

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 trophoblast), 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

- ^aDerived from the inner cell mass/epiblast of the blastocyst.
- ^aCapable 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.
- ^aClonogenic, 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 ES 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 cell 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.

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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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