

PART I

**Introduction to stem cells
and regenerative medicine**

Embryonic stem cells

Nicole Slawny and Gary D. Smith

1.1 Preimplantation embryo development sets the stage for pluripotency

Regenerative medicine has the potential to revolutionize health care by offering the promise of replacement cells, tissues, and organs to combat injury, disease, and aging. In an ideal setting, stem cell therapies would begin with a pluripotent cell that by definition is able to give rise to any cell formed in the embryo. Additionally this would most likely require that the stem cells could self-renew or were able to divide and give rise to either more pluripotent stem cells or progressively more differentiated cells under the control of extrinsic cues. Stem cells are biological cells found in multicellular organisms, that can mitotically divide and differentiate into specialized cell types and can self-renew to produce more stem cells. There are two broad types of stem cells: embryonic stem cells and adult stem cells. Embryonic stem cells originate from the inner cell mass of the preimplantation embryo and are considered pluripotent whereas *in situ* adult stem cells are considered multipotent. Embryonic stem cells (ESCs) possess characteristics that make them a potentially outstanding starting material for use in regenerative medicine. They are unique among cultured cells because they have an apparently limitless capacity to self-renew *in vitro*, as well as being pluripotent. Because of these extraordinary properties, ESCs have been an intense focus of research for more than 30 years.

In order to fully understand the basic properties of ESCs and how they are generated, it is important to consider the events of embryonic development that surround the timing of their formation. The events

and molecular signaling required for embryonic development have been explored to a large extent using the laboratory mouse as a model system due to there being very limited material for experimentation and the inherent moral complexities of studies utilizing human embryos (Vassena *et al.*, 2011; Cockburn and Rossant, 2010). Therefore early mouse development will be used to illustrate the events critical to generating pluripotency in ESCs and other cells. While some developmental events are certainly conserved among all mammalian species, other aspects of rodent embryologic development beyond the scope of this chapter have made their ESCs unique and more amenable for use as a model system (Brons *et al.*, 2007; Tesar *et al.*, 2007; Nichols and Smith, 2009; Rossant, 2008). A more complete understanding of these species-specific differences may be important if hESCs are to be utilized to their fullest potential to improve human health.

Following fertilization of the egg by sperm the preimplantation embryo undergoes a series of cell divisions that generate smaller cells known as blastomeres (Figure 1.1).

Early cleavage divisions result in an eight-cell embryo when compaction or an increase in intracellular adhesion is initiated, an event believed to create the first molecular differences in polarity between blastomeres. Continued cell divisions give rise to a 32-cell morula where the first cell-fate choice occurs in the embryo. Cells on the outside differentiate into trophoblast that gives rise to part of the placenta, while cells on the inside become the inner cell mass. The specification of trophoblast versus inner cell mass occurs

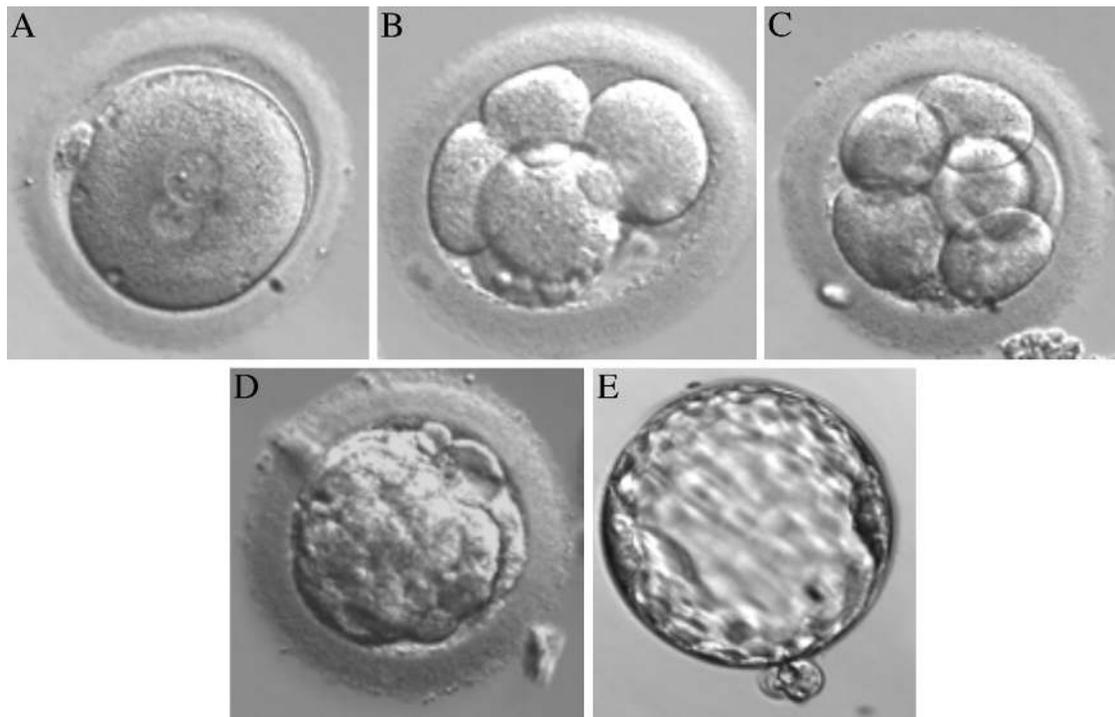


Figure 1.1 Cleavage- and blastocyst-stage human embryos. It is important to note that preimplantation mouse and human embryo development are extremely similar in morphological characteristics. A. Pronuclear stage embryo immediately following fertilization. The haploid nuclei of both the sperm and the egg are clearly visible. B. Four-cell embryo cultured for two days. C. Eight-cell embryo cultured for three days. D. Morula-stage embryo illustrating compaction of blastomeres cultured for four days. E. Human blastocyst, the stage at which the inner cell mass is removed to culture human embryonic stem cells (culture day six). All images kindly provided by Sandra Mojica, Gary Smith, and the University of Michigan Taubman Consortium for Stem Cell Therapies.

via a combination of asymmetrical/symmetrical cell divisions, cell polarity, and differential gene expression, the control of which is still debated (Arnold and Robertson, 2009; Rossant and Tam, 2009; Zernicka-Goetz *et al.*, 2009). The maintenance of the inner cell mass requires the formation of a blastocoel, a fluid-filled cavity in the center of the embryo now called a blastocyst: the stage from which ESCs are frequently derived.

Lineage allocation in the preimplantation mouse embryo continues with the segregation of blastocyst inner cell mass into the epiblast, which differentiates into the embryo proper, and subjacent primitive endoderm, which gives rise to extra-embryonic tissues that play key roles in further directing embryo

development. It was initially supposed that inner-cell-mass cells were uniform and assumed different cell fates based on undefined positional cues (Dziadek, 1979). More recently it was demonstrated that, prior to differentiation, cells of the inner cell mass express either Nanog (epiblast marker) or Gata6 (primitive endoderm marker) in a “salt and pepper” pattern, indicating that the inner cell mass is not a homogeneous population (Chazaud *et al.*, 2006). The model proposed on the basis of this work suggested that expression of Nanog versus Gata6 controlled the expression of different cell-surface adhesion molecules, allowing the cells to physically sort into two distinct populations. Recently, it has been shown that

up-regulation of fibroblast growth factor (FGF)/mitogen-activated protein kinase (MAPK) signaling in the inner cell mass converts the entire population to primitive endoderm cells, whereas blocking FGF/MAPK converts the entire population to epiblast cells (Yamanaka *et al.*, 2010). Cells within the inner cell mass express FGF4 (Nichols *et al.*, 1998; Yuan *et al.*, 1995) and the FGF4-null mouse completely lacks primitive endoderm development (Feldman *et al.*, 1995), suggesting that signaling within the inner cell mass promotes primitive endoderm fate (Yamanaka *et al.*, 2010). While a type of pluripotent stem cell can still be generated from the epithelized epiblast even after embryo implantation, differentiation of Gata6-expressing primitive endoderm has been shown to be inhibitory to the derivation of ESCs from the blastocyst (Brook and Gardner, 1997).

While they are not fully specified until after embryo implantation, primordial germ cells are embryonic precursors that differentiate into sex-specific gametes that come together to generate the pluripotent embryo of the next generation. Extra-embryonic tissues secrete bone morphogenic proteins that signal to roughly six cells in the adjacent epiblast to differentiate into primordial germ cells (Tam and Snow, 1981, Ohinata *et al.*, 2005; Ying *et al.*, 2001; Ying and Zhao, 2001). Thus, primordial germ cells begin to express germ-line-specific transcription factors, undergo rapid mitotic cell division, and migrate into the genital ridges between 10 and 13 days *post coitum* (dpc) (Godin *et al.*, 1990; Gomperts *et al.*, 1994; Molyneaux *et al.*, 2001). Once in the genital ridges, inherited epigenetic imprinting marks, for example X chromosome inactivation, are erased and sex-specific differentiation of gametes determines the timing and specificity of remethylation (Lucifero *et al.*, 2002, 2004; Davis *et al.*, 1999, 2000). These two embryonic populations of cells, inner cell mass and primordial germ cells, were the starting material from which the entire scientific investigation of pluripotent stem cells began.

1.2 A brief history of pluripotent cells

Pioneering work analyzing teratocarcinomas, malignant tumors now known to be initiated by embryonic

germ cells, demonstrated that these tumors contained poorly organized somatic cells derived from all three embryonic germ layers, namely ectoderm, endoderm, and mesoderm, in addition to harboring a stem cell component (Kleinsmith and Pierce, 1964). Embryonic carcinoma stem cells (Figure 1.2) provided the first tissue culture system of pluripotency and differentiation used to model embryologic development (Kahan and Ephrussi, 1970; Rosenthal *et al.*, 1970). Defining requirements for culturing embryonic carcinoma stem cells (Martin and Evans, 1974, 1975) and maintaining their pluripotency paved the way for the first derivations of mouse (mESCs) and later human (hESCs) embryonic stem cells (Figure 1.2) from the inner cell mass of preimplantation blastocysts (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Even more striking experiments demonstrated that embryonic carcinoma stem cells injected into mouse blastocysts could form not only tumors (Rossant and McBurney, 1982) but also normal cells in chimeric mice derived from both the recipient blastocyst and the injected embryonic carcinoma stem cells (Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975; Illmensee and Mintz, 1976). The ability of cells injected into the blastocyst to contribute to all tissues of the resulting mouse embryo is currently one of the more stringent tests used to demonstrate pluripotency (Jaenisch and Young, 2008; Kuijk *et al.*, 2011). While embryonic carcinoma cells have historical importance, it is relevant to note that they likely have little relevance for regenerative medicine.

Teratocarcinomas arise at high frequency in the 129 mouse strain (Stevens and Little, 1954), but also can be induced to form in other mouse strains by transplanting either 7-dpc mouse embryos (Solter *et al.*, 1970) or genital ridges from 12.5-dpc embryos (Stevens, 1967) into ectopic sites such as under the kidney capsule or into the testis. Recall that at 12.5 dpc the genital ridges contain primordial germ cells (Ewen and Koopman, 2010; Saga, 2008). Given that they can drive teratocarcinoma formation, it is not surprising that pluripotent embryonic germ cell lines have been derived from mouse and human primordial germ cells that closely resemble ESCs (Resnick *et al.*, 1992; Matsui *et al.*, 1992; Durcova-Hills *et al.*, 2001; Shambloott *et al.*, 1998).

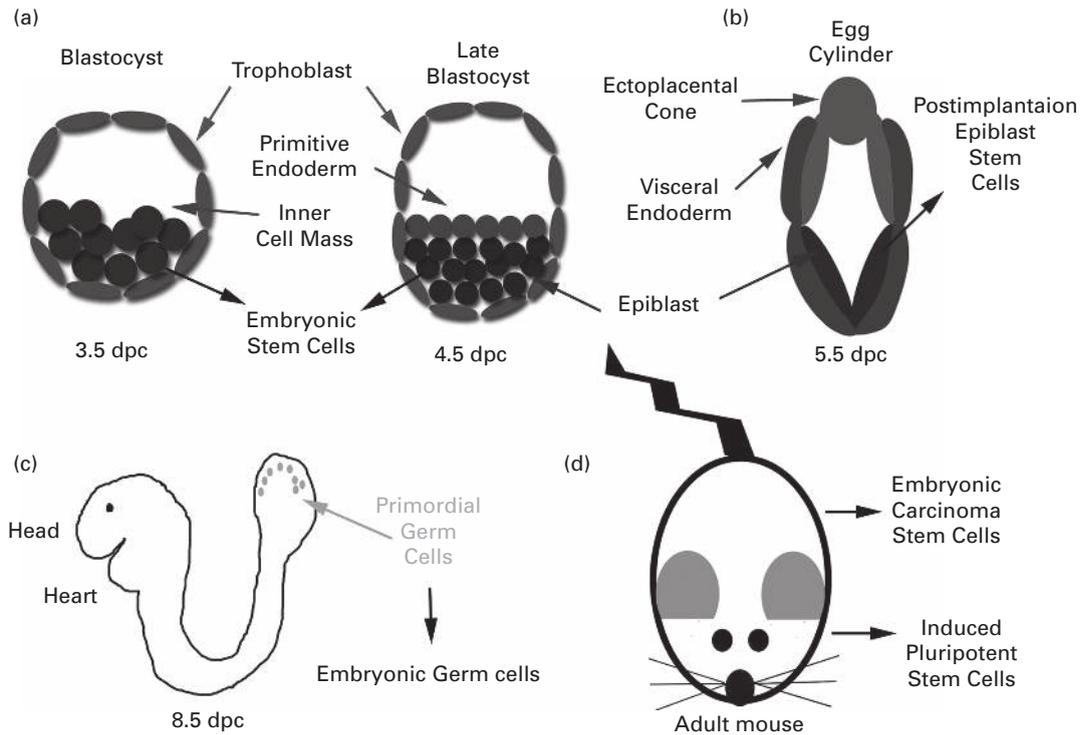


Figure 1.2 Embryonic stages of mouse stem cell derivation. (a) Embryonic stem cells can be derived from the inner cell mass of the blastocyst or from the preimplantation epiblast in the late blastocyst. (b) Postimplantation epiblast stem cells are derived from the epiblast of the egg cylinder stage. The ectoplacental cone is an extra-embryonic structure that forms a portion of the placenta. (c) Embryonic germ cell lines are derived from primordial germ cells harvested from the 8.5 dpc mouse embryo. (d) Embryonic carcinoma stem cell lines are derived from teratocarcinomas in adult mice. Induced pluripotency cells can be derived from nearly any somatic cell in the adult mouse.

More recently, pluripotent stem cells have been derived from perhaps unforeseen sources (Figure 1.2), for instance from the postimplantation mouse epiblast (Tesar *et al.*, 2007; Brons *et al.*, 2007). While postimplantation epiblast stem cells can differentiate into cells of all three germ layers *in vitro* and generate teratomas (benign tumors derived from embryonic germ cells or injected pluripotent stem cells containing all three germ layers when injected into immunocompromised mice) they are unable to contribute to chimeras, suggesting that there are some important functional differences from mESCs (Tesar *et al.*, 2007; Brons *et al.*, 2007). Even more unexpected was the demonstration that terminally differentiated cells such

as fibroblasts could be induced to form pluripotent stem cells by the expression of only four factors, namely Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007). Induced pluripotent stem cells (Figure 1.2) are morphologically and functionally similar to mESCs and can generate teratomas as well as contribute cells to chimeras when injected into the blastocyst. By comparing the properties and developmental potential of all of these pluripotent stem cells one can begin to draw conclusions about the origin of ESCs and how to improve their production and control their differentiation for use in regenerative medicine. It is also important to recognize that ESCs are pluripotent (can give rise to all

endoderm, mesoderm, and ectoderm cells) as well as being able to colonize the germline and contribute to germ cells.

1.3 Properties and origins of pluripotent stem cells

The pluripotency of ESCs is controlled at multiple levels, including a poised chromatin state with a large transcriptome, a core set of transcription factors that inhibit differentiation, a unique cell cycle that promotes proliferation and inhibits differentiation and extracellular signaling molecules that stimulate or inhibit key signal transduction pathways. The chromatin in ESC DNA tends to have an abundance of trimethylation of Lysine 4 of histone H3 (H3K4) and acetylation of histone 4 (H4Ac) that generally marks areas of open chromatin with concomitant gene activity (Azuara *et al.*, 2006). The large regions of open chromatin result in widespread gene expression, but expression is at low levels, leading to the suggestion that ESC are “primed” to differentiate (Efroni *et al.*, 2008). While histone marks associated gene inactivation, such as trimethylation of Lysine 27 of histone H3 (H3K27) are more rare, they are concentrated on promoters of lineage specific genes (Azuara *et al.*, 2006; Bernstein *et al.*, 2006). These promoter regions are also the targets of repression by the core transcription factors Oct3/4, Sox2, and Nanog discussed in detail below (Boyer *et al.*, 2005). Regions of DNA marked with both closed (H3K27) and open (H3K4) chromatin histone marks are considered bivalent and thought to represent the pluripotent state, creating the unique situation in which ESCs are poised for differentiation yet held in an undifferentiated state (Azuara *et al.*, 2006; Bernstein *et al.*, 2006).

Three core transcription factors maintain the undifferentiated state of ESCs: Oct3/4 (Nichols *et al.*, 1998), Sox2 (Nichols *et al.*, 1998; Avilion *et al.*, 2003), and Nanog (Mitsui *et al.*, 2003; Chambers *et al.*, 2003). Oct3/4 is a POU domain transcription factor that represses the trophoblast lineage and promotes the inner cell mass lineage by forming a repressive complex with the *cdx2* transcription factor (Niwa

et al., 2005; Ralston and Rossant, 2008). Sox2 is a member of the SRY-related HMG box gene family that, like Oct3/4, is required for survival of epiblast cells (Avilion *et al.*, 2003). Oct3/4 and Sox2 frequently co-regulate gene expression by binding adjacent sites (POU/HMG sites) within gene promoters, and can reciprocally regulate their own promoters (Chew *et al.*, 2005). Nanog is a homeobox protein, with no known homology to other proteins, that occupies many of the same gene promoters as Oct3/4 and Sox2 (Boyer *et al.*, 2005; Chambers *et al.*, 2003; Mitsui *et al.*, 2003), indicating that these three transcription factors work in concert to maintain the balance of pluripotency versus differentiation of mESCs. While these factors form the core of the pluripotency regulation machinery, there are many other factors that regulate the expression of the core factors, such as Tcf3 (Cole *et al.*, 2008; Pereira *et al.*, 2006; Tam *et al.*, 2008), Stat3 (Wu *et al.*, 2009), and Klf4 (Chen *et al.*, 2008).

The ESCs and the epiblast spend the majority of the cell cycle in S phase with a very short G1 phase (Burdon *et al.*, 2002; White *et al.*, 2005), unlike somatic cells, where G1 predominates. These differences not only produce the rapid cycling of the epiblast and ESCs, but also may play an important role in inhibiting differentiation and maintaining pluripotency. The most crucial decision point in the cell cycle, i.e. to proliferate, differentiate, quiesce, senesce, or apoptose, occurs at the G1 checkpoint (Blomen and Boonstra, 2007). Spending minimal amounts of time in the G1 phase had also been suggested to prevent differentiation by insulating cells from growth factor signaling (Orford and Scadden, 2008; Burdon *et al.*, 1999). In fact, many of the mechanisms that maintain ESC pluripotency are directed at keeping the cells rapidly dividing. Moreover, exit from the cell cycle is considered a prerequisite for differentiation due to differential requirements for organization of the cytoskeleton in both processes (Grosshans and Wieschaus, 2000).

Initially culture of mESCs relied on co-culture with a layer of mitotically inactivated fibroblast feeder cells (Evans and Kaufman, 1981; Martin, 1981) until it was discovered that they were providing the cytokine leukemia inhibitory factor (LIF) to activate Stat3 (Niwa

et al., 1998; Matsuda *et al.*, 1999), after which it became possible to simply supplement media with LIF (Smith *et al.*, 1988). While LIF was critical for mESC pluripotency, it required cooperation of proteins found in fetal calf serum including BMP4, a member of the TGF- β signaling family (Ying *et al.*, 2001), to induce the expression of inhibitor of differentiation (Id) genes. Subsequent investigations have demonstrated that inhibition of FGF/MAPK with and without inhibition of glycogen synthase kinase-3 (GSK-3) using small-molecule inhibitors can maintain pluripotency in serum-free conditions without LIF (Ying *et al.*, 2008). Alternatively, hESCs are routinely maintained on a fibroblast feeder layer because LIF is unable to prevent their differentiation (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Humphrey *et al.*, 2004). Addition of BMP-4 to hESC cultures induces their differentiation into trophoblast (Xu *et al.*, 2002), which is striking because mESCs are unable to form trophoblast unless there is a reduction in expression of Oct3/4 (Niwa *et al.*, 2000). Because exposure to BMP promotes differentiation, hESCs are cultured in knock-out serum replacement (KOSR; Invitrogen) with the addition of high levels of basic fibroblast growth factor (bFGF) (Xu *et al.*, 2005a, 2005b) to inhibit signaling by any BMP present in KOSR and maintain their pluripotency (Amit *et al.*, 2000). The striking differences in culture requirements for mESCs versus hESCs likely reflect differences in the developmental timing between mouse and human embryos that may enable mouse epiblast cells to linger in the pluripotent state longer than human epiblast cells (Nichols and Smith, 2009).

Growth factor requirements for hESCs more closely resemble those required for postimplantation epiblast stem cells: absence of LIF, but inclusion of FGF and activin (Brons *et al.*, 2007; Tesar *et al.*, 2007). In addition, both cell types have a relatively flattened morphology, can differentiate into trophoblast when exposed to BMP-4, and cannot be maintained at single-cell densities (Nichols and Smith, 2011). These observations have led to the hypothesis that mESCs represent an earlier state of pluripotency with greater developmental potential than hESCs, which may be more representative of the postimplantation epiblast and therefore have a more restricted developmental

potential (Rossant, 2008; Nichols and Smith, 2009). In fact, postimplantation epiblast stem cells cannot contribute to chimeras when injected into the blastocyst even though they can form all three germ layers in vitro (Brons *et al.*, 2007; Tesar *et al.*, 2007). Experiments involving injection of hESCs into human blastocysts cannot be performed due to obvious moral issues; however, they have been injected into mouse blastocysts (James *et al.*, 2006). Despite some rare but promising integration of hESCs into very early mouse embryos, there can be no conclusions about the ability of hESCs to colonize recipient blastocysts. Recent experiments have demonstrated that both hESCs and postimplantation epiblast stem cells can be converted to cells much more similar to mESCs by expression of Oct3/4, Klf4, and Klf2 in hESCs and by Klf4 alone in postimplantation epiblast stem cells (Hanna *et al.*, 2010; Guo *et al.*, 2009).

The observed similarities among mESCs, embryonic carcinoma stem cells, and embryonic germ cells, as well as experimental results suggesting that, while preimplantation epiblast (i.e. inner cell mass that is not primitive endoderm) cells were the definitive source of mESCs, only three cell lines could be derived from an entire epiblast (recall that just six epiblast cells give rise to primordial germ cells), led to a hypothesis that the embryologic origin of ESCs was germ cells (Gardner and Brook, 1997; Zwaka and Thomson, 2005). Indeed, mESCs and embryonic carcinoma stem cells express genes considered to be markers of primordial germ cells, and during induced pluripotent stem cell formation expression of primordial germ cell markers precedes expression of pluripotency genes (Xu *et al.*, 2011; Tang *et al.*, 2010). In turn, primordial germ cells and germ cells express the core pluripotency transcription factors: Oct3/4, Sox2, and Nanog (Yabuta *et al.*, 2006; Ohinata *et al.*, 2005; Avilion *et al.*, 2003; Chambers *et al.*, 2003; Yamaguchi *et al.*, 2005). However, refinement of mESC derivation conditions by including inhibitors of FGF/MAPK and GSK-3 signaling, likely driving more of the inner cell mass to adopt an epiblast fate instead of a primitive endoderm fate, illustrated that the number of mESC cell lines derived per embryo could be increased far above the number of primordial germ cells found in the epiblast (Nichols and Smith,

2011). Therefore there appear to be two distinct times during embryologic development when pluripotent cells are created, corresponding to the preimplantation inner cell mass and primordial germ cells which carry pluripotency to the next generation (Nichols and Smith, 2011). While a great deal has been learned about the pluripotent state, many questions still remain. In order to safely and effectively utilize ESCs for cell replacement therapies and regenerative medicine we will need to know exactly how a true pluripotent state is created as well as how to end that state and begin controlled differentiation. Only by studying all pluripotent cells, namely ESCs, embryonic germ cells, embryonic carcinoma stem cells, induced pluripotent stem cells, and postimplantation epiblast stem cells, will we be able to completely understand the pluripotent state and how to utilize this state for regenerative medicine.

1.4 Derivation of human embryonic stem cells

The first step in derivation of hESC lines is the ethical acquisition of appropriate starting material, which is most commonly supernumerary embryos from assisted reproductive technology (ART). These are either cryopreserved embryos donated by couples that are no longer pursuing family building or, alternatively, non-cryopreserved or frozen embryos that have been judged to be genetically abnormal by preimplantation genetic screening (Harper and Sengupta, 2012) for aneuploidy or preimplantation genetic diagnosis (Kuliev and Rechitsky, 2011) for single-gene disorders. Those embryos which are tested and found to be genetically abnormal can be considered unsuitable for transfer to a patient's uterus. To maintain the highest ethical standards regarding informed consent, the information given to donors should include that their embryos will be used for hESC derivation, that there are alternatives to donation for hESC derivation, that they might not receive direct medical benefit, that resulting hESC lines may result in a commercial product for which they will receive no payment, and, finally, that they may withdraw consent

until the embryos are used. There should be no monetary or medical compensation for embryo donation (Fraga *et al.*, 2011; Murdoch *et al.*, 2012; Hasegawa *et al.*, 2010). Also the embryo donor's personal information must be carefully protected to ensure confidentiality. Because there is significant debate about the morality of destroying embryos to derive hESC lines, it is of utmost importance that all embryo research is carried out observing the highest ethical standards.

Supernumerary embryos from ART are frequently frozen at the two pronuclear stages, namely early cleavage stage or blastocyst stage (Figures 1.1 and 1.3) and therefore need to be thawed and cultured until the inner cell mass appears within the blastocyst on day 5 or 6 in order to derive hESC lines. Appropriate culture conditions are critical for successful embryo maturation and have been extensively refined by clinics performing in-vitro fertilization (IVF) to ensure the highest possible rates of successful embryo maturation (Hasegawa *et al.*, 2010; Stojkovic *et al.*, 2004). There are several commercially available media and protocols used to culture human embryos using two sequential media (Ilic *et al.*, 2009; Bongso and Tan, 2005), a single medium (Biggers and Summers, 2008), or even co-culture with various types of supporting cells (Kattal *et al.*, 2008) all in an attempt to closely model the in-vivo niche of the fallopian tube. Once the embryos have reached the blastocyst stage they are graded on the basis of morphology (Bongso and Tan, 2005). The blastocysts with the best morphology routinely give rise to hESC lines at the highest frequency; however, several groups have been successful at deriving lines from clinically inferior embryos (Mitalipova *et al.*, 2003; Lerou *et al.*, 2008; Gavrilov *et al.*, 2011).

The next step in hESC derivation is to isolate the inner cell mass from the surrounding trophoblast cells (Figure 1.3). In many cases trophoblast cells are killed by immunosurgery utilizing antibodies and complement (Solter and Knowles, 1975). However, this procedure exposes xenomaterials to the hESC culture, which could introduce either disease causing agents or foreign proteins into the cells, potentially increasing the likelihood of rejection if they are used for regenerative therapies in the future (Fraga *et al.*, 2011; Hasegawa *et al.*, 2010; Vazin and Freed, 2010).

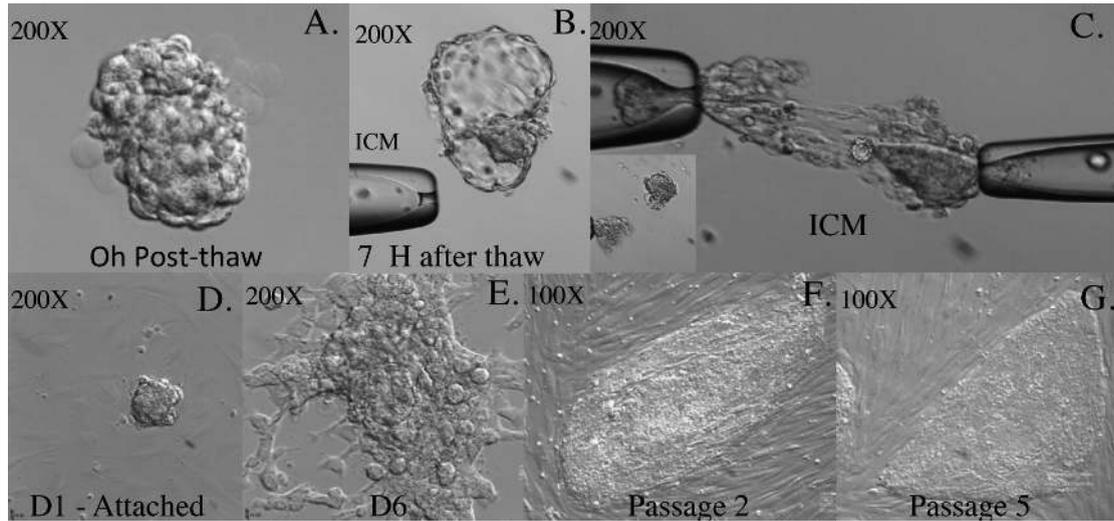


Figure 1.3 Derivation of human embryonic stem cells. A. Cryopreserved blastocyst-stage embryo immediately after thawing. B. Seven hours after thawing the inner cell mass (ICM) is clearly visible. C. Laser-assisted separation of the ICM from the trophoblast. The laser target used for aiming is visible in the center of the frame. Isolated ICM pictured in insert. D. After one day in culture, the ICM has attached to a layer of mitotically inactive mouse or human fibroblast feeder cells. E. Following 6 days of ICM expansion, the center of the explant is removed and plated onto a new layer of fibroblast feeder cells. F. Newly derived hESC line after two passages on inactivated fibroblasts. G. Newly derived hESC line after five passages on inactivated fibroblasts. All images kindly provide by Sandra Mojica, Gary Smith, and the University of Michigan Taubman Consortium for Stem Cell Therapies.

Alternatively, trophoblast cells can be removed by microdissection using fine needles (Strom *et al.*, 2007; Amit and Itskovitz-Eldor, 2002) or with lasers (Figure 1.3) originally used to remove single or multiple blastomeres for preimplantation genetic diagnosis/screening (Turetsky *et al.*, 2008). Because destruction of embryos to generate hESC is so controversial, techniques to generate cell lines from single blastomeres of human embryos that in theory would allow for continued development to term on the basis of results from embryos subjected to preimplantation genetic diagnosis/screening have been proposed (Klimanskaya *et al.*, 2006; Chung *et al.*, 2008). While this is not yet commonplace, one could imagine an opportunity for hESC banking for children conceived by IVF much as umbilical cord blood cells are currently banked.

Once the inner cell mass has been isolated it is typically plated on a layer of feeder cells to enable primary expansion. Since the original protocols

(Reubinoff *et al.*, 2000; Thomson *et al.*, 1998) were based on mESC derivation protocols, these were often mouse embryonic fibroblasts. Owing to the discovery that mouse feeder cells and fetal calf serum resulted in the expression of non-human surface antigens on hESCs that could precipitate immune rejection in humans (Martin *et al.*, 2005), current protocols now commonly use human embryonic fibroblasts or other human cell feeder layers (Amit *et al.*, 2003; Choo *et al.*, 2004; Lee *et al.*, 2004). As discussed earlier, hESC are characteristically maintained in Dulbecco's Modified Eagle's Medium-based medium with KOSR and bFGF. A great deal of investigation has centered on developing feeder-free, xeno-free, completely defined conditions in which to derive, expand, and maintain pluripotent hESCs, enabling adoption of good manufacturing practice standards (Ahrlund-Richter *et al.*, 2009; Unger *et al.*, 2008; Rajala *et al.*, 2010). Currently there are several commercially available defined

xeno-free media that can maintain hESC pluripotency in the absence of feeder layers (Hasegawa *et al.*, 2010). It should be noted that there is some evidence that the absence of feeder layers causes increased chromosomal instability, thereby necessitating increased karyotypic vigilance (Catalina *et al.*, 2008). However, recently numerous investigative groups have demonstrated long-term expansion of karyotypically normal, undifferentiated hESCs on fully defined and xeno-free substrates such as hydrogels (Villa-Diaz *et al.*, 2010; Melkounian *et al.*, 2010) and recombinant laminin (Rodin *et al.*, 2010).

Expanded hESCs are passaged mechanically by cutting larger colonies into smaller clumps and replating them. Differentiated cells can also be eliminated from cultures during passage. Manually dissecting colonies is labor-intensive and inhibitory to rapid expansion. However, use of enzymatic or chemical agents to passage hESCs has been shown to increase the risk of chromosomal instability (Catalina *et al.*, 2008; Mitilipova *et al.*, 2005). Therefore, currently mechanical dissection is the best way to maintain the bulk of a hESC line, with enzymatic and chemical passage methods being used only sparingly (Hasegawa *et al.*, 2010). Once established, hESC lines need to be characterized to determine whether they are pluripotent (Englund *et al.*, 2010). Immunohistochemistry or fluorescence-activated cell sorting (FACS) can be used to look for surface antigens found on pluripotent cells, such as SSEA-3, SSEA-4, and/or TRA-1-60, TRA-1-81 as well as expression of pluripotency markers such as Oct3/4 and Nanog. Karyotyping should be done on early, middle, and late passages to demonstrate chromosomal stability. The cells should be subjected to the in-vitro differentiation assay of embryoid body formation. When ESC are grown in suspension culture as embryoid bodies, floating aggregates of cells, they spontaneously differentiate into all three germ layers: ectoderm, mesoderm, and endoderm. The presence of ectoderm, mesoderm, and endoderm contributions in the embryoid body can be confirmed with immunocytochemistry with lineage-specific antibodies or with PCR and lineage-specific mRNA/cDNA primers. However, there are significant differences in the organization of the embryo and an embryoid body. Lineage

allocation in an embryoid body is far more chaotic; there is no organized geometry, formation of an anterior visceral endoderm (AVE) (an embryonic organizing center), node, or primitive streak. Finally, the ESCs should be used to generate teratomas in immunocompromised mice, considered the gold standard of pluripotency with hESCs.

1.5 Pluripotent stem cells in regenerative medicine

Currently a major disadvantage of using ESCs in cell replacement therapies is their potential to generate teratocarcinomas or teratomas, or simply undergo uncontrolled levels of proliferation that result in graft overgrowth. One of the most scientifically sound ways to approach this problem is to gain sufficient understanding of the embryonic development necessary to differentiate ESCs to the level of the resident tissue stem cell or even fully differentiated cell, then transplant those cells with a “suicide” transgene (e.g. thymidine kinase driven by a cyclin gene promoter) that could be used to kill cells if they re-enter cell cycle (Fareed and Moolten, 2002; Schuldiner *et al.*, 2003). Alternatively, several cell surface antigens have been identified that can be used in conjunction with FACS to remove tumor-initiating cells from a population of differentiated ESCs prior to implantation (Tang *et al.*, 2011).

Another major obstacle in the use of ESCs in regenerative medicine is immunogenicity of implanted cells. While elimination of foreign proteins and disease-causing agents from ESC culture conditions is important, major histocompatibility (MHC), minor histocompatibility (mHC), and blood group antigen (ABO) differences between donor ESC and graft recipients can all precipitate rejection of ESC transplants (Druker and Benvenisty, 2004; Lui *et al.*, 2009). There are several methods that may be employed to avoid rejection. Seemingly the most simple would be to match MHC, mHC, and ABO antigens between hESC lines and recipients, as is done for organ transplantation. Unfortunately, this would require an enormous bank of hESC lines because of the massive number of