EMBRYONIC STEM CELLS

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Embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass (ICM) of preimplantation embryos [1]. ES cells are invaluable tools to create genetic modifications in mice for the study of gene function and disease. The first true ES cell lines were derived from the 129 strain of mouse in 1981 by two groups simultaneously [2,3]. In 1998, Thomson and colleagues reported the isolation of the first human ES cell lines from human blastocysts left over from in vitro fertilization [4]. Putative ES or ES-like cells from other species, such as, bird, fish, monkey, dog, cow, and rat, have also been reported. However, only ES cells from mice have proved to be able to efficiently contribute to chimeras and reenter germline. There are other pluripotent cell lines including embryonic germ (EG) cells, which are derived from gonadal ridges of the embryo, and embryonic carcinoma (EC) cells, which are isolated from spontaneously arising teratocarcinomas and are therefore karyotypically abnormal. In this chapter, we will focus on mouse and human ES cells.

1.1 THE ORIGIN OF EMBRYONIC STEM (ES) CELLS

There appears to be a very limited period of embryonic development during which pluripotent ES cells can be established in culture. This period is termed the preimplantation blastocyst stage (Figure 1.1). From the one-cell to early eight-cell stage, the blastomeres of the embryo are equipotent. Each blastomere is considered to be totipotent; that is, they have the ability to differentiate into all cell types in an
organism, including the extraembryonic tissue associated with that organism, and are able to form an entire organism. However, blastomeres lack the capacity for self-renewal and therefore are not considered stem cells. As cleavage proceeds, there is a gradual restriction in the developmental potency of the cells, eventually resulting in the generation of the first two distinct lineages: the trophectoderm and the ICM, followed by the formation of a fully expanded blastocyst consisting of a hollow vesicle of trophectoderm surrounding a fluid-filled cavity and a small group of ICM cells. The preimplantation blastocyst is formed after about six cleavage divisions, which occur about 3.5 or 5.5 days after fertilization in the mouse and human, respectively. The trophectoderm cells are required for implantation and development of the placenta. The ICM is the foundation of all somatic tissues and germ cells in adults. The ICM comprises the inner pluripotent primitive ectoderm cells and the outer primitive endoderm cells. ES cells are derived from the primitive ectoderm cells when ICM is isolated and cultured in vitro under proper conditions. Little is known, however, about how pluripotent primitive ectoderm cells in the ICM are transformed into pluripotent ES cells in culture. It is thought that the pluripotent ES cells remain in a state in vitro that may occur only transiently in vivo, since the primitive ectoderm cells progress rapidly through development to form the epiblast cells. Although these epiblast cells are still pluripotent and can give rise to all three primary germ layers, ectoderm,
mesoderm, and endoderm, there is still no evidence suggesting that ES cells can be derived from the pluripotent epiblast cells of the implanted embryo. However, cells resembling ES cells in both morphology and growth characteristics have been obtained previously from preblastocyst stages [5,6]. Since attached embryos from progressively earlier developmental stages require longer periods in culture before they reach a state that yield ES cells, the possibility exists that preblastocyst embryos progress to an equivalent stage of blastocyst embryos in culture before ES cells can be obtained. It remains unclear, however, whether this state represents the sole timepoint from which ES cells can be obtained or whether it demarcates the beginning of a window of time during which the derivation of ES cells is possible.

1.2 DERIVATION OF ES CELLS

The research on embryonic carcinoma (EC) cells in the 1970s eventually paved the way for the establishment of the first ES cell lines [7–10]. When epiblast cells from early postimplantation embryos were grafted into adult mice, they produced teratocarcinomas. Teratocarcinomas are malignant multidifferentiated tumors containing a proportion of undifferentiated cells. These undifferentiated cells could be propagated in culture and established as cell lines termed EC cells. Clonally isolated EC cells retained the capacity for differentiation into derivatives of all three germ layers. EC cells could also participate in embryonic development when introduced into the ICM of blastocysts to generate chimeric mice [11]. Maintenance of the undifferentiated state of EC cells relied on cocultivation with feeder cells, usually mitotically inactivated mouse embryonic fibroblasts (MEFs) [12]. It was reasoned that these feeder cells were providing some critical factors to sustain the pluripotency of the EC cells. Since EC cells have undergone transformation and karyotypic changes prior to establishment as cell lines, they are almost always aneuploid and are not capable of proceeding through meiosis to produce mature gametes. Following the discovery of EC cells, the next logical step was to attempt to directly isolate pluripotent cells from embryos. In 1981, two groups succeeded in establishing pluripotent ES cell lines from mouse embryos [2,3]. The protocol for the derivation of ES cells is relatively simple, and most labs are still using the original protocol developed by Evans and Martin. In brief, the protocol involves plating intact embryos at the expanded blastocyst stage onto a mitotically inactivated feeder layer with either DMEM or GMEM as the basal culture medium supplemented with 10–20% fetal calf serum, 2-mercaptoethanol, nonessential amino acids, L-glutamine, and sodium pyruvate. After several days of culture, the cells from the ICM will expand to form a cell mass. After being disaggregated and replated onto fresh feeders, various types of differentiated colonies as well as colonies of a characteristic undifferentiated morphology will appear. The undifferentiated colonies can generally be expanded further to establish cell lines, now known as ES cells. The first human ES cell line was established in essentially the same method by Thomson and colleagues in 1998 [4]. Since ES cells were first created, it has become clear that different strains of mice vary considerably in their facility for derivation [13]. The inbred 129 and C57Bl/6 strains are the most permissive;
approximately 30% of 129 or C57Bl/6 embryos can be expected to give rise to ES cell lines. Most other strains rarely produce ES cell lines at all. The reason for this intriguing variability is not yet understood. The ES cell derivation efficiency can be increased by several modifications of the protocol. For example, subjecting the embryos to delayed implantation or diapause can improve the efficiency [14]. Removal of the extraembryonic tissues from blastocysts mechanically or by immunosurgery can also facilitate the derivation of ES cells. It has been reported that, by combining the two techniques, the ES cell yield rates can be increased up to 100% for 129 and to over 50% for CBA embryos [1]. In addition to removing the differentiative signals from the surrounding tissues of ICM, it is possible to encourage the self-renewal aspect of stem cell proliferation by preferentially inhibiting signaling pathways that promote differentiation. For example, commercially available inhibitors of the Erk-activating MEK pathway such as PD98059 have been used to promote ES cell self-renewal and improve the efficiency of ES cell derivation [15]. ES cell lines from 129 and C57Bl/6 strain mice can also be efficiently derived under serum and feeder-free conditions with the addition of both the leukemia inhibitory factor (LIF) and bone marrow morphogenetic protein 4 (BMP4) [16,17].

1.3 KEY PROPERTIES OF ES CELLS

Embryonic stem cells are a remarkable cell type, mainly because of the two key properties they possess: unlimited proliferation and unlimited differentiation. ES cells can be maintained in culture for an extended amount of time, perhaps even indefinitely. Additionally, even after many passages in culture, ES cells continue to maintain the ability to differentiate into any type of cell in the body. For this reason, ES cells are considered as pluripotent. Although there are some similarities between the various types of adult stem cells, pluripotent ES stem cells clearly differ from other adult stem cells in several ways. Adult stem cells are generally limited to differentiation into the cell types of their tissue of origin, making them only multipotent, and can be maintained in culture for only a very limited number of passages before they differentiate. However, pluripotent ES cells, when reintroduced into early-stage embryo, have the ability to reenter developmental processes and contribute to all cell lineages, including germ cells [14,18–20]. The capacity to generate all fetal and adult cell lineages in vitro and in vivo, combined with the facility of genetic manipulations, makes ES cells a very powerful tool for molecular dissection of tissue differentiation and cellular (patho)physiology [21]. Human ES cells also create a platform for a renewable source of differentiated cells for applications in pharmacogenomics and cell transplantation therapies [22,23]. ES cells have other functionally important or unique properties, including derivation without transformation or immortalization, stable diploid karyotype (prolonged culture will increase genetic or epigenetic abnormality), clonogenic (can be grown from single cells; it has proved difficult for human ES cells), absence of G1 cell cycle checkpoint, and absence of X inactivation (in XX lines).
1.4 MAINTENANCE OF ES CELL SELF-RENEWAL

When an ES cell divides, it has to decide whether to produce identical copies of itself (self-renewal) or to differentiate into specific cell types. This ES “cell fate” decision, that is, self-renewal versus differentiation, is greatly influenced by both extrinsic and intrinsic factors (Figure 1.2).

1.4.1 Extrinsic Factors in Mouse ES Cell Fate Determination

LIF When ES cells are cultured on feeders in medium supplemented with fetal calf serum, they can be sustained in an undifferentiated state indefinitely while retaining the ability to give rise to all the cell types of the embryo and adult. It has been shown that conditioned media from the feeders has the same effect on ES cell self-renewal as

![Diagram of mouse ES cell self-renewal regulation](image)

**FIGURE 1.2** Regulation of mouse ES cell self-renewal. There are at least two known independent pathways regulating mouse ES cell self-renewal. *(a)* Mouse ES cell self-renewal can be driven by the combined action of LIF/Stat3 and BMP/Smad/Id pathways. LIF activates Stat3 and blocks nonneural differentiation. BMP blocks neural differentiation by induction of Ids. *(b)* Mouse ES self-renewal can also be sustained by simple elimination of differentiation cues associated mainly with MAPK, GSK3, and FGF receptor signaling pathways. In both conditions, the proper function of Oct4 and Nanog remains important for the maintenance of ES cell identity.
the feeder layers. This observation eventually led to the identification of leukemia inhibitory factor (LIF) as the key cytokine secreted by feeder cells in supporting mouse ES cell self-renewal [24,25]. Feeders lacking functional LIF gene do not support ES propagation effectively [26]. LIF is a soluble glycoprotein and is a member of the interleukin-6 (IL6) family. LIF was initially identified by its activity to induce differentiation of M1 leukemia cells [27,28]. Other members of the IL6 family, including oncostatin M (OSM) [29], ciliary neurotrophic factor (CNTF) [30] and cardiotrophin 1 (CT1) [31], also have an effect similar to that of LIF in suppressing differentiation and sustaining self-renewal of mouse ES cells. LIF works by binding directly to a heterodimeric receptor complex containing two transmembrane glycoproteins, gp130 and the LIF-specific receptor subunit LIFRβ, bringing the associated JAKs into close proximity and causing their cross-phosphorylation and activation [32]. A wide range of downstream effectors can be activated via gp130. These include the signal transducer and activator of transcription (STAT) 1, 3, and 5, the mitogen-activated protein kinases (MAPK), extracellular regulated kinases (ERK) 1 and 2, and phosphoinositol-3 kinase (PI3K). In mouse ES cells LIF and other related cytokines sustain ES cell self-renewal mainly through activation of Stat3 [33]. Inhibition of Stat3 signaling directly, through the use of interfering mutant forms of the transcription factor Stat3F, provides the best evidence for an essential role of Stat3 in ES cell self-renewal mediated by LIF or related cytokines. Stat3F is a dominant-negative mutant of Stat3. Overexpression of Stat3F in ES cells resulted in induction of differentiation even in the presence of LIF, indicating that activation of Stat3 is essential to the LIF-mediated ES cell self-renewal. Studies using a chimaeric Stat3 molecule that can be activated directly by estradiol indicate that Stat3 activation is not only necessary but might be sufficient to block differentiation [34]. Several groups have tried to identify LIF/Stat3 target gene(s) that are responsible for ES cell self-renewal [35–37], but so far it has not been very successful. Downstream target genes of Stat3 in ES cells that mediate self-renewal are still largely unknown. In mouse ES cells, LIF/gp130 signaling also activates MAPK pathway. The ERK MAPKs Erk1 and Erk2 are strongly phosphorylated on stimulation of LIF. Phosphorylated ERKs then undergo nuclear translocation and modulate the activities of transcriptional regulators such as Elk, Myc, and the serum response factor (SRF). Inhibition of MAPK/ERK signaling by small molecule inhibitors PD98059 and U0126 limits the differentiation of ES cells and promotes self-renewal [15]. This strongly suggests that while LIF/Stat3 pathway sustains ES cell self-renewal, the LIF/gp130/ERK pathway is antagonistic to it. The overall balance of conflicting activation of Stat3 and ERKs might well determine the efficiency of mouse ES cell self-renewal.

**Serum/BMP** Feeder layers or LIF can support mouse ES cell self-renewal. However, these observations were made in the presence of fetal calf serum. In the absence of serum, LIF is not sufficient to sustain mouse ES cell self-renewal (Figure 1.2a). Instead, the ES cells will differentiate predominantly into neural phenotypes. Hence, there is another factor or factors needed in combination with LIF to achieve self-renewal. It has been demonstrated that bone morphogenetic protein 4 (BMP4) can replace the requirement for serum both during clonal propagation of
mouse ES cells and during their de novo derivation [17]. BMPs were originally isolated from demineralized bone matrix and identified as factors responsible for inducing bone formation in muscular tissues [38]. The critical contribution of BMP4 to self-renewal is to induce expression of the negative helix-loop-helix factors, inhibitors of differentiation (Ids). Id proteins lack a DNA binding domain and are not thought capable of inducing gene transcription. They act by binding and sequestering E proteins, thereby inhibiting the E–protein–dependent transcriptional activity of basic helix-loop-helix (bHLH) factors. Constitutive Id gene expression in ES cells replaces the need for BMP4 in the media. Serum also induces Id genes via multiple pathways, including integrin engagement by extracellular matrix molecules such as fibronectin [39,40]. A BMP-like factor was purified from serum that is responsible for both inhibition of myogenesis and stimulation of osteoblast differentiation in vitro. This BMP-like factor was identified as BMP4. In addition, BMP4 in serum was found to form a large complex with other molecules, resulting in potentiation of its activity [41]. Therefore it is not surprising that ES cells cultured in serum also show appreciable levels of Id gene expression [17]. These findings together suggest that the biological activities of serum in suppressing differentiation and sustaining self-renewal of mouse ES cells might be mediated at least in part by BMPs. Significantly, in the absence of LIF, both serum and BMP4 drive ES cells differentiation into nonneural fates (Figure 1.2a). Thus serum or BMP4 stimulation has a dual potential in ES cells, and the outcome is dictated by the presence or absence of LIF/Stat3 pathways. The ability of BMP4 to suppress differentiation and maintain ES cell self-renewal in collaboration with LIF/Stat3 is shared by other BMP family, BMP2 and GDF6, but not other transforming growth factor β (TGFβ) superfamily members, such as TGFβ1 and activin A. Mouse ES cells can also be maintained in serum-free medium supplemented with LIF and knockout serum replacement without addition of serum or BMPs. Under these culture conditions, it was reported that activin or Nodal, but not TGFβ1 or BMP4, can significantly enhance ES cell proliferation without affecting pluripotency [42]. Although serum replacement is thought to be better defined than serum, it is a proprietary product that cannot be regarded as fully defined. In fact, it has been shown that BMP-like activity is present in serum replacement. Indeed, both BMP2 and BMP4 proteins were detected in serum replacement [43].

**Inhibitors** ES cells exist in the artificial milieu of tissue culture, therefore the composition of the in vitro environment can obscure the actual requirements for maintaining pluripotency. Pathways that appear obligatory for ES cell propagation in culture may not, in fact, be core components of the pluripotent state, but accessories demanded by unrefined culture settings. It has been suggested that mouse ES cell self-renewal is not driven by activating signals but by the elimination of differentiation cues and consolidation of a basal program of cell growth and proliferation [44]. MAPK, FGFR, and GSK3 signaling pathways are involved in numerous cell functions including cell proliferation and differentiation. Under serum-free basal conditions mouse ES cells can be efficiently derived and maintained in an undifferentiated, proliferative, and pluripotent state through the actions of chemically defined inhibitors of these three signaling pathways without added growth factors or cytokines [44]. By
blocking these three pathways, it is even possible to derive Stat3-/- mouse ES cell lines, authentic ES cell lines from rats and nonpermissive strains of mice such as CBA, all at high efficiency in defined feeder-free conditions without adding any growth factors or cytokines [44]. Through a cell-base screen of chemical libraries, Chen et al. identified a small molecular inhibitor, pluripotin, that can maintain mouse ES cell self-renewal in the absence of feeders and exogenous factors [45]. Pluripotin may act by inhibition of both Erk1 and RasGAP-dependent signaling pathways. These findings indicate that mouse ES cell self-renewal may not be dependent on extrinsic stimulation and that elimination of differentiation cues is sufficient to sustain self-renewal (Figure 1.2b).

1.4.2 Extrinsic Factors in Human ES Cell Fate Determination

Currently human ES cells are routinely grown on mitotically inactivated feeder layers with medium containing either serum or serum replacements, both of which are ill-defined. Although progress has been made in delimiting culture environments for the derivation and propagation of human ES cells, it is still largely unclear what extrinsic factors are really required for sustaining human ES cell self-renewal. Human and mouse ES cells appear to be very different in terms of their requirements for sustaining self-renewal. For example, while the activation of LIF/Stat3 pathway has long been considered necessary for maintenance of mouse ES cell self-renewal, it has no obvious effect on human ES cells [46,47]. Instead, human ES cells require bFGF, unknown factor(s) secreted by feeder cells and cell–cell adhesion to sustain pluripotency in serum-free conditions. BMP4 can replace serum in supporting mouse ES cell self-renewal in collaboration with LIF/Stat3 pathway. However, BMP4 induces human ES cell differentiation toward trophectoderm lineage [48]. The question that still remains is why they are so different. Is it because of the mechanisms that regulate human and mouse ES cell self-renewal are fundamentally different, or is it because of subtle variations in the machinery regulating self-renewal modulating the response of the cell? It is worth noting that despite these differences, feeder cells support both mouse and human ES cell self-renewal. In fact, the first mouse and human ES cell lines were both derived in essentially the same culture conditions using mitotically inactivated MEFs as feeder layers in serum-containing media [2–4]. For mouse ES cells, the requirement for feeders can be partially replaced by LIF or related cytokines that activate the gp130 signal transducer and the downstream transcriptional effector Stat3 [24,25,33]. However, LIF has no obvious effect on human ES cell self-renewal. When mouse ES cells are “weaned off” feeder cells, they go through a “crisis” in which most ES cells die or differentiate, even in the presence of LIF. Although sublines of feeder-independent cells can be derived, they generally lose the hallmark of normal ES cells, which is competence for germline transmission. This suggests that feeder cells must produce a factor or factors, other than LIF, that may not yet be identified, that support both human and mouse ES cell growth. It may or may not, however, be the same factor(s) for human and mouse ES cells. Another major difference between mouse and human ES cells is that mouse ES cells can easily adhere to substrates such as gelatin, fibronectin, or laminin, while most human ES cells do not adhere to gelatin, and adhere
only poorly to fibronectin or laminin. Adherent growth appears to be important for maintenance of ES cell self-renewal, since ES cells aggregate in suspension culture, a state generally associated with differentiation into structures known as embryoid bodies. Feeder layers help to support the adhesion of human ES cells, which is possibly one of the major contributions of feeder layers in sustaining human ES cell self-renewal. There have been several reports that have investigated the effects of a broad range of factors, including Wnts, Noggin, Nodal, activin A, neurotrophins, or TGFβ1 [49–53], all of which have been claimed to be involved in maintenance of human ES cell self-renewal. However, bFGF is still the single most potent extrinsic factor identified for human ES cell self-renewal under serum-free conditions [54]. Why do human ES cells require bFGF? While bFGF has no obvious effect on wild-type mouse ES cells, it was found that Eras-null mouse ES cells need bFGF for efficient propagation in serum-free conditions (unpublished data). This finding is interesting because bFGF and Eras both activate PI3-kinase/Akt pathway and Eras is not expressed in human ES cells [55]. Eras is a member of the ras family that is expressed specifically in undifferentiated mouse ES cells [56]. It is constitutively active and is important for ES cell proliferation; therefore it would be interesting to see if lack of Eras function in human ES cells underlies the requirement for bFGF to sustain self-renewal.

1.4.3 Intrinsic Determinants

**Oct4** Oct4 is a POU family transcriptional factor that is encoded by *Pou5f1*. Oct4 plays a pivotal role in both human and mouse ES cell fate determination. Expression of Oct4 is restricted to pluripotent cell types, such as cells in early embryos; germline cells; and undifferentiated EC, EG, and ES cells [57]. *In vivo*, zygotic expression of Oct4 is essential for the initial development of pluripotential capacity in the ICM [58]. Oct4 deficient embryos fail to initiate fetal development because the prospective founder cells of the ICM do not acquire pluripotency and differentiate into the trophectoderm lineage. Investigation via conditional repression/expression in ES cells has suggested that the level of Oct4 is critical for ES cell fate determination. Artificial repression of Oct4 in mouse or human ES cells induces differentiation along the trophectodermal lineage [59–61], which suggests that Oct4 is continuously required by ES cells in order to maintain their pluripotent identity and may act as a lock that prevents differentiation into the trophoblast stage [61]. It has been shown that Oct4 directly prevents differentiation toward trophectoderm by forming a repressor complex with Cdx2, an inducer for trophectoderm differentiation [62]. This complex interferes with the autoregulation of these two factors, giving rise to a reciprocal inhibition system that establishes their mutually exclusive expression. The downregulation of Oct4 results in an upregulation of Cdx2, and vice versa. This might account for the segregation of the first two cell lineages in early embryonic development: pluripotent stem cells in ICM and trophectoderm cells. When the level of Oct4 was artificially raised by more than 50% of wild-type levels, with ES cells differentiated into endoderm and mesoderm, indicating that in ES cells, continuous Oct4 function at appropriate levels may be crucial to maintain pluripotency. However,
Oct4 cannot act alone to maintain pluripotency, and interaction with other factor(s) and signaling pathways like LIF/Stat3 and BMP/Smad is required. Many of the genes that are regulated by Oct4 signaling also contain Stat binding sites, suggesting that these Oct4 and LIF/Stat3 could act cooperatively in regulating the expression of ES cell-specific genes.

**Nanog** Nanog is a homeodomain-containing protein, and is expressed in a restricted range of cell types [63,64]. Nanog mRNA is present in pluripotent mouse and human ES cells, and absent from differentiated cells. In embryo, Nanog expression is first detected in morula, increases in the early blastocyst, and declines prior to implantation. Following implantation, Nanog is expressed in only a subset of epiblast cells and rapidly downregulated on entry into the primitive streak. Nanog is also expressed in developing germ cells. Deletion of Nanog prevents acquisition of pluripotency in the ICM of preimplantation mouse blastocyst. Downregulation of Nanog via siRNA in human ES cells leads to a significant downregulation of Oct4 and loss of ES cell surface antigens, and differentiation toward extraembryonic endodermal lineages [65]. Endogenous Nanog acts in parallel with cytokine stimulation of Stat3 to drive ES cell self-renewal. Nanog overexpression from transgene constructs is sufficient to maintain constitutive self-renewal in mouse ES cells, bypassing LIF/Stat3 and BMP/Smad/Id pathways [66]. It has been shown that overexpression of Nanog can also enable both human and primate ES cell self-renewal in the absence of feeder layers [67,68]. Perhaps appropriately, Nanog is considered a core element of the pluripotent state. However, Nanog function still requires the continued presence of Oct4. Elevated Nanog expression is not sufficient to prevent ES cell differentiation into trophectodermal lineage when Oct4 expression was repressed. Using a genome-scale analysis to identify a cohort of genes that respond to Oct4, Sox2, and Nanog in human ES cells, it has been observed that a majority of genes coregulated by Oct4 and Sox2 are also targets of Nanog. It will be interesting to see whether this regulatory circuitry is functionally important in both mouse and human ES cell fate regulation. More recently, the same group that initially identified the Nanog found that transient downregulation of Nanog in ES cells appears to predispose cells toward differentiation, yet is fully reversible. Permanent deletion of Nanog gene in ES cells reduces but does not eliminate clonogenic self-renewal. Nanog-null ES cells can reenter embryo development and contribute extensively to all three germ layers. Interestingly, Nanog-null ES cells can also be recruited to the germline. However, primordial germ cells lacking Nanog fail to mature on reaching the genital ridge. This suggests that Nanog also has a specific role in the formation of germ cells in addition to epiblast. Rather than being part of the integral machinery of pluripotency, it was proposed that Nanog may act primarily in establishing the unique states of epiblast and germ cells.

**Sox2 and FoxD3** Sox2 and FoxD3 are the two transcription factors that have been suggested to interact with Oct4 and contribute to pluripotency. Sox2 is a HMG DNA-binding domain containing transcription factor. ICMs from Sox2 knockout embryos cannot give rise to ES cell lines. Instead, they differentiate into both trophectodermal
and primitive endodermal cell types. This suggests that Sox2 also play a pivotal role in the establishment of ES cell identity [69]. Sox2 is one of the four transcription factors that were reported to be sufficient to establish pluripotency in the nuclei of fibroblasts when they were forcibly expressed [70]. The other three factors are Oct4, Klf4, and c-Myc. Sox2 seems to be essential in the regulation of several Oct4 target genes at the transcriptional level [71]. However, it has not yet been proved that Sox2 is required in order to enable Oct4 to block trophectodermal differentiation. FoxD3 is a forkhead transcription factor. Its expression is detectable in the blastocyst and later in the postimplantation egg cylinder epiblast. FoxD3 knockout embryos survive until about E6.5 [72], suggesting that Foxd3 is required at a stage beyond establishment and maintenance of a pluripotent ICM.

1.4.4 Negative Regulators of ES Cell Self-Renewal

**Socs3**  
Socs3 is a member of the family of suppressors of cytokine signaling, which act as classical negative regulators to attenuate the signal leading to their induction. In ES cells, Socs3 is an immediate target gene of the LIF/Stat3 pathway. Overexpression of Socs3 has been suggested to have an apoptotic effect on ES cells [73]. When ES cells are transfected with Socs3 transgenes, there is a significant decrease in colony formation. Among the formed colonies, the majority are morphologically differentiated even in the presence of LIF [66]. Therefore induction of Socs3 is considered a negative feedback of the LIF/Stat3 pathway in ES cell self-renewal regulation. However, a more recent study on Socs3-null ES cells suggested otherwise [74]. When cultured in LIF levels that sustain self-renewal of wild-type cells, Forrai and coworkers found that Socs3-null ES cell lines actually exhibited less self-renewal and greater differentiation into primitive endoderm. The absence of Socs3 enhanced JAK-STAT and Erk1/2 signal transduction via gp130 in response to LIF stimulation. Attenuation of ERK signaling by the addition of MAPK/ERK kinase inhibitors to Socs3-null ES cell cultures rescued the differentiation phenotype, but did not restore proliferation to wild-type levels. These data suggest that the level of Socs3 expression might be critical in the regulation of mouse ES cell self-renewal mediated by the LIF/Stat3 signaling pathway.

**Gata Factors**  
Gata4 and Gata6 are zinc finger-containing transcription factors that have been shown to play a pivotal role in the initiation and promotion of differentiation of extraembryonic endoderm. They are expressed in the primitive endoderm and its derivatives, the visceral endoderm and parietal endoderm [75]. Gata4-null mice die between E8 and E9 as a result of defects in heart morphogenesis [76,77]. Gata6 null mice die at E5.5 because of defects in visceral endoderm formation and subsequent extraembryonic development [78]. Gata6 is considered an upstream regulator of Gata4 because loss of Gata6 expression results in the absence of Gata4, whereas loss of Gata4 leads to the upregulation of Gata6 [76,78]. Gata6 and Gata4 mRNAs are detectable in undifferentiated ES cells by RT-PCR, but not by Northern blot analysis, indicating very weak expression of these genes. Gata4 mRNA is increased during ES cell differentiation induced by elevation of Oct4 or withdrawal of LIF [61]. Forced
expression of either Gata6 or Gata4 in mouse ES cells causes ES cells to differentiate uniformly into primitive endoderm even in the presence of LIF [79]. Interestingly, ES cells lacking Nanog also tend to differentiate into primitive endoderm [63,64].

1.5 DIFFERENTIATION OF ES CELLS

Embryonic stem cells have the capacity to produce every type of fetal and adult cell both in vitro and in vivo. In culture, when factors that sustain ES cell self-renewal are removed, ES cells will differentiate and, under appropriate conditions, will generate progeny consisting of derivatives of the three germ layers: mesoderm, endoderm, and ectoderm. In order to use ES cell-derived cells therapeutically, it is critical to know whether they are functional in vivo. There is increasing evidence suggesting that these in vitro–generated cells can integrate and function when transplanted into adult tissue. For example, cardiomyocytes from differentiating ES cells have been shown to form stable intracardiac grafts when injected into ischemic rat hearts [80]; and glia precursors derived from mouse ES cells interacted with host neurons and efficiently myelinated axons in brain and spinal cord when transplanted into a rat model of acute demyelination [81]; dopaminergic neurons from ES cells can show electrophysiological and behavioral properties expected of neurons from the midbrain and can restore cerebral function and behavior in an animal model of Parkinson’s disease [82,83].

Two general approaches are used to initiate ES cell differentiation (Figure 1.3). The primary approach is to allow ES cells to grow in suspension and form three-dimensional aggregates known as embryoid bodies (EBs) [84]; another approach is called monolayer differentiation. ES cells differentiate readily in monolayer culture when deprived of LIF or feeder support [85, 86]. Both approaches have been successful in the generation of certain cell types from ES cells.

Within the EBs, cellular differentiation proceeds on a schedule similar to that in the embryo but in the absence of proper axial organization or elaboration of a body plan [87]. Each EB develops multiple cell types and further differentiation is elaborated on subsequent attachment and outgrowth. It is possible to bias the differentiation for or against certain cell types by addition of different factors such as retinoic acid [88]. However, in the absence of understanding how to instruct ES cells uniformly to enter a lineage of choice, it is still a challenge to direct ES cells into specific pathways and then to support the viability and maturation of individual differentiated phenotypes. As a result, the differentiation products from ES cells by different protocols remain a mixture of cell types. Several strategies have been developed to isolate cells of interest from the mixed cell populations. One cell type from each germ layer will be chosen to elucidate such strategies.

1.5.1 Neural Differentiation (Ectoderm)

Both mouse and human ES cells differentiate efficiently into neural precursor cells on withdrawal of serum in adherent monolayer culture [86,89,90] or via treatment of embryoid bodies with retinoic acid [91–93]. Under appropriate conditions, each of the
three major neural cell types of the central nervous system, neurons, astrocytes, and oligodendrocytes, as well as subtypes of neurons, can be generated [94,95]. For instance, midbrain dopaminergic neurons were derived at relatively high efficiency from Nurr1 overexpressed ES cells or ES cells grown on stromal cell layers [83,96]. Addition of SHH and FGF8 seemed to dramatically increase the dopaminergic neuron production [83]. Nurr1, SHH, and FGF8 are required for the development of this class of neurons in the early embryo [97,98]. Oligodendrocytes can also been derived and enriched from both mouse and human ES cells and proved to have biological functionality [99,100].

Embryonic stem cell–derived neural cells provide an unlimited source for potential application in cell replacement therapies for neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease. However, before ES cell–derived neural cells can be used clinically, it is important to develop strategies to purify neural cell population of interest, as the resulting cell populations from ES cells are always heterogeneous. Neural stem cells or specific differentiated neural cells can be purified with restricted expression of marker genes, a process termed the lineage selection strategy [92,101], or through selective culture conditions and/or clonal expansion [102,103]. Both Sox1 and Sox2 have been used as markers to purify neural precursors

![Diagram of differentiation potential of ES cells](image)
from ES cell culture [101]. Sox1 and Sox2 are members of the B group of Sox family transcription factors [104]. Sox2 is expressed in neural precursors as well as in undifferentiated ES cells. In order to enrich Sox2-positive neural precursors and eliminate any residual Sox2-positive ES cells after differentiation, an ES cell line OS25 has been generated in which a bifunctional βgeo cassette was inserted into the Sox2 locus and an hptk was inserted into the Oct4 locus. The βgeo in the Sox2 locus will permit enrichment for neural precursors and the hptk in the Oct4 locus will allow to eliminate any residual ES cells by the addition of gancyclovir [101]. Sox1 is one of the best markers for neural stem cells. It is expressed specifically in neuroectoderm at the formation of the neural plate and downregulated at the onset of both neuronal and glial differentiation [105], and importantly, it is not expressed in undifferentiated ES cells. A Sox1-GFP ES cell line was generated by introducing the eGFP into the Sox1 locus, so the Sox1-positive neural precursors derived from ES cells can be purified by FACS sorting. The Sox1-GFP ES cells also harbor a pac gene that is coupled to Sox1-GFP via an internal ribosome entry site and is therefore co-expressed with Sox1. After neural differentiation, brief exposure to puromycin results in pure Sox1-positive neural precursors by rapid eliminating of Sox1-negative cells [86].

Conti’s group reported that neural precursors generated from both mouse and human ES cells can be maintained in culture for a prolonged time while retaining the capability to differentiate into all three types of neural cells [102]. Neural precursors are expanded readily in adherent conditions using a combination of EGF and bFGF in serum-free medium. Interestingly, they found that nonneural cell types and differentiated neural cells cannot grow under these conditions and thus are able to generate pure neural stem cell population without any genetic interventions. These cells are remarkably homogeneous and show similarities to radial glia.

1.5.2 Hematopoietic Differentiation (Mesoderm)

Embryonic stem cells can be induced to differentiate into several mesodermal cell lineages, including hematopoietic, cardiac [106], adipogenic [107], osteoblast [108], and myogenic cells [109]. Specific differentiation factors are required for the efficient generation of these cell types from pluripotent ES cells. Of all mesoderm lineages derived from ES cells, hematopoietic differentiation has been studied in most detail. Under appropriate culture conditions, development of the hematopoietic lineages has been demonstrated to be highly reproducible and efficient [84]. Hematopoietic commitment within these cultures can be monitored by gene expression pattern, the appearance of specific cell surface markers, and the development of clonable progenitors. With these assays, several groups have analyzed in details the early stages of hematopoietic commitment within EBs. Application of serum and cytokines such as IL3, IL1, and granulocyte macrophage colony stimulating factor (GM-CSF) to ES cells generates early hematopoietic precursor cells expressing both embryonic z globin (βH1) and adult β major globin RNAs. Different hematopoietic cell types including erythroid, myeloid, and lymphoid lineages and natural killer cells have also been generated from ES cells when cocultured with OP9 cells. In addition to phenotypic characterization of ES cell-derived hematopoietic cells by specific gene
expression patterns and by surface antigens, it is of even greater importance to demonstrate that these cells are indeed functional. ES cell–derived hematopoietic progenitors have been shown to have a long-term multilineage hematopoietic repopulating potential [110,111]. There is also strong evidence suggesting that regulation of hematopoietic development in the EBs is similar to that in the early embryo.

Several studies have documented hematopoietic development in human ES cells. By applying a combination of different factors, including BMP4, stem cell factor, and FLT3L, human ES cells have been induced to differentiate into hematopoietic lineages robustly [112–114]. Differentiation of hematopoietic colony-forming cells from human ES cells can also be achieved by coculture with mouse bone marrow stromal cells in the presence of serum [115].

### 1.5.3 Insulin-Producing Pancreatic Cells (Endoderm)

Efficient and reproducible generation of endoderm derivatives from ES cells, such as pancreatic β cells and hepatocytes, holds great promise for potential clinical treatment of type I diabetes and liver disease. Several groups have claimed to have derived endoderm lineages from ES cells, including pancreatic islets [116,117], hepatocytes [118–120], thyrocytes [121], lung [122], and intestinal cells [123]. Compared to the generation of mesoderm and ectoderm from ES cells, the progress in endoderm cell differentiation from ES cells has been slow. This is in part because there are no specific inducers of endoderm lineage and there are no good markers to identify or separate definitive endoderm from other lineages at different differentiation stages. Most of the markers currently used for definitive endoderm have an overlapping expression patterns with visceral endoderm, an extraembryonic tissue, and do not contribute to the formation of any tissue of embryo or adult.

Lumelsky and colleagues [124] developed a five-step protocol to induce ES cells differentiation into pancreatic islet-like cells. These cells expressed insulin and other pancreatic endocrine hormones. They self-assembled to form three-dimensional clusters similar in topology to normal pancreatic islets where pancreatic cells are in close association with neurons. Glucose triggers insulin release from these cell clusters by mechanisms similar to those employed in vivo. When injected into diabetic mice, the ES cell–derived insulin-producing cells undergo rapid vascularization and maintain a clustered, islet-like organization. However, when tested for their ability to function in vivo following transplantation into streptozotocin (STZ)-induced diabetic mice, these cells failed to correct the hyperglycemia of these animals [124]. This could be due to the fact that the cells were too immature or that they were not true islet cells. Subsequently several groups have modified this protocol and reported that the islet-like cells they derived from ES cells were functional and could improve the hyperglycemia in STZ-induced diabetic mice. These were achieved by forced expression of Pax4 [125] or pancreatic duodenal homeobox 1 (Pdx1) [126] during ES cell differentiation, or by treatment of cells with the inhibitor of phosphoinositide 3-kinase (PI3K), LY294002 [127] at the final stage. Pax4 and Pdx1 are transcription factors that play an essential role during β-cell development [128].
Kahan’s group [129] developed another approach to derive islet-like structure from ES cells. They found that, following growth and differentiation in nonselective medium containing serum, mouse ES cells spontaneously differentiated into cells individually expressing each of the four major islet hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide [129]. This system allows the investigation of many facets of islet development since it promotes the appearance of the complete range of islet phenotypes and reproduces important developmental stages of normal islet cytodifferentiation in differentiating ES cell cultures. However, the efficiency of generation of islet-like cells from ES cells is very low. Micallef and coworkers [130] developed a strategy to select for early pancreatic cell population from the mixed ES cell differentiation culture. They generated an embryonic stem (ES) cell line in which sequences encoding GFP were targeted to the locus of Pdx1 [130]. Pdx1 is expressed in the earliest stages of pancreatic development as the organ rudiments are specified from the gut endoderm [128].

Insulin-producing β cells have also been generated from human ES cells [131,132]. The approach used to induce human ES cell differentiation into islet-like structures is similar to that of mouse ES cells [124]. First human ES cells were grown in suspension to allow the formation EBs. The EBs were then cultured and plated in an insulin–transferring–selenium–fibronectin medium, followed by medium supplemented with N2, B27, and bFGF. Next, the glucose concentration in the medium was lowered, bFGF was withdrawn, and nicotinamide was added. Reverse transcription–polymerase chain reaction detected an enhanced expression of pancreatic genes in the differentiated cells. Immunofluorescence and in situ hybridization analyses revealed a high percentage of insulin-expressing cells in the clusters. In addition to insulin, most cells also coexpressed glucagon or somatostatin, indicating a similarity to immature pancreatic cells. These findings validate the human ES cell model system as a potential basis for enrichment of human β cells or their precursors, as a possible future source for cell replacement therapy in diabetes.

1.6 CONCLUSIONS

Embryonic stem cells have the ability to form all three embryonic germ layers and their differentiated derivatives in vitro and in vivo. They can be manipulated by controlling their growth conditions or by introducing genetic modifications, allowing the controlled direction of their differentiation into specific cell types. Because of these qualities, there are many ways in which ES cells might be used in basic and clinical research. The most obvious potential application of human ES cells would be the generation of cells and tissues for cell-based therapies. Although significant progress has been made toward an understanding of ES cell biology, the mechanisms by which the ES cell fate is regulated are still largely unknown. In order to fully realize the potential of ES cells, several fundamental questions need to be addressed. First, while some of the pathways controlling mouse ES cell self-renewal have been identified, none of these appear to function equivalently in both humans and mice. This raises the question as to whether the fundamental mechanisms underlying ES cell self-renewal
are shared or distinct among different species. We expect that cross-species comparisons using large-scale genomic analysis will provide a better understanding of the conserved and divergent pathways required for ES cell self-renewal. While numerous studies have demonstrated that ES cells have the potential to differentiate into nearly all the specialized cell types in the body, little is known about the routes by which specialized cell types arise from ES cells. Among other methods, gene expression profiling using whole-genome microarrays and epigenomics for the detection of “silenced” region of chromosomes known to be involved in cell fate decision, are likely to provide important insights into the basic mechanisms controlling ES cell differentiation.

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