. Strelchenko, N., and Stice, S. 1994. Bovine embryonic pluripotent cell lines derived from morula stage embryos. *Theriogenolgy* 41: 304

60. Sukoyan, M. A., Golubitsa, A. N., Zhelezova, A. I., Shilov, A., Vatolin, S., Maximovsky, L., Andreeva, L., McWhir, J., Pack, S., Bayborodin, S., Kerkis, A. Y., Kizilova, H. I., and Serov, O. L. 1992. Isolation and cultivation of blastocyst-derived stem cell lines from American mink (*Mustela vison*). *Mol. Reprod. Dev.* 33: 418-431.

61. Sukoyan, M. A., Vatolin, S.Y., Golubitsa, A. N., Zhelezova, Semenova, L. A., and Serov, O. L. 1993. Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: Comparisons of their pluripotencies. *Mol. Reprod. Dev.* 36: 148-158.

62. [Taber's Cyclopedic Medical Dictionary. 1981. Edition 14. Clayton L. Thomas, ed. F.A. Davis Company, Philadelphia.]

63. Talbot, N., Rexroad, C. E. Jr., Pursel, V., and Powell, A. 1993. Alkaline phosphatase staining of pig and sheep epiblast cells in culture. *Mol. Reprod. Dev.* 36: 139-147.

64. Tanne, J. H. 1999. Researchers implant tissue engineered bladders. *BMJ* 318: 350.

65. [Textbook of Medical Physiology. 1981. Sixth Edition. Arthur C. Guyton. W. B. Saunders Company, Philadelphia.]

66. Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P. Becker, R. A., and Hearn, J. P. 1995. Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. USA* 92: 7844-7848.

67. Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., and Hearn, J. P. 1996. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biol. Reprod*. 55: 254-259.

68. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.

69. Thomson, J. A., and Marshall, V. S. 1998. Primate embryonic stem cells. *Current Topics in Developmental Biology* 38: 133-165.

70. Van Blerkom, J. 1994. The history, current status and future direction of research involving human embryos. *Papers Commissioned for the Human Embryo Research Panel*, pp. 1-25.

71. Wade, N. 1998. Human Cells Revert to Embryo State, Scientists Assert. *The New York Times* November 12.

72. Weiss, S., Reynolds, B. A., Vescovi, A. L., Morshead, C., Craig, C. G. and van der Kooy, D. 1996. Is there a neural stem cell in the mammalian forebrain? *Trends in Neurosciences* 19(9): 387-393.

73. Wheeler, M. B. 1994. Develoment and validation of swine embryonic stem cells: A review. *Reprod. Fertil. Dev.* 6: 563-568.

74. Wobus, A., Wallukat, G., and Hescheler, J. 1991. Pluipotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation* 48: 173-182.

75. Wylie, C. 1999. Germ cells. *Cell* 96: 165-174.