Embryonic Stem Cells

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Contents

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Abstract

Embryonic stem cells (ESCs) are pluripotent stem cells derived from a preimplantation embryo. ESCs are distinguished by two major properties: their pluripotency (the ability to differentiate into all derivatives of the three primary germ layers) and their ability to replicate indefinitely under defined conditions. Human ESCs (hESCs) can be used to study early human development and genetic disease and for in vitro toxicology testing. Because of their plasticity and potentially unlimited capacity for self-renewal, clinical-grade hESC therapies have been proposed for tissue replacement after injury or disease. In this chapter we summarize the process of hESC derivation, discuss characterization (the standard tests that are performed during the cell culture process to check that the cells exhibit the fundamental properties that make them ESCs), and provide examples of protocols that are used to induce hESCs to differentiate into specific cell types.

1   Introduction

Embryonic stem cells (ESCs) are pluripotent stem cells derived from preimplantation embryos. Several features define ESCs, but the two key properties that make these cells remarkable are their capacity for indefinite self-renewal and their ability to give rise to all cell types derived from the three embryonic germ layers. The characteristics and culture methods used for ESCs vary greatly between species; as such, this review will focus exclusively on human ESCs (hESCs), although we will reference discoveries in other species such as mouse for historical context and development of the field. In addition, induced pluripotent stem cell (iPSC) lines will be referred to throughout for comparative purposes. Human ESCs and iPSCs will be referred to collectively as human pluripotent stem cells (hPSCs).

2   Origin and Derivation of hESCs

hESCs are typically derived from the inner cell mass (ICM) of blastocyst stage embryos on days 5–8 after fertilization. The blastocyst is composed of two distinct cell types: the ICM, which later becomes the epiblast and goes on to give rise to the fetus, and the trophectoderm (TE) (Fig. 1a). The principal role of the TE in early development is to regulate the microenvironment in which the ICM develops. As development continues the TE will form the extraembryonic support structures required for successful development of the embryo, such as the placenta and extraembryonic membranes.
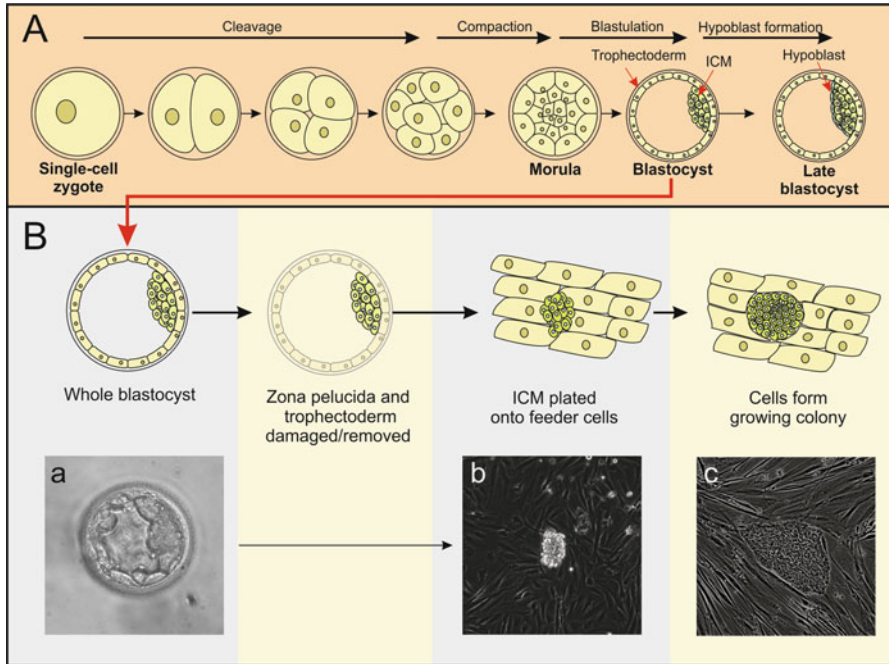
Fig. 1 Origin and derivation of hESCs. (**a**) Early human embryonic development, progressing from single-cell zygote to late blastocyst. Cells for hESC derivation are taken from early blastocyst stage embryos, typically prior to formation of the hypoblast, as indicated. (**b**) Derivation of hESCs from the blastocyst. Cells are derived from the whole blastocyst as shown in the phase contrast image (**a**); the zona pellucida is then commonly removed enzymatically before the isolated ICM is separated from the TE and plated onto feeder cells (**b**). After some days, these ICM cells begin to form a hESC colony among the feeder cells (**c**)

The ICM is formed from the inner cells of the morula, while the TE forms from the outer cells. During blastocyst formation the ICM becomes localized to one side of the TE vesicle, adjacent to the forming fluid-filled blastocyst cavity. The ICM is later separated from the blastocyst cavity by the formation of a second extraembryonic layer of cells, the hypoblast, becoming the early epiblast (Fig. 1a). While the cells of the TE begin to form a specialized support structure, the ICM cells remain undifferentiated, fully pluripotent and proliferative and progress to form all of the tissues and structures of the human body. It is from the pluripotent cells of the ICM that hESC cells are derived.

2.1   Derivation

ESCs are typically derived using a variety of techniques from microsurgery to antibody or even laser-assisted methodologies (Solter and Knowles 1975; Thomson et al. 1998; Reubinoff et al. 2000; Strom et al. 2007; Turetsky et al. 2008). Although

there are differences in these methods, the aim of each is the same: to remove and separate the ICM from the TE so that the cells of the ICM can be cultured and expanded in vitro (Fig. 1b). Derivation without separation of the ICM and TE has been accomplished; however, the presence of highly proliferative TE cells can suppress growth of ESC cells and their co-culture is typically avoided (Heins et al. 2004; Inzunza et al. 2005). All of the above methodologies require the destruction of the blastocyst, which has raised concerns among some religious groups. Alternative methodologies have been developed which generate hESC lines from stages other than blastocyst, including as early as the four-cell embryo (Geens et al. 2009), and indeed using only single blastomeres from the morula, a technique which leaves a potentially viable embryo (Chung et al. 2008; Klimanskaya et al. 2006, 2007), While use of blastomeres biopsied from the embryo in this way to generate hESC lines might overcome one objection to the use of human embryos, arguably the potential damage incurred to the embryo raises another equally valid objection. A number of other efforts have been made to mitigate any concerns surrounding stem cell derivation, by utilizing eggs or embryos which would otherwise be discarded from the IVF process. These include deriving cells from oocytes unsuitable for IVF following parthenogenetic activation (De Sousa et al. 2009; Camarasa et al. 2012), from growth-arrested IVF embryos (Zhang et al. 2006), and from otherwise nonviable or poor-quality embryos (Gavrilov et al. 2011; Lerou et al. 2008; Ye et al. 2017).

Conventionally, the separated, expanded ICM-derived cells are then cultured in medium which promotes pluripotent stem cell growth, on a substrate formed of a lawn of non-mitotic "feeder" cells, such as mitotically inactivated mouse embryonic fibroblasts (MEFs) (Thomson et al. 1998; Crocco et al. 2013). Islands of cells emerge with the classical ESC morphology of small cells with a high nucleus to cytoplasm ratio and prominent nucleoli. If derivation is successful, then the ESCs will form a growing colony of pluripotent stem cells within the lawn of feeder cells (Fig. 1b). As this colony grows care must be taken to only expand pluripotent stem cells, as the cells are prone to spontaneous differentiation, particularly at the periphery of the colony (Rosowski et al. 2015). Once the colony reaches a threshold size, the cells can be passaged manually by physical dissection of the colony into smaller pieces, which allows the separation of morphologically ESC-like cells from those showing evidence of differentiation. Passaged cells are moved to a fresh plate of feeder cells where they form fresh colonies, and expansion continues this way until the stability of the ESC line has been established, usually defined as the ability to cryopreserve and successfully resuscitate the line (Masters and Stacey 2007; Murdoch et al. 2012).

3   Culture of hESCs

In general the conditions and methods of hESC culture are similar to those for other mammalian cells; cells are cultured in plates or flasks; in an isotonic, nutrient-rich medium; and in a humid 37 °C incubator at typically 5% CO_2 in air. However, hESC

culture differs from many standard mammalian cell culture systems in three key attributes which are essential to maintaining pluripotency: media, substrate, and passaging method.

3.1 Media and Substrates

3.1.1 Substrates

The principal point of variance in the way hESCs can be grown is that of feeder-dependent or feeder-free culture. hESCs require a combination of signaling molecules, both soluble and adherent, to maintain their pluripotency and growth. Initially the provision of the, then unknown, signaling factors was facilitated by growing colonies of hESCs on a lawn of non-mitotic MEFs.

The dependence on the use of MEFs was problematic as they are of nonhuman origin, and the composition of signaling and extracellular proteins that they secrete and present can be highly variable between batches and labs (Lim and Bodnar 2002; Chin et al. 2007; Crocco et al. 2013). Despite many advances in substrates and media, there is not yet a robust, universally accepted feeder-free protocol for the derivation and culture of karyotypically normal hESC lines (International Stem Cell Initiative et al. 2011). In order to avoid the potential contamination of these cultures with animal-derived pathogens, a wide variety of xeno-free feeder cells have been developed such as human dermal, foreskin, placental, or embryonic fibroblasts (HDFs, HFFs, HPFs, or HEFs) (Crocco et al. 2013; Mckay et al. 2011). These cell lines have been demonstrated to reliably replace MEFs, removing our reliance on them when deriving hESC lines.

Once hESC lines have been stably derived onto feeder cells, they can be moved to feeder-free conditions, in which tissue culture plastic is pre-coated with proteins or protein mixtures prior to the addition of cells. Matrigel, an extracellular matrix (ECM) protein preparation derived from Engelbreth–Holm–Swarm mouse sarcoma cells, has been used for over 30 years to culture ECM-dependent cells (Kleinman et al. 1982; Bissell et al. 1987) and for over 15 years to culture hESCs in feeder-free conditions (Xu et al. 2001). While Matrigel has allowed hESCs to be cultured feeder-free, it is still an animal-derived, undefined product, containing a variable mixture of laminins, collagen IV, proteoglycans, and signaling molecules. To avoid this variability, researchers looked into use of cell-binding proteins, or protein motifs, which could be recombinantly produced and be immobilized onto tissue culture plastic, such as vitronectin (Braam et al. 2008) or laminins (Rodin et al. 2010). These proteins enable initial cell attachment allowing cells to then produce their own extracellular matrix permitting pluripotent growth of cells (Soteriou et al. 2013) with activation of focal adhesion kinase (Vitillo et al. 2016). The recombinant N-terminal vitronectin domain (VTN-N) is a particularly noteworthy substrate due to the cost-efficiency of its production (Braam et al. 2008). VTN-N is now used routinely with media such as E8 (see below) to culture pluripotent stem cells in completely xeno-free, defined conditions (Chen et al. 2011).

3.1.2 Media

As with all cell lines, hESCs require a complex mixture of nutrients and growth factors for successful in vitro culture. This medium generally needs to be changed daily. Initial success in culturing hESCs in vitro was achieved in medium containing serum (a complex, undefined mixture of nutrients and signaling factors). While these conditions allow the robust expansion and culture of pluripotent hESCs, with the use of MEFs and Matrigel, as discussed above (Sect. 3.1.1), there is a batch-to-batch variation in the content of serum, and the use of animal-derived products adds additional technical and safety considerations for applications in regenerative medicine. As such there has been a consistent drive toward synthesizing a fully xeno-free and defined medium.

The progress away from undefined animal-derived sera has been rapid. Initially the culture-staple fetal calf serum (FCS) was improved upon by utilizing partly defined or synthetic serum replacements such as knockout serum replacement (KOSR), synthetic serum substitute (SSS), or StemPro (Weathersbee et al. 1995; Lee et al. 2006; Swistowski et al. 2009). Fully defined medium such as TeSR reduced and defined the number of components in hESC medium to a supplement of 18 components added to a DMEM/F12 basal media of 52 components (Chen et al. 2011; Ludwig et al. 2006b). This formula was later improved in TeSR2 by making all components xeno-free in origin (Meng et al. 2012).

TeSR medium was formulated methodically by removing individual components from stem cell culture medium and determining whether their removal had any negative impact on cell pluripotency (Ludwig et al. 2006b). This reductive methodology was improved through a combinatorial approach taking into account component interactions, and it was revealed that it was possible to remove further factors without negatively affecting the cells. This resulted in the simplest medium to date containing only seven components added to a DMEM/F12 basal media, Essential 8 (Chen et al. 2011). These components are DMEM/F12 medium, L-ascorbic acid, selenium, transferrin, $NaHCO_3$, insulin, FGF2, and TGFβ1.

3.1.3 Passaging Methods

In understanding the behavior of hESCs in culture, it is informative to consider that a growing hESC culture is poised to continue the developmental progress of the ICM and begin differentiation. The pluripotent stem cell population is present only transiently in the embryo; for about 24–48 h before being lost to cell commitment. In order to maintain the pluripotency of a hESC culture, the cells are prevented from becoming over confluent. Cells are typically allowed to proliferate for only 2–3 days, at which point they are "passaged" disaggregating them into small clumps which will form new colonies. All passaging methods work by dissociating the cells from the culture surface and/or each other and then seeding a portion of these cells into a fresh culture vessel. hESC cultures are passaged either as clumps or as single cells and consequently will either grow in colonies or in a continuous culture monolayer (Fig. 2).
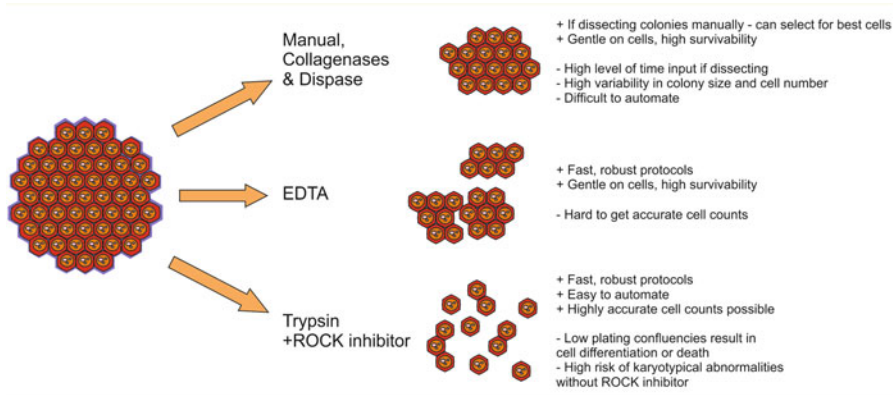
Fig. 2 Common methods of hESC passage (Based on Beers et al. 2012). Once a hESC culture reaches a threshold density, it is disaggregated into smaller clusters of cells, or to single cells, a portion of which are then plated onto a new culture surface; this process is called cell passaging. Each passaging method has advantages (+) and disadvantages (−), some of which are listed above

Manual Passage

The presence of cell–cell contacts between hESCs in culture helps to maintain their pluripotency (Studeny et al. 2002). Therefore, hESCs are typically passaged and cultured in clumps and colonies, rather than being individualized by dissociation to single cells as is common with other mammalian cells (including mouse ESCs). Manual passage of hESCs is often used in early stages of cell line derivation and is particularly advantageous as it can allow for the enrichment of healthy, pluripotent stem cells in a colony of mixed quality. Cells can be passaged manually either by dissection, which can very precisely enrich for specific groups of cells, or by use of a cell scraper which is a less labor-intensive method employed when selection of certain cells is not necessary.

Enzymatic Dissociation

Enzymatic methods of dissociation utilize enzymes such as trypsin to digest the extracellular adhesion domains and proteins which bind cells to each other and/or to their substrate. The use of enzymes which target exclusively the extracellular matrix produced by cells provides a gentler dissociation method which maintains cells in large clumps and colonies; however, their use is often restricted to particular substrates. Collagenases, for example, can be used to gently dissociate colonies cultured on feeder cells, whereas dispases are more effective in feeder-free culture.

Trypsin

While the above methods allow for healthy passage of hESCs, the operator variability in the size of colonies generated and the number of cells passaged make it difficult to accurately quantify cell number when cells are passaged as clumps. Enzymatic passage using trypsin avoids this and is a robust, reproducible, and easily automated method of hESC passage. Additionally, GMP-grade recombinant trypsin is available

and widely used in the field, which is advantageous for translating cultures to regenerative therapies (Ellerstrom et al. 2007). Trypsin breaks down the extracellular connections both between cells and between cells and their substrate. However, culturing hESCs in this manner has been reported to decrease viability and pluripotency in some studies (Brimble et al. 2004), and the surviving culture may become enriched for cells with karyotypic abnormalities (Sect. 4.4). The loss of viability and pluripotency by single-cell passage can be avoided by supplementing media with an inhibitor of the protein Rho-associated protein kinase (ROCK) for 24 h following passage (Watanabe et al. 2007). It has been suggested that ROCK inhibition disrupts extracellular cues that would normally induce detachment-induced apoptosis (anoikis) when the cells are dissociated and enhances cell–cell interaction through modulation of cadherins and GAP junctions, leading to the formation of small aggregates of hESCs in suspension (Krawetz et al. 2009; Wang et al. 2009). The predominant mechanism appears to be thorough myosin light chain phosphorylation (Chen et al. 2010) and so inhibition of actin–myosin contraction. There are persistent concerns in the field that long-term culture in the presence of ROCK inhibitor may carry the risk of an increased incidence of karyotypic abnormalities, although a definitive study demonstrating this has yet to appear.

Ethylenediaminetetraacetic Acid (EDTA)

Rather than digesting extracellular proteins, EDTA treatment functions by chelating calcium, which is required for cell–cell adhesion and integrin binding. This results in a less harsh passage, with retention of clusters of up to tens of cells and minimal damage to cell surface proteins. The larger clusters of cells resulting from EDTA passage remove the necessity of ROCK inhibitor, by avoiding viability loss associated with individualization (Beers et al. 2012). Similarly to other aggregate-passaging methods, EDTA passage makes precise quantification of cells more difficult, which is a serious drawback for automation and scale-up.

3.2   Culture Developments

In the two decades since hESCs were first cultured via manual passage with xeno-derived, undefined media and substrates, the field has advanced substantially to achieve fully defined, xeno-free culture with enzymatic, automation compatible passage. These advances continue, with the aim of reducing the cost, complexity and reproducibility of hPSC culture. One avenue for such progress is the removal of the necessity for substrate coating of tissue culture plastic and the treatment of cells with ROCK inhibitor at passage. Pijuan-Galito and colleagues (2016) reported that the supplementation of E8 with 50 μl/ml of the protein inter-α-inhibitor (IαI) when cells were passaged allowed for culture without prior substrate coating, or ROCK inhibition. Their data also suggested that IαI passage had a protective effect against trypsin carry-over after passage which would provide increased margin for error in an automated scale-up culture system. Similar advances could pave the way for a more robust and economically viable future for hPSC therapies. Others have

reported successful nonadherent culture (Chen et al. 2012; Steiner et al. 2010), but his has not been achieved with systematic success across the field so far.

3.3   Stem Cell Derivation for Regenerative Medicine

To ensure defined quality and safety in cell transplantation, hESCs need to be derived and maintained using good manufacturing practice (GMP) standards, set down by both the European Medicines Agency and the Food and Drug Administration. Thus, before hESC lines can be effectively deployed in regenerative medicine, a defined set of methodologies and criteria needs to be established for their safety and quality. The protocols for the establishment and in vitro culture of hESC are currently varied and have in the past necessitated the use of animal-derived products or support cells (International Stem Cell Initiative et al. 2011). Cell lines from these protocols are restricted in their usefulness in regenerative medicine, because of both the risk of introducing animal antigens and pathogens to a patient and the absence of other quality assurance measures (Martin et al. 2005; Nukaya et al. 2015). As such, only a small number of existing hESC lines are suitable for clinical use. There has been a recent push to establish cell lines using both xeno-free and GMP methodologies from the outset (Vaskova et al. 2013; Ye et al. 2017). These lines should be derived from fresh embryos, sourced from GMP standard in vitro fertilization (IVF) facilities that are surplus to patient clinical requirements (Murdoch et al. 2012). Once the ICM has been separated from the TE, cells can be initially cultured on qualified human feeder cell lines, such as human dermal fibroblast (HDF) cells, and fed with medium in which all nonhuman reagents have been replaced with xeno-free equivalents (Hewitson et al. 2016; Ilic et al. 2012). GMP-grade hESCs can be derived from the ICM of good-quality frozen IVF embryos plated onto recombinant cell extracellular matrix components such as laminin-521 and maintained in xeno-free medium (Rodin et al. 2014). There are several commonly used xeno-free culture systems that support undifferentiated growth of hPSCs consisting of a xeno-free growth medium ideally with xeno-free substratum, including TeSR2 with human recombinant laminin (LN-511), NutriStem with LN-511, RegES with human foreskin fibroblasts (HFFs), KO-SR Xeno-Free/GF cocktail with CELLstart matrix, Essential E8 (Chen et al. 2011) with recombinant vitronectin, and StemFit medium, among others. A recent study has demonstrated a fully GMP-compliant derivation system whereby fresh discarded surplus IVF embryos were cultured onto GMP-grade human feeder cells with the sequential use of commercially available media, HES-V2 and TeSR2 (Ye et al. 2017).

In order to provide cost-effective treatments, HLA-matched allogeneic tissue banks of the highest-quality clinical-grade hESCs will be required (Sect. 5.2). The UK establishment of quality standards, as well as the cataloguing and banking of high quality lines, has been greatly facilitated by national initiatives such as the human embryonic stem cell coordinators' (hESCCO) network and the UK Clinical Stem Cell Forum (Murdoch et al. 2012), which has allowed hESC derivation centers to coordinate efforts with the UK Stem Cell Bank (UKSCB – http://www.nibsc.org/

ukstemcellbank). As a result the UK has now established a bank of "ethically approved, quality-controlled stem cell lines for medical research and treatment" to help progress the dissemination and regulation of high-quality stem cells for regenerative medicine (Stacey and Hunt 2006; Geraghty et al. 2014). There are also many other national and international initiatives and companies, such as the National Stem Cell Bank in the USA, that provide PSC lines for medical research and treatment and the International Society of Stem Cell Research recently published guidelines for clinical translation (Daley et al. 2016).

4   Characteristics of hESCs and How to Assess Them

Stem cell banks such as the UK Stem Cell Bank (UKSCB) and others store, characterize, and supply ethically approved stem cells for medical research and treatment. Information about these can be found in the Human Pluripotent Stem Cell Registry (https://hpscreg.eu/). More than a thousand hESC lines have been generated worldwide (Seltmann et al. 2016). These lines exhibit a variety of differences based upon their origin, derivation protocol, passage number, and culture conditions; all these factors can impact the gene expression, epigenetics and karyotype, as well as the ability of the cells to self-renew and differentiate (Abeyta et al. 2004; Draper et al. 2004; Enver et al. 2005; Bock et al. 2011). Therefore, during hESC maintenance it is vital to regularly and comprehensively test karyotypic stability, functional pluripotency, identity, quality, safety, and suitability for intended purpose. Analyses currently performed to characterize ESCs are depicted in Fig. 3 (Singh et al. 2012; Marti et al. 2013).

Basic identification of maintenance or loss of pluripotency is possible through observation of the cells under phase contrast microscopy, to identify the characteristic morphology exhibited by pluripotent cultures. Pluripotent cell status is commonly also assessed by examining expression of self-renewal markers by immunostaining, flow cytometry, or quantitative reverse transcription polymerase chain reaction (RT-qPCR) (International Stem Cell Banking Initiative 2009; Pistollato et al. 2012; Singh et al. 2012; Marti et al. 2013; International Stem Cell Initiative et al. 2007).

Functional pluripotency is defined as the capacity for cells to differentiate into each of the three germ layers (endoderm, ectoderm, and mesoderm). While molecular expression of pluripotency-associated markers is a strong indicator of functional pluripotency, the latter can only truly be verified through assessment of three germ layer differentiation by one of the following: assessment of spontaneous differentiation, in vitro embryoid body (EB) formation (Itskovitz-Eldor et al. 2000; Peterson and Loring 2012), in vitro-directed differentiation using growth factors or small molecules, or in vivo through teratoma formation that also allows evaluation of
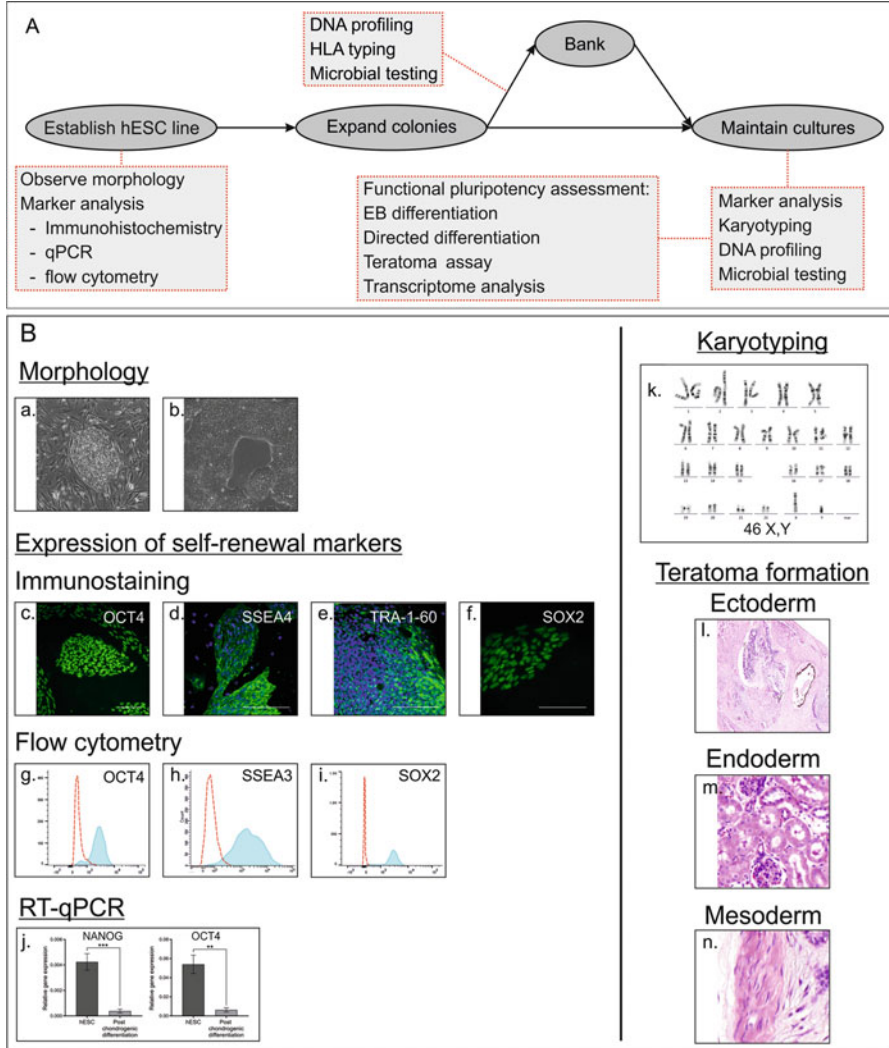
Fig. 3 Characterization of hESC lines. (**a**) Flowchart showing steps that may be undertaken when characterizing hESC lines. (**b**) Typical morphology of hESCs grown on MEFs (**a**) and vitronectin-N (**b**); immunostaining: fluorescence images of hESCs stained with OCT4 (**c**), SSEA4 (**d**), TRA-1-60 (**e**), and SOX2 (**f**) in green and DAPI in blue. Scale bars represent 100 μm; flow cytometry analysis: representative overlay histograms showing the profile of OCT4 (**g**), SSEA3 (**h**), and SOX2 (**i**) reactivity on hESCs in blue with the unstained cells in red; RT-qPCR showing relative expression of NANOG and OCT4 in hESCs and differentiated cells (**j**). Karyotyping: G-banding of hESCs showing a normal male karyotype (**k**). Hematoxylin and eosin staining of the three germ layers in a teratoma formed from a hESC line (**l**–**n**)

ability of cells to form tissues (Gertow et al. 2007). The teratoma assay (discussed further under Sect. 4.5, below) currently remains the gold standard for assessing the ability of stem cells to form tissues from all three germ layers. Stem cell biologists have recognized the need to standardize methodologies in the field, resulting in global initiatives such as the International Stem Cell Initiative (ISCI, http://www.stem-cell-forum.net/initiatives/isci).

4.1   Morphology of hESCs

An important skill in successful culturing of hESCs is the ability to recognize the morphology of undifferentiated cells under a variety of conditions. Undifferentiated hESCs and iPSCs cultured on feeders display a distinct morphology with a prominent nucleolus and a high nucleus to cytoplasm ratio. Cells are arranged in flat, tightly organized colonies with defined edges (Amit and Itskovitz-ELDOR 2012). Observing hPSC morphology is a quick and inexpensive way to assess good colonies because colonies that have started to differentiate develop rough edges with loosely organized cells. This is especially true in a research setting where the number of cell lines is small enough to be manageable. However, for scale-up, it is not feasible to examine all cells by the eye (Rosowski et al. 2015), thus generating a need for automated imaging software that can quickly and reliably examine the morphology of large numbers of PSCs (Kerz et al. 2016 ; Perestrelo et al. 2017). It may be that automated measurement of cell size, density, nucleus to nucleolus ratio, and nucleus to cytoplasm ratio will be useful metrics that can be used by imaging software to judge the quality of PSCs. However, since PSCs cultured under different conditions can have slightly different morphologies (Ludwig et al. 2006a), significant experience in inspecting PSC cultures is required, and it is important for laboratories to keep representative images of ideal colonies for each cell line and examples of poor-quality cultures that have areas of differentiation as an aide-memoire and for training purposes. In order to facilitate scale-up and medical application, feeder-free systems of culturing hESCs have been developed. In feeder-free systems, PSC colonies often appear looser soon after plating, but as the cells divide, they compact to form the typical PSC colony morphology (Healy and Ruban 2014; Vestergaard et al. 2016). This is partly a function of the absence of feeder cells but also of the manner in which these cells are often passaged. While feeder-dependent cells are typically passaged in large aggregates, feeder-free cells are more often passaged in smaller aggregates or even single cells (Sect. 3.1.3). As a result, PSCs spread more diffusely across the culture surface, migrate actively, and proliferate in the initial hours of culture and then form the more typical PSC culture morphology as their density increases.

While the assessment of PSC morphology is a valuable method of regularly observing broad colony quality trends during culture progression, proper evaluation of pluripotency requires assessment of self-renewal/pluripotency-associated markers ("Assessing Pluripotent Cells").

4.2   Molecular Assessment of Pluripotent Cells

Many studies have utilized transcriptome analyses to compare different undifferentiated hESCs and hiPSCs to their differentiated counterparts (Sato et al. 2003; Dvash et al. 2004; Gifford et al. 2013) (Sect. 4.5.3). These studies have found that hESCs are enriched in hundreds of genes including OCT4, SOX2, NANOG, REX1, FLJ10713, DNMT3B, FOXD3, SALL2, GABRB3, and TDGF1, suggesting that they play a role in maintaining pluripotency. It is accepted that a core triad of these transcription factors regulates the pluripotent state: OCT4 (POU5F1), SOX2, and NANOG (De los Angeles et al. 2015; Li and Izpisua Belmonte 2016), but the regulation of pluripotency is not limited to protein-coding genes. Several micro-RNAs (miRNAs) and long noncoding RNAs (lncRNAs) are differentially enriched in PSC prior to and after differentiation and contribute to controlling stemness (Houbaviy et al. 2003; Lakshmipathy et al. 2007; Li and Izpisua Belmonte 2016). The publication of these gene expression signatures has paved the way for the development of in silico tools that predict pluripotency based on transcriptome data and have revealed potential markers that can positively identify hPSCs.

ISCI has suggested a core set of markers that play a role in pluripotency or are consistently expressed in 59 hESC lines, including NANOG, TDGF, OCT4, GABRB3, GDF3, DNMT3B, the keratan sulfate antigens Trafalgar (TRA)-1-60 and TRA-1-81, and the glycolipid antigens stage-specific embryonic antigen (SSEA)3 and SSEA4 (Draper et al. 2002; Henderson et al. 2002; Chambers et al. 2003; Sperger et al. 2003; Dvash et al. 2004; Richards et al. 2004; International Stem Cell Initiative et al. 2007). hPSCs may also be identified through the detection of SOX2, REX1, and hTERT expression, as well as alkaline phosphatase (AP) activity (Singh et al. 2012; Marti et al. 2013). As can be seen from the list above, a wide range of markers whose expression is associated with pluripotency have been used as surrogate evidence of hPSC pluripotency, but some markers are more informative than others. For instance, during the directed differentiation of hESCs into the three germ lineages, OCT4, SSEA3, and TRA-1-60 were consistently downregulated earlier than AP and NANOG (Ramirez et al. 2011). Levels of SOX2 and OCT4 expression need to be precisely regulated to maintain pluripotency, with either higher or lower expression leading to the loss of pluripotency in ESCs (Fong et al. 2008; Kopp et al. 2008).

Various methods are used to detect self-renewal markers. Live fluorescent substrate-based staining is used to detect AP activity (Singh et al. 2012; Marti et al. 2013). At the protein level, antibody-based methods such as imaging and flow cytometry are often used; immunostaining of intracellular proteins like OCT4, NANOG, and SOX2 requires cell permeabilization and the termination of the culture. Alternatively, intracellular markers can be detected in live cells through the use of molecular beacons – short single-stranded oligos that bind to complementary mRNA, allowing the attached reporter to fluoresce (Santangelo et al. 2006; King et al. 2011). A limitation of using molecular beacons is that they need to be delivered into the cells and the delivery method may be inefficient or even toxic. In addition, RT-qPCR is useful to generate quantitative or semiquantitative expression data.

It is important to keep in mind that no single marker is sufficient to identify PSCs. Many genes are expressed in multiple tissues. For instance, SOX2 is expressed in neural progenitor cells and PSCs (Graham et al. 2003). Also, pluripotency-regulating genes may have splicing variants with different functions, as illustrated by OCT4, which has an A isoform relevant to pluripotency and a B isoform that is not (Marti et al. 2013), TCF3, FOXP1, NANOG, and MBD2 (reviewed: Li and Izpisua Belmonte 2016). Moreover, several processed OCT4 pseudogenes exist in the human genome with high homology to OCT4 (Suo et al. 2005) that may cause a false-positive signal in gene expression experiments. OCT4 distal enhancer activity is considered a molecular signature of ground state (Sect. 4.3) pluripotency, a pluripotent state that may resemble the preimplantation embryonic configuration in mouse (Ying et al. 2008). However, both distal and proximal enhancer elements of OCT4 are active in naïve and primed pluripotent states and cannot be used as a binary distinguishing marker since it is their relative activity level and dominance that determine if the cells are pluripotent (Buecker and Wysocka 2012; Karwacki-Neisius et al. 2013). Therefore, it is considered good practice to carefully select a panel of markers and analyze them using validated detection methods. It is important to note that even the presence of several markers does not guarantee that a cell is a PSC; for example, OCT4, NANOG, and SOX2 must be expressed to the right level and in a specific equilibrium to maintain pluripotency (Kashyap et al. 2009). Therefore, using more markers increases the likelihood of identifying pluripotent cells accurately, and this has driven the trend toward using multigene expression panels.

Positive marker panels only confirm the presence or absence of PSCs, but they do not detect contamination by differentiated cells (Muller et al. 2008; Marti et al. 2013). Accordingly, when characterizing PSCs, it is also useful to include a selection of negative markers of early differentiation such as SSEA1, A2B5, CD56, GD2, GD3, and CD13 (Draper et al. 2002; International Stem Cell Initiative et al. 2007).

4.3   Naïve Pluripotency

Studies on murine ESCs have provided a great deal of insight and facilitated the pioneering derivation and characterization of the first hESCs. However, there are a number of profound differences between hESCs and mESCs, including the apparent different developmental status of the ICM/epiblast at the point of derivation. While both mouse ESCs and hESCs are derived from the inner cell mass of the preimplantation blastocyst, mouse ESCs are in a pluripotent state termed "naïve" or ground state pluripotency (Bar-NUR et al. 2011; Hackett and Surani 2014), while human ESC cells are in a state of pluripotency which shows similarity but not identity with that of the "primed" postimplantation mouse epiblast rather than the ICM (Brons et al. 2007; Tesar et al. 2007). Naïve pluripotent cells seem to exist transiently during mouse development, between ICM formation and their priming in preparation for lineage specification. The murine ESCs are considered to represent a developmentally "blank slate," while their "primed" pluripotent counterparts in humans are considered to be poised for differentiation (Polo et al. 2010). This state of naïve

pluripotency is not only characterized by the expression of key pluripotency factors but critically hypomethylation and a derestricted epigenome free of developmental bias (Leitch et al. 2013).

It is not yet known whether cells of the human ICM possess the biological characteristics of naïve pluripotency during normal development. Researchers have replicated aspects of the naïve state in hESCs artificially, both through the use of transgene expression and more recently through induction by small molecules (Buecker et al. 2010; Duggal et al. 2015; Guo et al. 2016). These induced-naïve hESCs have potential to improve regenerative therapies as their open chromatin configuration increases the success rate of gene-editing technologies in these cells (Hackett and Surani 2014) which could allow gene correction for personalized therapies. Additionally, it has been reported that when iPSCs are induced to a more naïve state and directed to differentiate, the culture increases expression of desired differentiation markers compared to the iPSC parent cell line (Duggal et al. 2015). While there is potential for improvements in regenerative therapies through the induction of naïve pluripotency in hESCs, there is still a great deal of research still to be done. There are molecular and functional differences between pluripotent cell types, which subsequently influence their characteristics, function, and safety; however it remains to be elucidated whether there are drawbacks to maintaining PSCs under naïve conditions and if there are additional safety concerns associated with naïve pluripotency. For instance, it is currently unknown whether naïve stem cells have an increased tendency to acquire genomic abnormalities due to their open chromatin configuration and reduced repressive epigenetic marks (Weinberger et al. 2016).

4.4   Genomic Stability of hESCs

hESCs need to be screened carefully to rule out the occurrence of genetic abnormalities, which can arise either from the source embryonic material or occur during long-term ESC culture (International Stem Cell Initiative et al. 2011; Laurent et al. 2011). hESC lines should be screened initially since a large proportion of early human embryos are chromosomally abnormal as a result of meiotic errors arising in the gametes or post-zygotic errors arising during cleavage resulting in mosaic embryos containing both euploid and aneuploid cells (Harper et al. 2004). Ongoing screening is equally important as both pluripotency and genetic stability may change during adaptation to different passaging methods and culture conditions (Brimble et al. 2004; International Stem Cell Initiative et al. 2011). Weissbein and colleagues (2014) summarized the common genetic abnormalities in cultured PSCs in their review. These chromosomal modifications, such as those on chromosomes 1, 12, 17q, and 20, are reminiscent of those observed in cancers (for instance, chromosome 20 duplication resulting in enhanced expression of BCL2L1, a classical cancer gene). They typically accumulate by predisposing cells toward increased survival and replication, such that PSC cultures become overgrown with abnormal cells but often retain a minority of normal karyotypes (Draper et al. 2004; International Stem

Cell Initiative et al. 2011; Na et al. 2014). It is, therefore, important to continually monitor the quality of ESC lines. Tests that determine cell identity and ensure quality and safety also need to be carried out, especially if the cell lines are intended for banking or therapeutic use (Stacey 2012). These additional tests include more detailed genetic profiling, human leukocyte antigen (HLA) typing, and microbial testing (Young et al. 2010; Stacey 2011). Karyotyping will only reveal large deletions or translocations, and hence more refined analyses such as comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays may also be needed (De Sousa et al. 2009; Elliott et al. 2010; Canham et al. 2015), although complete sequencing of the genome has little clinical value at the moment. In summary, tests that confirm functional pluripotency and normal genetic status are important for documenting the characteristics of hESC lines and need to be performed periodically throughout the cell line expansion: every ten passages may be a realistic goal with repeated return to early passage stocks to avoid acquisition and enrichment of genetic changes selected through culture adaptation.

4.4.1 Epigenetic Characterization and Regulation

Pluripotent stem cells are governed by a network of transcription factors whose own transcription is regulated at the epigenetic level, notably by histone modification. In general terms the core pluripotent complex acts by repressing developmental genes, particularly though upregulation of polycomb proteins. A subset of the polycomb group (including Ezh2 as methylase) complex with the dimethylated histone 3 lysine 27 (H3K27me2) to induce trimethylation and hence repression of developmentally regulated genes (Shirane et al. 2016; Boyer et al. 2006). The trithorax proteins, particularly Wdr5, interact with H3K4me2 converting it to me3, an activating mark which is found associated with active genes in pluripotent stem cells (Ang et al. 2011). The demethylation of the inhibitory H3K9me3 mark is also involved in the maintenance of pluripotent cells (Becker et al. 2016). Additionally, histone acetylation is important, although less well documented, and histone deacetylase inhibitors have been shown to improve reprogramming from somatic cells. It has been proposed that pluripotent stem cells are poised for differentiation since they carry bivalent marks, both activating H3K4me3 and inhibiting H27K27me3 (Steward et al. 2006; Hochedlinger and Plath 2009), and this forms another very characteristic feature of hESCs. This is combined with and interacts with DNA methylation: globally DNA is hypomethylated (Hackett and Surani 2014) although the situation is far more complex with differences between pluripotent cells and somatic in the methylation at CpG islands (Lister et al. 2009) and a predominance of hydroxymethylated cytosine (Krishnakumar and Blelloch 2013).

4.5   Assessing Functional Pluripotency

To test PSC lines for pluripotency, it is important to confirm ability to give rise to cells of all three germ layers: ectoderm, mesoderm, and endoderm. In mice, it is possible to do this directly by testing chimera formation, germline transmission, and

tetraploid complementation in vivo. For ethical reasons, it is not possible to conduct similar experiments with human PSCs although injection of hESCs (and hiPSCs) into gastrula stage mouse embryos suggests some early tissue integration is possible (Mascetti and Pedersen 2016). The closest alternative to chimera assays in the hPSC field is the teratoma formation assay (Damjanov and Andrews 2016). In vitro assays for determining functional pluripotency involve spontaneous differentiation with EB formation or directed differentiation. Alternative 'omics or similar assays for determining pluripotency have been developed in recent years.

4.5.1 Teratoma Formation Assay

The teratoma assay is a qualitative assay that involves injecting PSCs most commonly under the kidney capsule or testis capsule or subcutaneously around the scapula or flank of an immune-deficient host mouse (Gertow et al. 2007). Over the weeks, the PSCs form differentiated tumor-containing cells from the different germ lineages, often forming higher-order organoids and tissues through a combination of signals from the three-dimensional environment, cell to cell interactions, and exposure to morphogens (Przyborski 2005). Much of the tissue is immature but recognizable; e.g., cartilage, bone, neuroepithelium, kidney tubules, and gastrointestinal tract tissues can be recognized (Thomson et al. 1998; Gertow et al. 2004, 2011; Damjanov and Andrew 2016). Histological analyses are performed to assist in the identification of tissues, and employment of a fetal pathologist is useful since many of the tissues are fetal-like (Lim et al. 2015). Also, donor and host cells have been known to form combined structures and histology cannot differentiate donor versus host cells. In situ hybridization, immunostaining or transcriptomic analyses need to be undertaken to definitively show that the donor hESCs are capable of forming the specific tissues of interest. Human nuclear antigen and human cadherin-1 may be used to aid donor cell identification (Vescovi et al. 1999; Heins et al. 2004). Markers commonly used to detect ectodermal tissue include class III β-tubulin (TUJ1) for neurons, keratin for keratinocytes, and dopamine beta-hydroxylase for adrenal cells. Markers used to detect mesodermal derivatives include smooth muscle actin for myocytes, cartilage matrix protein for the bone, alcian blue for the cartilage, kallikrein 1 for the kidney, Wilms tumor protein for the kidney/urogenital tract, and cardiac muscle α-actin for the heart. For endoderm tissue, α-1 antitrypsin and insulin/C-peptide, which are expressed by hepatocytes and the pancreas, respectively, are often used (Przyborski 2005).

The teratoma assay is considered the gold standard for demonstrating pluripotency of human PSCs because it shows the pluripotent lineage range under physiological conditions and actual tissue formation and has been used for the characterization of many newly derived hESC and iPSC lines (Thomson et al. 1998; Yu et al. 2007; Tannenbaum et al. 2012; Ye et al. 2017). However, less than half of the published ESC and iPSC lines have been validated using teratoma assays (Muller et al. 2010), and the published teratoma assay methods vary greatly in terms of the number and preparation of cells, the site of injection, and the length of the incubation period. Cells can respond to their direct environment, preferring to differentiate into specific lineages depending on the injection site (Miyazono et al.

1995; Wakitani et al. 2003; Gertow et al. 2004). Since the injection site, cell number, and incubation period can all influence the outcome, and results are not quantitative, teratoma assays are vulnerable to inconsistencies due to differences in protocol, making it difficult to interpret and compare published teratoma studies.

Other disadvantages of the teratoma assay are that teratoma formation is lengthy non-quantitative and time-consuming and carries the heavy cost of housing and monitoring of host mice for the duration of the experiment (Muller et al. 2008). Thus, its utility and position as a gold standard have been called into question, with many turning to lower-burden, time-saving alternatives which avoid animal use such as in vitro differentiation assays and high-throughput profiling coupled with computer predictions (Muller et al. 2011; Buta et al. 2013; Roost et al. 2015; Tsankov et al. 2015; Avior et al. 2015) (Sect. 4.5.3).

4.5.2   Embryoid Body (EB) Formation

In vitro, hESCs in suspension can undergo spontaneous differentiation by forming EBs. EBs form when PSCs grown in suspension are deprived of pluripotency signals and start to differentiate in suspended aggregates (Itskovitz-Eldor et al. 2000; Peterson and Loring 2012). Initially, PSCs form simple EBs and densely packed aggregates of hESCs, and with time these form large cystic EBs with a lumen similar to the proamniotic cavity in the murine epiblast. EBs can be formed in a number of ways (Kurosawa 2007). The simplest method for generating EBs involves scoring PSC colonies or using mild trituration to generate smaller cell aggregates and culturing them in a nonadherent polystyrene dish (Marti et al. 2013). This results in aggregates with heterogeneous shapes and sizes that lead to asynchronous differentiation and development (Bauwens et al. 2008). Alternatively, the hanging drop method that involves harvesting hESCs as a single-cell suspension in the presence of ROCK inhibitor and allowing a defined number of cells to collect and aggregate at the bottom of the drops generates EBs with homogeneous and defined sizes (Watanabe et al. 2007). EBs can also be formed through forced aggregation, where prescribed numbers of single cells are placed in round-bottom wells or triangular microwells and cells are allowed to collect through gravity or centrifugation (Ng et al. 2008; Nakazawa et al. 2013).

Fully formed EBs can be allowed to spontaneously differentiate or can be used for directed differentiation. EBs kept in suspension or transferred onto an adherent surface and grown without FGF2 over 7–21 days will differentiate spontaneously (Kaur and Tilkins 2013; Lin and Chen 2014). Thereafter, immunostaining or RT-PCR of lineage-specific markers is used to confirm tri-lineage differentiation. Commonly used markers include TUJ1 for (neur)ectoderm, α-smooth muscle actin (α-SMA) for mesoderm, and alpha-fetoprotein (AFP) or GATA4 for endoderm (Skalli et al. 1989; Katsetos et al. 2003; Kwon et al. 2006). At present, there are no standard methods for generating, differentiating, or analyzing EBs. Different methods, media compositions, and the size of EBs can all influence the differentiation trajectory (Kurosawa 2007; Bauwens et al. 2008). For instance, the forced aggregation method in microwells simulates hypoxic conditions and tends to favor cardiac differentiation (Nakazawa et al. 2013), which has been exploited to direct differentiation into cardiomyocytes (Pesl et al. 2014). EBs generated using different methods have

also been used for the directed differentiation, e.g., to pancreatic cells (Phillips et al. 2007), osteoclasts (Grigoriadis et al. 2010), cartilage (Koay et al. 2007; Toh et al. 2007; Hwang et al. 2008), skeletal muscle cells (Hwang et al. 2013), and dopaminergic neurons (Datta et al. 2013).

4.5.3 Gene Expression Approaches

The methods described in the previous three sections are time-consuming and mostly qualitative. Gene expression profiling can be performed to confirm a cell type or cell state. Transcriptome studies are generally performed using qPCR, microarrays, or RNA sequencing. Recent studies have investigated using gene expression signatures as a more quantitative, efficient way to assess the quality and potential of hPSCs, resulting in the development of novel in silico tools that can be used to characterize stem cells. Examples of these platforms are briefly described in this section: PluriTest (Muller et al. 2011), ScoreCard (Tsankov et al. 2015), TeratoScore (Avior et al. 2015), and KeyGenes (Roost et al. 2015).

The teratoma assay gives a qualitative assessment of germ layer contributions to the ES-derived tissue, but it is also possible to quantitatively assess the differentiation potential of hPSCs by evaluating the gene expression pattern in a teratoma using the TeratoScore online tool (Avior et al. 2015). PluriTest can be used to assess the pluripotency of cells with a high degree of sensitivity and specificity based on gene expression profiles (Muller et al. 2011). The ScoreCard assay can also be used to predict the in vitro lineage bias of PSC lines; it uses qPCR measurements of a set of 96 genes to evaluate the molecular signature of pluripotency and expression signatures that indicate functional pluripotency (Tsankov et al. 2015). The KeyGenes algorithm evaluates tissue differentiation efficiency in vitro and can be used to assign developmental stages to differentiated hPSC derivatives (Roost et al. 2015). None of these technologies have yet become a "standard" method of characterizing pluripotency, either by research labs or across different stem cell banks, and currently standard metrics for pluripotency are typically antibody-based assays which vary significantly lab to lab ("Assessing Pluripotent Cells").

5 Alternative Sources of Cells for Regenerative Medicine

Although hESCs are the only cell lines which are pluripotent from their initial derivation, other pluripotent stem cell lines are important in regenerative medicine and disease modeling, such as iPSCs and the multipotent mesenchymal stem cells (MSCs). Though each of these cell types is applicable in regenerative medicine, for the purposes of this chapter, we will focus on the relative advantages and disadvantages in the application of hESCs and iPSCs.

5.1 Induced Pluripotent Stem Cells (iPSCs)

Although pluripotency is only naturally present in embryonic stem cells, both multipotent stem cells and terminally differentiated somatic cells can be returned

to the pluripotent state through reprogramming, either by the induced expression of transcription factors or by treatment with small molecules ("IPSC Book Chapter"). iPSCs therefore offer an excellent method of achieving the developmental potential of hESCs but without the prerequisite of having to acquire these cells from blasto-cyst-stage embryos.

Since the discovery of inducible pluripotency via expression of the 'Yamanaka transcription factors' (Oct4, Sox2, Klf-4, cMyc), much work has been done to establish the functional and molecular differences between hESCs and iPSCs (Takahashi and Yamanaka 2006). All pluripotent stem cell lines are, by definition, able to generate cells of the three germ layers; however it has been demonstrated that different hESC and iPSC cell lines may exhibit biases in their ability to form different tissue types (Mehta et al. 2010). This has been demonstrated both in the relative proportions of each lineage produced after differentiation in unbiased EB or teratoma experiments and also in their relative efficiency at forming cell types by directed differentiation (Mikkola et al. 2006; Burridge et al. 2007; Kim et al. 2010) although the diverse genetic background of these cells needs to be taken into account.

During the course of development from pluripotent hESCs and ICM cells to terminally differentiated somatic cells, major changes in the epigenetic signature of these cells take place in order to permit or restrict transcription of genes relevant to each cell type. When somatic cells are reprogrammed by transcription factors, this epigenetic architecture has to be overhauled in order to reestablish pluripotency (Maherali et al. 2007); however, cells retain a considerable amount of the genomic methylation profile of their tissue of origin. This carry-over of somatic methylation profiles into the resulting iPSC has been termed "epigenetic carry-over" and leads to detectible differences in transcription, resulting in observable differences in functional pluripotency (Kim et al. 2010; Ohi et al. 2011). Indeed, it has been demonstrated that iPSCs more readily differentiate back into their tissue of somatic origin than to other tissues (Kim et al. 2010). Interestingly the carry-over of somatic cell of origin-memory in iPSC cell lines has been shown to diminish with increased passage number (Kim et al. 2010; Polo et al. 2010). One study demonstrated that at passage four, the transcriptome profile of iPSCs clearly clustered by their somatic origin; however by passage 16 these same cell lines were transcriptionally indistinguishable (Kim et al. 2010).

This epigenetic carry-over is not necessarily a bad thing for regenerative medicine but has consequences for the application of iPSCs. While the ideal iPSCs would be functionally indistinguishable from hESC cell lines, biased iPSC lines from different somatic origins could be employed to increase the efficiency of generating certain cell types, particularly those that are currently proving more challenging to achieve. Unfortunately for both regenerative medicine and in vitro disease modeling, the necessity to increase passage number to overcome this bias only adds duration to an already long process of generating lines. It may also lead to an increased likelihood of culture adaptation and/or selection of advantageous adapted karyotypic variants (Gokhale et al. 2015; International Stem Cell Initiative et al. 2011).

While both hESCs and hiPSCs have great value in regenerative medicine applications, there are advantages and disadvantages associated with each cell type, as illustrated in the succeeding table. The choice between hESCs and hiPSCs will also depend on whether autologous or allogeneic cells are needed, the former only being available for patient iPSCs. As hiPSC reprogramming takes weeks, existing cells made from HLA-matched clinical-grade hESCs may be better utilized, for acute applications such as a heart attack or skin burns. For chronic conditions such as diabetes, where time is less intensive, there may be a benefit in deriving hiPSCs from a patient and utilizing these for therapies, as they should be fully histocompatible. On the other hand, if such chronic diseases are of genetic origin, then gene correction of patient iPSCs would be needed, increasing the risks in culture time and cost. Patient-specific therapies of this type will present challenges to current models of healthcare funding.

	hESCs	hiPSCs
Advantages	Low cost	Easy to obtain
	Well established and characterized	HLA histocompatibility
	Realistic HLA spectrum	Disease modeling possible
	MHC downregulation possible	Drug/toxicity profiles possible
		Autologous use possible
Disadvantages	Immunosuppressants may be needed	Cost of reprogramming cells for individualized therapies
	Tissue rejection	Retroviral gene delivery
	Carcinogen risk	Oncogene activation risk
		Mutagenesis risk
		Retention of alterations
		Inability (time and expense) to characterize patient-specific hiPSC lines as fully as a banked HLA-matched line

5.2 Histocompatibility

A major issue for the use of hPSCs in regenerative medicine is histocompatibility. Unless a transplant is tissue matched to the patient, the use of antirejection medications is necessary to prevent the transplanted tissues from being rejected by the host immune system, as used for many years in organ transplant programs. iPSC and MSC lines can be readily generated from a particular patient and as such can theoretically be derived, expanded, and differentiated into the desired cell or tissue type and then reintroduced into the same patient. This methodology has outstanding potential for the field of regenerative medicine as such personalized therapies would drastically reduce the risk of rejection. Personalized hESC lines can also be derived,

by transferring somatic cell nuclei from a patient into an enucleated donor oocyte (known as somatic cell nuclear transfer or SCNT) which reprograms the nucleus to develop a blastocyst with a conventional ICM from which a hESC line genetically identical to the somatic nucleus can be derived (Wolf et al. 2016; Tachibana et al. 2013). Up until now the supply of donated oocytes has limited serious consideration of this method for application in regenerative medicine, but the recent demonstration that mouse ESCs and iPSCs can be differentiated into functional oocytes (Hikabe et al. 2016) may change this in the future should the technology prove transferable to human oocytes.

While promising, individualized therapies come with the caveat of having to successfully derive and rigorously quality- control several iPSC lines per patient to confirm that the cells are high quality, safe, and functionally viable. Such personalized iPSC therapies would therefore always come at an increased cost and time required to produce clinically relevant cell numbers of high-quality cells compared to being able to utilize a central bank of rigorously screened and characterized lines.

A variety of methods have been investigated to attempt to avoid the logistical issues caused by generating personalized stem cell lines for each patient. Immunosuppression has been used widely for transplantation for decades; however, as immunosuppression is both a nonspecific and long-term therapy, it is associated with an increased risk of both opportunistic infections and of malignancies overcoming a weakened immune system. Additionally, immunosuppression is not a guarantee of long-term success, and the proportion of transplantations which eventually fail despite immunosuppression increases with the degree of HLA mismatch between patient and donor (Williams et al. 2016). It is also notable that hESC-derived retinal pigment epithelium (RPE) cells have been transplanted safely and successfully without concern for histocompatibility and without immunosuppression (Schwartz et al. 2015). Healthy RPE is typically considered to be immune-privileged; however whether this privilege extends to the diseased conditions in which RPE transplantation is necessary, or indeed when the retinal barrier is breached during RPE cell transplantation, is a subject of debate (Whiting et al. 2015).

A possible solution to this problem of histocompatibility lies in generating a pool of high-quality stem cell lines which are compatible with a vast majority of the population. It has been estimated, and later demonstrated, that a PSC bank of only 150 selected homozygous HLA-typed cell lines would be required to HLA match the 93% of the UK population with zero HLA mismatch (Taylor et al. 2005, 2012). By collecting, HLA typing, expanding, and quality controlling this limited number of cell lines with a suitably immunologically diverse range of HLA types (Solomon et al. 2015), it would be possible to avoid the increased costs of personalized therapies while still reaping the benefits of HLA-matched transplantation.

Alternatively the issue of HLA matching could be bypassed entirely by generation of HLA universal cells. It has recently been postulated that by silencing expression of major histocompatibility complexes I and II (MHCI and II, the major proteins which specify immune compatibility) in pluripotent stem cells, the regenerative medicine cell products could be made to be "HLA universal," that is,

able to be transplanted into any host without the risk of host rejection (Figueiredo and Blasczyk 2015). An excellent test case for this technology is in the mass production of HLA-universal platelets. After repeated platelet transfusions, patients can develop anti-HLA antibodies which result in an insufficient platelet count after transfusion (Schiffer 2001). As demonstrated in mouse models by Borger and colleagues (2016), the differentiation of megakaryocytes from iPSCs in which HLA Class I has been silenced allows for the development of functional HLA-universal platelets which can bypass the problem of immune action against trans-fused platelets. Problematically, complete silencing of the HLA Class 1 in cells leaves them vulnerable to the innate immune response of natural killer (NK) cells. Some groups are endeavoring to overcome this problem by forcing expression of minimally polymorphic HLA-E molecules, an approach which has been demon-strated to be highly successful by Gornalusse and colleagues (2017). Even if further advancements in this method prove successful, there are a variety of stumbling blocks to overcome especially as other antigens are involved in rejection, not just the MHC complex (French et al. 2015). However, there remains potential for the development of universal donor hESC cell lines which, after thorough quality control and safety assessments, could circumvent the limitations of individualized therapies utilizing iPSCs and MSCs. High-quality HLA universal hESCs could be produced en mass, to be differentiated into the tissues required for therapies at a fraction of the cost of doing so for individual patients. Some groups are endeavoring to generate universally compatible PSC lines which would not be recognized by the immune system (e.g., Gornalusse et al. 2017) to solve this problem.

6   Translating ES Cells for Therapy

6.1   Scaled-Up Culture of hESCs

In order to use hESCs for regenerative medicine, it will be necessary to generate a significant number of cells, depending on application. For some applications, for example, for blood cell transplantations, as many as 10^8 healthy, pluripotent stem cells may be required, which is a considerable scale-up from a typical six-well plate culture of 10^6 cells. For other applications the numbers needed are significantly smaller, e.g., for the recent hESC-retinal pigment epithelium, as few as $5–15 \times 10^4$ cells were used per eye (Schwartz et al. 2015). Any viable culture system for hESC scale-up must have fully defined, xeno-free, cost-effective substrate and medium which permits a method of culture and passage easily performed by a specialized robot. Additionally, in-line characterization of the health and pluripotency of cells would be ideal in order to ensure the quality of the cells.

At the time of writing, the most promising medium/substrate combination for this purpose appears to be E8/VTN-N (Sect. 3). This combination has been made available commercially by two independent companies (Life Technologies, Califor-nia, USA; STEMCELL Technologies, Vancouver, Canada) and is in wide use within the scientific community. Cells cultured adherently in E8/VTN can be passaged

either in aggregates or in single cells, with the caveat that hESCs passaged as single cells in E8/VTN require a 24-h incubation in ROCK inhibitor at plating in order to maintain viability (Wang et al. 2013). E8/VTN-N has also demonstrated success in stirred microcarrier culture systems which are another potential avenue for increasing the efficiency of pluripotent stem cell culture scale-up (Badenes et al. 2016).

Several groups have demonstrated successful expansion of hPSCs in stirred-tank bioreactors either as aggregates in suspension or as adhered colonies on different types of microcarriers (Serra et al. 2010; Krawetz et al. 2010; Almutawaa et al. 2016; Kehoe et al. 2010; Chen et al. 2012). This technology may offer advantages in process scale-up over the standard two-dimensional static culture by providing a high surface area/volume ratio in a more controlled and homogeneous media microenvironment.

6.2   Automation of hESC Scale-Up

Automation of hESC culture is a crucial step in increasing efficiency of scale-up, bringing down costs while retaining or improving reproducibility and quality of cultures. A variety of cell culture robots are currently available commercially TAPBiosystems (n.d., TECAN), and companies interested in being part of the stem cell manufacture industry are pursuing their continued innovation and improvement. An ideal robot specifically for hESC scale-up in a closed system free of the risk of contamination would be able to:

– Feed cells.
– Assess the pluripotent status and health of cells.
– Assess whether cells require passage.
– Passage cells.
– Quantify passaged cell number and viability.
– Seed cells in fresh culture vessels.

Through the efforts of groups such as the AUTOSTEM consortium (AUTO-STEM – http://www.autostem2020.eu/), it is hoped that scientists will soon be able to take a sample of somatic tissue and derive and manufacture clinically relevant numbers of iPSCs without any human contact in an efficient, semiautomated manner ("IPSC Section/Chapter").

6.3   Assessing Pluripotency and Health of Cells

Methods such as immunocytochemistry, flow cytometry, and RT-PCR are well-established, and robust ways of assessing cell pluripotency and health in manual culture (see above) are already available as semiautomated systems. These methods of assessment, however, require all of the cultured cells to be passaged and a proportion to be removed and destroyed, decreasing the efficiency of scale-up.

Additionally, these methods only identify molecular markers associated with pluripotency, rather than functional pluripotency (evidenced by ability to differentiate into cells of all three germ layers). Offline methods such as teratoma formation or molecular array methods are still needed. In vitro techniques such as automated EB formation and directed differentiation are being developed by multiple laboratories to attempt to establish industry-accepted protocols and quantification techniques to confirm functional pluripotency. Directed differentiation has great potential as an upscalable functional pluripotency assessment technique. With the recent development of commercial kits, for instance, the STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies Inc., Vancouver, Canada), it seems likely that this approach will become standard to assess functional pluripotency of lines before and during scale-up.

6.4   Cell Passage

The most complex aspect of hESC culture to automate is culture passage, both the act of passaging and seeding cells and identifying when cells are ready to passage. Automated passage via trypsin dissociation has the benefit of allowing for rapid, accurate quantification of cell number and viability, allowing cells to be seeded with minimal cell or reagent wastage. Trypsin dissociation is also easily mechanized as protocols are simple and reproducible, and dissociation requires no physical contact with cells. However, single-cell passage remains stressful for cells requiring short-term ROCK inhibitor supplementation to prevent large-scale cell death. Long-term passage in E8:VTN with ROCK inhibitor supplementation only during passage has not been shown to cause karyotypic abnormalities or loss of pluripotency, but the goal is still to remove ROCK inhibitor from stem cell culture protocols and reduce cell stress during passage.

During routine culture of stem cells, pluripotency, cell health, and density are assessed daily via phase contrast microscopy. Trying to quantitate and automate the subjective assessments stem cell scientists make when assessing their cells in this way is a challenging proposition (Sect. 4.1).

6.5   Directed Differentiation

It is possible to mimic the signals that cells receive during successive stages of development, starting from the initial specification of ESCs or iPSCs into one of the embryonic germ lineages, followed by specification and patterning (Cohen and Melton 2011). Understanding and triggering the signaling pathways necessary and sufficient for induction of specific cell types in hESCs is a critical goal in cell regeneration therapy. Although the relevant signaling pathways to manipulate in vitro can be gleaned from developmental studies, directing differentiation to specific cells has proved challenging as it requires considerable optimization of the precise concentrations, timing, and combinations of growth factors and small molecules.

Numerous protocols for differentiating cells from progenitors of each of the three germ layers have been published in recent years. These include ectodermal lineages such as motor neurons (Wichterle et al. 2002), cerebral cortex neurons (Shi et al. 2012), forebrain interneurons (Liu et al. 2013; Nicholas et al. 2013), and cortical interneurons (Maroof et al. 2013); mesodermal lineages such as chondrocytes (Oldershaw et al. 2010), cardiomyocytes (Burridge and Zambidis 2013; Lian et al. 2013), and renal cells (Xia et al. 2013; Takasato et al. 2014); and endodermal lineages such as hepatocytes (Cai et al. 2007; Roelandt et al. 2010), intestinal cells (Spence et al. 2011), lung cells (Kadzik and Morrisey 2012; Huang et al. 2014), foregut epithelia (Kearns et al. 2013), and pancreatic cells (Van Hoof and LIKU 2013). Cell biologists are also interacting with tissue engineers to generate culture systems that will more accurately mimic important three-dimensional aspects of organogenesis. Indeed, some laboratories have taken the PSC differentiation process a step further to generate organoids or other three-dimensional structures that mimic human tissues (Mccracken et al. 2011; Spence et al. 2011; Lancaster et al. 2013; Tieng et al. 2014; Sinagoga and Wells 2015).

The starting material for directed differentiation may be cell aggregates similar to those used during spontaneous differentiation or PSC monolayers that are technically simpler to generate. During early development the pluripotent cells of the ICM differentiate during gastrulation, resulting in the formation of ectoderm, mesoderm, and endoderm progenitors. Many directed differentiation protocols mimic this using growth factors or small molecules to induce the conversion of ESCs or iPSCs into cells of the appropriate progenitor that gives rise to the desired cell type.

Developmental studies have revealed that variations in the signal intensity of members of signaling molecule families are required in the establishment and patterning of the germ layers in vivo: transforming growth factor-β (TGFβ) superfamily (including activin/nodal and bone morphogenetic proteins, BMPs) (Moustakas and Heldin 2009), the WNT family (Rao and Kuhl 2010), and fibroblast growth factors (FGFs) (Turner and Grose 2010). However, since the precise concentration of each factor to which a developing cell is exposed in the human embryo and the period of exposure are unknown, each candidate factor has to be tested at various concentrations and applied for various durations. For hESCs, transient Wnt followed by lower concentrations of the TGFβ family member activin A induce mesoderm lineage initiation (Yang et al. 2008; Oldershaw et al. 2010), whereas higher activin A concentrations induce endoderm formation (Kroon et al. 2008; Vallier et al. 2004). BMP4 is also required for efficient mesoderm induction (Yang et al. 2008). Noggin and DKK1, or small-molecule antagonists of endogenous BMP and WNT signaling, respectively, are used for ectoderm induction of hESCs (Lamba et al. 2006; Oshima et al. 2010; Efthymiou et al. 2014).

Following successful induction of germ layer progenitors, growth factors and signaling molecules, often in combination with small molecules, have been used to direct the cells further along the desired differentiation pathway. Researchers exploit the fact that similar signaling pathways are used at different stages and for different purposes during development. By sequential use of combinations of signaling molecules with titration of concentration and duration of application, a wide variety

of cell fates can be generated. Recent research indicates that early temporally refined stimulation of individual selected growth factor pathways (TGFβ, BMP, FGF, Wnt, and Hedgehog) combined with strategic pathway inhibition to remove unwanted lineages regulates binary switches in lineage commitment and can direct to specific mesodermal lineages in 3–5 days (Loh et al. 2016).

Recombinant factors are often produced in engineered bacterial or mammalian cells, traces of which may contaminate the final preparation, thus preventing their use in clinical application. Furthermore, the high cost of recombinant growth factors may limit their application in larger-scale differentiation procedures. Small molecules which activate or block signaling pathways offer an attractive alternative; they are more stable, less expensive, have less lot-to-lot variability, and are generally non-immunogenic. Hence, cells differentiated using small molecules might be more suitable to therapeutic transplantation than those treated with recombinant proteins (Ding and Schultz 2004; Rubin 2008). As a result, high-throughput chemical screening approaches have been used to identify novel molecules that produce the desired effect without the use of growth factors (Ding and Schultz 2004). An example of such a screen that aimed to identify small-molecule inducers of endoderm to replace activin A produced two molecules termed inducer of endoderm-1 and endoderm-2, both of which induced endoderm more robustly than activin A treatment by activating the TGFβ signaling pathway (Borowiak et al. 2009). However it is unlikely that all components of a differentiation pathway will be able to be replaced by small molecules.

Small-molecule agonists and antagonists of the Hedgehog pathway have proved very effective for motor neuron differentiation (Frank-Kamenetsky et al. 2002; Wichterle et al. 2002). Similarly, SB-431542, which antagonizes the nodal receptors ALK4, ALK5, and ALK7 (Laping et al. 2002), can substitute for protein antagonists of TGFβ in the differentiation of neurons and hepatocytes from hESCs (Smith et al. 2008; Chambers et al. 2009; Touboul et al. 2010). Some small molecules, such as KAAD-cyclopamine (D'Amour et al. 2006; Chen et al. 2009) and SU5402 (Oshima et al. 2010; Turner and Grose 2010), inhibit signaling through pathways for which an endogenous inhibitor is not known (Hedgehog and FGF signaling, respectively). Endogenous small molecules with roles in embryonic development may also be used in vitro to induce differentiation. For example, retinoic acid, a morphogen that is important in the patterning of the central nervous system in vivo (Oshima et al. 2010), has been used successfully to generate retinal cells from hESCs (Osakada et al. 2009). Similarly, the naturally occurring small molecule taurine has been used to direct the differentiation of retinal cells (Osakada et al. 2009).

Many directed differentiation protocols have a relatively low efficiency of desired cell generation, and there are safety and cost concerns posed by the reagents used to direct cell fate. It is important that hESC-derived progeny are functionally mature. It has been shown that differentiated products of hESCs and iPSCs retain an immature phenotype even when apparently terminally differentiated (Patterson et al. 2012). Although it is critical to find the appropriate culture conditions and microenvironments to ensure sufficient maturity and functionality of hESC-derived progeny, some plasticity may actually be an advantage for adaptation and continued differentiation

of transplanted cells ("Translation"). Thus, regardless of the protocol used for directed differentiation, it is necessary to monitor the efficiency, specificity, and functional maturity achieved by the various differentiation protocols. Finally, demonstrating that cells produced in vitro are functionally equivalent to those produced in vivo remains a challenging but essential element of any directed differentiation protocol.

Extracellular matrix-mediated signals include mechanical stimuli such as strain, shear stress, substrate rigidity, and topography, all of which have an impact on stem cell phenotype (Kshitiz et al. 2012). Therefore, the appropriate culture environment includes both precisely controlled biochemical and biophysical signals, some of which are provided by 2D or 3D matrices, to guide hESC differentiation toward specialized cells and development of functional tissue substitutes. For instance, substrate stiffness and application of uniaxial strain affect differentiation of mesenchymal stem cells down to osteogenic and chondrogenic lineages (Engler et al. 2006; Kurpinski et al. 2006), suggesting that the biophysical environment would also play a role in the production of osteoblasts and chondrocytes from hESCs. For bone tissue engineering, osteogenic cells are combined with biomaterial scaffolds and signaling molecules and, in some cases, subjected to dynamic in vitro culture in bioreactors in order to construct three-dimensional bone substitutes (De Peppo and MAROLT 2013; Curtis and Riehle 2001).

6.6 Directing Differentiation to Specific Lineages

Due to space constraints, it is not possible to detail here all of the directed differentiation protocols in the literature. Instead, we highlight some of the most successful protocols that have been developed so far, using one example cell type for each of the germ layers: dopaminergic neurons (ectoderm), pancreatic beta cells (endoderm), and chondrocytes (mesoderm), with an example of a directed differentiation protocol for each of these shown in Fig. 4.

6.6.1 Generation of Dopaminergic Neurons from hESCs

Stem cell therapy is being explored as a clinically viable treatment option for a number of CNS disorders (Bjorklund and Lindvall 2000). Here we summarize the work undertaken to generate dopaminergic neurons for cell replacement therapy for Parkinson's disease (PD). PD is a neurodegenerative disorder caused by decreased stimulation of the motor cortex due to progressive degeneration of dopaminergic neurons of the substantia nigra, leading to motor dysfunction characterized by muscle rigidity, tremor, bradykinesia, and akinesia (Kalia and Lang 2015). Clinical improvements have been reported following the transplantation of fetal mesencephalic grafts into PD patients (Lindvall and Bjorklund 2004; Barker et al. 2013; Kefalopoulou et al. 2014). However, several ethical and logistical issues with the use
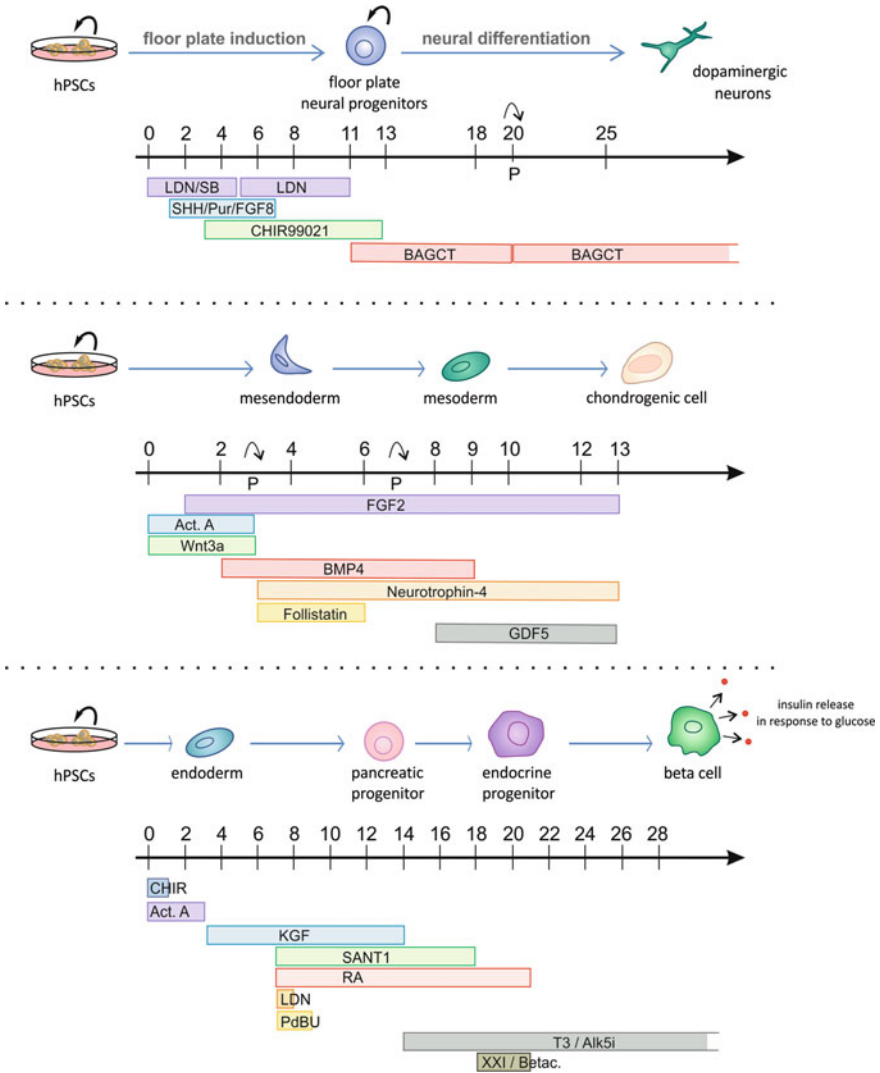
Fig. 4 Examples of directed differentiation protocols for dopaminergic neurons (Kriks et al. 2011), chondrogenic progenitors (Oldershaw et al. 2010), and pancreatic beta cells (Pagliuca et al. 2014). *LDN* LDN193189, *SB* SB431542, *SHH* purmorphamine + SHH C25II, *BAGCT* BDNF + ascorbic acid + GDNF + dbcAMP + TGFβ3, *FGF2* basic fibroblast growth factor, *Act. A* activin A, *BMP* bone morphogenetic protein 4, *GDF5* growth/differentiation factor 5, *CHIR* CHIR99021, *KGF* keratinocyte growth factor, *RA* retinoic acid, *SANT1* Sonic Hedgehog pathway antagonist, *PdbU* phorbol 12,13-dibutyrate, *Alk5i* Alk5 receptor inhibitor II, T3, triiodothyronine, *XXI* g-secretase inhibitor, *Betac* betacellulin

of aborted fetal tissue limit this approach. hESCs may provide an alternative source of dopaminergic neurons for transplantation (Kim et al. 2002; Perrier et al. 2004; Lindvall and Kokaia 2009; Barker 2014). Using protocols based on extrinsic patterning cues that mimic fetal midbrain development, it is now possible to generate dopaminergic neurons with an authentic midbrain phenotype from human PSCs that survive transplantation and can restore motor deficits in animal models of PD (Kriks et al. 2011; Kirkeby et al. 2012; Grealish et al. 2014).

Early studies showed that through the combined use of FGF8 and Sonic Hedgehog, hESC-derived neural progenitors could be differentiated in vitro into dopamine neuron-like cells expressing tyrosine hydroxylase, which is required for dopamine synthesis, and transcription factors such as Pax2, Pax5, and En1 that are indicative of dopamine synthesis (Perrier et al. 2004; Yan et al. 2005; Martinat et al. 2006), in addition to demonstrating the electrophysiological properties of midbrain dopamine neurons (Kim et al. 2002, 2007). However, most of the early protocols included undefined reagents due to co-culture with stromal cell lines or the use of conditioned media, thus limiting their translational potential. As a result, subsequent studies focused on generating dopaminergic neurons from hESCs using completely defined factors (Lukovic et al. 2017 SCTM).

Human ESCs can be differentiated into tri-lineage neural progenitors that are capable of giving rise to neurons, astrocytes, and oligodendrocytes by growing them as neurospheres in the presence of EGF and FGF2 (Joannides et al. 2007). A commonly used protocol for inducing PAX6+ neural ectoderm with >80% efficiency involves dual-SMAD inhibition, inhibiting the BMP and TGFβ pathways in a PSC monolayer using Noggin and the small molecule SB431542 (Chambers et al. 2009).

The midbrain floorplate contains A9 dopaminergic neurons that are lost in PD. In the developing midbrain, the floor plate marker FOXA2 and the roof plate marker LMX1A are co-expressed. Floor plate precursors have been derived from hESCs (Fasano et al. 2010) using a modified dual-SMAD inhibition protocol (Chambers et al. 2009). To induce LMX1A expression in FOXA2+ floorplate precursors derived from hPSC, Kriks et al. utilized small-molecule activators of Sonic Hedgehog and canonical WNT signaling using a time-specific application of GSK3β inhibitor CHIR99021 (Kriks et al. 2011). This induced LMX1A expression and neurogenic conversion of hPSC-derived midbrain FOXA2+ floorplate precursors toward dopaminergic neuron fate. Following floor plate induction, further maturation was carried out in Neurobasal/B27 medium supplemented with ascorbate, BDNF, GDNF, TGFβ3, and dbcAMP, generating engraftable midbrain DA neurons by day 25 that could be maintained in vitro for several months (Kriks et al. 2011).

Although much progress has been made in generating human dopaminergic neurons, several issues remain to be addressed before stem cell-based therapies will be feasible for PD. Differentiation protocols need to be further optimized to reliably produce pure and homogeneous populations of midbrain dopaminergic neurons that survive, reinnervate, and restore dopaminergic neurotransmission in the striatum. Long-term efficacy in vivo and safety of the procedure need to be fully assessed. Furthermore, patient selection, postoperative rehabilitation, and immunosuppression need to be optimized to maximize therapeutic benefits.

6.6.2   Generation of Pancreatic Beta Cells from hESCs (Endoderm)

Pancreatic beta cells regulate metabolic homeostasis by sensing glucose and producing the glucose-lowering hormone insulin. Type 1 diabetes mellitus is a metabolic disease in which the loss of pancreatic beta cells results in elevated blood glucose levels. Currently, type 1 diabetes is managed through daily injection of insulin (or through insulin pumps) matched to meals; however exogenous insulin replacement does not adequately control glucose levels, resulting in complications such as retinopathy, nephropathy, neuropathy, and increased risk of cardiovascular disease.

In this section we highlight recent progress in directed differentiation of hESCs toward a beta cell fate. Human PSCs, with their vast expansion capacity and differentiation potential, might provide sufficient fully functional insulin-secreting beta cells to treat diabetes. Importantly, replacement can occur away from the pancreas, meaning that cells could be transplanted subcutaneously in minimally invasive surgeries.

Directed differentiation studies have attempted to mimic pancreatic islet cell development, first focusing on the production of pancreatic and duodenal homeobox 1 (PDX1)-positive pancreatic endoderm with increasing use of defined culture conditions in later studies (Pagliuca and Melton 2013). The first step in beta cell generation is endodermal specification (D'Amour et al. 2005). After endodermal specification, signals from adjacent developing tissues induce specification of pancreatic progenitors that have the potential to generate all three pancreatic cell types: ductal, acinar, and endocrine. After selection of endocrine fate, endocrine progenitors are specified to become insulin-producing cells.

Several protocols generated beta cells that were responsive to direct depolarization, but not to high glucose concentrations (Basford et al. 2012; Takeuchi et al. 2014; D'Amour et al. 2006) meaning that these cells lacked the coupling of glucose influx to electrical activity. Similar protocols were developed to enhance differentiation into PDX1+ and Nkx6.1+ pancreatic progenitors through the addition of BMP inhibitors and protein kinase C (PKC) activators (Kroon et al. 2008; Chen et al. 2009; Nostro et al. 2011; Rezania et al. 2012). With the idea that the final stages of differentiation could be completed naturally in vivo, several groups pursued a strategy of transplanting PDX1-positive progenitor cells. One of the key advances in this field was the demonstration that the pancreatic progenitors generated can further differentiate into functional, glucose-responsive β-cells that co-express insulin and key transcription factors (PDX1, NKX6.1, and MAFA) in vivo when transplanted into a mouse and are sufficient to restore regular glucose levels in murine models of diabetes (Kroon et al. 2008; Rezania et al. 2012).

These promising data paved the way for a clinical trial testing the approach of progenitor cell transplantation (Sect. 6.7). In 2014, ViaCyte received approval for a phase I/II trial (NCT02239354) to test the safety, cell survival, and insulin secretion of a product that combines progenitor cells (PEC-01) and a macro-encapsulation device called Encaptra (Schulz 2015). Encaptra is a device that encapsulates the implanted cells, protecting them from a patient's alloimmunity and autoimmunity, while allowing oxygen, nutrients, and proteins to freely transport across the system's

membrane, thus allowing the implanted cells to sense glucose in the bloodstream and release insulin accordingly.

Recently, researchers recorded the first cellular-level evidence of glucose responsiveness, albeit at a superphysiological level, in stem cell-derived beta cells (Rezania et al. 2014). However, these cells generally failed to inactivate their calcium responses, which is an essential feature of mature beta cells that prevents hypoglycemia in the postprandial state. A similar protocol was published producing functional beta cells in vitro termed SC-β (Pagliuca et al. 2014). Importantly, glucose-responsive insulin secretion was reported from 2 weeks after transplantation into immunocompromised mice. A 2016 follow-up paper reported that encapsulated SC-β cells could reverse hyperglycemia in streptozotocin-injected C57BL/6 mice (Vegas et al. 2016).

These recent publications provide the first convincing evidence of glucose responsiveness from hESC-derived beta cells in vitro *and* in vivo (Pagliuca et al. 2014; Rezania et al. 2014; Vegas et al. 2016). Exciting progress has therefore been made toward the ultimate aim of creating fully functional β-cells from pluripotent cells. However, additional studies are required to convincingly show that these are fully functional, responding to glucose in a quantitatively similar manner to healthy human primary cells. Moreover, the different assessments of beta cell functionality, showing dynamic insulin secretion in response to multiple stimuli, need to be standardized to allow direct comparison of differentiation protocols. Finally, even after fully functional pancreatic beta cells are produced, it remains to be determined whether encapsulation can protect these cells from recurrent autoimmune attack in people with type 1 diabetes (Vendrame et al. 2010; Schulz 2015).

6.6.3   Generation of Chondrocytes from hESCs (Mesoderm)

Hyaline articular cartilage is an avascular tissue that lines synovial joints, allowing bones to glide over each other with little friction. Due to its avascular nature, articular cartilage has a low intrinsic capacity to repair itself. Articular chondral lesions are a major risk factor for the development of osteoarthritis (OA), a common and debilitating joint disease (Goldring and Goldring 2007). The current treatment option for OA that reduces pain and improves function is total joint arthroplasty; however, this is not a suitable treatment option for younger patients since they would outlive their implant and require multiple surgeries (Widuchowski et al. 2007; Jones and Pohar 2012; Aggarwal et al. 2014). Guided by knowledge of development, and physiology of native cartilage, tissue engineering efforts have sought to address this problem through the generation of chondrogenic cells/tissue that can be implanted to replace damaged tissue in order to treat symptomatic patients. The aim is to circumvent the onset of OA among individuals with small lesions, so predisposed to developing it, or repair full-blown cartilage lesions. Although multiple adult cell-based strategies have been attempted to restore joint cartilage and prevent progressive degeneration, the formation and long-term maintenance of permanent hyaline cartilage have not yet been achieved (Hunziker et al. 2015).

One of the major research goals for tissue engineering for cartilage regeneration is to expand chondroprogenitors to yield large numbers without the loss of chondrogenic activity. Because of their unlimited proliferative capacity and

pluripotency, human PSCs are a suitable alternative source. Here we focus on the recent progress using development-informed paradigms to control the differentiation of hESCs to an articular chondrocyte fate.

The process of epithelial-to-mesenchymal transition mediated by recombinant activin A, BMP4, VEGF, and FGF2 treatment of hESCs gives rise to a CD326–CD56+ population of multipotent mesoderm-committed progenitors (Evseenko et al. 2010). Subsequent, stage-specific modulation of multiple signaling pathways downstream of the early mesendoderm population can recapitulate the human developmental chondrogenic program in human ESCs and iPSCs (Toh et al. 2009; Oldershaw et al. 2010; Wu et al. 2013; Cheng et al. 2014; Craft et al. 2015; Lee et al. 2015). Most early studies employed spontaneous differentiation methods to generate mesenchymal cells, followed by expansion culture using serum-containing MSC media to enrich them. In 2010, Oldershaw and colleagues developed a three-stage, chemically defined directed differentiation protocol which has been developed further by multiple groups for efficient production of differentiated chondrocytes from independent lines of hESCs and iPSCs (Wu et al. 2013; Cheng et al. 2014; Craft et al. 2015; Lee et al. 2015; Oldershaw et al. 2010). In this protocol, human ESCs were directed through a transient primitive streak/mesendoderm stage, followed by controlled differentiation to a multipotent mesoderm and subsequent differentiation of the mesoderm intermediates to chondrogenic cells arranged in aggregates (Oldershaw et al. 2010). By applying temporal supplementation of activin A, bFGF, Wnt3a, BMP4, neurotrophin-4, follistatin, and GDF5, an 8.5-fold expansion of the cell population and 95% expression of the key chondrogenic transcription factor SOX9 are achievable (Oldershaw et al. 2010). Craft and colleagues developed a protocol for chondrogenesis in 3D and provided critical insights into the signaling involved in the controlled specification of hESCs to articular versus hypertrophic chondrocytes (Craft et al. 2015). Moreover, Yamashita and Tsumaki produced scaffoldless cartilage tissue from human iPSCs by use of an extended suspension culture method, although this protocol involved the use of serum (Yamashita et al. 2015).

Developing clinically relevant protocols for directing the differentiation of hESCs into definitive, homogenous populations of chondrogenic cells still faces several challenges. Although hESCs are expandable, it may be efficient to develop methods for the expansion of hPSC-derived chondroprogenitors, which would allow for the generation of chondrocytes from intermediate batches of expandable high-quality controlled cells. Additionally we need to generate tissue in a form optimal for subsequent integration with endogenous cartilage while ensuring quality in the physical properties and long-term durability of the de novo generated cartilage tissue.

6.7   hESC Clinical Trials

The power of hESCs is to generate a platform for the manufacture of potentially all the cell types of the human body. It is the differentiated progeny of hESCs that would be utilized for regenerative medicine and drug screening/toxicity studies, not the

hESCs themselves. Transplanted hESC-derived cells should survive in the recipient without being rejected and differentiate in a site-specific manner, integrate within the target tissue and host circuitry, and restore function. A lot of work is still needed to determine at which stage of differentiation the cells will work best to repair a particular damaged or diseased tissue and how to get those cells to the right place in the body. It is important that the cells are not too immature so avoiding teratoma or more likely progenitor-derived tumor formation, but they should not be too mature either: plastic enough to respond to signals from surrounding cells and host tissue. Currently eight clinical trials using hESC-derived cells are yielding promising results (Ilic and Ogilvie 2017) involving treatment for spinal cord injury, macular degeneration of retina, type 1 diabetes, heart failure, and a hESC-derived dendritic cell for a cancer vaccine. There was a small clinical trial by Geron investigating hESC-derived oligodendrocytes for treatment of spinal cord injury; however, no official results of this trial were published (ClinicalTrials.gov identifier: NCT01217008) and the trial was discontinued for financial reasons. Subsequently, BioTime company Asterias Biotherapeutics acquired all of Geron's stem cell assets and was granted a $14.3 million Strategic Partnership Award by the California Institute for Regenerative Medicine (CIRM) to reinitiate clinical development of hESC-derived OPC1 cells in a dose-escalating trial (ClinicalTrials.gov identifier: NCT02302157). They also acquired the hESC-modified dendritic cell cancer vaccine at the same time.

hESC-derived retinal pigment epithelial (RPE) cells have been tested for treatment of Stargardt's macular dystrophy and dry age-related macular degeneration (AMD) (Schwartz et al. 2012) (ClinicalTrials.gov identifiers: NCT01344993, NCT01345006, NCT01469832), and a phase I/II trial has been approved for testing these cells for the treatment of severe myopia (ClinicalTrials.gov identifier: NCT02122159). Long-term follow-up studies of 15 years for both dry AMD and Stargardt's macular dystrophy trials in the USA are currently ongoing (ClinicalTrials.gov identifiers: NCT02463344 and NCT02445612). A clinical study of a fibrin patch embedded with hESC-derived cardiac-committed progenitors transplanted into patients with severe heart failure commenced in autumn 2014 in France (ClinicalTrials.gov identifier: NCT02057900). Following the treatment, the first patient improved and remained stable 6 months after the intervention (Menasche et al. 2015). The FDA has also approved the ViaCyte phase I clinical trial for the treatment of diabetes using hESC-derived beta cells (ClinicalTrials.gov identifier: NCT02239354) ("Pancreatic Beta Cells").

7   Conclusion

Here we have summarized the progress made to date in the field of human embryonic stem cells from generation, through culture methods and understanding their characteristics to developing strategies to control cell fate. Derivation and culture methods have been significantly improved and simplified, hESC lines can be derived from single biopsied cells of embryos without destroying the embryo, and many directed differentiation protocols are fully chemically defined and have moved away

from using components that would make them unsuitable for human use. Much work still remains to be done in terms of scaling up current protocols to produce sufficient cells for regenerative cell therapies. In addition, more efficient protocols still need to be developed that produce a high proportion of mature desired cells and selection methods to separate these from other lineages in culture without loss of product need to be improved. It is encouraging to note that several clinical trials of human ESC derivatives are under way (Trounson and Dewitt 2016; Ilic and Ogilvie 2017). The medium-term to long-term safety of hESC-derived cells has already been demonstrated in a phase I/II clinical trial (Schwartz et al. 2015) for Stargardt's macular dystrophy and atrophic age-related macular degeneration. However off-target effects still need to be researched. For successful translation, it is important to produce hESC derivatives in a scalable and GMP-compliant manner. Directed differentiation protocols are becoming ever more efficient, and with the increasing availability of GMP reagents, researchers are working toward using fully standardized protocols with xeno-free reagents and defined culture media, carried out under GMP-compliant conditions (Ausubel et al. 2011). Differentiations protocols are now available from many cell types, and hESCs will continue to contribute to the study of human development and disease and to be major tools in the future of regenerative medicine.

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