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Isolation and Characterization of Human Embryonic Stem Cells and Future Applications in Tissue Engineering Therapies

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1.1 Derivation of human embryonic stem cells from the ICM

1.1.1 Early development of the ICM: the cells of origin for hESCs

The mammalian zygote (fertilized ovum) is defined as being totipotent, as it is capable of developing into a new offspring and the placenta required for full gestation. The zygote initially undergoes cleavage-stage cell division, forming cells (early blastomeres) that remain totipotent. With further development to the preimplantation blastocyst stage, a primary cell differentiation results in outside trophectoderm cells (TE) and an inside aggregate of inner cell mass (ICM) cells. The TE forms placental tissue and membranes, while the

Principles of Stem Cell Biology and Cancer: Future Applications and Therapeutics, First Edition.
Edited by Tarik Regad, Thomas J. Sayers and Robert C. Rees.

ICM forms the foetus and extra-embryonic membranes. Therefore, ICM cells are defined as being pluripotent, forming all cells of the developing offspring other than the complete placenta (unless genetically manipulated). Embryonic stem cells (ESCs) are derived *in vitro* from ICM cells, which adapt to specific conducive conditions that enable indefinite cell proliferation (self-renewal) without further differentiation and thereby confer a pluripotent capacity. This *in vitro* pluripotent state is due principally to the induction and maintenance of expression of key 'gate-keeper' genes, including Oct4, Nanog and Sox2, which then regulate one another (Silva & Smith, 2008). The capacity for self-renewal is sustained by high telomerase activity, which protects chromosome telomeres from degradation during mitosis (Blasco, 2007).

Mammalian ESCs were first derived in the mouse (mESC) (Evans and Kaufman, 1981; Martin, 1981). When mESCs are integrated into an embryo and returned to a recipient, they can contribute to all cell lineages, including germ cells. Their utility soon became invaluable for many transgenic procedures. Successful derivation of human (hESC) lines was reported by Thomson *et al.* (1998), who essentially followed the same procedure as used for the mouse. ICMs isolated from preimplantation human blastocysts were plated on to mitotically inactivated mouse embryonic feeders in culture medium with basic fibroblast growth factor (bFGF) and foetal calf serum (FCS). This culture medium was also supplemented with leukaemia inhibitory factor (LIF), a cytokine necessary to maintain mESCs (Smith *et al.*, 1988), although (as is now known) not necessary for standard hESC derivation. Human ESCs display (or lose on differentiation) plasma membrane expression of stage-specific embryonic antigens (SSEAs) that correlate with the preimplantation morphological development of human embryos (Henderson *et al.*, 2002) and form teratomas (benign tumours) in immune-deficient mice that can contain cell phenotypes from the three major cell lineages (endoderm, mesoderm and ectoderm), as well as trophoblast. The differentiation of trophoblast cells indicates that hESCs are not entirely equivalent to mESCs, as usually defined, but align with slightly later LIF-independent mouse epiblast pluripotent stem cells, which have the propensity to differentiate to trophoblast *in vitro* (Brons *et al.*, 2007).

1.1.2 Derivation of hESCs

Success in the derivation of hESCs depends in part on the quality of the human embryos used (usually blastocysts from days 5 to 8), although cell lines have been generated from morphologically poor embryos. Numerous hESC lines have been derived (Figure 1.1) from normal, aneuploid and mutant embryos from patients undergoing treatment for assisted conception (IVF, ICSI) or preimplantation genetic diagnosis (PGD) who consent to donate them for stem cell research. Some of these cell lines have been extensively characterized and compared, enabling international standards to be established (Adewumi *et al.*, 2007).

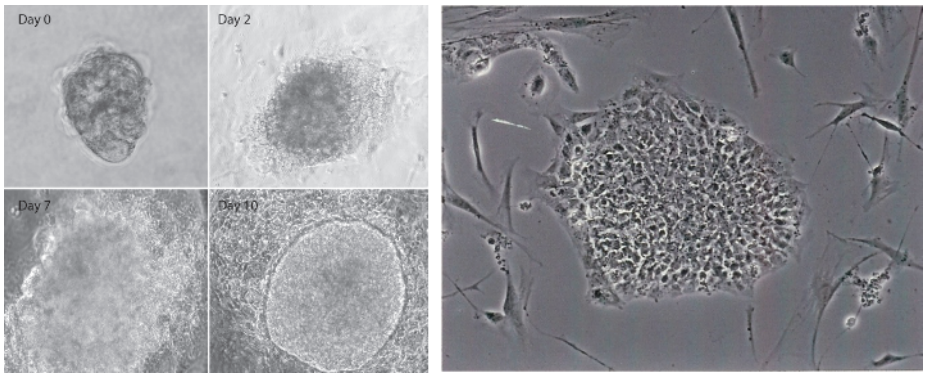

Figure 1.1 (A) Outgrowth of hESCs over 10 days of culture from ICM. In this instance, a clearly defined colony was observed by 10 days, which was mechanically passaged. (B) hESC line Shef1 plated on ECM.

1.1.2.1 Evolution to a more efficient and better-defined derivation method: drivers and technologies Over the last 15 years, continuous improvements have been made in the process of deriving and maintaining hESC lines. The emphasis initially was on improving efficiency and consistency in the stem cell laboratory. But as hESC lines have become readily available for research in many countries, the focus has changed to devising methods for deriving clinical-grade cell lines that comply with health care regulatory authorities (e.g. Federal Drug Administration, FDA; European Medicines Agency, EMA), which can be used as starting materials for potential cell-therapy trials. Xeno-free methods (free of nonhuman animal components) are preferable as they minimize the risk of cross-species contamination with adventitious agents. An important early improvement was the replacement of FCS with a serum extract (knockout serum replacement, KOSR) to reduce hESC differentiation. This modification also minimized batch variation (inherent in FCS) between culture media, and allowed consistency in the proliferation of the cells after passaging (transfer of cells to a new culture vessel). Subsequently, more defined culture media (xeno-free) have been devised, which, in combination with a variety of extracellular matrix (ECM) compositions, facilitate the proliferation and passage of pluripotent hESCs in the absence of feeder cells (mouse or human), which otherwise remain an ill-defined and inconsistent component of the cell culture. Manipulation of the embryo has also changed over time. Initially, the ICM was isolated according to mouse protocols using enzymatic (protease) removal of the zona pellucida (ECM surrounding blastocyst) and immunosurgical lysis of TE with antitrophoblast antibody to prevent TE culture outgrowth from inhibiting early ESC proliferation. However, xeno-free methods using laser-assisted removal of the zona and plating of the intact blastocyst or the ICM on to a defined matrix (e.g. laminin 521) with a defined culture medium is the method of choice, leading

to successful feeder/xeno-free cell line production in ~20–40% of attempts with good-quality human embryos (Hasegawa *et al.*, 2010). With further improvements to the cell adhesion matrix and cell medium, the efficiency of hESC line derivation is likely to increase further, although the quality of the embryo used to develop ICM cells remains a crucial factor.

Another important consideration is the genetic character and stability of the hESC line. Generally, most hESC outgrowths and initial cell lines derived from unselected embryos (i.e. not PGD selected) are determined to be karyotypically normal within the precision of the chromosomal analysis. However, hESCs acquire genetic mutations in culture, which may endow them with a selective cell culture advantage, so that mutated cells predominate (Baker *et al.*, 2007). Since derivation and ESC passage represent key stress events for ESC cultures, minimization of selective pressure on cells at these stages may help to maintain their normal karyotype. For example, the proliferation of cells by mechanical division of hESC colonies into smaller aggregates may be preferable to enzymatic disaggregation to single cells, which will initiate apoptotic stress pathways unless inhibited from doing so by a chemical inhibitor (i.e. ROCK inhibitor).

1.1.3 Regulation of embryo research and hESC derivation

The destruction of the preimplantation human embryo in order to derive hESC lines has prompted fierce ethical debate in many countries, especially on religious grounds, which to some extent remains unresolved and irresolvable. The result is the implementation of policies of ethical oversight, regulation and permission for hESC research, which vary from country to country, and even within a country (the United States). In the United Kingdom, early introduction of laws related to human embryo research and the formation of a regulatory body (Human Fertilisation of Embryology Authority, HFEA) provided a framework (and important public confidence) for continuation of hESC research. Clinical-grade hESCs must meet compliance with conditions set by the EMA and overseen in the United Kingdom by the Human Tissue Authority. In the United States, the FDA and National Institutes of Health (NIH) undertake this responsibility. Since the development of cell therapies using pluripotent stem cells is novel, it remains to be determined exactly how regulatory authorities will implement conditions of compliance.

The induction of pluripotency in mouse and human somatic cells in 2006–07 using retroviral vectors to introduce four genes to reprogramme the genome (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) and enable the derivation of induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007) radically changed the landscape of human pluripotent stem cell (hPSC) research (Yamanaka, 2012). This technology not only provides a potential route for the creation of patient-specific stem cell lines for use in cell therapies but also makes pluripotent cell lines available to many more laboratories, with seemingly

fewer ethical bottlenecks. However, hESCs remain the current gold standard as their cellular reprogramming events are those that are normally evoked in the early embryo, rather than artificially induced, and they are therefore less likely to be subject to aberrant epigenetic effects on their gene function. Moreover, ethical issues related to obtaining informed consent from donors to use tissue samples to derive iPSCs still persist. Progress in the use of hESCs (or iPSCs) for therapy will depend on whether robust protocols for their expansion and differentiation to a precise and economic manufacturing level can be devised, and a key aspect in meeting this objective is the implementation of reliable and accurate assays of cell type and quality.

1.2 Basic characterization of hESCs

Immediately following their derivation, hESCs are identified fundamentally on the basis of their indefinite capacity for self-renewal, their ability to form derivatives of all three embryonic germ layers and, usually, their ability to maintain a euploid karyotype over extended periods in culture. However, not every derivation procedure results in an established hESC line, and a variety of other cell types may grow out from isolated embryo cultures. Furthermore, hESCs may be derived at different stages of embryo development (i.e. early or late blastocyst) while still retaining pluripotency, which can alter the subsequent features of their cell population. While cell lines may be superficially similar in these aspects, they often show significant differences in stem cell surface antigen expression, DNA methylation status, X-chromosome inactivation, variation in specific gene expression, cell doubling time, and capacity to differentiate. The cause of this variation between cell lines is largely unknown, but it is likely, in part at least, to be due to the wide genetic background of human donors (mESCs, by contrast, are produced from inbred mouse strains); it also depends on environmental conditions and stresses, which can impart phenotypic changes on cells during derivation and culture. It is therefore essential that hESCs are characterized under a set of criteria which allows for accurate, valid and robust comparisons to be made both within and between laboratories. In this section, we look more closely at the characteristics that currently define hESCs.

1.2.1 hESC morphology

Human ESCs typically form compact flat colonies with defined colony borders (Figure 1.2). This morphology is like that of mouse epiblast stem cells, with which hESCs share most similarity, and in contrast to that of mESCs, which form characteristic discrete domed colonies. The hESC possesses a nucleus with distinctive nucleoli and little cytoplasm when viewed by phase-contrast microscopy. These characteristics, together with colony formation, provide effective initial identification. Although hESCs dissociate readily with a

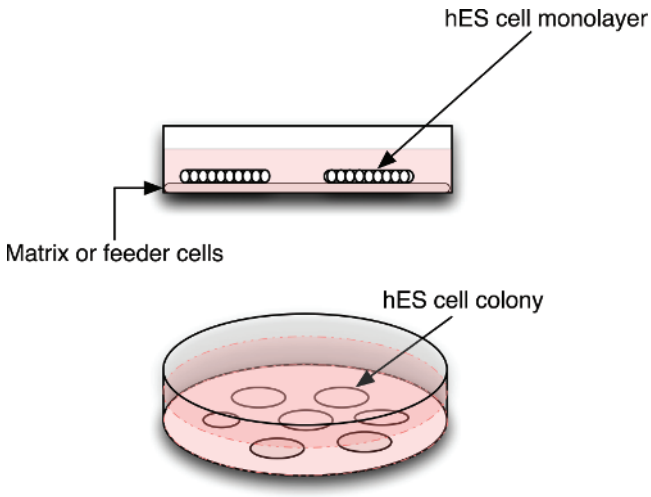

Figure 1.2 Human ESCs grow as flat colonies on a matrix- or feeder cell-coated dish.

variety of enzymes and protocols (i.e. low salt conditions) to disrupt cell–cell adhesion, their survival is poor, with single cell colony-forming capacity often less than 1%. For this reason, most standard passaging involves clumps or sheets of hESCs to limit apoptosis. In contrast, human embryonic germ cells (hEGCs), which are also pluripotent (Shamblott *et al.*, 1998), form spherical colonies, which unlike hESCs are refractory to standard cell dissociation methods.

1.2.2 Stem cell markers

Besides the typical cell/colony morphology, which is a routine check during cell culture, hESCs are characterized mainly by their expression of a variety of specific cell-surface and intracellular protein markers using antibodies (usually monoclonal), often in combination with flow cytometry or high-content image analysis. These cell-surface markers were first identified in the preimplantation mouse embryo or in embryonal carcinoma cells (ECCs; pluripotent cancer cell lines). The phenotypic morphology of a hESC may alter as spontaneous differentiation occurs during cell culture, with cells gradually losing expression of markers associated with pluripotency and upregulating those associated with differentiation; therefore, a panel of markers can rapidly identify subpopulations of cells. If quantitative analysis is used, the stability of a hESC culture can be monitored accurately over time. Surface markers indicative of an undifferentiated hESC state include SSEA-3, SSEA-4 and the high-molecular-weight glycoproteins TRA-1-60 and TRA-1-81 (Thomson *et al.*, 1998). HESCs also express the intracellular markers OCT4, Nanog and REX1 and stain positive for alkaline phosphatase activity (Figure 1.3).

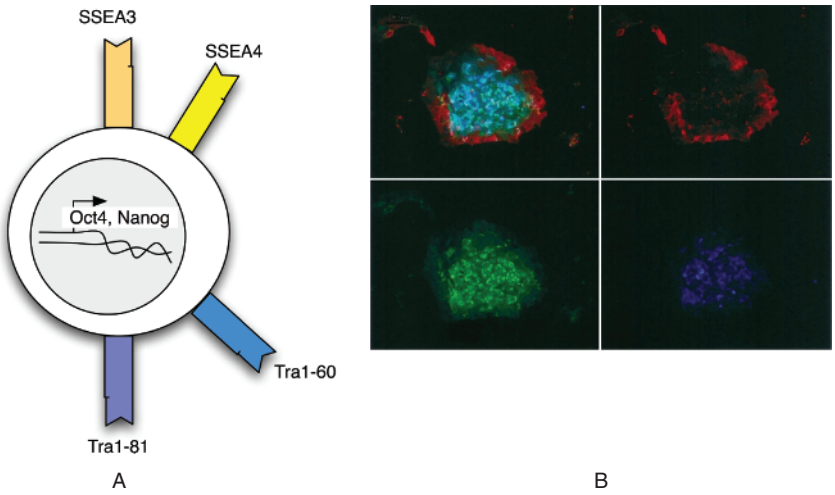

Figure 1.3 (A) Main intracellular and extracellular markers used to identify hESCs. (B) A colony of Shef1 hESCs plated on ECM (Matrigel). Immunofluorescent localization of cell-surface markers Tra-1-60 (green), SSEA3 (blue) and SSEA4 (red). Although all three markers identify pluripotent cells, the expression patterns in the colony differ.

Significantly, mESCs differ in their surface-antigen profile, failing to express SSEA-3 or SSEA-4 but expressing SSEA-1, a cell-surface marker characteristic of differentiated hESCs. The markers display differences in sensitivity to shifts in the differentiation status of the cell, which can be exploited to some extent to forecast developmental changes. For example, SSEA-3 expression is the first to downregulate upon early differentiation while markers such as SSEA-4 and TRA1-60 lag behind (Henderson *et al.*, 2002).

1.2.3 Function characterization: differentiation potential

ESCs are unique in their ability to self-renew and differentiate into all three embryonic germ layers, in principal forming any fully terminally differentiated cell within the body. In the mouse, ESC pluripotency is defined by the ability to generate chimeric offspring and contribute to the germ line. However, for ethical and practical reasons, in humans and some nonhuman primate species, the ability of ESCs to form chimeras is not a testable property, and alternative protocols on which to base functional pluripotency must be used. In the absence of the natural stem cell niche of the embryo, hESCs are in a dynamic balance between cell fates and are highly susceptible to environmental cues, which can induce spontaneous cell differentiation or, in the correct combination, can be employed to drive a more 'directed' cell differentiation. Therefore, pluripotency is measured either *in vitro* by differentiation of cells

as aggregates in suspension culture (called embryoid bodies, EBs) or *in vivo* by their formation in the mouse as benign tumours called teratomas.

1.2.3.1 *In vitro*: EBs Human ESCs can be induced to differentiate *in vitro* by the process of EB formation (Figure 1.4). The process involves growing hESCs in suspension to form cell aggregates on a nonadhesive substrate to prevent their dissociation. As the EBs mature, hESCs alter their morphological appearance and acquire molecular markers characteristic of differentiated derivatives. Markers specific to each embryonic lineage can include neurofilament 68Kd (ectoderm), β-globin (mesoderm) and α-fetoprotein (endoderm) (Itskovitz-Eldor *et al.*, 2000). However, more markers per germ layer are usually analysed, to illustrate a more global picture of differentiation ability. Initial testing of differentiation capacity is commonly done by spontaneous EB differentiation in medium supplemented with serum. Methods have become more refined, however, using defined number of cells and defined media formulations (Ng *et al.*, 2005). An EB formation assay should always be part of the basic hESC characterization, and should clearly show either upregulation of markers from the three germ layers in the EBs or outgrowth from them.

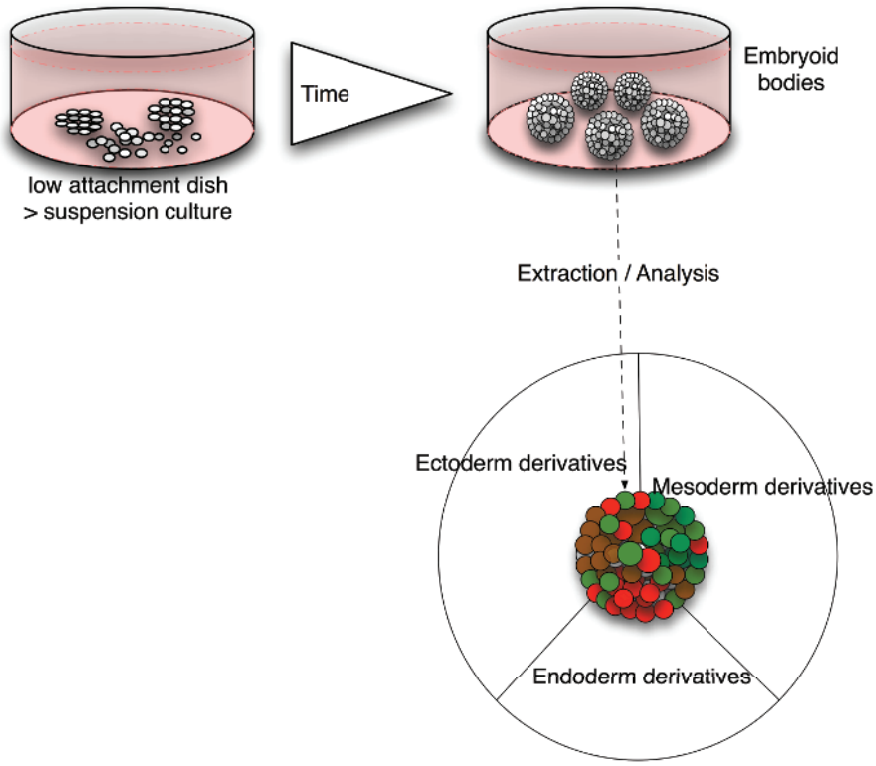

Figure 1.4 Simple overview of EB formation from hESCs. EBs from hESCs should contain tissues derived from all three embryonic germ layers.

1.2.3.2 ***In vivo*: teratoma formation** The formation of a teratoma is a formal demonstration of pluripotency of hESCs *in vivo*. Teratomas are benign tumours that contain different types of developmental tissue derived from all three germ layers. They are formed after injection of undifferentiated hESCs into the hind leg, testis or kidney capsule of immunocompromised mice (i.e. nonobese diabetic severe combined-immunodeficient, NOD/SCID). They are then usually analysed by histological evaluation of the tumour mass for the presence of representatives of all three germ layers (Figure 1.5)

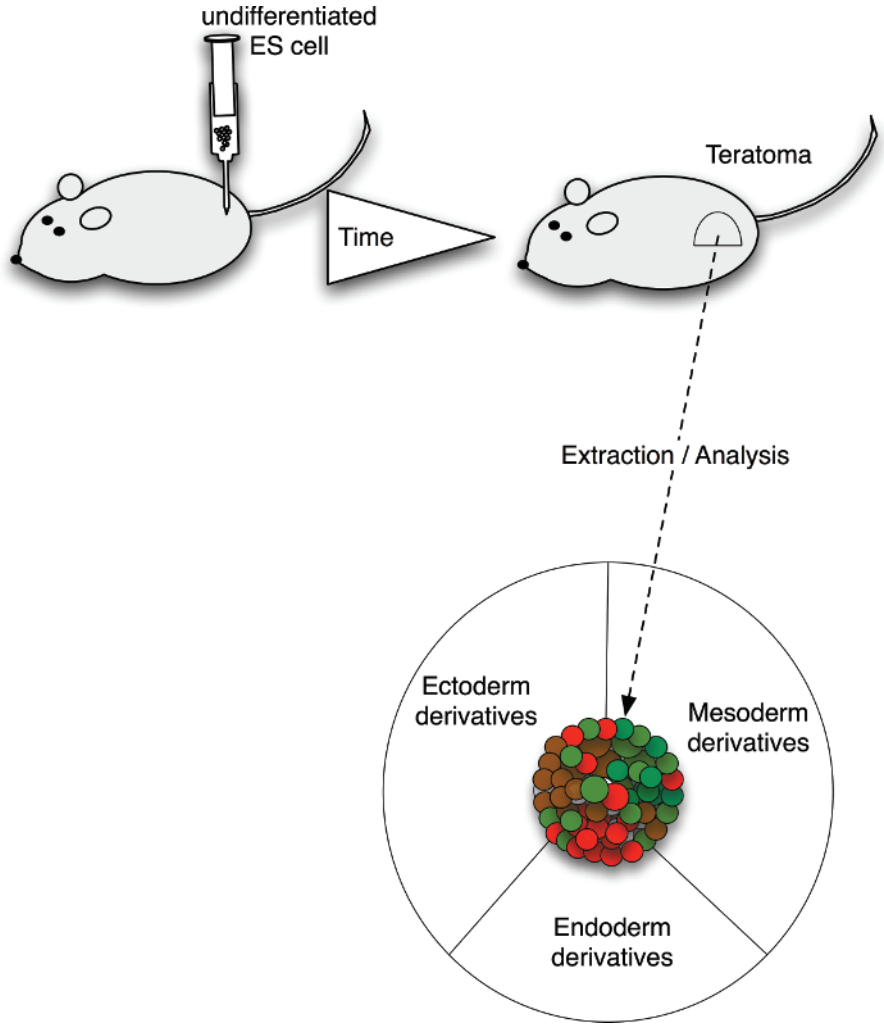

Figure 1.5 Simple overview of teratoma formation in immunocompromised mice. Teratomas from hESCs contain tissues derived from all three embryonic germ layers.

(Thomson *et al.*, 1998). On occasion, after injection, hESCs fail to form teratomas; therefore, injection of more than one mouse is often necessary to account for any variability. This may be due to the abnormal environment in which hESCs are placed, residual immune reactivity, the quality of the hESCs or the scientific methodology. Efficiencies can be improved by adding ECMs such as inactivated mouse embryonic fibroblasts (MEFs) or Matrigel with the hESCs and by using more severely immunocompromised mice (Gropp *et al.*, 2012).

While teratoma formation is an expected part of the basic characterization panel for new hESC lines, a search for less expensive and shorter surrogate assays is ongoing. In particular, more streamlined and time-efficient methods are required for the mass generation of iPSCs (Muller *et al.*, 2011).

1.3 Stem cell quality and culture adaptation with reference to cancer

In the embryo, during normal development, the cells of the ICM usually exist for just a few days before differentiating into more mature cell types to form the three germ layers. During the derivation of human embryonic stem cell lines, cells from the ICM of a blastocyst are transferred to a culture dish and need to adapt to this *in vitro* environment. Prolonged culture of these cells exposes them to various stress factors, which can then lead to further selection of the most adapted cells. Initially, this process of adaptation occurs mostly through epigenetic mechanisms, as *in vitro*-cultured mESCs can convert back to form a normal mouse embryo *in vivo*. However, with extended laboratory culture for months or years (as is possible with pluripotent stem cells), selection of cells that have increased survival may occur, further helping their culture adaptation. This can lead to not only epigenetic but also genomic changes in the cell population.

Any genetic or epigenetic changes that occur in hESCs over extended culture may alter their developmental potential, function or behaviour and should therefore be avoided, if possible. In particular, nonreversible genomic changes need to be tracked and controlled to minimize effects on experimental studies or treatments.

1.3.1 Genomic abnormalities

Genomic abnormalities that have been observed in pluripotent stem cell cultures range from large chromosomal changes to single-nucleotide mutations.

1.3.1.1 Chromosomal aberrations The study of large chromosomal aberrations has been possible since chromosomal banding methods were established in the late 1960s. 'Karyotyping', in which metaphase chromosomes are stained with either quinacrine mustard (q-banding) (Caspersson *et al.*, 1970)

or Giemsa (g-banding) (Sumner *et al.*, 1971) to give a characteristic banding pattern to each chromosome, is now a routine method. Depending on the chromosomal region, a resolution of 5–10 megabases can be achieved. The detection of aneuploidy in patient cells can be an indicator or marker for disease; for example, trisomy 21 is found in Down syndrome.

Initial studies revealed that hESC lines could maintain a normal diploid set of chromosomes during extended periods in culture (>6 months) (Thomson *et al.*, 1998). However, follow-up studies soon revealed that hESC lines could also acquire chromosomal changes (Draper *et al.*, 2003) and thereby emphasized the need for genome monitoring.

Recurrent large aberrations in hESCs after extended culture are mostly gains of regions in chromosomes 1, 12, 17 and X. Interestingly, the most frequent gain of human chromosome 17 (Figure 1.6) is also syntenic to the distal part of mouse chromosome 11, which is most often gained in mESCs (Ben-David and Benvenisty, 2012). Such changes are nonrandom gains that seem to be selected for by *in vitro* culture systems, and have been seen to occur at a rate of 10–20%. However, the general frequency of changes, including subchromosomal changes, is at a rate of 30–35%; this includes aberrations that are selected against during culture and those that are introduced at derivation or come from the embryo (Amps *et al.*, 2011). The observed frequency of chromosomal abnormalities clearly reiterates the need to monitor cells over time, with karyotyping being the most commonly used method.

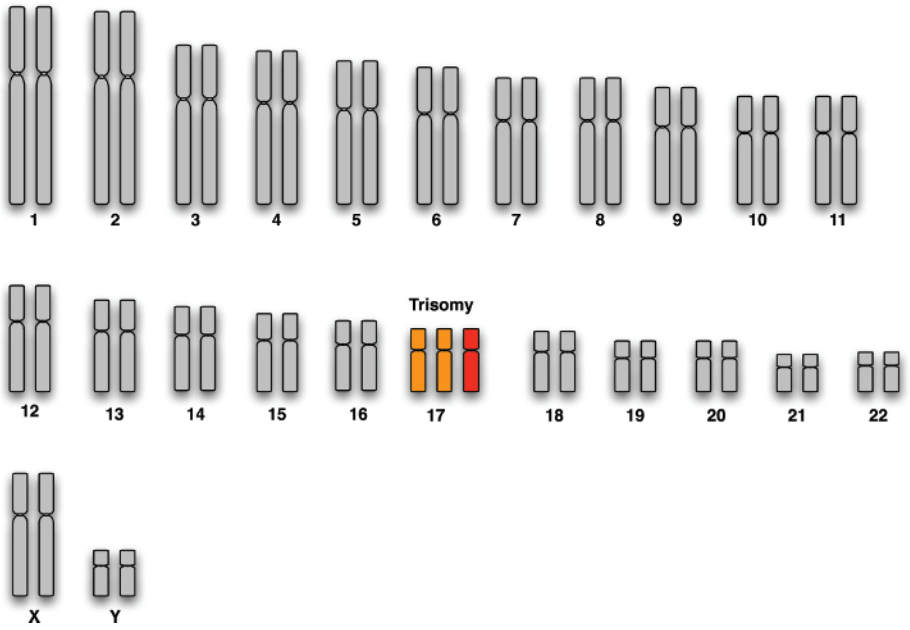

Figure 1.6 Illustration of karyotype with an extra chromosome 17. Trisomy 17 is one of the most common chromosome changes acquired during hESC culture.

1.3.1.2 Copy-number variations While karyotyping initially identified large chromosomal changes, recent application of higher-resolution technologies has both confirmed such large deviations and revealed additional changes on a subchromosomal level. Several studies using single-nucleotide polymorphism (SNP) data have established that all hESC lines exhibit copy-number variations (CNVs) of various sizes, many of which are specific to hESCs (Figure 1.7). At a higher resolution, changes that naturally exist in the human population must be differentiated from changes that have been acquired during *in vitro* culture. Analysis conducted on early and late passage cell populations revealed several regions with gain or loss of heterozygosity (Narva *et al.*, 2010; Hanahan and Weinberg, 2011; Avery *et al.*, 2013). In particular, a minimal amplicon in chromosome 20q11.21 was found in more than 20% of cell lines (Werbowetski-Ogilvie *et al.*, 2009; Amps *et al.*, 2011). Furthermore, it was revealed that the gain of this minimal amplicon introduces a resistance to apoptosis, most likely caused by one specific gene, BCL2L1. A simple genomic quantitative polymerase chain reaction (qPCR)-based approach or fluorescence *in situ* hybridization (FISH) on karyotyping slides should be a good measure to verify that this region has not changed in a particular set of hESC cultures (Avery *et al.*, 2013).

1.3.1.3 Single-nucleotide variations With the advent of whole-genome sequencing, a few studies on iPSCs have been able to increase their resolution

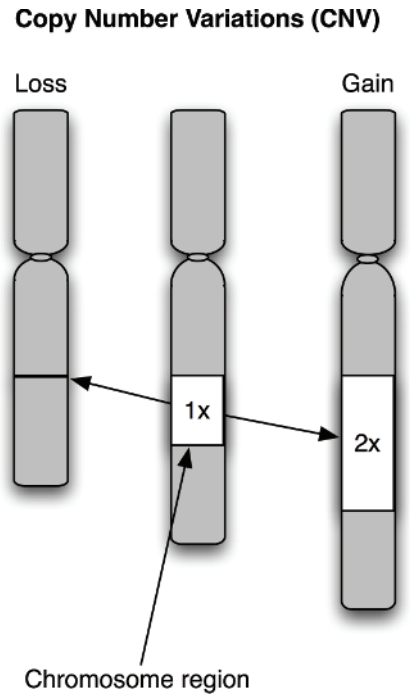

Figure 1.7 Illustration of a possible CNV in hESs.

to the single base pair level and have thus identified single-nucleotide variations (SNVs) (Gore *et al*., 2011). An average of five to six mutations in coding regions have been reported, but many of these likely derive from the parental cell lines. It is important to bear in mind that only a few complete human genomes have been sequenced to date, so the extent of normal variation amongst our population is unclear. However, over time, and with further advanced sequencing technology, bigger data sets will reveal more answers with regards to genome stability. More scientific studies using whole-genome sequencing on hESC lines will be very interesting and may reveal significant SNVs that cannot be detected with other methods and which impact the quality of hESC lines.

1.3.2 Epigenetics

Epigenetics is the study of changes acting upon but not altering the DNA sequence, namely such mechanisms as imprinting, DNA methylation and histone modification to regulate gene expression. The epigenetic characterization of hESC lines, and other cell types, is much less established than genomic analysis, because of its higher level of complexity. While we have sequenced the whole human genome, we do not yet have the same understanding of our epigenome. There are hundreds of epigenomes for every genome, because every person has hundreds of cell types, each of which has different DNA modifications. Another reason is that epigenetic changes are dynamic: able to adapt to changes in the cellular environment over time. High-throughput methods for the analysis of a full set of methylations are now available, but the technology is still new and expensive, and it still needs to develop reliable references for hESCs.

Epigenetic mechanisms give specific cell types their identity by allowing only a subset of genes to be active. Faulty regulation in early embryonic development can result in embryo mortality or distort differentiation and should be evaluated and deselected from hESC cultures, if necessary.

Human ESCs are derived from an early blastocyst stage – a developmental time point at which cells are fragile because processes like X-chromosome inactivation (XCI) are still ongoing (van den Berg *et al*., 2009).

1.3.2.1 Imprinting and XCI Expression of a number of genes is necessarily mono-allelic, which means that one of the parental alleles needs to be silenced (in a process termed 'imprinting') for proper development to occur. For example, mono-allelic expression is important for X-linked genes and hence requires XCI in female embryos. With regards to imprinting and XCI, the gene dosage is important, and failure to properly silence one allele can result in lethality or developmental disorders. Several studies have indicated that prolonged hESC culture can affect the XCI pattern of pluripotent cell lines (Lengner *et al*., 2010; Tchieu *et al*., 2010; Nazor *et al*., 2012). Cell culture under low oxygen allows for the derivation of female ESC

lines with two active X chromosomes, while normal oxygen will produce mixed cultures, indicating that the cell culture environment has a profound effect on XCI (Lengner *et al.*, 2010). Consequently, if a certain X-linked expression is required for disease modelling, the activation or inactivation should be evaluated during ESC characterization. A PCR for X-inactive specific transcript (XIST) expression, which is expressed from the inactivated X chromosome, will give an initial idea of whether XCI has occurred and is maintained in a particular culture system.

1.3.2.2 Methylation pattern DNA methylation silences promoter regions and prevents gene expression where it is not required. New technologies have now started to evaluate genome-wide DNA methylation patterns and are building a reference map for ESCs. While many promoter regions are equally methylated and demethylated between ESC lines, other genes appear to be variably methylated (Bock *et al.*, 2011). Processes that give rise to variation include underlying human variability, cell culture methods, the time point, the method of derivation and other stress factors. What seems clear is that these changes in methylation patterns are impacting the differentiation capacity of ESCs and could be used to predict their ability to differentiate along certain lineages (Bock *et al.*, 2011). Hence, methylation analysis on promoter regions for genes that are important for lineage-specific differentiation can give important insight into the selection of a cell line for a specific purpose and may be included in the characterization of a line. Established methods such as methylation-specific polymerase chain reaction (MSP), pyrosequencing or array-based methylation analysis, together with a reference map, can give clues as to whether a particular cell line is able to differentiate towards all lineages equally.

1.3.2.3 Histone modifications Histones are proteins that package the DNA in eukaryotic cells and play a role in gene regulation, by rendering DNA active or inactive. They can be highly modified through various modifying enzymes and thereby affect gene regulation. For example, promoters occupied by a histone H3 lysine 4 trimethylation (H3K4me3) or histone H3 lysine 27 trimethylation (H3K27me3) are associated with gene activation and repression, respectively. Histone modifications can be affected by cell culture adaptation, and may lead to higher proliferation and differential expression of tumour suppressor genes with parallels to cancer cells. While analysis of histone modification is not commonly carried out for hESC characterization, they impact many genes that are linked to severe developmental disorders and cancers (Lund *et al.*, 2013).

1.3.3 hESC culture adaptation with reference to cancer (genomic and epigenetic)

Cell culture can induce genomic and epigenetic changes in hESCs and should be controlled for. In fact, most cultured cells will have or acquire changes

over time, and it is important to find out whether these changes are in an acceptable range for normal functionality. The hESC field is still relatively new and much remains to be understood before the right conclusions can be drawn from particular changes, making it necessary to screen for such changes in order to increase our knowledge. Genome instability and resistance of cell death through abnormalities are a hallmark of cancer (Hanahan and Weinberg, 2011), so introducing such abnormalities might be a big risk for future clinical applications. Some abnormalities enriched in hESC cultures are also found in tumours and might therefore carry a higher risk of inducing cancer-like changes.

Primordial germ cells and ESCs are closely related cell types as they originate from a similar developmental stage. Their similarity is partly mirrored in the abnormalities they acquire, with germ cell tumours (GCTs) most often amplifying chromosome 12p and gaining material from chromosome 17, much like culture-adapted hESCs (Summersgill *et al.*, 2001); it has therefore been proposed that hESC culture adaptation may be used as a model for GCT malignancy (Harrison *et al.*, 2007). During the malignant evolution of ECCs, differentiation capacity is lost in favour of proliferation proficiency, eventually leading to nullipotent ECCs with a high self-renewal capability. Culture-adapted cells may therefore lose some or all of their differentiation capacity and cause embryonal carcinoma-like tumours if undifferentiated cells are contaminating the differentiated cells used in clinical protocols.

1.3.3.1 Impact of hESC culture-induced genomic and epigenetic changes in differentiated cells Considering that hESCs can differentiate into any cell type found in the human body, there is a real risk that genomic or epigenetic abnormalities in these early stem cells cause more mature cell types to acquire cancer phenotypes. For example, trisomy 12, the most common abnormality in hESCs, is also associated with chronic lymphoid leukaemia (Juliusson *et al.*, 1990), while gain of chromosome 17, particularly the long arm of 17, is strongly associated with neuroblastoma (Plantaz *et al.*, 1997) and CNVs on chromosome locus 20q11 are associated with a variety of cancers (Beroukhim *et al.*, 2010).

Epigenetically, there are many links between hESC abnormalities and neoplasia. The methylation of tumour-suppressor genes or the activation of oncogenes through epigenetic mechanisms might be a prime reason for the transformation of benign cells.

1.4 Future applications in tissue-engineering therapies

Tissue engineering is a concept that evolved from organ transplantation and has existed since the mid 1980s – over a decade before the isolation of the first hESC lines. It aims to maintain or restore function to tissues whose failure is threatening illness. This can be done in three different ways: the support of

preexisting tissues to prevent loss of function; the encouragement of damaged tissues to regain lost function; and the replacement of lost or damaged tissue. The main approaches have included treatment with bio-active molecules such as inhibitors and growth factors, the use of structural biomaterials as scaffolds and the introduction of new cells or tissues, as well as various combinations of these methods.

Many approaches to cell or tissue transplantation have met with significant levels of success. One of the best established of these procedures is the autologous transplantation of hematopoietic stem cells to restore blood cell production after chemotherapy-induced bone marrow ablation. Other cell therapies include the implantation of foetal dopaminergic neurons into patients suffering from Parkinson's disease (Ali *et al*., 2013), the grafting of a retinal pigmented epithelium (RPE)-choroid patch to treat age-related macular degeneration (Buchholz *et al*., 2013) and transplantation of Islet cells or the whole pancreas to treat diabetes (Pavlakis and Khwaja, 2007). Each of these examples provides good proof of concept for cell-replacement therapy, but restricted levels of source tissue prevent many such therapies from being commonly applied in a clinical setting.

Human pluripotent stem cells, such as hESCs and hiPSCs, have the potential to solve this issue. Theoretically, they can differentiate into virtually any cell type in the human body, allowing a single source of cells to be applied to multiple different clinical uses. Furthermore, unlike many primary cell types, hPSCs can be easily maintained in culture and it is possible to scale up their cell numbers exponentially. This means that they have the potential to solve the problem of tissue supply faced by cell-replacement tissue engineering and even to provide previously unobtainable cell types for regenerative purposes.

So far, very few clinical trials that aim to utilize hPSC-derived tissues for replacement therapies have been announced. The differentiation potential and long-term *in vitro* culture of hPSCs introduces a level of complexity to the engineering process that makes an understanding of early human development, and subsequent control of cell phenotype, an essential key to success. Issues facing the implementation of hPSC-derived replacement therapies are: production and maintenance of high-quality and safe source hPSCs; development of efficient protocols for generating the cell type of interest; acquisition of pure differentiated cells of interest, without contamination of undifferentiated or other unwanted cell types; and circumvention of immune-compatibility issues, to prevent immune-rejection (Figure 1.8).

1.4.1 Efficient differentiation and purification of the cell type of interest

Protocols need to be devised that can recapitulate the embryology of a specific cell type *in vitro*. It is unlikely to be possible to produce just one phenotype, so methods have to be in place to recognize the cell of interest and enrich the population for that particular cell type. This all needs to be done with enough

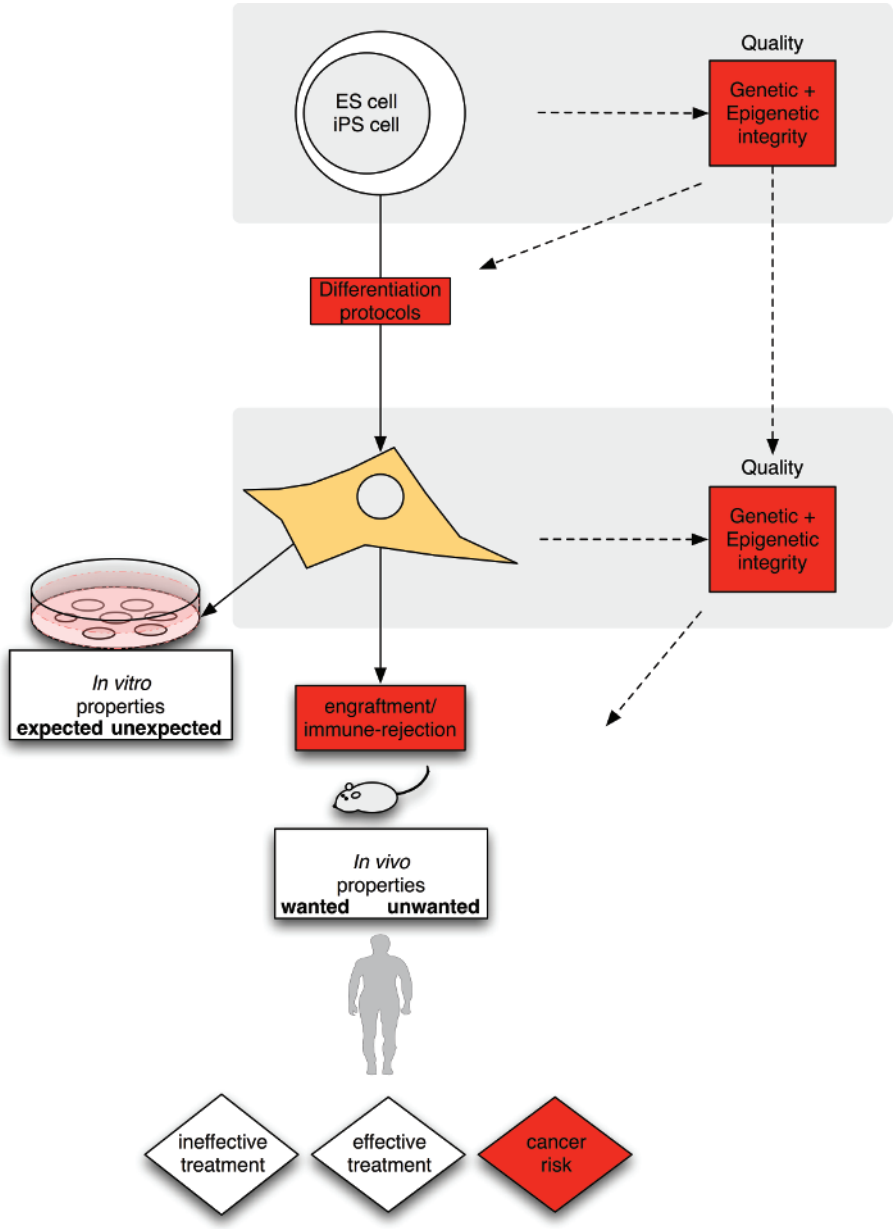

Figure 1.8 Simplified research and development pathway for future therapies from ES/iPSCs to differentiated, potentially therapeutically applicable cells, highlighting factors that can affect cancer risk (in red), as described in this chapter.

cells to allow the required function to be elicited from their transplantation into the patient.

In the developing embryo, differentiation of pluripotent cells is spatially, temporally and chemically regulated. When cellular transplantation from a multipotent or terminally differentiated source is used for clinical purposes, the cells already have the required function and can be identified easily through tissue location and marker expression. When hPSC derivatives are used, they first have to be differentiated *in vitro*. For example, in order to replace foetal dopaminergic neuron treatment of Parkinson's disease with hPSC derivatives, this process has been mimicked with a defined protocol that drives differentiation in the correct direction. Use of small-molecule inhibitors (SMIs) to block or stimulate specific signalling pathways has allowed the development of well-defined culture conditions that do not require co-culture with secondary cell types (Ali *et al*., 2013).

When intrastriatal transplantation of foetal neurons is carried out, the ventral mesencephalon of the foetus is dissected and dissociated before it is implanted into the courdate and putamen of the patient. When this is done, knowledge of dissection location combined with approximately 15% of cells expressing tyrosine hydroxylase is enough to be confident of cell type (Widner *et al*., 1992). The range of different derivatives that can be produced from hPSCs raises the possibility that contaminating phenotypes, even with comparable marker expression, may be present in any differentiated culture. For this reason, the dopaminergic differentiation protocol for hPSCs can be used in concert with serum-free culture methods that remove any extrinsic signalling from the system, reducing contaminating cell types and enriching the final cell population for the required phenotype. These cells are able to respond to the axon guidance cues that allow innervation and connection to host neurons (Cord *et al*., 2010) and have been shown to alleviate symptoms of Parkinson's disease in Rat models (Ali *et al*., 2013). All of this work has brought us to a stage where there is a real possibility of clinical trials using hPS derived neurons to treat Parkinson's disease.

Some cell types require more than clever combinations of signalling molecules and SMIs for successful differentiation from hPSCs. Production of glucose-sensitive, insulin-producing β cells for use in the treatment of diabetes has been a much sought-after goal ever since the isolation of the first hESCs. Human PSCs can be induced to form definitive endoderm by Activin A/Nodal signalling, or more efficiently by using the SMIs IDE1 and IDE2. Following this, there are a number of different methods for differentiation and enrichment of the culture for pancreatic cells, assessed on the basis of the pancreatic marker PDX1 (Aguayo-Mazzucato and Bonner-Weir, 2010). Insulin-expressing cells have been produced in this manner, paving the way for a cell-replacement therapy for diabetes, but none of these methods appears to be capable of producing cells that secrete insulin in a glucose-sensitive manner. This is a characteristic that they share with foetal β cells, perhaps identifying them as a nascent counterpart to the fully

functioning cell required for clinical use. The failure to achieve this last step of β-cell maturation has prevented a cell-replacement therapy for diabetes from reaching clinical trials and is driving research into methods for better recapitulating pancreatic development *in vitro* by spatially organizing the cells into 3D structures, with the hope that polarization of the cells will allow them to mature (Greggio *et al*., 2013).

Despite the establishment of specific and selective methods for producing a desired cell type, such protocols will rarely be able to exclude all other cell types that might develop from hPSCs. Because of this, most protocols, especially those for clinical applications, will include steps for purification (i.e. antibody-based magnetic-activated cell sorting). Importantly, undifferentiated hPSCs that can form teratomas and cells that might in any way negatively affect the treatment must be removed from a final cell-therapy product.

1.4.2 Genetic stability and tumourigenic potential

Antiapoptotic and proliferative selection pressures subjected to cells in culture can lead to the accumulation of genetic and epigenetic changes, which promote a phenotype analogous to that of a tumour cell. This process of culture adaptation is fully discussed in Section 1.3, but it should be noted that such changes present a significant barrier to the successful application of hPSCs in tissue engineering. If an adapted cell of this type were to be implanted into a person, there would be a significant danger of tumour formation. For this reason, any cell population that is used for such a purpose needs to undergo a thorough genetic analysis of the kind described in Section 1.3.

1.4.3 Immune compatibility

One of the greatest challenges facing any kind of cell-replacement therapy is the response of the host's own immune system to a graft. Immune reactions to grafts and graft-versus-host disease (GVHD) can be so severe as to not only render the transplant useless but also kill the patient. In some cases, immunosuppressant drugs originally developed for organ transplantation can circumvent this, but these can produce their own complications. The intricate play between host and graft has been widely studied since the advent of hematopoietic stem cell transplantations. While engraftment of many cell types can strongly benefit from a limited host immune response, it must be considered that residual host response in allogeneic transplantation might also have applications in therapies.

The advent of cellular reprogramming holds the potential to make allogeneic transplants a thing of the past, circumventing the issue of immune-rejection altogether. By taking a small skin biopsy or blood sample, it is now possible to create patient-specific iPSCs that can be used in place of hESCs in all of the aforementioned examples of tissue engineering. Derivatives of such reprogrammed cells will be a perfectly immune-compatible match to a

host system, and considered as the host's 'own' cells. It is of note that this will deprive the host of any means of immune detection, should such cells carry abnormalities that could cause cancer.

1.5 Conclusions

One of the most promising applications to date for stem cell-based tissue engineering is the treatment of the dry form of age-related macular degeneration (AMD) with hPSC-derived RPE. This is the first approach using hPSCs to move to clinical trials, partly because of the ease with which differentiation into RPE cells was first achieved. Human PSCs will spontaneously develop into RPE cells at low efficiencies and can be easily identified via pigmentation. Recent advances in differentiation protocols have increased efficiencies to ~60%, allowing purification of enough cells for transplantation into patients, through either the injection of a single cell suspension or insertion of a 'patch' of cells grown in a monolayer on a scaffold (Buchholz *et al.*, 2013)*. These first clinical trials may successfully cure AMD and push hPSCs into a clinical era. However, they might also uncover unwanted side effects, such as those described with the first attempts to cure severe combined immunodeficiency (SCID-X1) by using gene therapy to genetically modify cells (Hacein-Bey-Abina *et al.*, 2003). An early clinical trial conducted by the Geron Corporation attempted to use hESC derivatives to treat spinal cord injury, but it ended prematurely, partly because of the unanticipated cost of the project. This is an example of the unforeseen hurdles that can still slow advancement to treatments.

The use of hPSCs for tissue engineering could circumvent issues of tissue supply, regress symptoms of neurodegenerative diseases rather than just alleviate them, and provide replacement cells that were previously unobtainable. The approaches described in this chapter represent only those that are closest to clinical use. In the future, it may be possible to use gene therapy to cure genetic defects in the iPSCs of patients before re-implanting them as an autologous cell-replacement therapy, and even to use additive manufacturing to create whole organs from hPSC derivatives (Orlando *et al.*, 2013). Currently, the costs of these therapies are prohibitive, but, although the need for extensive preclinical analysis of cells will always be present, optimization of these procedures alongside differentiation, transplantation and reprogramming will eventually bring costs down.

As our understanding of human embryology expands and we learn to better recapitulate it *in vitro*, we can expect to see the number of applications for hPSCs in tissue engineering rise exponentially.

* Since the time of writing trials have shown improvements in the eyesight of patients taking part in trials for this treatment of AMD (Schwartz *et al.*, 2014).

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