1. THE STEM CELL

WHAT IS A STEM CELL?

A stem cell is a cell that has the ability to divide (self replicate) for indefinite periods—often throughout the life of the organism. Under the right conditions, or given the right signals, stem cells can give rise (differentiate) to the many different cell types that make up the organism. That is, stem cells have the potential to develop into mature cells that have characteristic shapes and specialized functions, such as heart cells, skin cells, or nerve cells.

THE DIFFERENTIATION POTENTIAL OF STEM CELLS: BASIC CONCEPTS AND DEFINITIONS

Many of the terms used to define stem cells depend on the behavior of the cells in the intact organism (*in vivo*), under specific laboratory conditions (*in vitro*), or after transplantation *in vivo*, often to a tissue that is different from the one from which the stem cells were derived.

For example, the fertilized egg is said to be totipotent—from the Latin *totus,* meaning entire—because it has the potential to generate all the cells and tissues that make up an embryo and that support its development in utero. The fertilized egg divides and differentiates until it produces a mature organism. Adult mammals, including humans, consist of more than 200 kinds of cells. These include nerve cells (neurons), muscle cells (myocytes), skin (epithelial) cells, blood cells (erythrocytes, monocytes, lymphocytes, etc.), bone cells (osteocytes), and cartilage cells (chondrocytes). Other cells, which are essential for embryonic development but are not incorporated into the body of the embryo, include the extra-embryonic tissues, placenta, and umbilical cord. All of these cells are generated from a single, totipotent cell—the zygote, or fertilized egg.

Most scientists use the term pluripotent to describe stem cells that can give rise to cells derived from all three embryonic germ layers—mesoderm, endoderm, and ectoderm. These three germ layers are the embryonic source of all cells of the body (see Figure 1.1. Differentiation of Human Tissues). All of the many different kinds of specialized cells that make up the body are derived from one of these germ layers (see Table 1.1. Embryonic Germ Layers From Which Differentiated Tissues Develop). "Pluri"—derived from the Latin *plures*—means several or many. Thus, pluripotent cells have the potential to give rise to any type of cell, a property observed in the natural course of embryonic development and under certain laboratory conditions.

Unipotent stem cell, a term that is usually applied to a cell in adult organisms, means that the cells in question are capable of differentiating along only one lineage. "Uni" is derived from the Latin word *unus,* which means one. Also, it may be that the adult stem cells in many differentiated, undamaged tissues are typically unipotent and give rise to just one cell type under normal conditions. This process would allow for a steady state of self-renewal for the tissue. However, if the tissue becomes damaged and the replacement of multiple cell types is required, pluripotent stem cells may become activated to repair the damage [2].

The embryonic stem cell is defined by its origin—that is from one of the earliest stages of the development of the embryo, called the blastocyst. Specifically, embryonic stem cells are derived from the inner cell mass of the blastocyst at a stage before it would implant in the uterine wall. The embryonic stem cell can self-replicate and is pluripotent—it can give rise to cells derived from all three germ layers.

The adult stem cell is an undifferentiated (unspecialized) cell that is found in a differentiated (specialized)

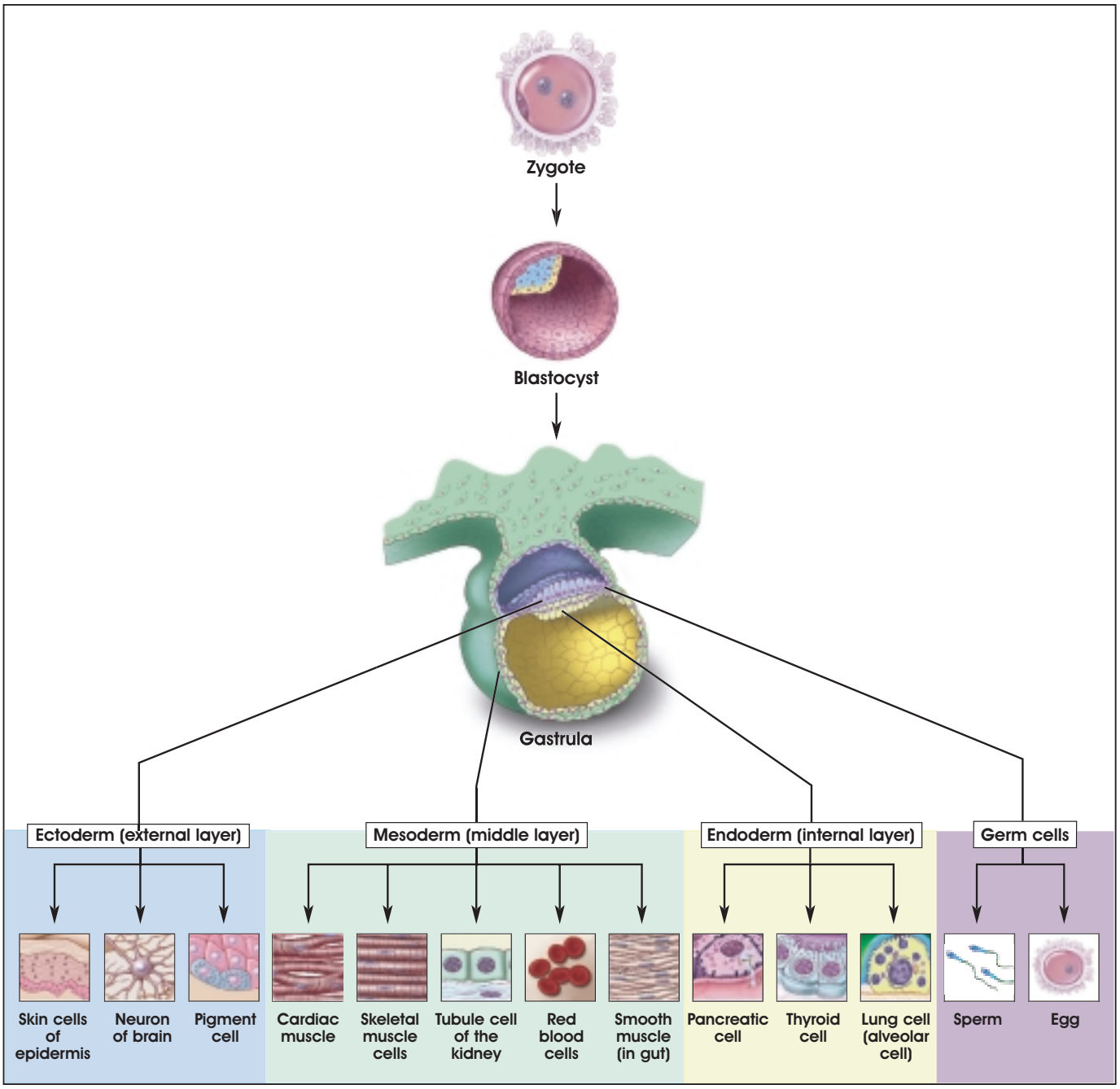

Figure 1.1. Differentiation of Human Tissues.

tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated. Adult stem cells are capable of self-renewal for the lifetime of the organism. Sources of adult stem cells have been found in the bone marrow, blood stream, cornea and retina of the eye, the dental pulp of the tooth, liver, skin, gastrointestinal tract, and pancreas. Unlike embryonic stem cells, at this point in time, there are no isolated adult stem cells that are capable of forming all cells of the body. That is, there is no evidence, at this time, of an adult stem cell that is pluripotent.

Table 1.1. Embryonic Germ Layers From Which Differentiated Tissues Develop

Embryonic Germ Layer	Differentiated Tissue
Endoderm	Thymus Thyroid, parathyroid glands Larynx, trachea, lung Urinary bladder, vagina, urethra Gastrointestinal (GI) organs (liver, pancreas) Lining of the GI tract Lining of the respiratory tract
Mesoderm	Bone marrow (blood) Adrenal cortex Lymphatic tissue Skeletal, smooth, and cardiac muscle Connective tissues (including bone, cartilage) Urogenital system Heart and blood vessels (vascular system)
Ectoderm	Skin Neural tissue (neuroectoderm) Adrenal medulla Pituitary gland Connective tissue of the head and face Eyes, ears

[1]

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2. THE EMBRYONIC STEM CELL

As stated in the first chapter, an embryonic stem cell (ES cell) is defined by its origin. It is derived from the blastocyst stage of the embryo. The blastocyst is the stage of embryonic development prior to implantation in the uterine wall. At this stage, the preimplantation embryo of the mouse is made up of 150 cells and consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel), and a cluster of cells on the interior (the inner cell mass).

Studies of ES cells derived from mouse blastocysts became possible 20 years ago with the discovery of techniques that allowed the cells to be grown in the laboratory. Embryonic–like stem cells, called embryonic germ (EG) cells, can also be derived from primordial germ (PG) cells (the cells of the developing fetus from which eggs and sperm are formed) of the mouse [20] and human fetus [30].

In this chapter the discussion will be limited to mouse embryonic stem cells. Chapter 3 describes the human embryonic stem cell.

DO EMBRYONIC STEM CELLS ACTUALLY OCCUR IN THE EMBRYO?

Some scientists argue that ES cells do not occur *in* the embryo as such. ES cells closely resemble the cells of the preimplantation embryo [3], but are not in fact the same [32]. An alternative perspective is that the embryos of many animal species contain stem cells. These cells proliferate extensively in the embryo, are capable of differentiating into all the types of cells that occur in the adult, and can be isolated and grown *ex vivo* (outside the organism), where they continue to replicate and show the potential to differentiate [18].

For research purposes, the definition of an ES cell is more than a self-replicating stem cell derived from the embryo that can differentiate into almost all of the cells of the body. Scientists have found it necessary to develop specific criteria that help them better define the ES cell. Austin Smith, whose studies of mouse ES cells have contributed significantly to the field, has offered a list of essential characteristics that define ES cells [18, 32].

DEFINING PROPERTIES OF AN EMBRYONIC STEM CELL

- [a]Derived from the inner cell mass/epiblast of the blastocyst.
- [a]Capable of undergoing an unlimited number of symmetrical divisions without differentiating (long-term self-renewal).
- Exhibit and maintain a stable, full (diploid), normal complement of chromosomes (karyotype).
- Pluripotent ES cells can give rise to differentiated cell types that are derived from all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm).
- [a,b]Capable of integrating into all fetal tissues during development. (Mouse ES cells maintained in culture for long periods can still generate any tissue when they are reintroduced into an embryo to generate a chimeric animal.)
- [a,b]Capable of colonizing the germ line and giving rise to egg or sperm cells.
- [a]Clonogenic, that is a single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell.

- Expresses the transcription factor Oct-4, which then activates or inhibits a host of target genes and maintains ES cells in a proliferative, non-differentiating state.
- Can be induced to continue proliferating or to differentiate.
- Lacks the G1 checkpoint in the cell cycle. ES cells spend most of their time in the S phase of the cell cycle, during which they synthesize DNA. Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.
- Do not show X inactivation. In every somatic cell of a female mammal, one of the two X chromosomes becomes permanently inactivated. X inactivation does not occur in undifferentiated ES cells.

[[a] Not shown in human EG cells. [b] Not shown in human ES cells. All of the criteria have been met by mouse ES cells.]

ARE EMBRYONIC STEM CELLS TRULY PLURIPOTENT?

Pluripotency—that is the ability to give rise to differentiated cell types that are derived from all three primary germ layers of the embryo, endoderm, mesoderm, and ectoderm—is what makes ES cells unique. How do we know that these cells are, indeed, pluripotent? Laboratory-based criteria for testing the pluripotent nature of ES cells derived from mice include three kinds of experiments [19]. One test is conducted by injecting ES cells derived from the inner cell mass of one blastocyst into the cavity of another blastocyst. The "combination" embryos are then transferred to the uterus of a pseudopregnant female mouse, and the progeny that result are chimeras. Chimeras are a mixture of tissues and organs of cells derived from both donor ES cells and the recipient blastocyst.

This test has been extended in studies designed to test whether cultured ES cells can be used to replace the inner cell mass of a mouse blastocyst and produce a normal embryo. They can, but the process is far less efficient than that of using cells taken directly from the inner cell mass. Apparently, the ability of ES cells to generate a complete embryo depends on the number of times they have been passaged *in vitro* [21, 22]. A passage is the process of removing cells from one culture dish and replating them into fresh culture dishes. Whether the number of passages affects the differentiation potential of human ES cells remains to be determined. (For a detailed discussion of the techniques for maintaining mouse ES cells in culture, see Appendix B. Mouse Embryonic Stem Cells.)

A second method for determining the pluripotency of mouse ES cells is to inject the cells into adult mice (under the skin or the kidney capsule) that are either genetically identical or are immune-deficient, so the tissue will not be rejected. In the host animal, the injected ES cells develop into benign tumors called teratomas. When examined under a microscope, it was noted that these tumors contain cell types derived from all three primary germ layers of the embryo—endoderm, mesoderm, and ectoderm. Teratomas typically contain gut-like structures such as layers of epithelial cells and smooth muscle; skeletal or cardiac muscle (which may contract spontaneously); neural tissue; cartilage or bone; and sometimes hair. Thus, ES cells that have been maintained for a long period *in vitro* can behave as pluripotent cells *in vivo.* They can participate in normal embryogenesis by differentiating into any cell type in the body, and they can also differentiate into a wide range of cell types in an adult animal. However, normal mouse ES cells do not generate trophoblast tissues *in vivo* [32].

A third technique for demonstrating pluripotency is to allow mouse ES cells *in vitro* to differentiate spontaneously or to direct their differentiation along specific pathways. The former is usually accomplished by removing feeder layers and adding leukemia inhibitory factor (LIF) to the growth medium. Within a few days after changing the culture conditions, ES cells aggregate and may form embryoid bodies (EBs). In many ways, EBs in the culture dish resemble teratomas that are observed in the animal. EBs consist of a disorganized array of differentiated or partially differentiated cell types that are derived from the three primary germ layers of the embryo—the endoderm, mesoderm, and ectoderm [32].

The techniques for culturing mouse ES cells from the inner cell mass of the preimplantation blastocyst were first reported 20 years ago [9, 19], and versions of these standard procedures are used today in laboratories throughout the world. It is striking that, to date, only three species of mammals have yielded

long-term cultures of self-renewing ES cells: mice, monkeys, and humans [27, 34, 35, 36] (see Appendix B. Mouse Embryonic Stem Cells).

HOW DOES A MOUSE EMBRYONIC STEM CELL STAY UNDIFFERENTIATED?

As stated earlier, a true stem cell is capable of maintaining itself in a self-renewing, undifferentiated state indefinitely. The undifferentiated state of the embryonic stem cell is characterized by specific cell markers that have helped scientists better understand how embryonic stem cells—under the right culture conditions—replicate for hundreds of population doublings and do not differentiate. To date, two major areas of investigation have provided some clues. One includes attempts to understand the effects of secreted factors such as the cytokine leukemia inhibitory factor on mouse ES cells *in vitro*. The second area of study involves transcription factors such as Oct-4. Oct-4 is a protein expressed by mouse and human ES cells *in vitro*, and also by mouse inner cell mass cells *in vivo*. The cell cycle of the ES also seems to play a role in preventing differentiation. From studies of these various signaling pathways, it is clear that many factors must be balanced in a particular way for ES cells to remain in a self-renewing state. If the balance shifts, ES cells begin to differentiate [18, 31]. (For a detailed discussion of how embryonic stem cells maintain their pluripotency, see Appendix B. Mouse Embryonic Stem Cells.)

CAN A MOUSE EMBRYONIC STEM CELL BE DIRECTED TO DIFFERENTIATE INTO A PARTICULAR CELL TYPE IN VITRO?

One goal for embryonic stem cell research is the development of specialized cells such as neurons, heart muscle cells, endothelial cells of blood vessels, and insulin secreting cells similar to those found in the pancreas. The directed derivation of embryonic stem cells is then vital to the ultimate use of such cells in the development of new therapies.

By far the most common approach to directing differentiation is to change the growth conditions of the ES cells in specific ways, such as by adding growth factors to the culture medium or changing the chemical composition of the surface on which the ES cells are growing. For example, the plastic culture dishes used to grow both mouse and human ES cells can be treated with a variety of substances that allow the cells either to adhere to the surface of the dish or to avoid adhering and instead float in the culture medium. In general, an adherent substrate helps prevent them from interacting and differentiating. In contrast, a nonadherent substrate allows the ES cells to aggregate and thereby interact with each other. Cell-cell interactions are critical to normal embryonic development, so allowing some of these "natural" *in vivo* interactions to occur in the culture dish is a fundamental strategy for inducing mouse or human ES cell differentiation *in vitro*. In addition, adding specific growth factors to the culture medium triggers the activation (or inactivation) of specific genes in ES cells. This initiates a series of molecular events that induces the cells to differentiate along a particular pathway.

Another way to direct differentiation of ES cells is to introduce foreign genes into the cells via transfection or other methods [6, 39]. The result of these strategies is to add an active gene to the ES cell genome, which then triggers the cells to differentiate along a particular pathway. The approach appears to be a precise way of regulating ES cell differentiation, but it will work only if it is possible to identify which gene must be active at which particular stage of differentiation. Then, the gene must be activated at the right time—meaning during the correct stage of differentiation—and it must be inserted into the genome at the proper location.

Another approach to generate mouse ES cells uses cloning technology. In theory, the nucleus of a differentiated mouse somatic cell might be reprogrammed by injecting it into an oocyte. The resultant pluripotent cell would be immunologically compatible because it would be genetically identical to the donor cell [25].

All of the techniques just described are still highly experimental. Nevertheless, within the past several years, it has become possible to generate specific, differentiated, functional cell types by manipulating the growth conditions of mouse ES cells *in vitro*. It is not possible to explain *how* the directed differentiation occurs, however. No one knows how or when gene expression is changed, what signal-transduction systems are triggered, or what cell-cell interactions

must occur to convert undifferentiated ES cells into precursor cells and, finally, into differentiated cells that look and function like their *in vivo* counterparts.

Embryonic stem cells have been shown to differentiate into a variety of cell types. For example, mouse ES cells can be directed *in vitro* to yield vascular structures [40], neurons that release dopamine and serotonin [14], and endocrine pancreatic islet cells [16]. In all three cases, proliferating, undifferentiated mouse ES cells provide the starting material and functional, differentiated cells were the result. Also, the onset of mouse ES cell differentiation can be triggered by withdrawing the cytokine LIF, which promotes the division of undifferentiated mouse ES cells. In addition, when directed to differentiate, ES cells aggregate, a change in their three-dimensional environment that presumably allowed some of the cell-cell interactions to occur *in vitro* that would occur *in vivo* during normal embryonic development. Collectively, these three studies provide some of the best examples of directed differentiation of ES cells. Two of them showed that a single precursor cell can give rise to multiple, differentiated cell types [16, 40], and all three studies demonstrated that the resulting differentiated cells function as their *in vivo* counterparts do. These two criteria—demonstrating that a single cell can give rise to multiple cells types and the functional properties of the differentiated cells—form the basis of an acid test for all claims of directed differentiation of either ES cells or adult stem cells. Unfortunately, very few experiments meet these criteria, which too often makes it impossible to assess whether a differentiated cell type resulted from the experimental manipulation that was reported. (For a detailed discussion of the methods used to differentiate mouse embryonic stem cells, see Appendix B. Mouse Embryonic Stem Cells.)

Table 2.1 provides a summary of what is known today about the types of cells that can be differentiated from mouse embryonic stem cells.

Table 2.1. Reported differentiated cell types from mouse embryonic stem cells *in vitro

Cell Type	Reference
Adipocyte	[7]
Astrocyte	[11]
Cardiomyocyte	[8, 17]
Chondrocyte	[13]
Definitive hematopoietic	[23, 24, 38]
Dendritic cell	[10]
Endothelial cell	[28, 40]
Keratinocyte	[1, 40]
Lymphoid precursor	[26]
Mast cell	[37]
Neuron	[2, 33]
Oligodendrocyte	[4, 15]
Osteoblast	[5]
Pancreatic islets	[16]
Primitive haematopoietic	[8, 23]
Smooth muscle	[40]
Striated muscle	[29]
Yolk sac endoderm	[8]
Yolk sac mesoderm	[8]

**Adapted with permission from reference [32].*

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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

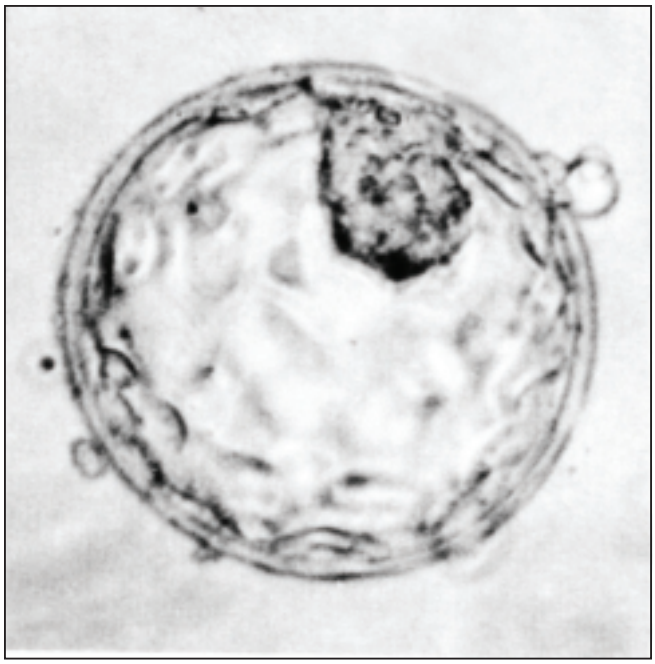

Photo Credit: Mt. J. Conaghan

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

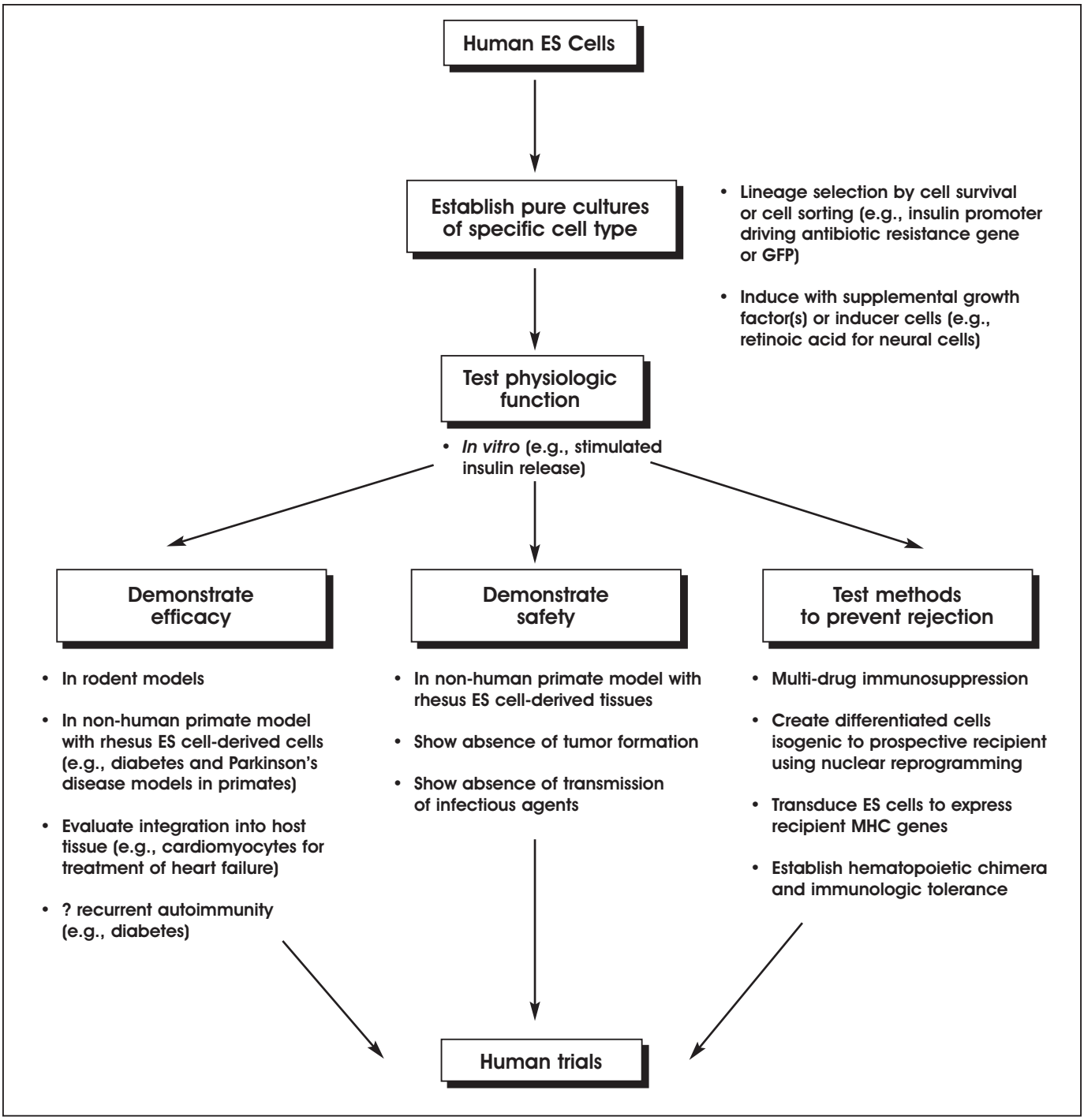

Figure 3.2. Major Goals in the Development of Transplantation Therapies from Human ES Cell Lines. (Reproduced with permission from Stem Cells, 2001)

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

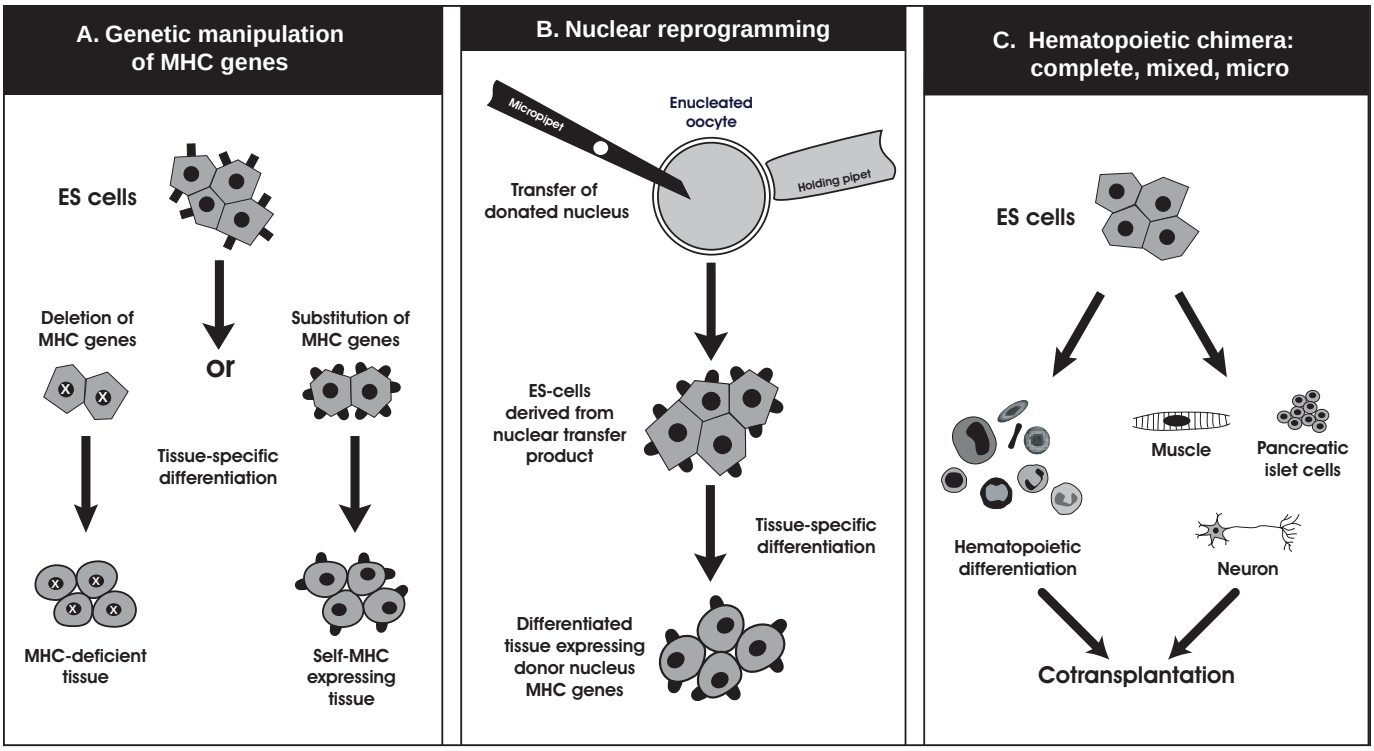

Figure 3.3. Genetic Manipulation of Human Embryonic Stem Cells. (Reproduced with permission from Stem Cells, 2001)

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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4. THE ADULT STEM CELL

For many years, researchers have been seeking to understand the body's ability to repair and replace the cells and tissues of some organs, but not others. After years of work pursuing the how and why of seemingly indiscriminant cell repair mechanisms, scientists have now focused their attention on adult stem cells. It has long been known that stem cells are capable of renewing themselves and that they can generate multiple cell types. Today, there is new evidence that stem cells are present in far more tissues and organs than once thought and that these cells are capable of developing into more kinds of cells than previously imagined. Efforts are now underway to harness stem cells and to take advantage of this new found capability, with the goal of devising new and more effective treatments for a host of diseases and disabilities. What lies ahead for the use of adult stem cells is unknown, but it is certain that there are many research questions to be answered and that these answers hold great promise for the future.

WHAT IS AN ADULT STEM CELL?

Adult stem cells, like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies (shapes) and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway, although this characteristic may not be as definitive as once thought [82] (see Figure 4.1. Distinguishing Features of Progenitor/Precursor Cells and Stem Cells).

Adult stem cells are rare. Their primary functions are to maintain the steady state functioning of a cell—called homeostasis—and, with limitations, to replace cells that die because of injury or disease [44, 58]. For example, only an estimated 1 in 10,000 to 15,000 cells in the bone marrow is a hematopoietic (blood-forming) stem cell (HSC) [105]. Furthermore, adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment. For example, HSCs are constantly being generated in the bone marrow where they differentiate into mature types of blood cells. Indeed, the primary role of HSCs is to replace blood cells [26] (see Chapter 5. Hematopoietic Stem Cells). In contrast, stem cells in the small intestine are stationary, and are physically separated from the mature cell types they generate. Gut epithelial stem cells (or precursors) occur at the bases of crypts—deep invaginations between the mature, differentiated epithelial cells that line the lumen of the intestine. These epithelial crypt cells divide fairly often, but remain part of the stationary group of cells they generate [93].

Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), adult stem cells share no such definitive means of characterization. In fact, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. Definitions of adult stem cells vary in the scientific literature range from a simple description of the cells to a rigorous set of experimental criteria that must be met before characterizing a

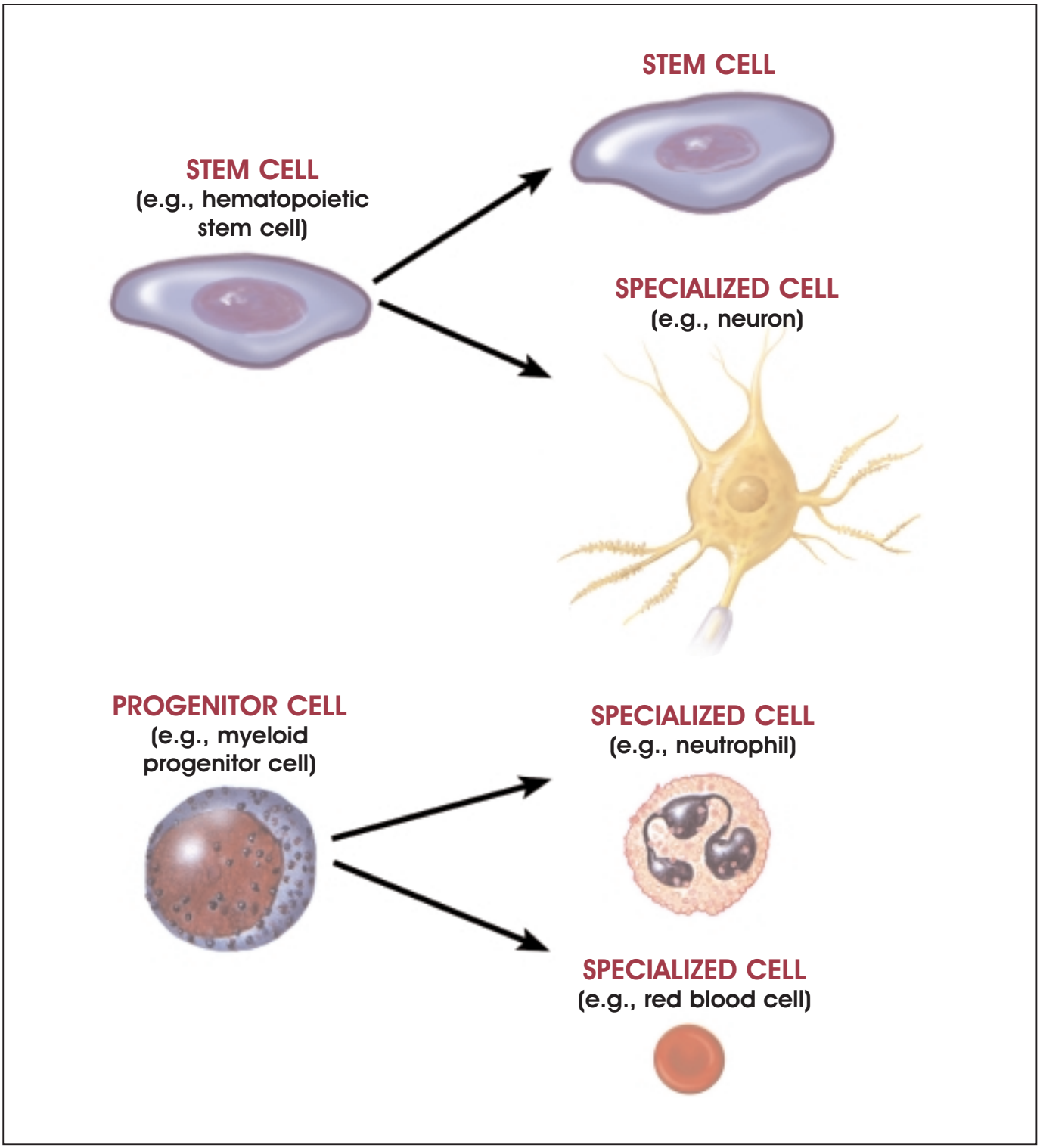

Figure 4.1. Distinguishing Features of Progenitor/Precursor Cells and Stem Cells. A stem cell is an unspecialized cell that is capable of replicating or self renewing itself and developing into specialized cells of a variety of cell types. The product of a stem cell undergoing division is at least one additional stem cell that has the same capabilities of the originating cell. Shown here is an example of a hematopoietic stem cell producing a second generation stem cell and a neuron. A progenitor cell (also known as a precursor cell) is unspecialized or has partial characteristics of a specialized cell that is capable of undergoing cell division and yielding two specialized cells. Shown here is an example of a myeloid progenitor/precursor undergoing cell division to yield two specialized cells (a neutrophil and a red blood cell).

particular cell as an adult stem cell. Most of the information about adult stem cells comes from studies of mice. The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas.

In order to be classified as an adult stem cell, the cell should be capable of self-renewal for the lifetime of the organism. This criterion, although fundamental to the nature of a stem cell, is difficult to prove *in vivo*. It is nearly impossible, in an organism as complex as a human, to design an experiment that will allow the fate of candidate adult stem cells to be identified *in vivo* and tracked over an individual's entire lifetime.

Ideally, adult stem cells should also be clonogenic. In other words, a single adult stem cell should be able to generate a line of genetically identical cells, which then gives rise to all the appropriate, differentiated cell types of the tissue in which it resides. Again, this property is difficult to demonstrate *in vivo*; in practice, scientists show either that a stem cell is clonogenic *in vitro*, or that a purified population of candidate stem cells can repopulate the tissue.

An adult stem cell should also be able to give rise to fully differentiated cells that have mature phenotypes, are fully integrated into the tissue, and are capable of specialized functions that are appropriate for the tissue. The term phenotype refers to all the observable characteristics of a cell (or organism); its shape (morphology); interactions with other cells and the non-cellular environment (also called the extracellular matrix); proteins that appear on the cell surface (surface markers); and the cell's behavior (e.g., secretion, contraction, synaptic transmission).

The majority of researchers who lay claim to having identified adult stem cells rely on two of these characteristics—appropriate cell morphology, and the demonstration that the resulting, differentiated cell types display surface markers that identify them as belonging to the tissue. Some studies demonstrate that the differentiated cells that are derived from adult stem cells are truly functional, and a few studies show that cells are integrated into the differentiated tissue *in vivo* and that they interact appropriately with neighboring cells. At present, there is, however, a paucity of research, with a few notable exceptions, in which researchers were able to conduct studies of genetically identical (clonal) stem cells. In order to fully characterize the regenerating and self-renewal capabilities of the adult stem cell, and therefore to truly harness its potential, it will be important to demonstrate that a single adult stem cell can, indeed, generate a line of genetically identical cells, which then gives rise to all the appropriate, differentiated cell types of the tissue in which it resides.

EVIDENCE FOR THE PRESENCE OF ADULT STEM CELLS

Adult stem cells have been identified in many animal and human tissues. In general, three methods are used to determine whether candidate adult stem cells give rise to specialized cells. Adult stem cells can be labeled *in vivo* and then they can be tracked. Candidate adult stem cells can also be isolated and labeled and then transplanted back into the organism to determine what becomes of them. Finally, candidate adult stem cells can be isolated, grown *in vitro* and manipulated, by adding growth factors or introducing genes that help determine what differentiated cells types they will yield. For example, currently, scientists believe that stem cells in the fetal and adult brain divide and give rise to more stem cells or to several types of precursor cells, which give rise to nerve cells (neurons), of which there are many types.

It is often difficult—if not impossible—to distinguish adult, tissue-specific stem cells from progenitor cells, which are found in fetal or adult tissues and are partly differentiated cells that divide and give rise to differentiated cells. These are cells found in many organs that are generally thought to be present to replace cells and maintain the integrity of the tissue. Progenitor cells give rise to certain types of cells—such as the blood cells known as T lymphocytes, B lymphocytes, and natural killer cells—but are not thought to be capable of developing into all the cell types of a tissue and as such are not truly stem cells. The current wave of excitement over the existence of stem cells in many adult tissues is perhaps fueling claims that progenitor or precursor cells in those tissues are instead stem cells. Thus, there are reports of endothelial progenitor cells, skeletal muscle stem cells, epithelial precursors in the skin and digestive system, as well as some reports of progenitors or stem cells in the pancreas and liver. A detailed summary of some of the evidence for the existence of stem

cells in various tissues and organs is presented later in the chapter.

ADULT STEM CELL PLASTICITY

It was not until recently that anyone seriously considered the possibility that stem cells in adult tissues could generate the specialized cell types of another type of tissue from which they normally reside—either a tissue derived from the same embryonic germ layer or from a different germ layer (see Table 1.1. Embryonic Germ Layers From Which Differentiated Tissues Develop). For example, studies have shown that blood stem cells (derived from mesoderm) may be able to generate both skeletal muscle (also derived from mesoderm) and neurons (derived from ectoderm). That realization has been triggered by a flurry of papers reporting that stem cells derived from one adult tissue can change their appearance and assume characteristics that resemble those of differentiated cells from other tissues.

The term plasticity, as used in this report, means that a stem cell from one adult tissue can generate the differentiated cell types of another tissue. At this time, there is no formally accepted name for this phenomenon in the scientific literature. It is variously referred to as "plasticity" [15, 52], "unorthodox differentiation" [10] or "transdifferentiation" [7, 54].

Approaches for Demonstrating Adult Stem Cell Plasticity

To be able to claim that adult stem cells demonstrate plasticity, it is first important to show that a cell population exists in the starting tissue that has the identifying features of stem cells. Then, it is necessary to show that the adult stem cells give rise to cell types that normally occur in a different tissue. Neither of these criteria is easily met. Simply proving the existence of an adult stem cell population in a differentiated tissue is a laborious process. It requires that the candidate stem cells are shown to be self-renewing, and that they can give rise to the differentiated cell types that are characteristic of that tissue.

To show that the adult stem cells can generate other cell types requires them to be tracked in their new environment, whether it is *in vitro* or *in vivo*. In general, this has been accomplished by obtaining the stem cells from a mouse that has been genetically engineered to express a molecular tag in all its cells. It is then necessary to show that the labeled adult stem cells have adopted key structural and biochemical characteristics of the new tissue they are claimed to have generated. Ultimately—and most importantly—it is necessary to demonstrate that the cells can integrate into their new tissue environment, survive in the tissue, and function like the mature cells of the tissue.

In the experiments reported to date, adult stem cells may assume the characteristics of cells that have developed from the same primary germ layer or a different germ layer (see Figure 4.2. Preliminary Evidence of Plasticity Among Nonhuman Adult Stem Cells). For example, many plasticity experiments involve stem cells derived from bone marrow, which is a mesodermal derivative. The bone marrow stem cells may then differentiate into another mesodermally derived tissue such as skeletal muscle [28, 43], cardiac muscle [51, 71] or liver [4, 54, 97].

Alternatively, adult stem cells may differentiate into a tissue that—during normal embryonic development—would arise from a different germ layer. For example, bone marrow-derived cells may differentiate into neural tissue, which is derived from embryonic ectoderm [15, 65]. And—reciprocally—neural stem cell lines cultured from adult brain tissue may differentiate to form hematopoietic cells [13], or even give rise to many different cell types in a chimeric embryo [17]. In both cases cited above, the cells would be deemed to show plasticity, but in the case of bone marrow stem cells generating brain cells, the finding is less predictable.

In order to study plasticity within and across germ layer lines, the researcher must be sure that he/she is using only one kind of adult stem cell. The vast majority of experiments on plasticity have been conducted with adult stem cells derived either from the bone marrow or the brain. The bone marrow-derived cells are sometimes sorted—using a panel of surface markers—into populations of hematopoietic stem cells or bone marrow stromal cells [46, 54, 71]. The HSCs may be highly purified or partially purified, depending on the conditions used. Another way to separate population of bone marrow cells is by fractionation to yield cells that adhere to a growth substrate (stromal cells) or do not adhere (hematopoietic cells) [28].

To study plasticity of stem cells derived from the brain, the researcher must overcome several problems. Stem cells from the central nervous system (CNS),

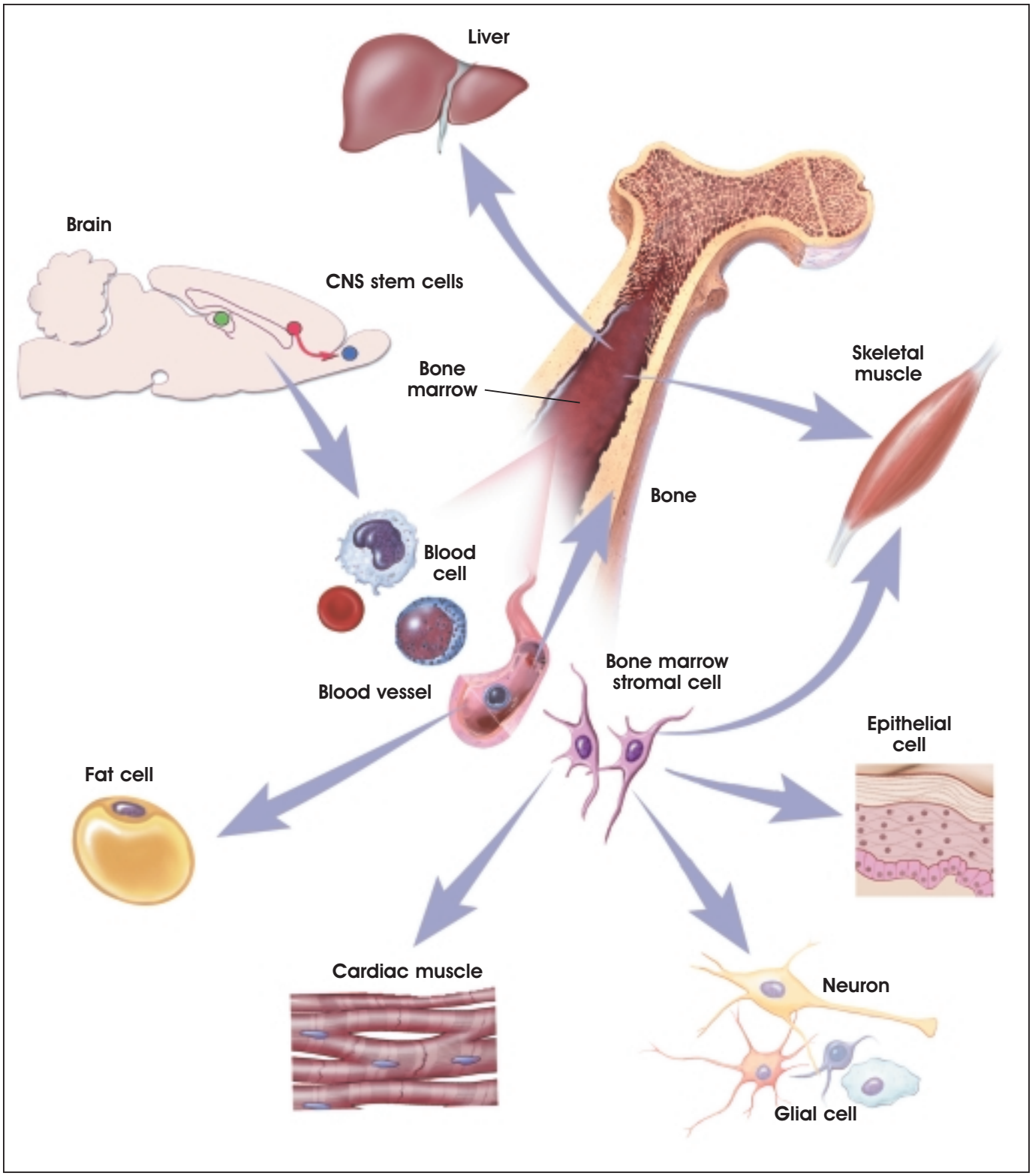

Figure 4.2. Preliminary Evidence of Plasticity Among Nonhuman Adult Stem Cells.

unlike bone marrow cells, do not occur in a single, accessible location. Instead, they are scattered in three places, at least in rodent brain—the tissue around the lateral ventricles in the forebrain, a migratory pathway for the cells that leads from the ventricles to the olfactory bulbs, and the hippocampus. Many of the experiments with CNS stem cells involve the formation of neurospheres, round aggregates of cells that are sometimes clonally derived. But it is not possible to observe cells in the center of a neurosphere, so to study plasticity *in vitro*, the cells are usually dissociated and plated in monolayers. To study plasticity *in vivo*, the cells may be dissociated before injection into the circulatory system of the recipient animal [13], or injected as neurospheres [17].

What is the Evidence for Plasticity?

The differentiated cell types that result from plasticity are usually reported to have the morphological characteristics of the differentiated cells and to display their characteristic surface markers. In reports that transplanted adult stem cells show plasticity *in vivo*, the stem cells typically are shown to have integrated into a mature host tissue and assumed at least some of its characteristics [15, 28, 51, 65, 71]. Many plasticity experiments involve injury to a particular tissue, which is intended to model a particular human disease or injury [13, 54, 71]. However, there is limited evidence to date that such adult stem cells can generate mature, fully functional cells or that the cells have restored lost function *in vivo* [54]. Most of the studies that show the plasticity of adult stem cells involve cells that are derived from the bone marrow [15, 28, 54, 65, 77] or brain [13, 17]. To date, adult stem cells are best characterized in these two tissues, which may account for the greater number of plasticity studies based on bone marrow and brain. Collectively, studies on plasticity suggest that stem cell populations in adult mammals are not fixed entities, and that after exposure to a new environment, they may be able to populate other tissues and possibly differentiate into other cell types.

It is not yet possible to say whether plasticity occurs normally *in vivo*. Some scientists think it may [14, 64], but as yet there is no evidence to prove it. Also, it is not yet clear to what extent plasticity can occur in experimental settings, and how—or whether—the phenomenon can be harnessed to generate tissues that may be useful for therapeutic transplantation. If the phenomenon of plasticity is to be used as a basis for generating tissue for transplantation, the techniques for doing it will need to be reproducible and reliable (see Chapter 10. Assessing Human Stem Cell Safety). In some cases, debate continues about observations that adult stem cells yield cells of tissue types different than those from which they were obtained [7, 68].

EXPERIMENTAL EVIDENCE OF ADULT STEM CELLS AND PLASTICITY

Adult Stem Cells of the Nervous System

More than 30 years ago, Altman and Das showed that two regions of the postnatal rat brain, the hippocampus and the olfactory bulb, contain dividing cells that become neurons [5, 6]. Despite these reports, the prevailing view at the time was that nerve cells in the adult brain do not divide. In fact, the notion that stem cells in the adult brain can generate its three major cell types—astrocytes and oligodendrocytes, as well as neurons—was not accepted until far more recently. Within the past five years, a series of studies has shown that stem cells occur in the adult mammalian brain and that these cells can generate its three major cell lineages [35, 48, 63, 66, 90, 96, 104] (see Chapter 8. Rebuilding the Nervous System with Stem Cells).

Today, scientists believe that stem cells in the fetal and adult brain divide and give rise to more stem cells or to several types of precursor cells. Neuronal precursors (also called neuroblasts) divide and give rise to nerve cells (neurons), of which there are many types. Glial precursors give rise to astrocytes or oligodendrocytes. Astrocytes are a kind of glial cell, which lend both mechanical and metabolic support for neurons; they make up 70 to 80 percent of the cells of the adult brain. Oligodendrocytes make myelin, the fatty material that ensheathes nerve cell axons and speeds nerve transmission. Under normal, *in vivo* conditions, neuronal precursors do not give rise to glial cells, and glial precursors do not give rise to neurons. In contrast, a fetal or adult CNS (central nervous system—the brain and spinal cord) stem cell may give rise to neurons, astrocytes, or oligodendrocytes, depending on the signals it receives and its three-dimensional environment within the brain tissue.

There is now widespread consensus that the adult mammalian brain does contain stem cells. However, there is no consensus about how many populations of CNS stem cells exist, how they may be related, and how they function *in vivo*. Because there are no markers currently available to identify the cells *in vivo*, the only method for testing whether a given population of CNS cells contains stem cells is to isolate the cells and manipulate them *in vitro*, a process that may change their intrinsic properties [67].

Despite these barriers, three groups of CNS stem cells have been reported to date. All occur in the adult rodent brain and preliminary evidence indicates they also occur in the adult human brain. One group occupies the brain tissue next to the ventricles, regions known as the ventricular zone and the subventricular zone (see discussion below). The ventricles are spaces in the brain filled with cerebrospinal fluid. During fetal development, the tissue adjacent to the ventricles is a prominent region of actively dividing cells. By adulthood, however, this tissue is much smaller, although it still appears to contain stem cells [70].

A second group of adult CNS stem cells, described in mice but not in humans, occurs in a streak of tissue that connects the lateral ventricle and the olfactory bulb, which receives odor signals from the nose. In rodents, olfactory bulb neurons are constantly being replenished via this pathway [59, 61]. A third possible location for stem cells in adult mouse and human brain occurs in the hippocampus, a part of the brain thought to play a role in the formation of certain kinds of memory [27, 34].

Central Nervous System Stem Cells in the Subventricular Zone. CNS stem cells found in the forebrain that surrounds the lateral ventricles are heterogeneous and can be distinguished morphologically. Ependymal cells, which are ciliated, line the ventricles. Adjacent to the ependymal cell layer, in a region sometimes designated as the subependymal or subventricular zone, is a mixed cell population that consists of neuroblasts (immature neurons) that migrate to the olfactory bulb, precursor cells, and astrocytes. Some of the cells divide rapidly, while others divide slowly. The astrocyte-like cells can be identified because they contain glial fibrillary acidic protein (GFAP), whereas the ependymal cells stain positive for nestin, which is regarded as a marker of neural stem cells. Which of these cells best qualifies as a CNS stem cell is a matter of debate [76].

A recent report indicates that the astrocytes that occur in the subventricular zone of the rodent brain act as neural stem cells. The cells with astrocyte markers appear to generate neurons *in vivo*, as identified by their expression of specific neuronal markers. The *in vitro* assay to demonstrate that these astrocytes are, in fact, stem cells involves their ability to form neurospheres—groupings of undifferentiated cells that can be dissociated and coaxed to differentiate into neurons or glial cells [25]. Traditionally, these astrocytes have been regarded as differentiated cells, not as stem cells and so their designation as stem cells is not universally accepted.

A series of similar *in vitro* studies based on the formation of neurospheres was used to identify the subependymal zone as a source of adult rodent CNS stem cells. In these experiments, single, candidate stem cells derived from the subependymal zone are induced to give rise to neurospheres in the presence of mitogens—either epidermal growth factor (EGF) or fibroblast growth factor-2 (FGF-2). The neurospheres are dissociated and passaged. As long as a mitogen is present in the culture medium, the cells continue forming neurospheres without differentiating. Some populations of CNS cells are more responsive to EGF, others to FGF [100]. To induce differentiation into neurons or glia, cells are dissociated from the neurospheres and grown on an adherent surface in serum-free medium that contains specific growth factors. Collectively, the studies demonstrate that a population of cells derived from the adult rodent brain can self-renew and differentiate to yield the three major cell types of the CNS cells [41, 69, 74, 102].

Central Nervous System Stem Cells in the Ventricular Zone. Another group of potential CNS stem cells in the adult rodent brain may consist of the ependymal cells themselves [47]. Ependymal cells, which are ciliated, line the lateral ventricles. They have been described as non-dividing cells [24] that function as part of the blood-brain barrier [22]. The suggestion that ependymal cells from the ventricular zone of the adult rodent CNS may be stem cells is therefore unexpected. However, in a recent study, in which two molecular tags—the fluorescent marker DiI, and an adenovirus vector carrying *lacZ* tags—were used to label the ependymal cells that line the entire CNS ventricular system of adult rats, it was shown that these cells could, indeed, act as stem cells. A few

days after labeling, fluorescent or *lacZ*$^+$ cells were observed in the rostral migratory stream (which leads from the lateral ventricle to the olfactory bulb), and then in the olfactory bulb itself. The labeled cells in the olfactory bulb also stained for the neuronal markers βIII tubulin and Map2, which indicated that ependymal cells from the ventricular zone of the adult rat brain had migrated along the rostral migratory stream to generate olfactory bulb neurons *in vivo* [47].

To show that DiI$^+$ cells were neural stem cells and could generate astrocytes and oligodendrocytes as well as neurons, a neurosphere assay was performed *in vitro*. DiI-labeled cells were dissociated from the ventricular system and cultured in the presence of mitogen to generate neurospheres. Most of the neurospheres were DiI$^+$; they could self-renew and generate neurons, astrocytes, and oligodendrocytes when induced to differentiate. Single, DiI$^+$ ependymal cells isolated from the ventricular zone could also generate self-renewing neurospheres and differentiate into neurons and glia.

To show that ependymal cells can also divide *in vivo*, bromodeoxyuridine (BrdU) was administered in the drinking water to rats for a 2- to 6-week period. Bromodeoxyuridine (BrdU) is a DNA precursor that is only incorporated into dividing cells. Through a series of experiments, it was shown that ependymal cells divide slowly *in vivo* and give rise to a population of progenitor cells in the subventricular zone [47]. A different pattern of scattered BrdU-labeled cells was observed in the spinal cord, which suggested that ependymal cells along the central canal of the cord occasionally divide and give rise to nearby ependymal cells, but do not migrate away from the canal.

Collectively, the data suggest that CNS ependymal cells in adult rodents can function as stem cells. The cells can self-renew, and most proliferate via asymmetrical division. Many of the CNS ependymal cells are not actively dividing (quiescent), but they can be stimulated to do so *in vitro* (with mitogens) or *in vivo* (in response to injury). After injury, the ependymal cells in the spinal cord only give rise to astrocytes, not to neurons. How and whether ependymal cells from the ventricular zone are related to other candidate populations of CNS stem cells, such as those identified in the hippocampus [34], is not known.

Are ventricular and subventricular zone CNS stem cells the same population? These studies and other leave open the question of whether cells that directly line the ventricles—those in the ventricular zone—or cells that are at least a layer removed from this zone—in the subventricular zone are the same population of CNS stem cells. A new study, based on the finding that they express different genes, confirms earlier reports that the ventricular and subventricular zone cell populations are distinct. The new research utilizes a technique called representational difference analysis, together with cDNA microarray analysis, to monitor the patterns of gene expression in the complex tissue of the developing and postnatal mouse brain. The study revealed the expression of a panel of genes known to be important in CNS development, such as *L3-PSP* (which encodes a phosphoserine phosphatase important in cell signaling), *cyclin D2* (a cell cycle gene), and *ERCC-1* (which is important in DNA excision repair). All of these genes in the recent study were expressed in cultured neurospheres, as well as the ventricular zone, the subventricular zone, and a brain area outside those germinal zones. This analysis also revealed the expression of novel genes such as A16F10, which is similar to a gene in an embryonic cancer cell line. A16F10 was expressed in neurospheres and at high levels in the subventricular zone, but not significantly in the ventricular zone. Interestingly, several of the genes identified in cultured neurospheres were also expressed in hematopoietic cells, suggesting that neural stem cells and blood-forming cells may share aspects of their genetic programs or signaling systems [38]. This finding may help explain recent reports that CNS stem cells derived from mouse brain can give rise to hematopoietic cells after injection into irradiated mice [13].

Central Nervous System Stem Cells in the Hippocampus. The hippocampus is one of the oldest parts of the cerebral cortex, in evolutionary terms, and is thought to play an important role in certain forms of memory. The region of the hippocampus in which stem cells apparently exist in mouse and human brains is the subgranular zone of the dentate gyrus. In mice, when BrdU is used to label dividing cells in this region, about 50% of the labeled cells differentiate into cells that appear to be dentate

gyrus granule neurons, and 15% become glial cells. The rest of the BrdU-labeled cells do not have a recognizable phenotype [90]. Interestingly, many, if not all the BrdU-labeled cells in the adult rodent hippocampus occur next to blood vessels [33].

In the human dentate gyrus, some BrdU-labeled cells express NeuN, neuron-specific enolase, or calbindin, all of which are neuronal markers. The labeled neuron-like cells resemble dentate gyrus granule cells, in terms of their morphology (as they did in mice). Other BrdU-labeled cells express glial fibrillary acidic protein (GFAP) an astrocyte marker. The study involved autopsy material, obtained with family consent, from five cancer patients who had been injected with BrdU dissolved in saline prior to their death for diagnostic purposes. The patients ranged in age from 57 to 72 years. The greatest number of BrdU-labeled cells were identified in the oldest patient, suggesting that new neuron formation in the hippocampus can continue late in life [27].

Fetal Central Nervous System Stem Cells. Not surprisingly, fetal stem cells are numerous in fetal tissues, where they are assumed to play an important role in the expansion and differentiation of all tissues of the developing organism. Depending on the developmental stage of an animal, fetal stem cells and precursor cells—which arise from stem cells—may make up the bulk of a tissue. This is certainly true in the brain [48], although it has not been demonstrated experimentally in many tissues.

It may seem obvious that the fetal brain contains stem cells that can generate all the types of neurons in the brain as well as astrocytes and oligodendrocytes, but it was not until fairly recently that the concept was proven experimentally. There has been a long-standing question as to whether or not the same cell type gives rise to both neurons and glia. In studies of the developing rodent brain, it has now been shown that all the major cell types in the fetal brain arise from a common population of progenitor cells [20, 34, 48, 80, 108].

Neural stem cells in the mammalian fetal brain are concentrated in seven major areas: olfactory bulb, ependymal (ventricular) zone of the lateral ventricles (which lie in the forebrain), subventricular zone (next to the ependymal zone), hippocampus, spinal cord, cerebellum (part of the hindbrain), and the cerebral cortex. Their number and pattern of development vary in different species. These cells appear to represent different stem cell populations, rather than a single population of stem cells that is dispersed in multiple sites. The normal development of the brain depends not only on the proliferation and differentiation of these fetal stem cells, but also on a genetically programmed process of selective cell death called apoptosis [76].

Little is known about stem cells in the human fetal brain. In one study, however, investigators derived clonal cell lines from CNS stem cells isolated from the diencephalon and cortex of human fetuses, 10.5 weeks post-conception [103]. The study is unusual, not only because it involves human CNS stem cells obtained from fetal tissue, but also because the cells were used to generate clonal cell lines of CNS stem cells that generated neurons, astrocytes, and oligodendrocytes, as determined on the basis of expressed markers. In a few experiments described as "preliminary," the human CNS stem cells were injected into the brains of immunosuppressed rats where they apparently differentiated into neuron-like cells or glial cells.

In a 1999 study, a serum-free growth medium that included EGF and FGF2 was devised to grow the human fetal CNS stem cells. Although most of the cells died, occasionally, single CNS stem cells survived, divided, and ultimately formed neurospheres after one to two weeks in culture. The neurospheres could be dissociated and individual cells replated. The cells resumed proliferation and formed new neurospheres, thus establishing an *in vitro* system that (like the system established for mouse CNS neurospheres) could be maintained up to 2 years. Depending on the culture conditions, the cells in the neurospheres could be maintained in an undifferentiated dividing state (in the presence of mitogen), or dissociated and induced to differentiate (after the removal of mitogen and the addition of specific growth factors to the culture medium). The differentiated cells consisted mostly of astrocytes (75%), some neurons (13%) and rare oligodendrocytes (1.2%). The neurons generated under these conditions expressed markers indicating they were GABAergic, [the major type of inhibitory neuron in the mammalian CNS responsive to the amino acid neurotransmitter, gamma-aminobutyric acid (GABA)]. However, catecholamine-like cells that express tyrosine hydroxylase (TH, a critical enzyme in the dopamine-synthesis pathway)

could be generated, if the culture conditions were altered to include different medium conditioned by a rat glioma line (BB49). Thus, the report indicates that human CNS stem cells obtained from early fetuses can be maintained *in vitro* for a long time without differentiating, induced to differentiate into the three major lineages of the CNS (and possibly two kinds of neurons, GABAergic and TH-positive), and engraft (in rats) *in vivo* [103].

Central Nervous System Neural Crest Stem Cells. Neural crest cells differ markedly from fetal or adult neural stem cells. During fetal development, neural crest cells migrate from the sides of the neural tube as it closes. The cells differentiate into a range of tissues, not all of which are part of the nervous system [56, 57, 91]. Neural crest cells form the sympathetic and parasympathetic components of the peripheral nervous system (PNS), including the network of nerves that innervate the heart and the gut, all the sensory ganglia (groups of neurons that occur in pairs along the dorsal surface of the spinal cord), and Schwann cells, which (like oligodendrocytes in the CNS) make myelin in the PNS. The non-neural tissues that arise from the neural crest are diverse. They populate certain hormone-secreting glands—including the adrenal medulla and Type I cells in the carotid body—pigment cells of the skin (melanocytes), cartilage and bone in the face and skull, and connective tissue in many parts of the body [76].

Thus, neural crest cells migrate far more extensively than other fetal neural stem cells during development, form mesenchymal tissues, most of which develop from embryonic mesoderm as well as the components of the CNS and PNS which arises from embryonic ectoderm. This close link, in neural crest development, between ectodermally derived tissues and mesodermally derived tissues accounts in part for the interest in neural crest cells as a kind of stem cell. In fact, neural crest cells meet several criteria of stem cells. They can self-renew (at least in the fetus) and can differentiate into multiple cells types, which include cells derived from two of the three embryonic germ layers [76].

Recent studies indicate that neural crest cells persist late into gestation and can be isolated from E14.5 rat sciatic nerve, a peripheral nerve in the hindlimb. The cells incorporate BrdU, indicating that they are dividing *in vivo*. When transplanted into chick embryos, the rat neural crest cells develop into neurons and glia, an indication of their stem cell-like properties [67]. However, the ability of rat E14.5 neural crest cells taken from sciatic nerve to generate nerve and glial cells in chick is more limited than neural crest cells derived from younger, E10.5 rat embryos. At the earlier stage of development, the neural tube has formed, but neural crest cells have not yet migrated to their final destinations. Neural crest cells from early developmental stages are more sensitive to bone morphogenetic protein 2 (BMP2) signaling, which may help explain their greater differentiation potential [106].

Stem Cells in the Bone Marrow and Blood

The notion that the bone marrow contains stem cells is not new. One population of bone marrow cells, the hematopoietic stem cells (HSCs), is responsible for forming all of the types of blood cells in the body. HSCs were recognized as a stem cells more than 40 years ago [9, 99]. Bone marrow stromal cells—a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation—were described shortly after the discovery of HSCs [30, 32, 73]. The mesenchymal stem cells of the bone marrow also give rise to these tissues, and may constitute the same population of cells as the bone marrow stromal cells [78]. Recently, a population of progenitor cells that differentiates into endothelial cells, a type of cell that lines the blood vessels, was isolated from circulating blood [8] and identified as originating in bone marrow [89]. Whether these endothelial progenitor cells, which resemble the angioblasts that give rise to blood vessels during embryonic development, represent a bona fide population of adult bone marrow stem cells remains uncertain. Thus, the bone marrow appears to contain three stem cell populations—hematopoietic stem cells, stromal cells, and (possibly) endothelial progenitor cells (see Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation).

Two more apparent stem cell types have been reported in circulating blood, but have not been shown to originate from the bone marrow. One population, called pericytes, may be closely related to bone marrow stromal cells, although their origin remains elusive [12]. The second population of blood-born stem cells, which occur in four species of

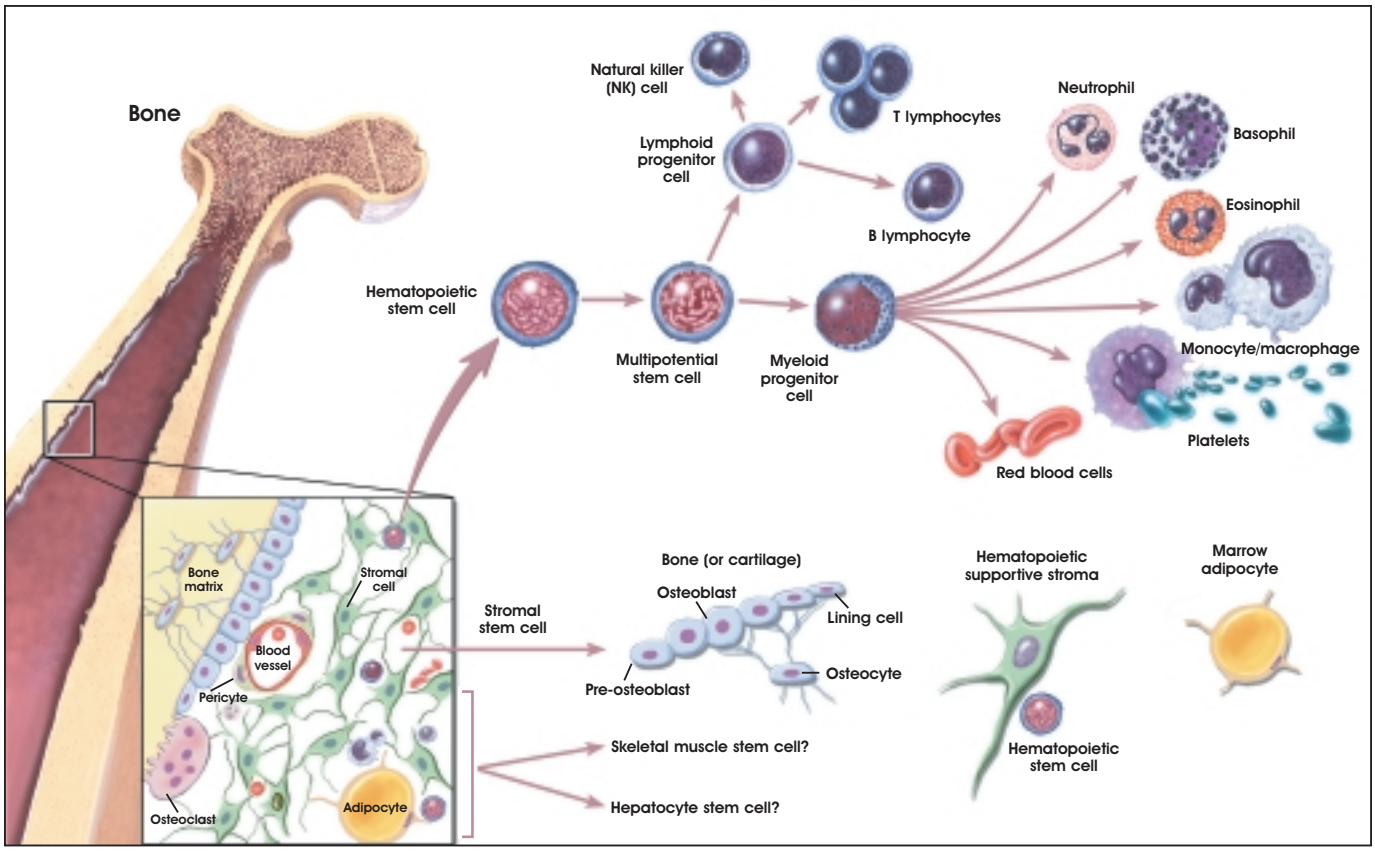

Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation.

animals tested—guinea pigs, mice, rabbits, and humans—resemble stromal cells in that they can generate bone and fat [53].

Hematopoietic Stem Cells. Of all the cell types in the body, those that survive for the shortest period of time are blood cells and certain kinds of epithelial cells. For example, red blood cells (erythrocytes), which lack a nucleus, live for approximately 120 days in the bloodstream. The life of an animal literally depends on the ability of these and other blood cells to be replenished continuously. This replenishment process occurs largely in the bone marrow, where HSCs reside, divide, and differentiate into all the blood cell types. Both HSCs and differentiated blood cells cycle from the bone marrow to the blood and back again, under the influence of a barrage of secreted factors that regulate cell proliferation, differentiation, and migration (see Chapter 5. Hematopoietic Stem Cells).

HSCs can reconstitute the hematopoietic system of mice that have been subjected to lethal doses of radiation to destroy their own hematopoietic systems. This test, the rescue of lethally irradiated mice, has become a standard by which other candidate stem cells are measured because it shows, without question, that HSCs can regenerate an entire tissue system—in this case, the blood [9, 99]. HSCs were first proven to be blood-forming stem cells in a series of experiments in mice; similar blood-forming stem cells occur in humans. HSCs are defined by their ability to self-renew and to give rise to all the kinds of blood cells in the body. This means that a single HSC is capable of regenerating the entire hematopoietic system, although this has been demonstrated only a few times in mice [72].

Over the years, many combinations of surface markers have been used to identify, isolate, and purify HSCs derived from bone marrow and blood. Undifferentiated HSCs and hematopoietic progenitor cells express c-kit, CD34, and H-2K. These cells usually lack the lineage marker Lin, or express it at very low levels ($Lin^{-/low}$). And for transplant purposes, cells that are $CD34^{+}$ $Thy1^{+}$ Lin^{-} are most likely to contain stem cells and result in engraftment.

Two kinds of HSCs have been defined. Long-term HSCs proliferate for the lifetime of an animal. In young adult mice, an estimated 8 to 10 % of long-term HSCs enter the cell cycle and divide each day. Short-term HSCs proliferate for a limited time, possibly a few months. Long-term HSCs have high levels of telomerase activity. Telomerase is an enzyme that helps maintain the length of the ends of chromosomes, called telomeres, by adding on nucleotides. Active telomerase is a characteristic of undifferentiated, dividing cells and cancer cells. Differentiated, human somatic cells do not show telomerase activity. In adult humans, HSCs occur in the bone marrow, blood, liver, and spleen, but are extremely rare in any of these tissues. In mice, only 1 in 10,000 to 15,000 bone marrow cells is a long-term HSC [105].

Short-term HSCs differentiate into lymphoid and myeloid precursors, the two classes of precursors for the two major lineages of blood cells. Lymphoid precursors differentiate into T cells, B cells, and natural killer cells. The mechanisms and pathways that lead to their differentiation are still being investigated [1, 2]. Myeloid precursors differentiate into monocytes and macrophages, neutrophils, eosinophils, basophils, megakaryocytes, and erythrocytes [3]. *In vivo*, bone marrow HSCs differentiate into mature, specialized blood cells that cycle constantly from the bone marrow to the blood, and back to the bone marrow [26]. A recent study showed that short-term HSCs are a heterogeneous population that differ significantly in terms of their ability to self-renew and repopulate the hematopoietic system [42].

Attempts to induce HSC to proliferate *in vitro*—on many substrates, including those intended to mimic conditions in the stroma—have frustrated scientists for many years. Although HSCs proliferate readily *in vivo*, they usually differentiate or die *in vitro* [26]. Thus, much of the research on HSCs has been focused on understanding the factors, cell-cell interactions, and cell-matrix interactions that control their proliferation and differentiation *in vivo*, with the hope that similar conditions could be replicated *in vitro*. Many of the soluble factors that regulate HSC differentiation *in vivo* are cytokines, which are made by different cell types and are then concentrated in the bone marrow by the extracellular matrix of stromal cells—the sites of blood formation [45, 107]. Two of the most-studied cytokines are granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) [40, 81].

Also important to HSC proliferation and differentiation are interactions of the cells with adhesion molecules in the extracellular matrix of the bone marrow stroma [83, 101, 110].

Bone Marrow Stromal Cells. Bone marrow (BM) stromal cells have long been recognized for playing an important role in the differentiation of mature blood cells from HSCs (see Figure 4.3. Hematopoietic and Stromal Stem Cell Differentiation). But stromal cells also have other important functions [30, 31]. In addition to providing the physical environment in which HSCs differentiate, BM stromal cells generate cartilage, bone, and fat. Whether stromal cells are best classified as stem cells or progenitor cells for these tissues is still in question. There is also a question as to whether BM stromal cells and so-called mesenchymal stem cells are the same population [78].

BM stromal cells have many features that distinguish them from HSCs. The two cell types are easy to separate *in vitro*. When bone marrow is dissociated, and the mixture of cells it contains is plated at low density, the stromal cells adhere to the surface of the culture dish, and the HSCs do not. Given specific *in vitro* conditions, BM stromal cells form colonies from a single cell called the colony forming unit-F (CFU-F). These colonies may then differentiate as adipocytes or myelosupportive stroma, a clonal assay that indicates the stem cell-like nature of stromal cells. Unlike HSCs, which do not divide *in vitro* (or proliferate only to a limited extent), BM stromal cells can proliferate for up to 35 population doublings *in vitro* [16]. They grow rapidly under the influence of such mitogens as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) [12].

To date, it has not been possible to isolate a population of pure stromal cells from bone marrow. Panels of markers used to identify the cells include receptors for certain cytokines (interleukin-1, 3, 4, 6, and 7) receptors for proteins in the extracellular matrix, (ICAM-1 and 2, VCAM-1, the alpha-1, 2, and 3 integrins, and the beta-1, 2, 3 and 4 integrins), etc. [64]. Despite the use of these markers and another stromal cell marker called Stro-1, the origin and specific identity of stromal cells have remained elusive. Like HSCs, BM stromal cells arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated and identified.

One theory about their origin is that a common kind of progenitor cell—perhaps a primordial endothelial cell that lines embryonic blood vessels—gives rise to both HSCs and to mesodermal precursors. The latter may then differentiate into myogenic precursors (the satellite cells that are thought to function as stem cells in skeletal muscle), and the BM stromal cells [10].

In vivo, the differentiation of stromal cells into fat and bone is not straightforward. Bone marrow adipocytes and myelosupportive stromal cells—both of which are derived from BM stromal cells—may be regarded as interchangeable phenotypes [10, 11]. Adipocytes do not develop until postnatal life, as the bones enlarge and the marrow space increases to accommodate enhanced hematopoiesis. When the skeleton stops growing, and the mass of HSCs decreases in a normal, age-dependent fashion, BM stromal cells differentiate into adipocytes, which fill the extra space. New bone formation is obviously greater during skeletal growth, although bone "turns over" throughout life. Bone forming cells are osteoblasts, but their relationship to BM stromal cells is not clear. New trabecular bone, which is the inner region of bone next to the marrow, could logically develop from the action of BM stromal cells. But the outside surface of bone also turns over, as does bone next to the Haversian system (small canals that form concentric rings within bone). And neither of these surfaces is in contact with BM stromal cells [10, 11].

Adult Stem Cells in Other Tissues

It is often difficult—if not impossible—to distinguish adult, tissue-specific stem cells from progenitor cells. With that caveat in mind, the following summary identifies reports of stem cells in various adult tissues.

Endothelial Progenitor Cells. Endothelial cells line the inner surfaces of blood vessels throughout the body, and it has been difficult to identify specific endothelial stem cells in either the embryonic or the adult mammal. During embryonic development, just after gastrulation, a kind of cell called the hemangioblast, which is derived from mesoderm, is presumed to be the precursor of both the hematopoietic and endothelial cell lineages. The embryonic vasculature formed at this stage is transient and consists of blood islands in the yolk sac. But hemangioblasts, per se, have not been isolated from the embryo and their existence remains in question. The process of forming new blood vessels in the embryo is called vasculogenesis. In the adult, the process of forming blood vessels from pre-existing blood vessels is called angiogenesis [50].

Evidence that hemangioblasts do exist comes from studies of mouse embryonic stem cells that are directed to differentiate *in vitro*. These studies have shown that a precursor cell derived from mouse ES cells that express Flk-1 [the receptor for vascular endothelial growth factor (VEGF) in mice] can give rise to both blood cells and blood vessel cells [88, 109]. Both VEGF and fibroblast growth factor-2 (FGF-2) play critical roles in endothelial cell differentiation *in vivo* [79].

Several recent reports indicate that the bone marrow contains cells that can give rise to new blood vessels in tissues that are ischemic (damaged due to the deprivation of blood and oxygen) [8, 29, 49, 94]. But it is unclear from these studies what cell type(s) in the bone marrow induced angiogenesis. In a study which sought to address that question, researchers found that adult human bone marrow contains cells that resemble embryonic hemangioblasts, and may therefore be called endothelial stem cells.

In more recent experiments, human bone marrow-derived cells were injected into the tail veins of rats with induced cardiac ischemia. The human cells migrated to the rat heart where they generated new blood vessels in the infarcted muscle (a process akin to vasculogenesis), and also induced angiogenesis. The candidate endothelial stem cells are $CD34^+$ (a marker for HSCs), and they express the transcription factor GATA-2 [51]. A similar study using transgenic mice that express the gene for enhanced green fluorescent protein (which allows the cells to be tracked), showed that bone-marrow-derived cells could repopulate an area of infarcted heart muscle in mice, and generate not only blood vessels, but also cardiomyocytes that integrated into the host tissue [71] (see Chapter 9. Can Stem Cells Repair a Damaged Heart?).

And, in a series of experiments in adult mammals, progenitor endothelial cells were isolated from peripheral blood (of mice and humans) by using antibodies against CD34 and Flk-1, the receptor for VEGF. The cells were mononuclear blood cells (meaning they have a nucleus) and are referred to as MB^{CD34+} cells and MB^{Flk1+} cells. When plated in tissue-culture

dishes, the cells attached to the substrate, became spindle-shaped, and formed tube-like structures that resemble blood vessels. When transplanted into mice of the same species (autologous transplants) with induced ischemia in one limb, the MB^{CD34+} cells promoted the formation of new blood vessels [8]. Although the adult MB^{CD34+} and MB^{Flk1+} cells function in some ways like stem cells, they are usually regarded as progenitor cells.

Skeletal Muscle Stem Cells. Skeletal muscle, like the cardiac muscle of the heart and the smooth muscle in the walls of blood vessels, the digestive system, and the respiratory system, is derived from embryonic mesoderm. To date, at least three populations of skeletal muscle stem cells have been identified: satellite cells, cells in the wall of the dorsal aorta, and so-called "side population" cells.

Satellite cells in skeletal muscle were identified 40 years ago in frogs by electron microscopy [62], and thereafter in mammals [84]. Satellite cells occur on the surface of the basal lamina of a mature muscle cell, or myofiber. In adult mammals, satellite cells mediate muscle growth [85]. Although satellite cells are normally non-dividing, they can be triggered to proliferate as a result of injury, or weight-bearing exercise. Under either of these circumstances, muscle satellite cells give rise to myogenic precursor cells, which then differentiate into the myofibrils that typify skeletal muscle. A group of transcription factors called myogenic regulatory factors (MRFs) play important roles in these differentiation events. The so-called primary MRFs, MyoD and Myf5, help regulate myoblast formation during embryogenesis. The secondary MRFs, myogenin and MRF4, regulate the terminal differentiation of myofibrils [86].

With regard to satellite cells, scientists have been addressing two questions. Are skeletal muscle satellite cells true adult stem cells or are they instead precursor cells? Are satellite cells the only cell type that can regenerate skeletal muscle. For example, a recent report indicates that muscle stem cells may also occur in the dorsal aorta of mouse embryos, and constitute a cell type that gives rise both to muscle satellite cells and endothelial cells. Whether the dorsal aorta cells meet the criteria of a self-renewing muscle stem cell is a matter of debate [21].

Another report indicates that a different kind of stem cell, called an SP cell, can also regenerate skeletal muscle may be present in muscle and bone marrow. SP stands for a side population of cells that can be separated by fluorescence-activated cell sorting analysis. Intravenously injecting these muscle-derived stem cells restored the expression of dystrophin in *mdx* mice. Dystrophin is the protein that is defective in people with Duchenne's muscular dystrophy; *mdx* mice provide a model for the human disease. Dystrophin expression in the SP cell-treated mice was lower than would be needed for clinical benefit. Injection of bone marrow- or muscle-derived SP cells into the dystrophic muscle of the mice yielded equivocal results that the transplanted cells had integrated into the host tissue. The authors conclude that a similar population of SP stem cells can be derived from either adult mouse bone marrow or skeletal muscle, and suggest "there may be some direct relationship between bone marrow-derived stem cells and other tissue- or organ-specific cells" [43]. Thus, stem cell or progenitor cell types from various mesodermally-derived tissues may be able to generate skeletal muscle.

Epithelial Cell Precursors in the Skin and Digestive System. Epithelial cells, which constitute 60 percent of the differentiated cells in the body are responsible for covering the internal and external surfaces of the body, including the lining of vessels and other cavities. The epithelial cells in skin and the digestive tract are replaced constantly. Other epithelial cell populations—in the ducts of the liver or pancreas, for example—turn over more slowly. The cell population that renews the epithelium of the small intestine occurs in the intestinal crypts, deep invaginations in the lining of the gut. The crypt cells are often regarded as stem cells; one of them can give rise to an organized cluster of cells called a structural-proliferative unit [93].

The skin of mammals contains at least three populations of epithelial cells: epidermal cells, hair follicle cells, and glandular epithelial cells, such as those that make up the sweat glands. The replacement patterns for epithelial cells in these three compartments differ, and in all the compartments, a stem cell population has been postulated. For example, stem cells in the bulge region of the hair follicle appear to give rise to multiple cell types. Their progeny can migrate down to the base of the follicle where they become matrix cells, which may then give rise to different cell types in the hair follicle, of which there are seven [39]. The

bulge stem cells of the follicle may also give rise to the epidermis of the skin [95].

Another population of stem cells in skin occurs in the basal layer of the epidermis. These stem cells proliferate in the basal region, and then differentiate as they move toward the outer surface of the skin. The keratinocytes in the outermost layer lack nuclei and act as a protective barrier. A dividing skin stem cell can divide asymmetrically to produce two kinds of daughter cells. One is another self-renewing stem cell. The second kind of daughter cell is an intermediate precursor cell which is then committed to replicate a few times before differentiating into keratinocytes. Self-renewing stem cells can be distinguished from this intermediate precusor cell by their higher level of β1 integrin expression, which signals keratinocytes to proliferate via a mitogen-activated protein (MAP) kinase [112]. Other signaling pathways include that triggered by β-catenin, which helps maintain the stem-cell state [111], and the pathway regulated by the oncoprotein c-Myc, which triggers stem cells to give rise to transit amplifying cells [36].

Stem Cells in the Pancreas and Liver. The status of stem cells in the adult pancreas and liver is unclear. During embryonic development, both tissues arise from endoderm. A recent study indicates that a single precursor cell derived from embryonic endoderm may generate both the ventral pancreas and the liver [23]. In adult mammals, however, both the pancreas and the liver contain multiple kinds of differentiated cells that may be repopulated or regenerated by multiple types of stem cells. In the pancreas, endocrine (hormone-producing) cells occur in the islets of Langerhans. They include the beta cells (which produce insulin), the alpha cells (which secrete glucagon), and cells that release the peptide hormones somatostatin and pancreatic polypeptide. Stem cells in the adult pancreas are postulated to occur in the pancreatic ducts or in the islets themselves. Several recent reports indicate that stem cells that express nestin—which is usually regarded as a marker of neural stem cells—can generate all of the cell types in the islets [60, 113] (see Chapter 7. Stem Cells and Diabetes).

The identity of stem cells that can repopulate the liver of adult mammals is also in question. Recent studies in rodents indicate that HSCs (derived from mesoderm) may be able to home to liver after it is damaged, and demonstrate plasticity in becoming into hepatocytes (usually derived from endoderm) [54, 77, 97]. But the question remains as to whether cells from the bone marrow normally generate hepatocytes *in vivo*. It is not known whether this kind of plasticity occurs without severe damage to the liver or whether HSCs from the bone marrow generate oval cells of the liver [18]. Although hepatic oval cells exist in the liver, it is not clear whether they actually generate new hepatocytes [87, 98]. Oval cells may arise from the portal tracts in liver and may give rise to either hepatocytes [19, 55] and to the epithelium of the bile ducts [37, 92]. Indeed, hepatocytes themselves, may be responsible for the well-know regenerative capacity of liver.

SUMMARY

What Do We Know About Adult Stem Cells?

- Adult stem cells can proliferate without differentiating for a long period (a characteristic referred to as long-term self-renewal), and they can give rise to mature cell types that have characteristic shapes and specialized functions.
- Some adult stem cells have the capability to differentiate into tissues other than the ones from which they originated; this is referred to as plasticity.
- Adult stem cells are rare. Often they are difficult to identify and their origins are not known. Current methods for characterizing adult stem cells are dependent on determining cell surface markers and observations about their differentiation patterns in test tubes and culture dishes.
- To date, published scientific literature indicates that adult stem cells have been derived from brain, bone marrow, peripheral blood, dental pulp, spinal cord, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas; thus, adult stem cells have been found in tissues that develop from all three embryonic germ layers.
- Hematopoietic stem cells from bone marrow are the most studied and used for clinical applications in restoring various blood and immune components to the bone marrow via transplantation. There are at least two other populations of adult stem cells that have been identified from bone marrow and blood.

- Several populations of adult stem cells have been identified in the brain, particularly the hippocampus. Their function is unknown. Proliferation and differentiation of brain stem cells are influenced by various growth factors.
- There are now several reports of adult stem cells in other tissues (muscle, blood, and fat) that demonstrate plasticity. Very few published research reports on plasticity of adult stem cells have, however, included clonality studies. That is, there is limited evidence that a single adult stem cell or genetically identical line of adult stem cells demonstrates plasticity.
- Rarely have experiments that claim plasticity demonstrated that the adult stem cells have generated mature, fully functional cells or that the cells have restored lost function *in vivo*.

What Do We Need to Know About Adult Stem Cells?

- What are the sources of adult stem cells in the body? Are they "leftover" embryonic stem cells, or do they arise in some other way? And if the latter is true—which seems to be the case—exactly how do adult stem cells arise, and why do they remain in an undifferentiated state, when all the cells around them have differentiated?
- Is it possible to manipulate adult stem cells to increase their ability to proliferate *in vitro*, so that adult stem cells can be used as a sufficient source of tissue for transplants?
- How many kinds of adult stem cells exist, and in which tissues do they exist? Evidence is accumulating that, although they occur in small numbers, adult stem cells are present in many differentiated tissues.
- What is the best evidence that adult stem cells show plasticity and generate cell types of other tissues?
- Is it possible to manipulate adult stem cells to increase their ability to proliferate *in vitro* so that adult stem cells can be used as a sufficient source of tissue for transplants?
- Is there a universal stem cell? An emerging concept is that, in adult mammals, there may be a population of "universal" stem cells. Although largely theoretical, the concept has some experimental basis. A candidate, universal adult stem cell may be one that circulates in the blood stream, can escape from the blood, and populate various adult tissues. In more than one experimental system, researchers have noted that dividing cells in adult tissues often appear near a blood vessel, such as candidate stem cells in the hippocampus, a region of the brain [75].
- Do adult stem cells exhibit plasticity as a normal event *in vivo*? If so, is this true of all adult stem cells? What are the signals that regulate the proliferation and differentiation of stem cells that demonstrate plasticity?

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5. HEMATOPOIETIC STEM CELLS

With more than 50 years of experience studying blood-forming stem cells called hematopoietic stem cells, scientists have developed sufficient understanding to actually use them as a therapy. Currently, no other type of stem cell, adult, fetal or embryonic, has attained such status. Hematopoietic stem cell transplants are now routinely used to treat patients with cancers and other disorders of the blood and immune systems. Recently, researchers have observed in animal studies that hematopoietic stem cells appear to be able to form other kinds of cells, such as muscle, blood vessels, and bone. If this can be applied to human cells, it may eventually be possible to use hematopoietic stem cells to replace a wider array of cells and tissues than once thought.

Despite the vast experience with hematopoietic stem cells, scientists face major roadblocks in expanding their use beyond the replacement of blood and immune cells. First, hematopoietic stem cells are unable to proliferate (replicate themselves) and differentiate (become specialized to other cell types) in vitro (in the test tube or culture dish). Second, scientists do not yet have an accurate method to distinguish stem cells from other cells recovered from the blood or bone marrow. Until scientists overcome these technical barriers, they believe it is unlikely that hematopoietic stem cells will be applied as cell replacement therapy in diseases such as diabetes, Parkinson's Disease, spinal cord injury, and many others.

INTRODUCTION

Blood cells are responsible for constant maintenance and immune protection of every cell type of the body. This relentless and brutal work requires that blood cells, along with skin cells, have the greatest powers of self-renewal of any adult tissue.

The stem cells that form blood and immune cells are known as hematopoietic stem cells (HSCs). They are ultimately responsible for the constant renewal of blood—the production of billions of new blood cells each day. Physicians and basic researchers have known and capitalized on this fact for more than 50 years in treating many diseases. The first evidence and definition of blood-forming stem cells came from studies of people exposed to lethal doses of radiation in 1945.

Basic research soon followed. After duplicating radiation sickness in mice, scientists found they could rescue the mice from death with bone marrow transplants from healthy donor animals. In the early 1960s, Till and McCulloch began analyzing the bone marrow to find out which components were responsible for regenerating blood [56]. They defined what remain the two hallmarks of an HSC: it can renew itself and it can produce cells that give rise to all the different types of blood cells (see Chapter 4. The Adult Stem Cell).

WHAT IS A HEMATOPOIETIC STEM CELL?

A hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis—a process by which cells that are detrimental or unneeded self-destruct.

A major thrust of basic HSC research since the 1960s has been identifying and characterizing these stem cells. Because HSCs look and behave in culture like ordinary white blood cells, this has been a difficult challenge and this makes them difficult to identify by morphology (size and shape). Even today, scientists must rely on cell surface proteins, which serve, only roughly, as markers of white blood cells.

Identifying and characterizing properties of HSCs began with studies in mice, which laid the groundwork for human studies. The challenge is formidable as about 1 in every 10,000 to 15,000 bone marrow cells is thought to be a stem cell. In the blood stream the proportion falls to 1 in 100,000 blood cells. To this end, scientists began to develop tests for proving the self-renewal and the plasticity of HSCs.

The "gold standard" for proving that a cell derived from mouse bone marrow is indeed an HSC is still based on the same proof described above and used in mice many years ago. That is, the cells are injected into a mouse that has received a dose of irradiation sufficient to kill its own blood-producing cells. If the mouse recovers and all types of blood cells reappear (bearing a genetic marker from the donor animal), the transplanted cells are deemed to have included stem cells.

These studies have revealed that there appear to be two kinds of HSCs. If bone marrow cells from the transplanted mouse can, in turn, be transplanted to another lethally irradiated mouse and restore its hematopoietic system over some months, they are considered to be *long-term stem cells* that are capable of self-renewal. Other cells from bone marrow can immediately regenerate all the different types of blood cells, but under normal circumstances cannot renew themselves over the long term, and these are referred to as *short-term progenitor or precursor cells.* Progenitor or precursor cells are relatively immature cells that are precursors to a fully differentiated cell of the same tissue type. They are capable of proliferating, but they have a limited capacity to differentiate into more than one cell type as HSCs do. For example, a blood progenitor cell may only be able to make a red blood cell (see Figure 5.1. Hematopoietic and Stromal Stem Cell Differentiation).

Harrison et al. write that short-term blood-progenitor cells in a mouse may restore hematopoiesis for three to four months [36]. The longevity of short-term stem cells for humans is not firmly established. A true stem cell, capable of self-renewal, must be able to renew itself for the entire lifespan of an organism. It is these

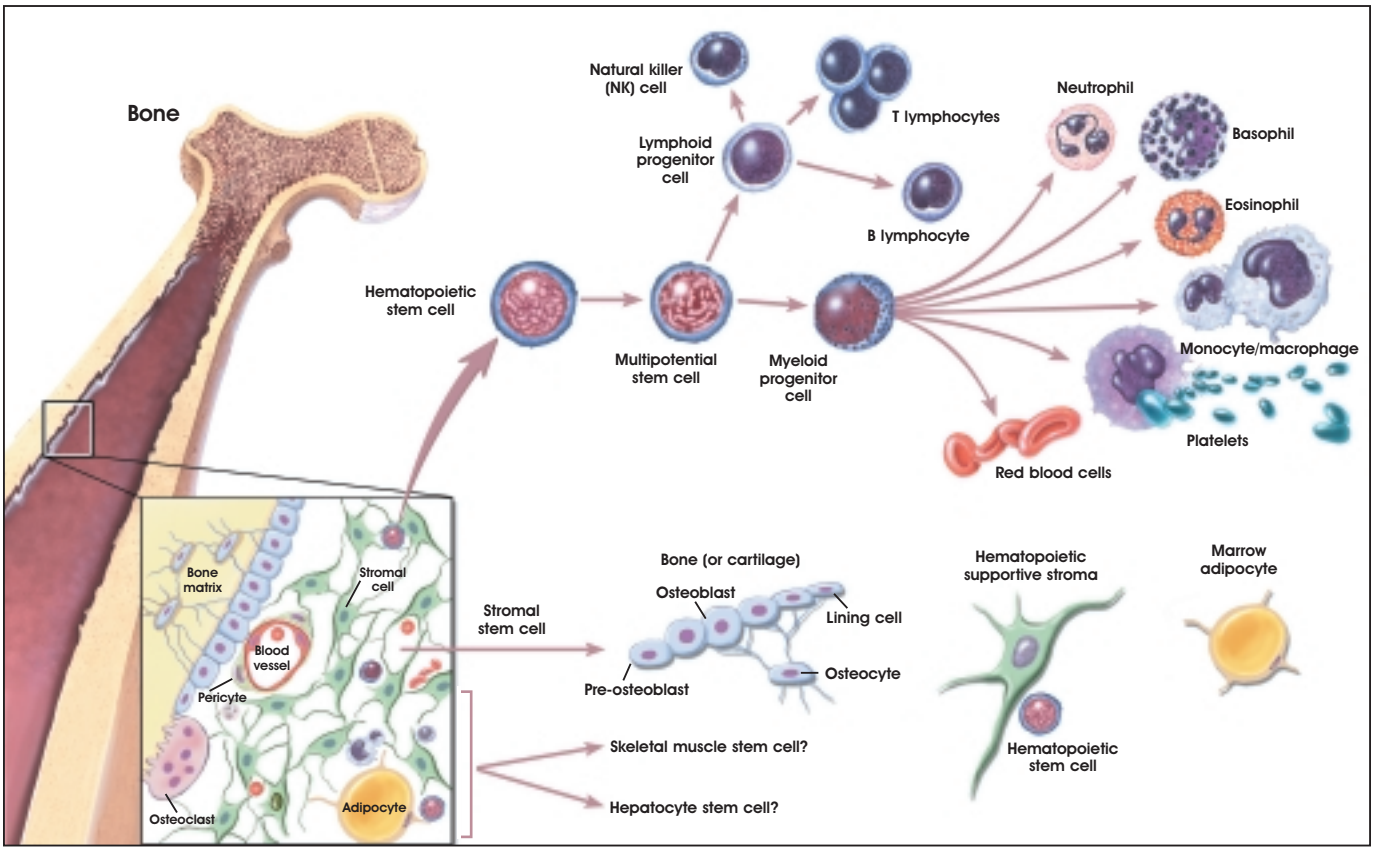

Figure 5.1. Hematopoietic and Stromal Stem Cell Differentiation.

long-term replicating HSCs that are most important for developing HSC-based cell therapies. Unfortunately, to date, researchers cannot distinguish the long-term from the short-term cells when they are removed from the bloodstream or bone marrow.

The central problem of the assays used to identify long-term stem cells and short-term progenitor cells is that they are difficult, expensive, and time-consuming and cannot be done in humans. A few assays are now available that test cells in culture for their ability to form primitive and long-lasting colonies of cells, but these tests are not accepted as proof that a cell is a long-term stem cell. Some genetically altered mice can receive transplanted human HSCs to test the cells' self-renewal and hematopoietic capabilities during the life of a mouse, but the relevance of this test for the cells in humans—who may live for decades—is open to question.

The difficulty of HSC assays has contributed to two mutually confounding research problems: definitively identifying the HSC and getting it to proliferate, or increase its numbers, in a culture dish. More rapid research progress on characterizing and using HSCs would be possible if they could be readily grown in the laboratory. Conversely, progress in identifying growth conditions suitable for HSCs and getting the cells to multiply would move more quickly if scientists could reliably and readily identify true HSCs.

CAN CELL MARKERS BE USED TO IDENTIFY HEMATOPOIETIC STEM CELLS?

HSCs have an identity problem. First, the ones with long-term replicating ability are rare. Second, there are multiple types of stem cells. And, third, the stem cells look like many other blood or bone marrow cells. So how do researchers find the desired cell populations? The most common approach is through markers that appear on the surface of cells. (For a more detailed discussion, see Appendix E.i. Markers: How Do Researchers Use Them to Identify Stem Cells?) These are useful, but not perfect tools for the research laboratory.

In 1988, in an effort to develop a reliable means of identifying these cells, Irving Weissman and his collaborators focused attention on a set of protein markers on the surface of mouse blood cells that were associated with increased likelihood that the cell was a long-term HSC [50]. Four years later, the laboratory proposed a comparable set of markers for the human stem cell [3]. Weissman proposes the markers shown in Table 5.1 as the closest markers for mouse and human HSCs [62].

Table 5.1. Proposed cell-surface markers of undifferentiated hematopoietic stem cells.

Listed here are cell surface markers found on mouse and human hematopoietic stem cells as they exist in their undifferentiated state *in vivo* and *in vitro*. As these cells begin to develop as distinct cell lineages the cell surface markers are no longer identified.

Mouse	Human
$CD34^{low/-}$	$CD\ 34^{+}$
$SCA\text{-}1^{+}$	$CD59^{+*}$
$Thy1^{+/low}$	$Thy1^{+}$
$CD38^{+}$	$CD38^{low/-}$
$C\text{-}kit^{+}$	$C\text{-}kit^{-/low}$
lin^{-*}	lin^{-**}

** Only one of a family of CD59 markers has thus far been evaluated.*

*** Lin- cells lack 13 to 14 different mature blood-lineage markers.*

Such cell markers can be tagged with monoclonal antibodies bearing a fluorescent label and culled out of bone marrow with fluorescence-activated cell sorting (FACS).

The groups of cells thus sorted by surface markers are heterogeneous and include some cells that are true, long-term self-renewing stem cells, some shorter-term progenitors, and some non-stem cells. Weissman's group showed that as few as five genetically tagged cells, injected along with larger doses of stem cells into lethally irradiated mice, could establish themselves and produce marked donor cells in all blood cell lineages for the lifetime of the mouse. A single tagged cell could produce all lineages for as many as seven weeks, and 30 purified cells were sufficient to rescue mice and fully repopulate the bone marrow without extra doses of backup cells to rescue the mice [49]. Despite these efforts, researchers remain divided on the most consistently expressed set of HSC markers [27, 32]. Connie Eaves of the University of British Columbia says none of the markers are tied to unique stem cell functions or truly define the stem cell [14]. "Almost every marker I am aware of has been shown to be fickle," she says.

More recently, Diane Krause and her colleagues at Yale University, New York University, and Johns Hopkins University, used a new technique to home in on a single cell capable of reconstituting all blood cell lineages of an irradiated mouse [27]. After marking bone marrow cells from donor male mice with a nontoxic dye, they injected the cells into female recipient mice that had been given a lethal dose of radiation. Over the next two days, some of the injected cells migrated, or homed, to the bone marrow of the recipients and did not divide; when transplanted into a second set of irradiated female mice, they eventually proved to be a concentrated pool of self-renewing stem cells. The cells also reconstituted blood production. The scientists estimate that their technique concentrated the long-term stem cells 500 to 1,000-fold compared with bone marrow.

WHAT ARE THE SOURCES OF HEMATOPOIETIC STEM CELLS?

Bone Marrow

The classic source of hematopoietic stem cells (HSCs) is bone marrow. For more than 40 years, doctors performed bone marrow transplants by anesthetizing the stem cell donor, puncturing a bone—typically a hipbone—and drawing out the bone marrow cells with a syringe. About 1 in every 100,000 cells in the marrow is a long-term, blood-forming stem cell; other cells present include stromal cells, stromal stem cells, blood progenitor cells, and mature and maturing white and red blood cells.

Peripheral Blood

As a source of HSCs for medical treatments, bone marrow retrieval directly from bone is quickly fading into history. For clinical transplantation of human HSCs, doctors now prefer to harvest donor cells from peripheral, circulating blood. It has been known for decades that a small number of stem and progenitor cells circulate in the bloodstream, but in the past 10 years, researchers have found that they can coax the cells to migrate from marrow to blood in greater numbers by injecting the donor with a cytokine, such as granulocyte-colony stimulating factor (GCSF). The donor is injected with GCSF a few days before the cell harvest. To collect the cells, doctors insert an intravenous tube into the donor's vein and pass his blood through a filtering system that pulls out $CD34^+$ white blood cells and returns the red blood cells to the donor. Of the cells collected, just 5 to 20 percent will be true HSCs. Thus, when medical researchers commonly refer to peripherally harvested "stem cells," this is something of a misnomer. As is true for bone marrow, the $CD34^+$ cells are a mixture of stem cells, progenitors, and white blood cells of various degrees of maturity.

In the past three years, the majority of autologous (where the donor and recipient are the same person) and allogeneic (where the donor and recipient are different individuals) "bone marrow" transplants have actually been white blood cells drawn from peripheral circulation, not bone marrow. Richard Childs, an intramural investigator at the NIH, says peripheral harvest of cells is easier on the donor—with minimal pain, no anesthesia, and no hospital stay—but also yields better cells for transplants [6]. Childs points to evidence that patients receiving peripherally harvested cells have higher survival rates than bone marrow recipients do. The peripherally harvested cells contain twice as many HSCs as stem cells taken from bone marrow and engraft more quickly. This means patients may recover white blood cells, platelets, and their immune and clotting protection several days faster than they would with a bone marrow graft. Scientists at Stanford report that highly purified, mobilized peripheral cells that have $CD34^+$ and $Thy\text{-}1^+$ surface markers engraft swiftly and without complication in breast cancer patients receiving an autologous transplant of the cells after intensive chemotherapy [41].

Umbilical Cord Blood

In the late 1980s and early 1990s, physicians began to recognize that blood from the human umbilical cord and placenta was a rich source of HSCs. This tissue supports the developing fetus during pregnancy, is delivered along with the baby, and, is usually discarded. Since the first successful umbilical cord blood transplants in children with Fanconi anemia, the collection and therapeutic use of these cells has grown quickly. The New York Blood Center's Placental Blood Program, supported by NIH, is the largest U.S. public umbilical cord blood bank and now has 13,000 donations available for transplantation into small patients who need HSCs. Since it began collecting umbilical cord blood in 1992, the center has provided thousands of cord blood units to patients. Umbilical cord blood recipients—typically children—have now lived in excess of eight years, relying on the HSCs from an umbilical cord blood transplant [31, 57].

There is a substantial amount of research being conducted on umbilical cord blood to search for ways to expand the number of HSCs and compare and contrast the biological properties of cord blood with adult bone marrow stem cells. There have been suggestions that umbilical cord blood contains stem cells that have the capability of developing cells of multiple germ layers (multipotent) or even all germ layers, e.g., endoderm, ectoderm, and mesoderm (pluripotent). To date, there is no published scientific evidence to support this claim. While umbilical cord blood represents a valuable resource for HSCs, research data have not conclusively shown qualitative differences in the differentiated cells produced between this source of HSCs and peripheral blood and bone marrow.

Fetal Hematopoietic System

An important source of HSCs in research, but not in clinical use, is the developing blood-producing tissues of fetal animals. Hematopoietic cells appear early in the development of all vertebrates. Most extensively studied in the mouse, HSC production sweeps through the developing embryo and fetus in waves. Beginning at about day 7 in the life of the mouse embryo, the earliest hematopoietic activity is indicated by the appearance of blood islands in the yolk sac (see Appendix A. Early Development). The point is disputed, but some scientists contend that yolk sac blood production is transient and will generate some blood cells for the embryo, but probably not the bulk of the HSCs for the adult animal [12, 26, 44]. According to this proposed scenario, most stem cells that will be found in the adult bone marrow and circulation are derived from cells that appear slightly later and in a different location. This other wave of hematopoietic stem cell production occurs in the AGM—the region where the *a*orta, *g*onads, and fetal kidney (*m*esonephros) begin to develop. The cells that give rise to the HSCs in the AGM may also give rise to endothelial cells that line blood vessels. [13]. These HSCs arise at around days 10 to 11 in the mouse embryo (weeks 4 to 6 in human gestation), divide, and within a couple of days, migrate to the liver [11]. The HSCs in the liver continue to divide and migrate, spreading to the spleen, thymus, and—near the time of birth—to the bone marrow.

Whereas an increasing body of fetal HSC research is emerging from mice and other animals, there is much less information about human fetal and embryonic HSCs. Scientists in Europe, including Coulombel, Peault, and colleagues, first described hematopoietic precursors in human embryos only a few years ago [20, 53]. Most recently, Gallacher and others reported finding HSCs circulating in the blood of 12- to 18-week aborted human fetuses [16, 28, 54] that was rich in HSCs. These circulating cells had different markers than did cells from fetal liver, fetal bone marrow, or umbilical cord blood.

Embryonic Stem Cells and Embryonic Germ Cells

In 1985, it was shown that it is possible to obtain precursors to many different blood cells from mouse embryonic stem cells [9]. Perkins was able to obtain all the major lineages of progenitor cells from mouse embryoid bodies, even without adding hematopoietic growth factors [45].

Mouse embryonic stem cells in culture, given the right growth factors, can generate most, if not all, the different blood cell types [19], but no one has yet achieved the "gold standard" of proof that they can produce long-term HSCs from these sources—namely by obtaining cells that can be transplanted into lethally irradiated mice to reconstitute long-term hematopoiesis [32].

The picture for human embryonic stem and germ cells is even less clear. Scientists from James Thomson's laboratory reported in 1999 that they were able to direct human embryonic stem cells—which can now be cultured in the lab—to produce blood progenitor cells [23]. Israeli scientists reported that they had induced human ES cells to produce hematopoietic cells, as evidenced by their production of a blood protein, gamma-globin [21]. Cell lines derived from human embryonic germ cells (cultured cells derived originally from cells in the embryo that would ultimately give rise to eggs or sperm) that are cultured under certain conditions will produce $CD34^+$ cells [47]. The blood-producing cells derived from human ES and embryonic germ (EG) cells have not been rigorously tested for long-term self-renewal or the ability to give rise to all the different blood cells.

As sketchy as data may be on the hematopoietic powers of human ES and EG cells, blood experts are intrigued by their clinical potential and their potential to answer basic questions on renewal and differentiation of HSCs [19]. Connie Eaves, who has made comparisons of HSCs from fetal liver, cord blood,

The Stem Cell Database

http://stemcell.princeton.edu

Ihor Lemischka and colleagues at Princeton University and the Computational Biology and Informatics Laboratory at the University of Pennsylvania are collaborating to record all the findings about hematopoietic stem cell (HSC) genes and markers in the Stem Cell Database.

The collaborators started the database five years ago. Its goal is listing and annotating all the genes that are differentially expressed in mouse liver HSCs and their cellular progeny. The database is growing to include human HSCs from different blood sources, and a related database, constructed in collaboration with Kateri A. Moore, also at Princeton University, will document all genes active in stromal cells, which provide the microenvironment in which stem cells are maintained. The combined power of the two databases, along with new tools and methods for studying molecular biology, will help researchers put together a complete portrait of the hematopoietic stem cell and how it works. The databases will continue to grow and take advantage of other efforts, such as those to complete the gene sequences of mammals. Data will be publicly available to researchers around the world.

and adult bone marrow, expects cells derived from embryonic tissues to have some interesting traits. She says actively dividing blood-producing cells from ES cell culture—if they are like other dividing cells—will not themselves engraft or rescue hematopoiesis in an animal whose bone marrow has been destroyed. However, they may play a critical role in developing an abundant supply of HSCs grown in the lab. Indications are that the dividing cells will also more readily lend themselves to gene manipulations than do adult HSCs. Eaves anticipates that HSCs derived from early embryo sources will be developmentally more "plastic" than later HSCs, and more capable of self-renewal [14].

HOW DO HSCs FROM VARYING SOURCES DIFFER?

Scientists in the laboratory and clinic are beginning to measure the differences among HSCs from different sources. In general, they find that HSCs taken from tissues at earlier developmental stages have a greater ability to self-replicate, show different homing and surface characteristics, and are less likely to be rejected by the immune system—making them potentially more useful for therapeutic transplantation.

Stem cell populations of the bone marrow

When do HSCs move from the early locations in the developing fetus to their adult "home" in the bone marrow? European scientists have found that the relative number of $CD34^+$ cells in the collections of cord blood declined with gestational age, but expression of cell-adhesion molecules on these cells increased. The authors believe these changes reflect preparations for the cells to relocate—from homing in fetal liver to homing in bone marrow [52].

The point is controversial, but a paper by Chen et al. provides evidence that at least in some strains of mice, HSCs from old mice are less able to repopulate bone marrow after transplantation than are cells from young adult mice [5]. Cells from fetal mice were 50 to 100 percent better at repopulating marrow than were cells from young adult mice were. The specific potential for repopulating marrow appears to be strain-specific, but the scientists found this potential declined with age for both strains. Other scientists find no decreases or sometimes increases in numbers of HSCs with age [51]. Because of the difficulty in identifying a long-term stem cell, it remains difficult to quantify changes in numbers of HSCs as a person ages.

Effectiveness of Transplants of Adult versus Umbilical Cord Blood Stem Cells

A practical and important difference between HSCs collected from adult human donors and from umbilical cord blood is simply quantitative. Doctors are rarely able to extract more than a few million HSCs from a placenta and umbilical cord—too few to use in a transplant for an adult, who would ideally get 7 to 10 million $CD34^+$ cells per kilogram body weight, but often adequate for a transplant for a child [33, 48].

Leonard Zon says that HSCs from cord blood are less likely to cause a transplantation complication called graft-versus-host disease, in which white blood cells

from a donor attack tissues of the recipient [65]. In a recent review of umbilical cord blood transplantation, Laughlin cites evidence that cord blood causes less graft-versus-host disease [31]. Laughlin writes that it is yet to be determined whether umbilical cord blood HSCs are, in fact, longer lived in a transplant recipient.

In lab and mouse-model tests comparing $CD34^+$ cells from human cord with $CD34^+$ cells derived from adult bone marrow, researchers found cord blood had greater proliferation capacity [24]. White blood cells from cord blood engrafted better in a mouse model, which was genetically altered to tolerate the human cells, than did their adult counterparts.

Effectiveness in Transplants of Peripheral Versus Bone Marrow Stem Cells

In addition to being far easier to collect, peripherally harvested white blood cells have other advantages over bone marrow. Cutler and Antin's review says that peripherally harvested cells engraft more quickly, but are more likely to cause graft-versus-host disease [8]. Prospecting for the most receptive HSCs for gene therapy, Orlic and colleagues found that mouse HSCs mobilized with cytokines were more likely to take up genes from a viral vector than were non-mobilized bone marrow HSCs [43].

WHAT DO HEMATOPOIETIC STEM CELLS DO AND WHAT FACTORS ARE INVOLVED IN THESE ACTIVITIES?

As stated earlier, an HSC in the bone marrow has four actions in its repertoire: 1) it can renew itself, 2) it can differentiate, 3) it can mobilize out of the bone marrow into circulation (or the reverse), or 4) it can undergo programmed cell death, or apoptosis. Understanding the how, when, where, which, and why of this simple repertoire will allow researchers to manipulate and use HSCs for tissue and organ repair.

Self-renewal of Hematopoietic Stem Cells

Scientists have had a tough time trying to grow—or even maintain—true stem cells in culture. This is an important goal because cultures of HSCs that could maintain their characteristic properties of self-renewal and lack of differentiation could provide an unlimited source of cells for therapeutic transplantation and study. When bone marrow or blood cells are observed in culture, one often observes large increases in the number of cells. This usually reflects an increase in differentiation of cells to progenitor cells that can give rise to different lineages of blood cells but cannot renew themselves. True stem cells divide and replace themselves slowly in adult bone marrow.

New tools for gene-expression analysis will now allow scientists to study developmental changes in telomerase activity and telomeres. Telomeres are regions of DNA found at the end of chromosomes that are extended by the enzyme telomerase. Telomerase activity is necessary for cells to proliferate and activity decreases with age leading to shortened telomeres. Scientists hypothesize that declines in stem cell renewal will be associated with declines in telomere length and telomerase activity. Telomerase activity in hematopoietic cells is associated with self-renewal potential [40].

Because self-renewal divisions are rare, hard to induce in culture, and difficult to prove, scientists do not have a definitive answer to the burning question: what puts—or perhaps keeps—HSCs in a self-renewal division mode? HSCs injected into an anemic patient or mouse—or one whose HSCs have otherwise been suppressed or killed—will home to the bone marrow and undergo active division to both replenish all the different types of blood cells and yield additional self-renewing HSCs. But exactly how this happens remains a mystery that scientists are struggling to solve by manipulating cultures of HSCs in the laboratory.

Two recent examples of progress in the culturing studies of mouse HSCs are by Ema and coworkers and Audet and colleagues [2, 15]. Ema et al. found that two cytokines—stem cell factor and thrombopoietin—efficiently induced an unequal first cell division in which one daughter cell gave rise to repopulating cells with self-renewal potential. Audet et al. found that activation of the signaling molecule gp130 is critical to survival and proliferation of mouse HSCs in culture.

Work with specific cytokines and signaling molecules builds on several earlier studies demonstrating modest increases in the numbers of stem cells that could be induced briefly in culture. For example, Van Zant and colleagues used continuous-perfusion culture and bioreactors in an attempt to boost human HSC numbers in single cord blood samples incubated for one to two weeks [58]. They obtained a 20-fold increase in "long-term culture initiating cells."

More clues on how to increase numbers of stem cells may come from looking at other animals and various developmental stages. During early developmental stages—in the fetal liver, for example—HSCs may undergo more active cell division to increase their numbers, but later in life, they divide far less often [30, 42]. Culturing HSCs from 10- and 11-day-old mouse embryos, Elaine Dzierzak at Erasmus University in the Netherlands finds she can get a 15-fold increase in HSCs within the first 2 or 3 days after she removes the AGM from the embryos [38]. Dzierzak recognizes that this is dramatically different from anything seen with adult stem cells and suggests it is a difference with practical importance. She suspects that the increase is not so much a response to what is going on in the culture but rather, it represents the developmental momentum of this specific embryonic tissue. That is, it is the inevitable consequence of divisions that were cued by that specific embryonic microenvironment. After five days, the number of HSCs plateaus and can be maintained for up to a month. Dzierzak says that the key to understanding how adult-derived HSCs can be expanded and manipulated for clinical purposes may very well be found by defining the cellular composition and complex molecular signals in the AGM region during development [13].

In another approach, Lemischka and coworkers have been able to maintain mouse HSCs for four to seven weeks when they are grown on a clonal line of cells (AFT024) derived from the stroma, the other major cellular constituent of bone marrow [39]. No one knows which specific factors secreted by the stromal cells maintain the stem cells. He says ongoing gene cloning is rapidly zeroing in on novel molecules from the stromal cells that may "talk" to the stem cells and persuade them to remain stem cells—that is, continue to divide and not differentiate.

If stromal factors provide the key to stem cell self-renewal, research on maintaining stromal cells may be an important prerequisite. In 1999, researchers at Osiris Therapeutics and Johns Hopkins University reported culturing and expanding the numbers of mesenchymal stem cells, which produce the stromal environment [46]. Whereas cultured HSCs rush to differentiate and fail to retain primitive, self-renewing cells, the mesenchymal stem cells could be increased in numbers and still retained their powers to generate the full repertoire of descendant lineages.

Differentiation of HSCs into Components of the Blood and Immune System

Producing differentiated white and red blood cells is the real work of HSCs and progenitor cells. M.C. MacKey calculates that in the course of producing a mature, circulating blood cell, the original hematopoietic stem cell will undergo between 17 and 19.5 divisions, "giving a net amplification of between ~170,000 and ~720,000" [35].

Through a series of careful studies of cultured cells—often cells with mutations found in leukemia patients or cells that have been genetically altered—investigators have discovered many key growth factors and cytokines that induce progenitor cells to make different types of blood cells. These factors interact with one another in complex ways to create a system of exquisite genetic control and coordination of blood cell production.

Migration of Hematopoietic Stem Cells Into and Out of Marrow and Tissues

Scientists know that much of the time, HSCs live in intimate connection with the stroma of bone marrow in adults (see Chapter 4. The Adult Stem Cell). But HSCs may also be found in the spleen, in peripheral blood circulation, and other tissues. Connection to the interstices of bone marrow is important to both the engraftment of transplanted cells and to the maintenance of stem cells as a self-renewing population. Connection to stroma is also important to the orderly proliferation, differentiation, and maturation of blood cells [63].

Weissman says HSCs appear to make brief forays out of the marrow into tissues, then duck back into marrow [62]. At this time, scientists do not understand why or how HSCs leave bone marrow or return to it [59]. Scientists find that HSCs that have been mobilized into peripheral circulation are mostly non-dividing cells [64]. They report that adhesion molecules on the stroma, play a role in mobilization, in attachment to the stroma, and in transmitting signals that regulate HSC self-renewal and progenitor differentiation [61].

Apoptosis and Regulation of Hematopoietic Stem Cell Populations

The number of blood cells in the bone marrow and blood is regulated by genetic and molecular mechanisms. How do hematopoietic stem cells know

when to stop proliferating? Apoptosis is the process of programmed cell death that leads cells to self-destruct when they are unneeded or detrimental. If there are too few HSCs in the body, more cells divide and boost the numbers. If excess stem cells were injected into an animal, they simply wouldn't divide or would undergo apoptosis and be eliminated [62]. Excess numbers of stem cells in an HSC transplant actually seem to improve the likelihood and speed of engraftment, though there seems to be no rigorous identification of a mechanism for this empirical observation.

The particular signals that trigger apoptosis in HSCs are as yet unknown. One possible signal for apoptosis might be the absence of life-sustaining signals from bone marrow stroma. Michael Wang and others found that when they used antibodies to disrupt the adhesion of HSCs to the stroma via VLA-4/VCAM-1, the cells were predisposed to apoptosis [61].

Understanding the forces at play in HSC apoptosis is important to maintaining or increasing their numbers in culture. For example, without growth factors, supplied in the medium or through serum or other feeder layers of cells, HSCs undergo apoptosis. Domen and Weissman found that stem cells need to get two growth factor signals to continue life and avoid apoptosis: one via a protein called BCL-2, the other from steel factor, which, by itself, induces HSCs to produce progenitor cells but not to self-renew [10].

WHAT ARE THE CLINICAL USES OF HEMATOPOIETIC STEM CELLS?

Leukemia and Lymphoma

Among the first clinical uses of HSCs were the treatment of cancers of the blood—leukemia and lymphoma, which result from the uncontrolled proliferation of white blood cells. In these applications, the patient's own cancerous hematopoietic cells were destroyed via radiation or chemotherapy, then replaced with a bone marrow transplant, or, as is done now, with a transplant of HSCs collected from the peripheral circulation of a matched donor. A matched donor is typically a sister or brother of the patient who has inherited similar human leukocyte antigens (HLAs) on the surface of their cells. Cancers of the blood include acute lymphoblastic leukemia, acute myeloblastic leukemia, chronic myelogenous leukemia (CML), Hodgkin's disease, multiple myeloma, and non-Hodgkin's lymphoma.

Thomas and Clift describe the history of treatment for chronic myeloid leukemia as it moved from largely ineffective chemotherapy to modestly successful use of a cytokine, interferon, to bone marrow transplants—first in identical twins, then in HLA-matched siblings [55]. Although there was significant risk of patient death soon after the transplant either from infection or from graft-versus-host disease, for the first time, many patients survived this immediate challenge and had survival times measured in years or even decades, rather than months. The authors write, "In the space of 20 years, marrow transplantation has contributed to the transformation of [chronic myelogenous leukemia] CML from a fatal disease to one that is frequently curable. At the same time, experience acquired in this setting has improved our understanding of many transplant-related problems. It is now clear that morbidity and mortality are not inevitable consequences of allogeneic transplantation, [and] that an allogeneic effect can add to the anti-leukemic power of conditioning regimens…"

In a recent development, CML researchers have taken their knowledge of hematopoietic regulation one step farther. On May 10, 2001, the Food and Drug Administration approved Gleevec™ (imatinib mesylate), a new, rationally designed oral drug for treatment of CML. The new drug specifically targets a mutant protein, produced in CML cancer cells, that sabotages the cell signals controlling orderly division of progenitor cells. By silencing this protein, the new drug turns off cancerous overproduction of white blood cells, so doctors do not have to resort to bone marrow transplantation. At this time, it is unknown whether the new drug will provide sustained remission or will prolong life for CML patients.

Inherited Blood Disorders

Another use of allogeneic bone marrow transplants is in the treatment of hereditary blood disorders, such as different types of inherited anemia (failure to produce blood cells), and inborn errors of metabolism (genetic disorders characterized by defects in key enzymes need to produce essential body components or degrade chemical byproducts). The blood disorders include aplastic anemia, beta-thalassemia, Blackfan-Diamond syndrome, globoid cell leukodystrophy, sickle-cell anemia, severe combined immunodeficiency, X-linked lymphoproliferative syndrome, and Wiskott-Aldrich syndrome. Inborn errors of metabolism that are treated with bone marrow

The National Marrow Donor Program

http://www.marrow.org

Launched in 1987, the National Marrow Donor Program (NMDP) was created to connect patients who need blood-forming stem cells or bone marrow with potential nonrelated donors. About 70 percent of patients who need a life-saving HSC transplant cannot find a match in their own family.

The NMDP is made up of an international network of centers and banks that collect cord blood, bone marrow, and peripherally harvested stem cells and that recruit potential donors. As of February 28, 2001, the NMDP listed 4,291,434 potential donors. Since its start, the Minneapolis-based group has facilitated almost 12,000 transplants—75 percent of them for leukemia. Major recruiting efforts have led to substantial increases in the number of donations from minorities, but the chance that African Americans, Native Americans, Asian/Pacific Islanders, or Hispanics will find a match is still lower than it is for Caucasians.

transplants include: Hunter's syndrome, Hurler's syndrome, Lesch Nyhan syndrome, and osteopetrosis. Because bone marrow transplantation has carried a significant risk of death, this is usually a treatment of last resort for otherwise fatal diseases.

Hematopoietic Stem Cell Rescue in Cancer Chemotherapy

Chemotherapy aimed at rapidly dividing cancer cells inevitably hits another target—rapidly dividing hematopoietic cells. Doctors may give cancer patients an autologous stem cell transplant to replace the cells destroyed by chemotherapy. They do this by mobilizing HSCs and collecting them from peripheral blood. The cells are stored while the patient undergoes intensive chemotherapy or radiotherapy to destroy the cancer cells. Once the drugs have washed out of a patient's body, the patient receives a transfusion of his or her stored HSCs. Because patients get their own cells back, there is no chance of immune mismatch or graft-versus-host disease. One problem with the use of autologous HSC transplants in cancer therapy has been that cancer cells are sometimes inadvertently collected and reinfused back into the patient along with the stem cells. One team of investigators finds that they can prevent reintroducing cancer cells by purifying the cells and preserving only the cells that are $CD34^+$, $Thy\text{-}1^+$ [41].

Graft-Versus-Tumor Treatment of Cancer

One of the most exciting new uses of HSC transplantation puts the cells to work attacking otherwise untreatable tumors. A group of researchers in NIH's intramural research program recently described this approach to treating metastatic kidney cancer [7]. Just under half of the 38 patients treated so far have had their tumors reduced. The research protocol is now expanding to treatment of other solid tumors that resist standard therapy, including cancer of the lung, prostate, ovary, colon, esophagus, liver, and pancreas.

This experimental treatment relies on an allogeneic stem cell transplant from an HLA-matched sibling whose HSCs are collected peripherally. The patient's own immune system is suppressed, but not totally destroyed. The donor's cells are transfused into the patient, and for the next three months, doctors closely monitor the patient's immune cells, using DNA fingerprinting to follow the engraftment of the donor's cells and regrowth of the patient's own blood cells. They must also judiciously suppress the patient's immune system as needed to deter his/her T cells from attacking the graft and to reduce graft-versus-host disease.

A study by Joshi et al. shows that umbilical cord blood and peripherally harvested human HSCs show antitumor activity in the test tube against leukemia cells and breast cancer cells [22]. Grafted into a mouse model that tolerates human cells, HSCs attack human leukemia and breast cancer cells. Although untreated cord blood lacks natural killer (NK) lymphocytes capable of killing tumor cells, researchers have found that at least in the test tube and in mice, they can greatly enhance the activity and numbers of these cells with cytokines IL-15 [22, 34].

Other Applications of Hematopoietic Stem Cells

Substantial basic and limited clinical research exploring the experimental uses of HSCs for other diseases is underway. Among the primary applications are autoimmune diseases, such as diabetes, rheumatoid

arthritis, and system lupus erythematosis. Here, the body's immune system turns to destroying body tissues. Experimental approaches similar to those applied above for cancer therapies are being conducted to see if the immune system can be reconstituted or reprogrammed. More detailed discussion on this application is provided in Chapter 6. Autoimmune Diseases and the Promise of Stem Cell-Based Therapies. The use of HSCs as a means to deliver genes to repair damaged cells is another application being explored. The use of HSCs for gene therapies is discussed in detail in Chapter 11. Use of Genetically Modified Stem Cells in Experimental Gene Therapies.

PLASTICITY OF HEMATOPOIETIC STEM CELLS

A few recent reports indicate that scientists have been able to induce bone marrow or HSCs to differentiate into other types of tissue, such as brain, muscle, and liver cells. These concepts and the experimental evidence supporting this concept are discussed in Chapter 4. The Adult Stem Cell.

Research in a mouse model indicates that cells from grafts of bone marrow or selected HSCs may home to damaged skeletal and cardiac muscle or liver and regenerate those tissues [4, 29]. One recent advance has been in the study of muscular dystrophy, a genetic disease that occurs in young people and leads to progressive weakness of the skeletal muscles. Bittner and colleagues used *mdx* mice, a genetically modified mouse with muscle cell defects similar to those in human muscular dystrophy. Bone marrow from non-*mdx* male mice was transplanted into female *mdx* mice with chronic muscle damage; after 70 days, researchers found that nuclei from the males had taken up residence in skeletal and cardiac muscle cells.

Lagasse and colleagues' demonstration of liver repair by purified HSCs is a similarly encouraging sign that HSCs may have the potential to integrate into and grow in some non-blood tissues. These scientists lethally irradiated female mice that had an unusual genetic liver disease that could be halted with a drug. The mice were given transplants of genetically marked, purified HSCs from male mice that did not have the liver disease. The transplants were given a chance to engraft for a couple of months while the mice were on the liver-protective drug. The drug was then removed, launching deterioration of the liver—and a test to see whether cells from the transplant would be recruited and rescue the liver. The scientists found that transplants of as few as 50 cells led to abundant growth of marked, donor-derived liver cells in the female mice.

Recently, Krause has shown in mice that a *single* selected donor hematopoietic stem cell could do more than just repopulate the marrow and hematopoietic system of the recipient [27]. These investigators also found epithelial cells derived from the donors in the lungs, gut, and skin of the recipient mice. This suggests that HSCs may have grown in the other tissues in response to infection or damage from the irradiation the mice received.

In humans, observations of male liver cells in female patients who have received bone marrow grafts from males, and in male patients who have received liver transplants from female donors, also suggest the possibility that some cells in bone marrow have the capacity to integrate into the liver and form hepatocytes [1].

WHAT ARE THE BARRIERS TO THE DEVELOPMENT OF NEW AND IMPROVED TREATMENTS USING HEMATOPOIETIC STEM CELLS?

Boosting the Numbers of Hematopoietic Stem Cells

Clinical investigators share the same fundamental problem as basic investigators—limited ability to grow and expand the numbers of human HSCs. Clinicians repeatedly see that larger numbers of cells in stem cell grafts have a better chance of survival in a patient than do smaller numbers of cells. The limited number of cells available from a placenta and umbilical cord blood transplant currently means that cord blood banks are useful to pediatric but not adult patients. Investigators believe that the main cause of failure of HSCs to engraft is host-versus-graft disease, and larger grafts permit at least some donor cells to escape initial waves of attack from a patient's residual or suppressed immune system [6]. Ability to expand numbers of human HSCs *in vivo* or *in vitro* would clearly be an enormous boost to all current and future medical uses of HSC transplantation.

Once stem cells and their progeny can be multiplied in culture, gene therapists and blood experts could combine their talents to grow limitless quantities of "universal donor" stem cells, as well as progenitors and specific types of red and white blood cells. If the cells were engineered to be free of markers that provoke rejection, these could be transfused to any recipient to treat any of the diseases that are now addressed with marrow, peripheral, cord, or other transfused blood. If gene therapy and studies of the plasticity of HSCs succeed, the cells could also be grown to repair other tissues and treat non-blood-related disorders [32].

Several research groups in the United States, Canada, and abroad have been striving to find the key factor or factors for boosting HSC production. Typical approaches include comparing genes expressed in primitive HSCs versus progenitor cells; comparing genes in actively dividing fetal HSCs versus adult HSCs; genetic screening of hematopoietically mutated zebrafish; studying dysregulated genes in cancerous hematopoietic cells; analyzing stromal or feeder-layer factors that appear to boost HSC division; and analyzing factors promoting homing and attachment to the stroma. Promising candidate factors have been tried singly and in combination, and researchers claim they can now increase the number of long-term stem cells 20-fold, albeit briefly, in culture.

The specific assays researchers use to prove that their expanded cells are stem cells vary, which makes it difficult to compare the claims of different research groups. To date, there is only a modest ability to expand true, long-term, self-renewing human HSCs. Numbers of progenitor cells are, however, more readily increased. Kobari et al., for example, can increase progenitor cells for granulocytes and macrophages 278-fold in culture [25].

Some investigators are now evaluating whether these comparatively modest increases in HSCs are clinically useful. At this time, the increases in cell numbers are not sustainable over periods beyond a few months, and the yield is far too low for mass production. In addition, the cells produced are often not rigorously characterized. A host of other questions remain—from how well the multiplied cells can be altered for gene therapy to their potential longevity, immunogenicity, ability to home correctly, and susceptibility to cancerous transformation. Glimm et al. [17] highlight some of these problems, for example, with their confirmation that human stem cells lose their ability to repopulate the bone marrow as they enter and progress through the cell cycle—like mouse stem cells that have been stimulated to divide lose their transplantability [18]. Observations on the inverse relationship between progenitor cell division rate and longevity in strains of mice raise an additional concern that culture tricks or selection of cells that expand rapidly may doom the cells to a short life.

Pragmatically, some scientists say it may not be necessary to be able to induce the true, long-term HSC to divide in the lab. If they can manipulate progenitors and coax them into division on command, gene uptake, and differentiation into key blood cells and other tissues, that may be sufficient to accomplish clinical goals. It might be sufficient to boost HSCs or subpopulations of hematopoietic cells within the body by chemically prodding the bone marrow to supply the as-yet-elusive factors to rejuvenate cell division.

Outfoxing the Immune System in Host, Graft, and Pathogen Attacks

Currently, the risks of bone marrow transplants—graft rejection, host-versus-graft disease, and infection during the period before HSCs have engrafted and resumed full blood cell production—restrict their use to patients with serious or fatal illnesses. Allogeneic grafts must come from donors with a close HLA match to the patient (see Chapter 6. Autoimmune Diseases and the Promise of Stem Cell-Based Therapies). If doctors could precisely manipulate immune reactions and protect patients from pathogens before their transplants begin to function, HSC transplants could be extended to less ill patients and patients for whom the HLA match was not as close as it must now be. Physicians might use transplants with greater impunity in gene therapy, autoimmune disease, HIV/AIDS treatment, and the preconditioning of patients to accept a major organ transplant.

Scientists are zeroing in on subpopulations of T cells that may cause or suppress potentially lethal host-versus-graft rejection and graft-versus-host disease in allogeneic-transplant recipients. T cells in a graft are a two-edged sword. They fight infections and help

the graft become established, but they also can cause graft-versus-host disease. Identifying subpopulations of T cells responsible for deleterious and beneficial effects—in the graft, but also in residual cells surviving or returning in the host—could allow clinicians to make grafts safer and to ratchet up graft-versus-tumor effects [48]. Understanding the presentation of antigens to the immune system and the immune system's healthy and unhealthy responses to these antigens and maturation and programmed cell death of T cells is crucial.

The approach taken by investigators at Stanford—purifying peripheral blood—may also help eliminate the cells causing graft-versus-host disease. Transplants in mouse models support the idea that purified HSCs, cleansed of mature lymphocytes, engraft readily and avoid graft-versus-host disease [60].

Knowledge of the key cellular actors in autoimmune disease, immune grafting, and graft rejection could also permit scientists to design gentler "minitransplants." Rather than obliterating and replacing the patient's entire hematopoietic system, they could replace just the faulty components with a selection of cells custom tailored to the patient's needs. Clinicians are currently experimenting with deletion of T cells from transplants in some diseases, for example, thereby reducing graft-versus-host disease.

Researchers are also experimenting with the possibility of knocking down the patient's immune system—but not knocking it out. A blow that is sublethal to the patient's hematopoietic cells given before an allogeneic transplant can be enough to give the graft a chance to take up residence in the bone marrow. The cells replace some or all of the patient's original stem cells, often making their blood a mix of donor and original cells. For some patients, this mix of cells will be enough to accomplish treatment objectives but without subjecting them to the vicious side effects and infection hazards of the most powerful treatments used for total destruction of their hematopoietic systems [37].

Understanding the Differentiating Environment and Developmental Plasticity

At some point in embryonic development, all cells are plastic, or developmentally flexible enough to grow into a variety of different tissues. Exactly what is it about the cell or the embryonic environment that instructs cells to grow into one organ and not another? Could there be embryological underpinnings to the apparent plasticity of adult cells? Researchers have suggested that a lot of the tissues that are showing plasticity are adjacent to one another after gastrulation in the sheet of mesodermal tissue that will go on to form blood—muscle, blood vessels, kidney, mesenchyme, and notochord. Plasticity may reflect derivation from the mesoderm, rather than being a fixed trait of hematopoietic cells. One lab is now studying the adjacency of embryonic cells and how the developing embryo makes the decision to make one tissue instead of another—and whether the decision is reversible [65].

In vivo studies of the plasticity of bone marrow or purified stem cells injected into mice are in their infancy. Even if follow-up studies confirm and more precisely characterize and quantify plasticity potential of HSCs in mice, there is no guarantee that it will occur or can be induced in humans.

SUMMARY

Grounded in half a century of research, the study of hematopoietic stem cells is one of the most exciting and rapidly advancing disciplines in biomedicine today. Breakthrough discoveries in both the laboratory and clinic have sharply expanded the use and supply of life-saving stem cells. Yet even more promising applications are on the horizon and scientists' current inability to grow HSCs outside the body could delay or thwart progress with these new therapies. New treatments include graft-versus-tumor therapy for currently incurable cancers, autologous transplants for autoimmune diseases, and gene therapy and tissue repair for a host of other problems. The techniques, cells, and knowledge that researchers have now are inadequate to realize the full promise of HSC-based therapy.

Key issues for tapping the potential of hematopoietic stem cells will be finding ways to safely and efficiently expand the numbers of transplantable human HSCs *in vitro* or *in vivo*. It will also be important to gain a better understanding of the fundamentals of how immune cells work—in fighting infections, in causing transplant rejection, and in graft-versus-host disease as well as master the basics of HSC differentiation. Concomitant advances in gene therapy techniques and the understanding of cellular plasticity could make HSCs one of the most powerful tools for healing.

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Adult stem cell—An undifferentiated cell found in a differentiated tissue that can renew itself and (with certain limitations) differentiate to yield all the specialized cell types of the tissue from which it originated.

Astrocyte—One of the large neuroglia cells of neural tissues.

Blastocoel—The cavity in the blastula of the developing embryo.

Blastocyst—A preimplantation embryo of about 150 cells. The blastocyst consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel), and a cluster of cells on the interior (the inner cell mass).

Bone marrow stromal cells—A stem cell found in bone marrow that generates bone, cartilage, fat, and fibrous connective tissue.

Cell division—Method by which a single cell divides to create two cells. This continuous process allows a population of cells to increase in number or maintain its numbers.

Cell-based therapies—treatment in which stem cells are induced to differentiate into the specific cell type required to repair damaged or depleted adult cell populations or tissues.

Cell culture—Growth of cells in vitro on an artificial medium for experimental research.

Clone—A line of cells that is genetically identical to the originating cell; in this case, a stem cell.

Culture medium—The broth that covers cells in a culture dish, which contains nutrients to feed the cells as well as other growth factors that may be added to direct desired changes in the cells.

Differentiation—The process whereby an unspecialized early embryonic cell acquires the features of a specialized cell such as a heart, liver, or muscle cell.

Directed differentiation—Manipulating stem cell culture conditions to induce differentiation into a particular cell type.

DNA—Deoxyribonucleic acid, a chemical found primarily in the nucleus of cells. DNA carries the instructions for making all the structures and materials the body needs to function.

Ectoderm—Upper, outermost layer of a group of cells derived from the inner cell mass of the blastocyst; it gives rise to skin nerves and brain.

Embryo—In humans, the developing organism from the time of fertilization until the end of the eighth week of gestation, when it becomes known as a fetus.

Embryoid bodies—Clumps of cellular structures that arise when embryonic stem cells are cultured.

Embryonic germ cells—Cells found in a specific part of the embryo/fetus called the gonadal ridge that normally develop into mature gametes.

Embryonic stem cells—Primitive (undifferentiated) cells from the embryo that have the potential to become a wide variety of specialized cell types.

Embryonic stem cell line—Embryonic stem cells, which have been cultured under in vitro conditions that allow proliferation without differentiation for months to years.

Endoderm—Lower layer of a group of cells derived from the inner cell mass of the blastocyst; it gives rise tolungs and digestive organs.

Feeder layer—Cells used in co-culture to maintain pluripotent stem cells. Cells usually consist of mouse embryonic fibroblasts.

Fertilization—The process whereby male and female gametes unite.

Fetus—A developing human from usually two months after conception to birth.

Gene—A functional unit of heredity that is a segment of DNA located in a specific site on a chromosome. A gene directs the formation of an enzyme or other protein.

Hematopoietic stem cell—A stem cell from which all red and white blood cells develop.

Human embryonic stem cell—A type of pluripotent stem cell derived from the inner cell mass of the blastocyst.

In vitro—Literally, "in glass"; in a laboratory dish or test tube; an artificial environment.

In vitro fertilization—An assisted reproduction technique in which fertilization is accomplished outside the body.

Inner cell mass—The cluster of cells inside the blastocyst. These cells give rise to the embryonic disk of the later embryo and, ultimately, the fetus.

Long-term self-renewal—The ability of stem cells to renew themselves by dividing into the same non-specialized cell type over long periods (many months to years) depending on the specific type of stem cell.

Mesenchymal stem cells—Cells from the immature embryonic connective tissue. A number of celltypes come from mesenchymal stem cells, includingchondrocytes, which produce cartilage.

Mesoderm—Middle layer of a group of cells derived from the inner cell mass of the blastocyst; it gives rise tobone, muscle, and connective tissue.

Microenvironment—The molecules and compounds such as nutrients and growth factors in the fluid surrounding a cell in an organism or in the laboratory, which are important in determining the characteristicsof the cell.

Neural stem cell—A stem cell found in adult neural tissue that can give rise to neurons, astrocytes, and oligodendrocytes.

Neurons—Nerve cells, the structural and functional unit of the nervous system. A neuron consists of a cell body and its processes, an axon, and one or more dendrites. Neurons function by the initiation and conduction of impulses and transmit impulses to other neurons or cells by releasing neurotransmitters at synapses.

Oligodendrocyte—A cell that provides insulation to nerve cells by forming a myelin sheath around axons.

Passage—A round of cell growth and proliferation in cell culture.

Plasticity—The ability of stem cells from one adult tissue to generate the differentiated cell types of another tissue.

Pluripotent—Ability of a single stem cell to develop into many different cell types of the body.

Proliferation—Expansion of a population of cells by the continuous division of single cells into two identical daughter cells.

Regenerative or reparative medicine—A treatment in which stem cells are induced to differentiate into the specific cell type required to repair damaged or depleted adult cell populations or tissues.

Signals—Internal and external factors that control changes in cell structure and function.

Somatic stem cells—Another name for adult stem cells.

Stem cells—Cells with the ability to divide for indefinite periods in culture and to give rise to specialized cells.

Stromal cells—Non-blood cells derived from blood organs, such as bone marrow or fetal liver, which are capable of supporting growth of blood cells in vitro. Stromal cells that make this matrix within the bone marrow are also derived from mesenchymal stem cells.

Subculturing—The process of growing and replating cells in tissue culture for many months.

Surface markers—Surface proteins that are unique to certain cell types, which are visualized using antibodies or other detection methods.

Teratoma—A tumor composed of tissues from the three embryonic germ layers. Usually found in ovary and testis. Produced experimentally in animals by injecting pluripotent stem cells, in order to determine the stem cells' abilities to differentiate into various types of tissues.

Transdifferentiation—The observation that stem cells from one tissue may be able to differentiate into cells of another tissue.

Trophoblast—The extraembryonic tissue responsible for implantation, developing into the placenta, and controlling the exchange of oxygen and metabolites between mother and embryo.

Undifferentiated—Not having changed to become a specialized cell type.