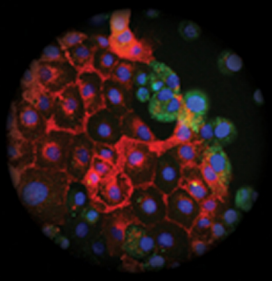
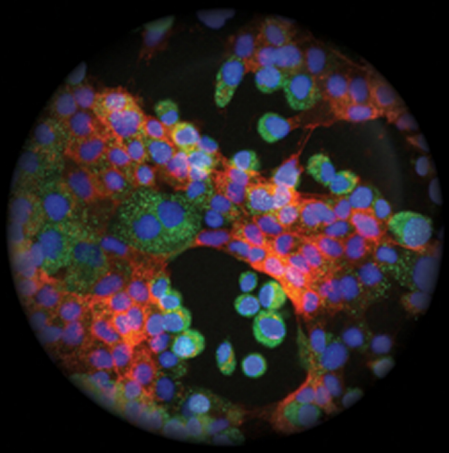
# PRINCIPLES OF STEM CELL BIOLOGY AND CANCER

## Future Applications and Therapeutics






*Editors*

**Tarik Regad**
**Thomas J. Sayers**
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# Principles of Stem Cell Biology and Cancer

# Principles of Stem Cell Biology and Cancer: Future Applications and Therapeutics

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# Preface

Stem cells are a population of cells capable of differentiating into diverse specialized cell types, or of undergoing self-renewal to produce more stem cells. There are two types of stem cells: embryonic stem cells, isolated from the blastocyst, and adult stem cells, found in different tissues of the body. These cells are essential in generating different cell lineages and thus maintaining the structural and functional integrity of tissues and organs. The decision whether to self-renew or to differentiate is tightly regulated and requires a strict control of cell division and cell-cycle exit. This level of control involves key molecules implicated in cell-cycle regulation, as well as several critical growth factors and cytokines. The balance between self-renewal and differentiation can be the target of oncogenic events, leading to cell transformation and the emergence of 'cancer stem cells', which are thought to be subpopulations of cancer cells responsible for tumour progression, development of metastases, tumour dormancy, cancer relapse and resistance to chemotherapy.

In recent years, the stem cell field has become a subject of extensive research, with many groups focusing on isolating and identifying cancer stem cell populations. This effort relies on identifying molecules expressed preferentially by cancer stem cells, with the aim of developing cancer therapies targeting these specific molecules in this cancer population without affecting the pool of normal healthy stem cells. Although some progress has been made, developing efficient therapies targeting cancer stem cells remains one of the important challenges facing the growing stem cell research community.

This book will provide a detailed introduction to stem cell biology. Part I focuses on the characterization of stem cells, the progress made towards their identification and their future therapeutic applications. Part II focuses on cancer stem cells and their role in cancer development, progression and chemoresistance, and presents an overview of recent progress in therapies targeting cancer stem cells. We believe that this book will be unique in providing compiled information about the link between stem cell biology and cancer.

The contributing authors are renowned experts in the field and will provide a timely book of high quality, outlining the current progress in and exciting future possibilities for stem cell research.

*Tarik Regad*
*Thomas J. Sayers*
*Robert C. Rees*

# Part I
# Stem Cells

# 1

# 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

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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).

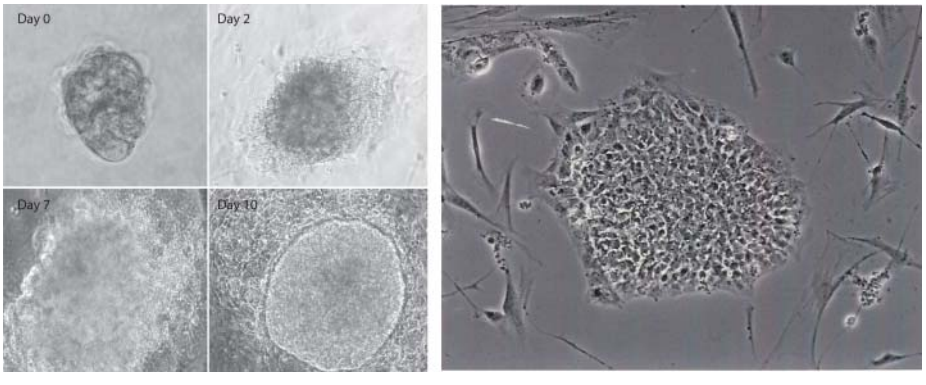


**Figure 1.1** (A) Outgrowth of hESCs over 10 days of culture from ICM. In this instance, a clearly defined colony was observed by 10 days, which was mechanically passaged. (B) hESC line Shef1 plated on ECM.

**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

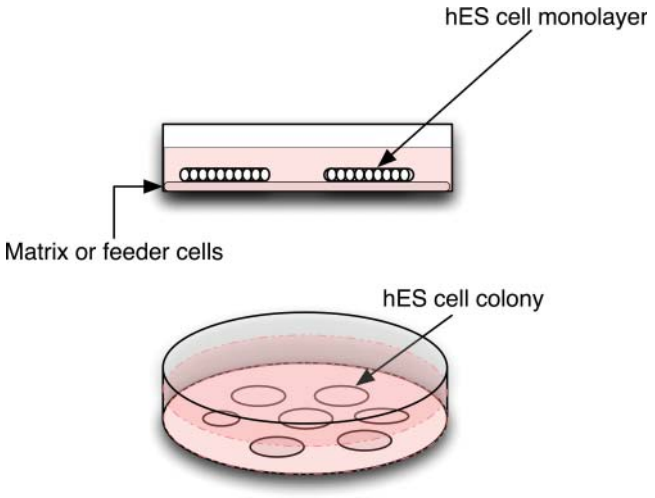


**Figure 1.2** Human ESCs grow as flat colonies on a matrix- or feeder cell-coated dish.

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).

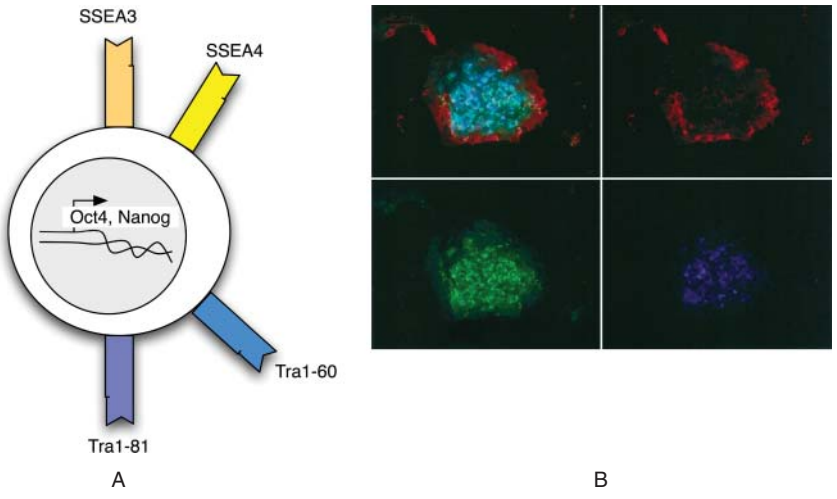


**Figure 1.3** (A) Main intracellular and extracellular markers used to identify hESCs. (B) A colony of Shef1 hESCs plated on ECM (Matrigel). Immunofluorescent localization of cell-surface markers Tra-1-60 (green), SSEA3 (blue) and SSEA4 (red). Although all three markers identify pluripotent cells, the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

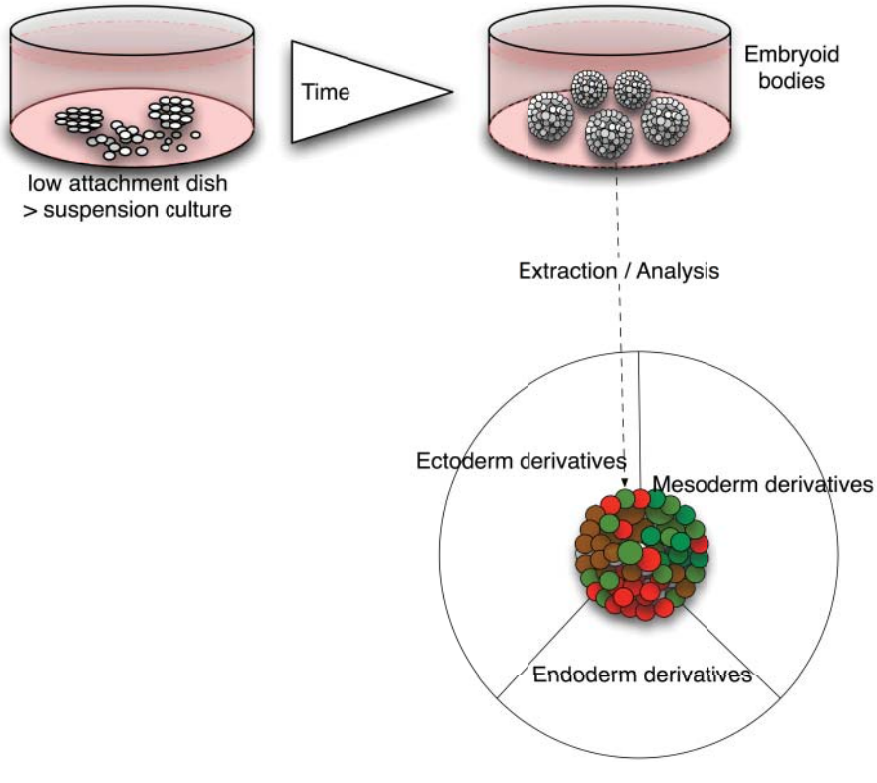


**Figure 1.4** Simple overview of EB formation from hESCs. EBs from hESCs should contain tissues derived from all three embryonic germ layers.

**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)

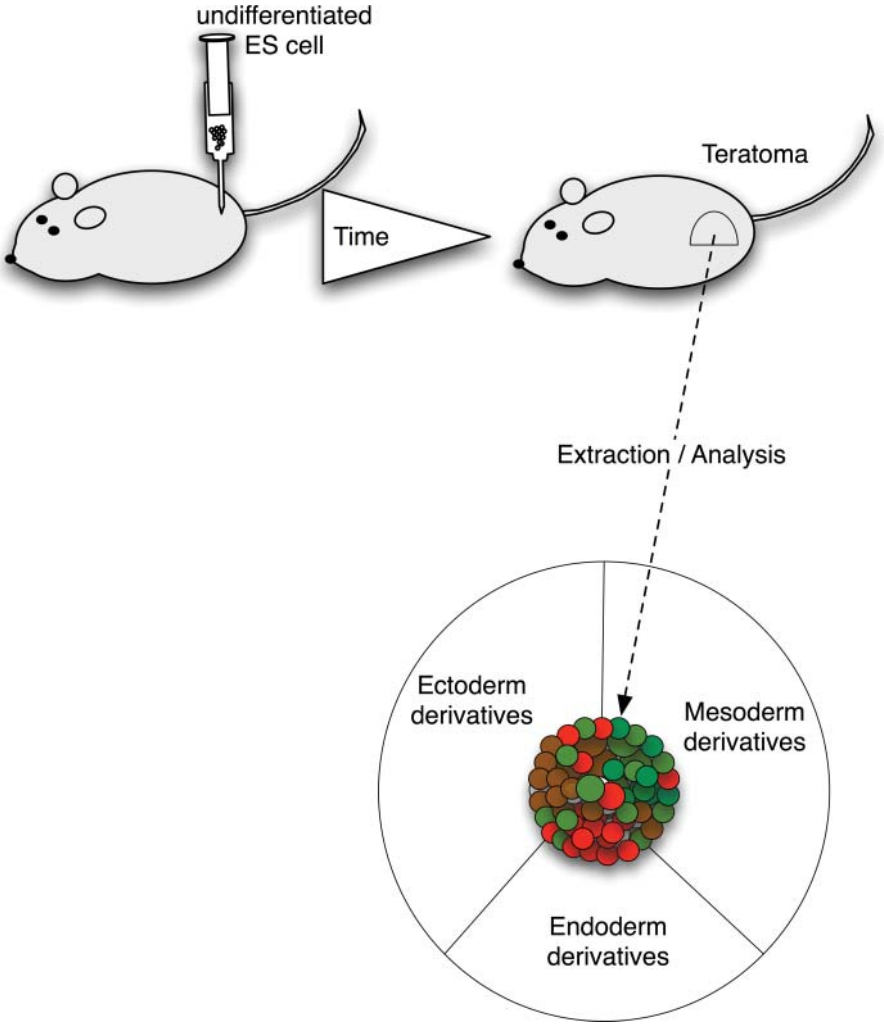


**Figure 1.5** Simple overview of teratoma formation in immunocompromised mice. Teratomas from hESCs contain tissues derived from all three embryonic germ layers.

(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.

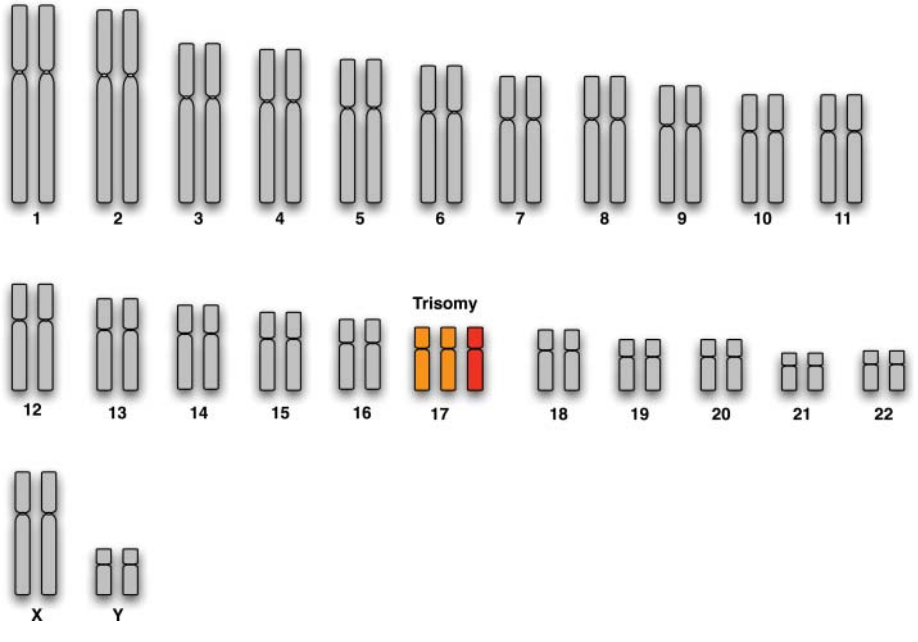


**Figure 1.6** Illustration of karyotype with an extra chromosome 17. Trisomy 17 is one of the most common chromosome changes acquired during hESC culture.

**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

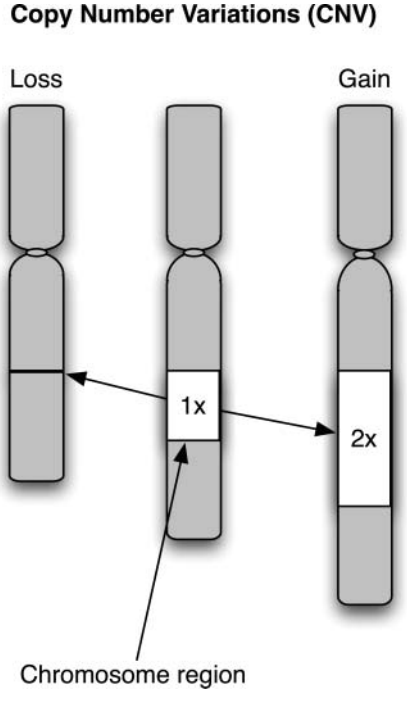


**Figure 1.7** Illustration of a possible CNV in hESs.