Human Reproduction Update, Vol.13, No.2 pp. 103–120, 2007 doi:10.1093/humupd/dml041
Advance Access publication August 26, 2006

Differences between human embryonic stem cell lines

C.Allegrucci[1] and L.E.Young[1,2]

[1]School of Human Development, University of Nottingham, Queens Medical Centre, Nottingham, UK

[2]To whom correspondence should be addressed at: School of Human Development, University of Nottingham, Queens Medical Centre, East Block, D Floor, Nottingham NG7 2UH, UK. E-mail: lorraine.young@nottingham.ac.uk

The promise of human embryonic stem cell (hESC) lines for treating injuries and degenerative diseases, for understanding early human development, for disease modelling and for drug discovery, has brought much excitement to scientific communities as well as to the public. Although all of the lines derived worldwide share the expression of characteristic pluripotency markers, many differences are emerging between lines that may be more associated with the wide range of culture conditions in current use than the inherent genetic variation of the embryos from which embryonic stem cells were derived. Thus, the validity of many comparisons between lines published thus far is difficult to interpret. This article reviews the evidence for differences between lines, focusing on studies of pluripotency marker molecules, transcriptional profiling, genetic stability and epigenetic stability, for which there is most evidence. Recognition and assessment of environmentally induced differences will be important to facilitate the development of culture systems that maximize stability in culture and provide lines with maximal potential for safety and success in the range of possible applications.

Key words: epigenetics/gene expression/human embryonic stem cells/inter-line variability/karyotype

Introduction

To date, human embryonic stem cell (hESC) lines have been obtained from fresh and frozen embryos of varying quality at morula and blastocyst stages of development (Hoffman and Carpenter, 2005a). Embryonic stem cells possess the unique ability of self-renewal that confers indefinite maintenance of the undifferentiated state *in vitro* while retaining the capacity to generate derivatives of the three embryonic germ layers that precede the formation of all of the tissues of the developing fetus (Semb, 2005; Trounson, 2006). As hESCs can differentiate potentially into all the cell types that make up a human body, they promise exciting potential as a source of cells for regenerative medicine, as well as valuable tools for drug discovery and for understanding human development and disease.

Since the establishment of the first hESC lines (Thomson *et al*., 1998), some progress has been made in elucidating the biology of these cells and in optimizing conditions for their maintenance in culture. To use hESCs in cell-based therapies, it is necessary not only to develop methods for the production of adequate numbers of differentiated cells for transplantation but also to have enough cell lines to cover the vast spectrum of transplant antigens to avoid rejection (Taylor *et al*., 2005). These requirements have been the driving force for the derivation of numerous cell lines worldwide. It is also a major advantage for drug discovery applications and toxicology screening that each line represents a unique human genetic complement. The interaction of compounds under test with the variable alleles that individuals inherit often results in adverse drug reactions in a subset of patients in clinical trials (Ingelman-Sundberg and Rodriguez-Antona, 2005). These effects are not identified in preclinical animal tests or in cell-based screens that utilize human, transformed cell lines that were derived from only a few individuals. Thus, the provision of primary human cell types from a wide range of hESC lines that represent genetic diversity represents a unique opportunity.

It was proposed initially that a minimum of 40–50 homozygous hESC lines would be necessary to cover ~50% of the HLA isotypes in the American population and thus to minimize the immune rejection of hESC-derived transplants (Faden *et al*., 2003). More recently, the number of hESC lines needed in a stem cell bank for HLA matching of the UK population has been calculated as ~150 (Taylor *et al*., 2005), but this number may underestimate the lines necessary to match a very ethnically diverse population (Rao and Auerbach, 2006; Rao and Civin, 2006). Two hundred hESC lines were estimated to have been derived worldwide in 2004 (Brimble *et al*., 2004), and this number is increasing rapidly as more and more embryo donors and fertility clinics become interested in collaborating with scientists. The increasing profile of hESC on national political agendas has also led to more permissive legislation for allowing hESC derivation in some countries. Notably, however, several countries including Italy, Ireland, Norway and Germany still have a complete ban (Rao and Civin, 2006). Of the derived hESC lines, only a limited number are readily available for distribution. These include 22 cell lines of the total 78 listed in the US NIH registry for commercial sale (http://stemcells.nih.gov) and the 17 cell lines derived in Harvard University

with funding from the Juvenile Diabetes Research Foundation (Cowan *et al*., 2004). The remaining lines reside largely within founding laboratories and have limited distribution between close collaborators, presumably due to the considerable resources required for cell expansion and characterization, commercial funding issues and difficulties over producing workable material transfer agreements. Although several national stem cell banks are being established, none are actively distributing hESC lines at the time of writing (March 2006).

Despite a widespread interest in defining the properties of hESCs, the beginnings of comprehensive characterization are available for only a subset of hESC lines (Thomson *et al*., 1998; Mitalipova *et al*., 2003; Amit *et al*., 2004; Brimble *et al*., 2004; Carpenter *et al*., 2004; Ginis *et al*., 2004; Heins *et al*., 2004; Rosler *et al*., 2004; Zeng *et al*., 2004; Xu *et al*., 2005). Notably, most of all publications pertaining to hESC focus on the first five lines derived in the University of Wisconsin, i.e. H1, H7, H9, H13 and H14 (Denning *et al*., 2006; Owen-Smith and McCormick, 2006). Although hESC lines are considered to be very similar in terms of self-renewal, expression of pluripotency markers and the ability to differentiate, it is becoming more and more evident that differences between lines also exist (Carpenter *et al*., 2003; Hoffman and Carpenter, 2005a). Differences in growth rate and genetic and epigenetic stability in long-term culture (Cowan *et al*., 2004; Hoffman and Carpenter, 2005b; Lee *et al*., 2005a) have been reported.

It is now been recognized by the scientific community that there is a need for much more comprehensive, parallel characterization of hESC lines than is currently being undertaken to assess their true potential. The safety and efficacy of lines for regenerative medicine applications is of prime concern. To this end, several initiatives are beginning to characterize multiple existing cell lines using standardized assay conditions to allow accurate comparison of the data generated. Such initiatives have been instigated by the International Stem Cell Forum (www.stemcellforum.org; Andrews *et al*., 2005), the NIH Stem Cell Unit (http://stemcells.nih.gov/research/nihresearch/scunit/) and the American Type Culture Collection (http://stemcells.atcc.org; Rao and Civin, 2005). However, the wide range of feeder cells, culture media, additives and passage methods used to derive lines still confounds the interpretation of inter-line differences and conclusions as to whether these are due to inherent genetic variation or environmental 'programming' of the cells (Allegrucci *et al*., 2005). No standard conditions that are optimal or generically applicable across lines have been established either for their culture or for their differentiation (Hoffman and Carpenter, 2005a). Characteristics described for cell lines cultured in certain conditions (and at a specific passage number) may only be applicable to those specific cells assayed within a specific laboratory. Although some success has been reported in standardizing conditions between independently-derived lines for maintenance and cardiomyocyte differentiation, media used to culture one line were often not transferable to others (Denning *et al*., 2006). Protocols published for some lines have also proved difficult to transfer to the lines cultured routinely within our laboratory (Figure 1).

The characterization of hESC lines: pluripotency assessment

Since the derivation of the first hESC lines, extensive effort has been devoted to compare hESCs with their mouse counterparts (Sato *et al*., 2003; Ginis *et al*., 2004; Wei *et al*., 2005). It is now well recognized that hESCs differ from murine embryonic stem cells (mESCs) in many biological aspects and that no direct extrapolation between the two systems is possible (Rao, 2004; Wobus and Boheler, 2005; Zhan *et al*., 2005). For this reason, extensive characterization of hESCs has been mandatory to understand the cellular requirements for long-term maintenance of hESC lines *in vitro* (Boyer *et al*., 2005; James *et al*., 2005; Vallier *et al*., 2005). Many studies have described comparisons between hESC lines derived in the same laboratory and cultured under the same conditions, with no study comparing more than four independently-derived lines (Thomson *et al*., 1998; Allegrucci *et al*., 2005). Fewer studies have investigated the characteristics of independently-derived and cultured lines (Abeyta *et al*., 2004; Amit *et al*., 2004; Bhattacharya *et al*., 2005), mainly because of the laborious and costly procedures of maintaining multiple cell lines with different culture requirements but also because of the complexity of interpreting data from cells cultured in different conditions. Despite variable inter-line culture conditions (Hoffman and Carpenter, 2005a), hESCs are considered to possess similar morphology, growth characteristics and expression of a range of 'pluripotency markers'. Nevertheless, different population doubling rates have been reported for different hESC lines, ranging from 28 to 48 h (Amit *et al*., 2000; Reubinoff *et al*., 2000; Xu *et al*., 2001; Amit *et al*., 2003; Cowan *et al*., 2004; Zeng *et al*., 2004; Kim *et al*., 2005; Sjogren-Jansson *et al*., 2005). Expression of SSEA (SSEA-3, SSEA-4) and TRA antigens (TRA-1-60, TRA-1-81), OCT4 and NANOG, is common between the hESC lines and is considered a general criterion for hESC characterization and evaluation of pluripotency (Hoffman and Carpenter, 2005a). However, not all hESC lines are generally tested for the same panels of markers, and differences in relative abundance of pluripotency markers have been described, raising the questions of whether hESC cultures are heterogeneous in nature or whether more than one 'state of pluripotency' may exist. For example, significant difference in SSEA-4 expression has been observed between the H1, H7, H9 and H14 lines, such that two populations of SSEA-4-high- and SSEA-4-low-expressing cells can be identified. These populations correlate with the differential expression of additional hESC markers, including TRA-1-60, TR1-81, CD9 and CD133 (Carpenter *et al*., 2004). Other examples of variable marker expression include the differential expression of *OCT4*, *NODAL* and *CTNNB1* (β-Catenin) between Miz-hES1 and SNU-hES3 cell lines (Rho *et al*., 2006) and of *OCT4*, *NANOG* and *GDF3* between HS181, HS235, HS237, FES21, FES22, FES29 and FES30 (Skottman *et al*., 2005a). The variable expression of pluripotency markers could result from the presence of early differentiated progenitors in culture because their down-regulation is gradual upon differentiation (Draper *et al*., 2002; Bhattacharya *et al*., 2005), raising the question of whether they can definitively characterize pluripotency. The physiological significance of expression of these markers is not clear, and it is likely that the limited panel of markers in current use may be insufficient to define the state of 'stemness' because many of them are not unique to embryonic stem cells. *OCT4* is also expressed in germ cells (Niwa *et al*., 2000), and *NANOG* has been reported in adult differentiated tissues (Hart *et al*., 2004). Furthermore, the difficulty in designing RT–PCR primers that distinguish *OCT4* and *NANOG* transcripts from related pseudogene transcripts (Pain *et al*., 2005) has not

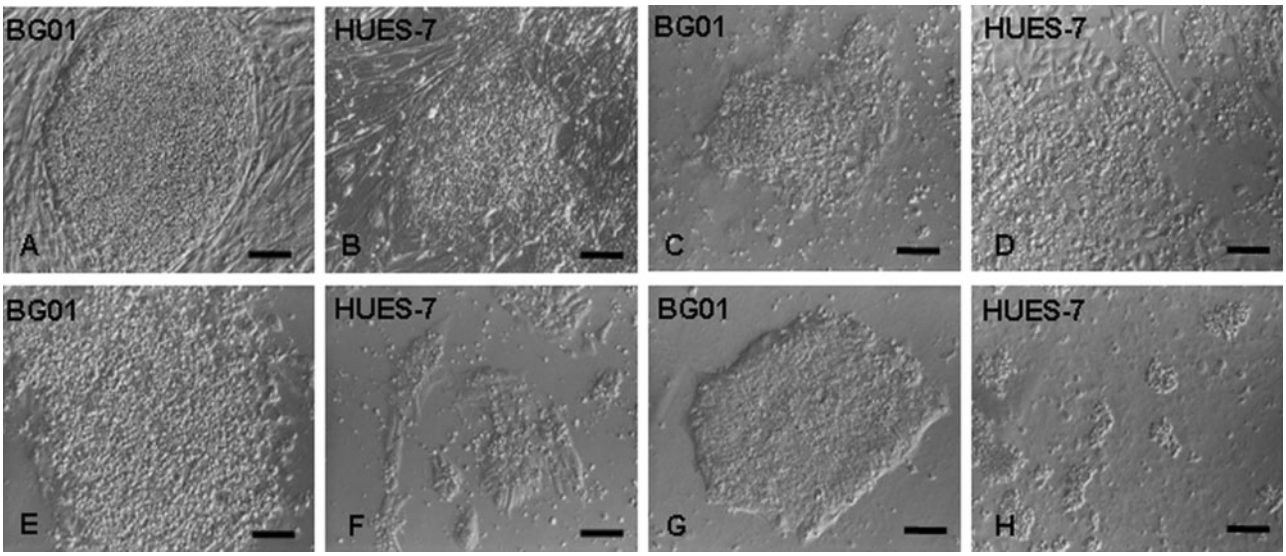

Figure 1. Morphology of BG01 and HUES-7 human embryonic stem cell (hESC) lines cultured under the proprietary standard protocols (**A**, **B**) and published feeder- and conditioned medium-free conditions (**C–H**). BG01 (**A**) and HUES-7 (**B**) cultured under proprietary standard conditions on feeders (Mitalipova *et al*., 2003; Cowan *et al*., 2004). BG01 (**C**) and HUES-7 (**D**) cultured according to Sato *et al*. (2004). Cultures of both cell lines could not be maintained due to extensive cell death. BG01 (**E**) and HUES-7 (**F**) cultured according to Vallier *et al*. (2005). Cultures of both cell lines could not be maintained for more than 10 days under these conditions. BG01 (**G**) and HUES-7 (**H**) cultured according to Klimanskaya *et al*. (2005). BG01 cultures could be maintained for at least 10 passages, whereas the same culture conditions were not suitable for HUES-7 maintenance beyond the first passage. Bar, 100 μm. All brightfield images were taken using Hoffman objective lenses. Magnification 160×.

been accounted for in many hESC studies, confounding data interpretation.

Considering that no hESC line has been derived clonally from a blastocyst, it is reasonable to hypothesize that hESC cultures represent a heterogeneous population of cells with different characteristics and potential. Studies on the positional effects of the inner cell mass cells within the blastocyst on the phenotype/potential are lacking in any mammalian species. If such positional effects exist, it is not known whether these would likely be reversed by *in vitro* culture. Some cell lines show differences either in their kinetics of expression of differentiation markers (Reubinoff *et al*., 2000; Abeyta *et al*., 2004; Lee *et al*., 2005a) or in their ability to form specific lineages *in vitro* (Lee *et al*., 2005a). Differences in ~40% of genes up-regulated in BG02 relative to a pool of H1, H7 and H9 hESC lines have also been described during differentiation into embryoid bodies (EBs) (Bhattacharya *et al*. 2005). Differentiation potential to any specific lineage *in vitro* is difficult to assay due to low typical efficiencies of forming most cell types and the inability to apply differentiation strategies across independently-derived lines cultured in different ways. The limited identification of molecular inducing factors and complex 3D tissue environment interactions mean that differentiation strategies are still largely in their infancy. The definitive test for hESC pluripotency is considered to be the ability to form derivatives of the three germ layers *in vivo* via the formation of teratomas in immunocompromised mice. The formation of teratomas has been reported for a subset of derived hESC lines (http://stemcells.nih.gov), sometimes with different outcomes (Heins *et al*., 2004). The type of differentiated cells obtained in teratomas is limited and variable and may not reflect the real capacity of hESCs to differentiate into all the cells of an organism, were they in the appropriate tissue environments (niche). To date, only keratinocytes, neurons, glia, cardiomyocytes, haematopoietic precursors, endothelial cells, osteogenic cells, hepatocyte-like cells, insulin-producing cells, adipocytes, trophoblast, germ cells, prostate tissue and melanocytes have been derived from hESCs (Allegrucci *et al*., 2005; Hoffman and Carpenter, 2005a; Hyslop *et al*., 2005; Fang *et al*., 2006; Taylor *et al*., 2006).

Gene expression signature of hESCs

The gene expression profile of hESCs has been explored by several techniques, including serial analysis of gene expression (SAGE), expressed sequence tag (EST) enumeration, microarray analysis and massively parallel signature sequencing (MPSS; Table I). Most of these studies have been undertaken to unravel the key genes that characterize the status of 'stemness', regulating pluripotency and maintaining the undifferentiated state. For this reason, research has been focused on those genes that are commonly expressed and enriched in several hESC lines, whereas much less attention has been given to the differences in gene expression observed (Sato *et al*., 2003; Sperger *et al*., 2003; Bhattacharya *et al*., 2004; Brandenberger *et al*., 2004a; Ginis *et al*., 2004; Rao *et al*., 2004; Richards *et al*., 2004; Cai *et al*., 2006; Wei *et al*., 2005).

To identify common genes between cell lines, a pool of the cell lines H1, H7, H9 was used in MPSS analysis. When the data generated were compared with other published datasets generated by microarrays and SAGE in different hESC cell lines, (Sato *et al*., 2003; Sperger *et al*., 2003; Abeyta *et al*., 2004; Ginis *et al*., 2004; Richards *et al*., 2004; Bhattacharya *et al*., 2005) similar results were observed with a concordance of ~70% (Brandenberger *et al*., 2004a). However, when a similar approach was undertaken for the generation of ESTs from pooled H1-H7-H9 cell lines (Brandenberger *et al*., 2004b), only a very limited match was observed in the expression of genes previously described in microarrays

Table I. Gene expression differences between human embryonic stem cell (hESC) lines

hESC lines	Passage number	Culture substrate	Gene expression assay	Number of transcripts analysed	Reference sample	Threshold difference	Difference between hESC lines or other hESC data sets	References
H1.1, H7, H9, H13, H14	NS	MEFs	Microarray	30300	SCL	≥3-fold	NS	Sperger *et al*. (2003)
H1	NS	Matrigel-CM	Microarray	22283-HG-U133A	NS	Regularized *t*-test	NS	Sato *et al*. (2003)
Pool H1-H7-H9, H1, I6, H9.2	NS	Matrigel-CM, MEFs	Microarray	96-GEArray Q	GAPDH	FET	NS	Ginis *et al*. (2004)
Pool H1-H7-H9	NS	Matrigel-CM	MPSS	22136	NS	FET	70% overall in common with enriched genes in Sato *et al*. (2003); Abeyta *et al*. (2004); Bhattacharya *et al*. (2004)	Brandenberger *et al*. (2004a)
Pool H1-H7-H9	H1 p32, H7 p29, H9 p26	Matrigel-CM	ESTs scan	32764	NS	FET	1.7% in common with Ramalho-Santos *et al*. (2002), 6% in common with Ivanova *et al*. (2002)	Brandenberger *et al*. (2004b)
H1, H7, H9	p28–37	Matrigel-CM	Microarray	4224-Research Genetics	NS	≥3-fold	NS	Carpenter *et al*. (2004)
H1, H7, H9	p28–37, p>48	Matrigel-CM	Microarray	4224-Research Genetics	NS	≥3-fold	NS	Rosler *et al*. (2004)
HES-3, HES-4	HES-3 p40 HES-4 p40	MEFs	SAGE	31852	AFT	Z-test> 4-fold	73% in common	Richards *et al*. (2004)
BG01, BG02	NS	MEFs	Microarray	16659	huURNA	≥3-fold	36 % in common	Zeng *et al*. (2004)
BG02, BG03	NS	Matrigel-CM	Microarray MPSS	454 (microarray) 14934 (MPSS)	NS	NS	85% in common with enriched genes in Bhattacharya *et al*. (2004)	Brimble *et al*. (2004)
H1, BG01	NS	Matrigel-CM, MEFs	Microarray	22283-HG-U133A	NS	>2-fold	75% in common	Rao *et al*. (2004b)
HSF-1, HSF-6, H9	HSF-1 p36, HSF-6 p46, H9 p51	MEFs	Microarray	39000-HG-U133A+B	NS	≥2-fold	52% in common	Abeyta *et al*. (2004)
H9, H13	p42–47	MEFs	Microarray	39000-HG-U133A+B	NS	>20-fold	>0.9 correlation	Dvash *et al*. (2004)
Pool H1-H7-H9, BG01, BG02, H1, H9, TE06	NS	Matrigel-CM, MEFs	Microarray	16659-Operon	huURNA	≥3-fold	>0.85 correlation	Bhattacharya *et al*. (2004)
BG01, BG02, BG03	NS	Fibronectin-CM, MEFs	Microarray	24000-Illumina RefSeq8	huURNA	>3-fold	≥0.9 correlation	Cai *et al*. (2006)

Table 1. *Continued*

hESC lines	Passage number	Culture substrate	Gene expression assay	Number of transcripts analysed	Reference sample	Threshold difference	Difference between hESC lines or other hESC data sets	References
Pool H1-H7-H9, HUES-2	NS	Matrigel-CM, MEFs	MPSS	23500	NS	≥10-fold	0.9 correlation	Wei *et al*. (2005)
H9.2	NS	MEFs	Microarray	22215-HG-U133A	AT	≥3-fold	NS	Golan-Mashiach *et al*. (2005)
Pool H1-H7-H9, BG02	Pool H1-H7-H9 p25–45	Matrigel-CM, MEFs	Microarray and MPSS	16659-Operon 26599 (MPSS)	huURNA	FET≥2-3-fold	94% in common with enriched genes in Bhattacharya *et al*. (2004)	Bhattacharya *et al*. (2005)
BG01V, Pool H1-H7-H9	NS	MEFs	Microarray	20170-G4110B Agilent 24000-Illumina RefSeq8	huURNA	≥3-fold	>0.9 correlation	Plaia *et al*. (2005)
HS181, HS235, HS237, FES21, FES22, FES29, FES30	HS181 p32–34, HS235 p50–56, HS237 p35–37, FES21 p20–30, FES22 p38–48, FES29 p23–33, FES30 p25–35	MEFs	Microarray	39000-HG-U133A+B	NL	>2-fold	80% in common	Skottman *et al*. (2005a)
HS237	P37	HFF	Microarray	39000-HG-U133A+B	NL	>2-fold	~10% in common with enriched genes in Sato *et al*. (2003); Sperger *et al*. (2003); Bhattacharya *et al*. (2004)	Skottman *et al*. (2005b)
H7-Adapted H7	Adapted H7 p>100	MEFs	Micrarray	22283-HG-U133A	NS	SAM	NS	Enver *et al*. (2005)
H1, H1T	NS	MEFs	Microarray	41000-Agilent	NS	≥1.5-fold	NS	Xiao *et al*. (2006)

AFT, adult and fetal peripheral tissues; AT, adult tissues; CM, mouse embryonic fibroblast conditioned medium; EST, expressed sequence tag; FET, Fisher exact test; HFF, human foreskin fibroblasts; huURNA, total human universal RNA; MEFs, mouse embryonic fibroblasts; MPSS, microarray analysis and massively parallel signature sequencing; ND, not determined; NL, non-lineage differentiated cells; NS, not specified; SAM, significance analysis of microarrays; SCL, somatic cell lines.

as characteristic of 'stemness' (Ramalho-Santos *et al*., 2002) or defining a 'stem cell signature' (Ivanova *et al*., 2002). Studies that compared independently-derived and cultured hESCs showed variation in numbers of differentially expressed genes between cell lines, such as 25% between H1 and BG01 (Rao and Stice, 2004), 48% between HSF-1, HSF-6 and H9 (Abeyta *et al*., 2004), and 20% between HS181, HS235, HS237, FES21, FES22, FES29 and FES30 (Skottman *et al*., 2005a; Table I). Variability in gene expression was found in housekeeping genes as well as in several important 'stem cell' genes, including *GP130*, *STAT3*, *FOXD3* and *RUNX1* (Abeyta *et al*., 2004). Quantitative differences in gene expression between hESC lines also exist in a variety of genes, but no direct correlation with function has been made yet (Abeyta *et al*., 2004; Skottman *et al*., 2005a). At least some of these differences may have important biological consequences, as demonstrated by the different developmental outcome resulting from a <2-fold variation in the expression of *Oct4* in mESCs (Niwa *et al*., 2000).

Sources of inter-line variation

Culture-related effects

Several suggestions have been made to explain the observed variation in gene expression profiles between hESC lines. One possibility is that the variation between independent hESC lines may be the result of different culture conditions and, if so, it would be predicted that hESC lines derived and cultured in the same laboratory may be more similar. Indeed, greater similarities have been observed in four cell lines from Finland (FES21, FES22, FES29 and FES30) compared with three from Sweden (HS181, HS235 and HS237; Skottman *et al*. 2005a), in HSF1/HSF6 compared with H9 (Abeyta *et al*., 2004) and in BG01/BG01-MEDII compared with H1 (Rao *et al*., 2004).

Independently-derived and cultured hESCs generally require different feeder cell types and densities, culture substrates, culture media, growth factors/other additives and passage methods (Allegrucci *et al*., 2005; Goh *et al*., 2005). That the culture environment can have a profound effect on the molecular signature of hESCs is shown by the differential expression of 1417 (13.5%) genes between HS237 hESCs cultured in medium containing serum or KnockOut™ Serum Replacement (Skottman *et al*., 2005b). Several lines have been exposed to multiple passaging methods during their early history in an attempt to improve their expansion before wide-scale distribution to researchers. Different culture conditions are also adopted frequently by investigators for a few passages to provide appropriate conditions for specific experiments. The long-term effects of these strategies need to be carefully investigated. Examples of such manipulations include the use of Matrigel™ substrate or lower-density feeders to facilitate genetic modification, enzymatic passaging methods for obtaining a suspension of single cells for freezing or transfection and the use of serum substitutes to provide a more defined and reproducible culture system (Reubinoff *et al*., 2001; Xu *et al*., 2001; Vallier *et al*., 2005; Denning *et al*., 2006; Ludwig *et al*., 2006a).

Another important issue to consider when comparing different expression profiles between hESC lines is the passage number at which the assay is performed. Although hESCs maintain the pluripotent state long-term in culture (Amit *et al*., 2000; Amit *et al*., 2003; Brimble *et al*., 2004; Rosler *et al*., 2004; Lee *et al*., 2005a), some time-related changes have been observed. Differences in gene expression after continuous passaging, which are independent of aneuploidy, have been reported (Rosler *et al*., 2004). Many studies do not take passage number into account (and often do not even report the passage number assayed), making the interpretation of inter-line comparisons within a study, as well as comparison between different studies, very difficult.

The presence of contaminating feeder cells typical in hESC culture may represent a further confounding factor in analysis of some parameters. There is also the aforementioned possibility that hESC cultures may not represent a homogeneous population of undifferentiated cells. These problems can be minimized by fluorescence-activated cell sorting (FACS) cells before analysis, as shown by the significant difference in gene expression observed between sorted cells highly positive for the pluripotency marker, SSEA-3, compared with the other fraction of cells that retain multilineage differentiation potential in the same culture but are SSEA-3 negative (Enver *et al*., 2005). Whether FACS with another 'pluripotency' marker, such as TRA-1-60 or TRA-1-81, would yield similar microarray profiles remains to be examined.

Genetic variation

Although more similarity in cell lines derived and cultured in the same laboratory has been observed, even with identical derivation conditions, hESC lines differ to some extent (Cowan *et al*., 2004; Zeng *et al*., 2004; Skottman *et al*., 2005a). SAGE analysis of HES-3 and HES-4 revealed a 27% difference in expressed genes, including the pluripotency-associated *REX-1* and *GAL* (Richards *et al*., 2004). Similarly, microarray analysis of the cell lines, BG01 and BG02, demonstrated only ~36% of genes expressed in common, and of these, only ~25% were found expressed in other hESC lines, including TE05, GE01, GE09 and the pooled H1-H7-H9 Wisconsin lines (Zeng *et al*., 2004). Because differences between lines have been detected despite the same culture conditions, it has been suggested that this could be due to the unique genetic identity of the embryos from which the hESCs were derived (Abeyta *et al*., 2004; Cai *et al*., 2006; Wei *et al*., 2005). Although hESC lines exhibit a distinctive genotype (Brimble *et al*., 2004; Carpenter *et al*., 2004; Kim *et al*., 2005; Lee *et al*., 2005b; Oh *et al*., 2005), it is unlikely that such a degree of difference could only be due to genetic variation as <2% variation in gene expression has been found in adult human tissues of different individuals (Hsiao *et al*., 2001). That hESC lines arise from different quality embryos, which were also cultured to the blastocyst stage in a variety of different media and were donated from patients of undefined infertility, may also contribute to varying degrees (Hoffman and Carpenter, 2005a).

Strategies to obviate comparison between cell lines include the adaptation of cells previously cultured under determined conditions to new ones for few passages before analysis (Skottman *et al*., 2005a). This approach does not discount epigenetically inherited modifications from previous culture history when differences are observed. Because universal protocols for hESC cultures are not available, it is at present difficult to discriminate the relative contribution of genetic, procedural and/or epigenetic variation to the gene expression signature of each hESC line.

The observed differences cannot be related solely to the gender of lines, as demonstrated by the observation that lines of the same

sex are not more similar (Abeyta *et al*., 2004) and that the expression differences between hESC lines do not cluster on any particular chromosome (Brandenberger *et al*., 2004a).

Experimental variation

Because of the effect of the microenvironment on hESCs, it is possible that variation in results may be encountered when the same experiment is repeated using a different dish of cells or a different batch post thaw.

However, although many of the studies are carried out with simultaneous replicates (i.e. technical repeats), there are no data on experiments repeated over time or from a different vial of thawed cells. This is likely due to the time scale involved in generating samples for analysis and the cost implications (Aldhous, 2006).

Variance in transcriptome profiles between studies may also be due to methodological variations. The validity of the first published bioinformatic comparisons between transcription profiling reports, undertaken in a search for common, 'stemness genes', has been questioned on the basis of differences in outcomes in different studies (Ivanova *et al*., 2002; Ramalho-Santos *et al*., 2002; Fortunel *et al*., 2003). Reproducibility of microarray experiments has been an object of discussion, and this could be due to difference in platforms (Kothapalli *et al*., 2002), differential gene annotation (Mecham *et al*., 2004) and variability due to biological material or operational procedures (Yauk *et al*., 2004). Different platforms for gene expression determination contain array of genes that do not represent the whole genome and may vary between different studies. In addition, variation may also be the result of using the same platform but different 'control' samples as reference. The use of a 'common reference pool' of relevant lines or cultures may be an appropriate method to limit variability across studies (Beqqali *et al*., 2006). Computational algorithms and statistical tests applied in microarray experiments are a limiting factor for cross comparisons between different studies, and sometimes the use of raw data may result in a more productive approach (Suarez-Farinas *et al*., 2005). Another source of variability may arise from the degree of difference in gene expression set as 'threshold' in different studies. Many of the experiments consider differential expression when the measurement is 2-fold or higher than the reference sample, but some studies use a higher threshold (Table I).

When considering all these genetic, environmental and methodological factors, directly comparing hESC lines becomes challenging. However, it must be recognized that all the gene expression studies performed so far have been very informative and valuable for the identification of those 'master' genes that are highly expressed in hESCs and that are likely to determine hESC function. The future development of standardized conditions for hESC culture and analysis together with stringent and uniform statistical data validation will allow better understanding of the unique ESC characteristics common across different cell lines.

Genetic stability

The preservation of genomic integrity in culture may be a major constraint for stem cell function *in vivo* and *in vitro*. Long-term maintenance of stem cells in an environment potentially suboptimal compared with the *in vivo* situation may lead to the accumulation of genetic defects. Even in optimal culture conditions, the fact that, *in vivo*, the inner cell mass exists in a pluripotent state for only a few days before forming the tissues of the fetus and placenta may render adaptive genetic changes (Draper *et al*., 2004), an inevitable consequence of long-term culture. This aspect is of particular relevance if stem cell derivatives are going to be used in cell therapies as the relationship between genomic instability and carcinogenesis is well established (Allegrucci *et al*., 2004; Rajagopalan and Lengauer, 2004; Storchova and Pellman, 2004).

hESC lines acquire abnormalities in specific chromosomes with extended time in culture, and higher incidence has been reported for aneuploidy of chromosomes 12, 17 and X (Table II; Brimble *et al.* 2004; Cowan *et al*., 2004; Draper *et al*., 2004; Inzunza *et al*., 2004; Hanson and Caisander, 2005; Maitra *et al*., 2005; Mitalipova *et al*., 2005). A propensity for the clonal selection of trisomy 12 was reported in HUES (Cowan *et al*., 2004) and H1 (Lakshmipathy *et al*., 2004) lines, whereas trisomy 17 was also observed (Brimble *et al*., 2004; Mitalipova *et al*., 2005) in BG01 and BG02. Preferential gains in 12 or 17 in H14 have also varied between laboratories (Liu *et al*., 1997), while Rosler *et al*. (2004) reported trisomy 20 as the prevalent aneuploidy in H1, H7 and H9. Trisomy of chromosomes 13 and 3 was only observed in SA002 (Heins *et al*., 2004; Caisander *et al*., 2006) and in Miz-hES13 (Kim *et al*., 2005), respectively.

Chromosomal abnormalities are not a general trend in every hESC culture (Buzzard *et al*., 2004; Darnfors *et al*., 2005; Mitalipova *et al*., 2005), and it is not clear whether certain cell lines are intrinsically prone to developing abnormalities or whether their instability is a consequence of certain culture methods. It has been proposed that passage of hESCs by mechanical dissection preserves genomic integrity better than enzymatic methods (Brimble *et al*., 2004; Buzzard *et al*., 2004; Mitalipova *et al*., 2005). This suggestion seems plausible as hESCs express high levels of gap junctions and cell adhesion molecules indicating that cell-to-cell contacts are essential for their function (Xu *et al*., 2001; Wong *et al*., 2004; Zeng *et al*., 2004; Caisander *et al*., 2006; Huettner *et al*., in press). Mechanical passage of hESC colonies allows preservation of these connections, given that only a portion of the colony is dissected and re-plated for successive culture with this method. On the contrary, enzymatic dissociation causes destruction of cell contacts to varying extents (e.g. trypsin more than collagenase) and imposes a stress pressure on the isolated hESCs in culture. However, sporadic aneuploidies can also occur in mechanically passaged cells (C. Allegrucci, unpublished data; Buzzard *et al*., 2004; Caisander *et al*., 2006), and this may be related to the selection of aberrant, faster-growing cells after passage and/or freezing/thawing—a phenomenon described as 'adaptation to culture' (Draper *et al*., 2004). During passage by manual dissection, larger colonies (with no visual evidence of differentiation) are generally selected for passage subjectively by the operator, even though there is evidence from mESCs that faster growing colonies are more likely to accumulate genetic alterations and do not give any germline contribution to chimeras (Liu *et al*., 1997). A chromosome decatenation checkpoint deficiency has been recently discovered in mESCs that could predispose to aneuploidy in conditions of selective pressure such as suboptimal, *in vitro* culture (Damelin *et al*., 2005; Damelin and Bestor, 2006). The variable expression of the *DNMT3B* enzyme observed in hESC lines (Sperger *et al*., 2003; Bhattacharya *et al*., 2004; Brandenberger *et al*.,

Table II. Karyotype reported for human embryonic stem cell (hESC) lines

hESC lines, passage number or time in culture	Culture substrate	Passage method	Karyotype analysis	Number of cells analysed	Replicates	Karyotype	References
H1 (NS) H7 (NS) H9 (NS) H13 (NS) H14 (NS)	MEFs	C-IV	G banding	NS	NS	46,XY-(H1) 46,XX-(H7) 46,XX-(H9) 46,XY-(H13) 46,XY-(H14)	Thomson *et al*. (1998)
H9 (122) H9.1 (NS) H9.2 (NS)	MEFs	C-IV	G banding SKY	20-G banding 5-SKY	NS	46,XX-(H9) 46,XX-(H9.1) 46,XX-(H9.2)	Amit *et al*. (2000)
I3 (>20) I4 (>20) I6 (>20)	MEFs	C-IV	G banding	NS	NS	46,XX-(I3) 46,XX-(I4) 46,XY-(I6)	Amit and Itskovitz-Eldor (2002)
WT3 (NS) HUES-3 (NS)	MEFs	M	CGH	100–200	NS	46,XX-(WT3) 46,XX-(HUES-3)	Pickering *et al*. (2003)
I3 (107) H9 (NS)	MEFs + HFF	C-IV	G banding	≥50	Yes	46,XX-(I3) 46,XX-(H9)	Amit *et al*. (2003)
HS181 (30)	HFF	M	G banding	NS	NS	46,XX	Hovatta *et al*. (2003)
BG01 (35) BG02 (35)	MEFs	M	G banding	NS	NS	46,XY-(BG01) 46,XY-(BG02)	Mitalipova *et al*. (2003)
HUES-1 (47,59,81) HUES-2 (94,104,118,140) HUES-3 (58,84,97,110) HUES-4 (25,54,91) HUES-5 (13,20,23,26,38,42,24) HUES-6 (13,27,34)	MEFs	M	G banding	20	NS	46,XX-(HUES-1) 46,XX-(HUES-2) 46,XX-(HUES-3) 46,XY-(HUES-4) 46,XY/46,Y,t(X;17)(p11;p13)-(HUES-5 only p38) 46,Y,t (X;17)(p11;p13)-(HUES-5 only p42) 46,XX-(HUES-6)	Buzzard *et al*. (2004)
HS181 (22,39) HS235 (39,59) HS237 (17,35,61)	HFF	M	CGH G banding	(HS181-24, HS235-14, HS237-12)-CGH	NS	46,XX/20% 46,X-(HS181-CGH p39) 46,XX-(HS181-G banding p22) 46,XX-(HS235-CGH p39) 46,XX-(HS235-G banding p59) 46,XX-(HS237-G banding p17) 46,XX-(HS237-CGH p35) Xq21, 46,X,idic (X)(q21) (HS237-CGH p61)	Inzunza *et al*. (2004)
H7 (44,53,55,56,68,80) H14 (30,48,53)	MEFs	C-IV	G banding FISH	≥20	NS	46,XX-(H7 p28, 32,35, 44,55) 47,XX,+17-(H7 p53,56,68) 46,XX, der(6)t(6;17q)-(H7 p80) 46,XY-(H14 p30,48) 47,XY,+17-(H14 p53)	Draper *et al*. (2004)
HUES-1 (20,30,39) HUES-2 (48,50) HUES-3 (23,33–47)	MEFs	TE	G banding	20	NS	46,XX-(HUES-1;46,XX,2q+ at p39) 46,XX-(HUES-2) 46,XY-(HUES-3; 47,XY,+12/46,XY at p33–47)	Cowan *et al*. (2004)

Table II. *Continued*

hESC lines, passage number or time in culture	Culture substrate	Passage method	Karyotype analysis	Number of cells analysed	Replicates	Karyotype	References
HUES-4 (20,22,29–47) HUES-5 (14) HUES-6 (12) HUES-7 (10) HUES-8 (10) HUES-9 (9) HUES-10 (7) HUES-11 (10) HUES-12 (12,15) HUES-13 (11) HUES-14 (10,11) HUES-15 (7) HUES-16 (8,9) HUES-17 (13)						46,XY-(HUES-4; 47,XY,+12/46,XY at p29–47) 46,XX,inv9-(HUES-5) 46,XX-(HUES-6) 46,XY-(HUES-7) 46,XY-(HUES-8) 46,XX,inv9-(HUES-9) 46,XY-(HUES-10) 46,XY-(HUES-11) 46,XX-(HUES-12) 46,XY-(HUES-13) 46,XX-(HUES-14) 46,XX-(HUES-15) 46,XY-(HUES-16) 46,XY-(HUES-17)	
BG01 (32,35,42,52,84) BG02 (19,28,32) BG03 (17,23,30,31,34)	MEFs, Matrigel-CM	M,TE	G banding	10–44	NS	46,XY-(BG01;47,XY,+17,60%-48,XY,+12,+17, 15%-49,XY+1,+12,+17,20% in TE p32; 47,XY,+17,87% in TE p42; 50,XXY,+12,+14,+17,91%-51,XXY,+7,+12, +14,+17,4.5%-51,XXY,+8,+12,+14,+17,4.5% in TE p84) 46,XY-(BG02) 46,XX-(BG03)	Brimble *et al*. (2004)
hUES-NCL1 (NS)	MEFs, Matrigel-CM	C-IV	G banding	NS	NS	46,XX	Stojkovic *et al*. (2004)
SA002 (NS) FC018 (NS) AS034 (NS) AS038 (NS) SA121 (NS) SA181 (NS) AS034.1 (NS)	MEFs	M	G banding FISH	≥200-FISH	NS	47,XX,+13-(SA002) 69,XXY-(FC018) 46,XY-(AS034) 46,XY-(AS038) 46,XY-(SA121) 46,XY-(SA181) 46,XY-(AS034.1)	Heins *et al*. (2004)
MB01 (>15) MB02 (>15) MB03 (>15) MB04 (>15) MB05 (>15) MB06 (>15) MB07 (>15) MB08 (>15) MB09 (>15)	STO	C-IV	G banding	NS	NS	46,XY-(MB01) 46,XX-(MB02) 46,XX-(MB03) 46,XY-(MB04) 46,XX-(MB05) 46,XY-(MB06) 46,XY-(MB07) 46,XX-(MB08) 46,XY-(MB09)	Park *et al*. (2004)
I9 (27,29)	MEFs	C-IV	G banding	66	NS	46,XX	Suss-Toby *et al*. (2004)
Royan H1 (5,29)	MEFs	M+D	G banding	≥20	NS	46,XX	Baharvand *et al*. (2004)

Table II. *Continued*

hESC lines, passage number or time in culture	Culture substrate	Passage method	Karyotype analysis	Number of cells analysed	Replicates	Karyotype	References
Miz-hES4 (37) Miz-hES5 (31) Miz-hES6 (33) Miz-hES7 (31) Miz-hES8 (31) Miz-hES10 (19) Miz-hES11 (19) Miz-hES12 (16) Miz-hES13 (16)	MEFs	M	G banding	≥50	NS	46,XY-(Miz-hES4) 46,XY-(Miz-hES5) 46,XX-(Miz-hES6) 46,XX-(Miz-hES7) 46,XX-(Miz-hES8) 46,XY-(Miz-hES10) 46,XX-(Miz-hES11) 46,XX-(Miz-hES12) 47,XX,+3-(Miz-hES13)	Kim *et al*. (2005)
HS293 (9,49) HS306 (32)	HFF	M	G banding	NS	NS	46,XY-(HS293) 46,XX-(HS306)	Inzunza *et al*. (2005)
SA002.5 (174,196) AS034.1.1 (164,167)	MEFs	M	G banding	6–19 100-FISH	NS	46,XX-(SA002.5) 46,XY-(AS034.1.1)	Heins *et al*. (2006)
BG01 (41,65) BG02 (50,52,58,59, 62,68,70,74,80,100,105)	MEFs	M,CDB,C-IV,T	G banding FISH	7–25	NS	46,XY-(BG01;48,XY,+12,+17,80%-49,XXY,+12,+17,20% in CDB p65) 46,XY-(BG02; 47,XY,+17,71%-47,XY, +inv(17)(q11.2q21),29% in CBD p70; 50,XXY,+12,+14,+17,85%-51,XXY,+12,+14,+17,+20,15% in T p68)	Mitalipova *et al*. (2005)
SA002 (87) AS038 (54) SA121 (35) SA167 (42)	MEFs+Matrigel-CM	M, C-IV	G banding FISH	3–17	NS	47,XX,+13-(SA002) 46,XY-(AS038) 46,XY-(SA121) 46,XX-(SA167)	Sjogren-Jansson *et al*. (2005)
H9 (>20) ACT-14 (6 months)	MEFs Matrix	M,T	G banding	NS	NS	46, XX-(H9) 46,XX-(ACT-14)	Klimanskaya *et al*. (2005)
Miz-hES-9 (6 months) Miz-hES14 (6 months) Miz-hES15 (6 months)	hUEC	M	G banding	>100	NS	46,XX-(Miz-hES-9) 46,XX-(Miz-hES-14) 46,XX-(Miz-hES-15)	Lee *et al*. (2005b)
VAL1 (44) VAL2 (24)	HPF	M	G banding	NS	NS	46,XX-(VAL1) 46,XX-(VAL2)	Simon *et al*. (2005)
SNUhES1 (12,15) SNUhES2 (12,15) SNUhES3 (12,15)	STO	M	G banding	NS	NS	46,XY-(SNUhES1) 46,XX-(SNUhES2) 46,XY-(SNUhES3)	Oh *et al*. (2005)
cHES-1 (16,43)	MEFs	C-IV	G banding	NS	NS	46,XX	Li *et al*. (2005)
H7 (>100)	MEFs	C-IV	G banding	NS	NS	47,XX,+1,der(6)t(6;17)(q27;q1)	Enver *et al*. (2005)
BG01V(25)	MEFs	C-IV	G banding	43	NS	49,XXY,+12,+17	Plaia *et al*. (2005)
WA15 (5,7 months) WA16 (5,7 months)	HM	D	G banding FISH	20-G banding 200-FISH	NS	46,XY-(WA15-5 months;47,XY,+12-7 months) 47,XXY-(WA16)	Ludwig *et al*. (2006b)
H1T(NS)	MEFs	T	G banding SKY	>20	NS	46,XY,der(10)t(10;17)	Xiao *et al*. (2006)

Table II. *Continued*

hESC lines, passage number or time in culture	Culture substrate	Passage method	Karyotype analysis	Number of cells analysed	Replicates	Karyotype	References
ReliCell®hES1 (15,37)	MFS	M	G binding	NS	NS	46,XX	Mandal *et al.* (2006)
SA002 (19–46) SA002.5 (17–214) AS034.1.1 (164–197) SA121 (35–148) SA461 (12–36)	MEFs	M	G binding FISH CGH	100–200-FISH 10-15-CGH	NS	47,XX,+13-(SA002 p19,23) 46,XX-(SA002.5) 46,XY-(AS034.1.1) 46,XY-(SA121) 46,XY-(SA461)	Caisander *et al*. (2006)
Royan H2 (10–30) Royan H3 (10–30) Royan H4 (10–30) Royan H5 (10–30) Royan H6 (10–30)	MEFs	M+D	G banding	≥20	NS	46,XX-(Royan H2) 69,XXY-(Royan H3) Mosaic 69,XXY-(Royan H4) 46,XX-(Royan H5) 46,XY-(Royan H6)	Baharvand *et al*. (2006)

CDB, cell dissociation buffer; CGH, comparative genomic hybridization; C-IV, collagenase IV; CM, mouse embryonic fibroblast conditioned medium; D, dispase; FISH, fluorescence *in situ* hybridization; HFF, human foreskin fibroblasts; HM, human matrix; HPF, human placental fibroblasts; hUEC, human uterine endometrial cells; M, mechanical; MEFs, mouse embryonic fibroblasts; ND, not determined; NS, not specified; p, passage; SAGE, serial analysis of gene expression; SKY, multicolour spectral analysis; STO, immortalized mouse fetal fibroblast line; T, Trypsin; TE, Trypsin/EDTA.

2004a; Rao *et al*., 2004; Richards *et al*., 2004; Skottman *et al*., 2005a) may also contribute to chromosomal instability due to alteration in genome-wide methylation (Chen *et al*., 1998; Dodge *et al*., 2005; Zvetkova *et al*., 2005). In terms of cryopreservation, vitrification appears to enhance post-thaw survival relative to conventional freezing techniques, but whether it is more likely to induce clonal selection of cell-harbouring defects that enhance survival in culture remains to be investigated. The relative importance of the passage method in the occurrence of chromosomal abnormalities in certain cell lines is not clear. hESCs are derived from supernumerary embryos generated by assisted reproductive technologies (ARTs), and they may be mosaic for aneuploidies from the time of derivation (Hardarson *et al*., 2003). The derivation of triploid Royan H3, Royan H4 and FC018 cell lines may be the result of such chromosomal mosaicism (Heins *et al*., 2004; Baharvand *et al*., 2006).

However, perhaps the major limitation to interpreting ploidy data is the lack of experimental replication. Most published karyotypes refer to only a single culture (Table II), often concluding effects of culture treatment on this limited data. In addition, no technical standards have been fixed for hESC karyotype assessment, and different methods are currently used in different laboratories. The conventional analysis is based on banding of metaphase spreads. With this technique, different conclusions may be reached by analysing variable and statistically insufficient numbers of spreads (Table II) when the aneuploidy does not represent the entire cell population—a typical scenario in hESC cultures (Brimble *et al*., 2004; Rosler *et al*., 2004; Mitalipova *et al*., 2005). According to Hook (1977), examination of 30 cells means that mosaicism at the level of ≥10% can be ruled out with 95% confidence interval (CI). In addition, G banding, although reliable when performed by an appropriately trained operator, does not permit high resolution. Superior methods, such as fluorescent *in-situ* hybridization (FISH) and comparative genome hybridization (CGH), have recently been implemented in hESC chromosomal analysis (Amit *et al*., 2000; Pickering *et al*., 2003; Heins *et al*., 2004; Inzunza *et al*., 2004; Mitalipova *et al*., 2005; Sjogren-Jansson *et al*., 2005; Caisander *et al*., 2006; Ludwig *et al*., 2006b), and only a combination of different techniques can guarantee ultimately good coverage of all possible genetic abnormalities (Pera, 2004; Speicher and Carter, 2005). For instance, the predominant mechanism of mutation in mESCs is loss and reduplication of chromosomes leading to uniparental disomy (UPD) (Cervantes *et al*., 2002), and this abnormality is generally not detected by standard karyotyping but can be identified by single-nucleotide polymorphism (SNP) arrays (Speicher and Carter, 2005).

The suggestion that genetic instability in hESCs may arise from the selection of cells with a growth advantage in culture (Draper *et al*., 2004) is supported by the observed high incidence of trisomies of chromosomes 12 and 17. The presence of isochromosome 12p is also a common feature of human teratocarcinoma cells (hECs), the malignant counterpart of hESCs (Skotheim *et al*., 2002; Clark *et al*., 2004; Draper *et al*., 2004), and the amplification of 17q is associated with neuroblastoma (Westermann and Schwab, 2002). Genes that control self-renewal differentiation and apoptosis, including *STELLAR*, *NANOG*, *GDF3*, *STAT3*, *GRB2*, *BIRC5*, *NT3* and $p75^{NGFR}$, are located on those chromosomes (Burdon *et al*., 2002; Chiou *et al*., 2003; Clark *et al*., 2004; Pyle *et al*., 2006), and *in vitro* constraint may provide the pressure to increase gene copy number. In fact, aneuploid hESC cultures grow faster (Cowan *et al*., 2004; Enver *et al*., 2005; Plaia *et al*., 2005; Herszfeld *et al*., 2006; Xiao *et al*., 2006) and are highly clonogenic. Aneuploid cultures also express higher levels of the hESC markers, SSEA-3 and SSEA-4 (Enver *et al*., 2005; Xiao *et al*., 2006), and pluripotency genes, *OCT4* and *NANOG*. They also up-regulate self-renewal signalling pathways involving Nodal/Activin, Wnt, FGF and Hedgehog and down-regulate differentiation pathways involving BMP and Notch (Enver *et al*., 2005; Mitalipova *et al*., 2005; Xiao *et al*., 2006). The expression of a truncated form of CD30 is also a feature of karyotypically abnormal hESCs and hECs, and it is associated with the inhibition of apoptosis and increased cell survival (Herszfeld *et al*., 2006).

Despite these imbalances, aneuploid hESCs seem to maintain the ability to differentiate *in vitro* (Brimble *et al*., 2004; Draper *et al*., 2004) and *in vivo* (Xiao *et al*., 2006). However, the presence and isolation of undifferentiated cells from a teratoma generated by the injection of the aneuploid BG01V and HUES-3 cell lines may indicate the formation of an undifferentiated teteratocarcinoma-like tumour and not the completely differentiated benign teratoma expected of euploid hESCs (Plaia *et al*., 2005; Herszfeld *et al*., 2006).

A recent, extensive, study demonstrated that 5 of 10 hESC lines analysed presented ploidy alterations (including deletions and amplifications) during prolonged time in culture (Maitra *et al*., 2005). Of note is the observed amplification of the proto-oncogene, *MYC*, that is likely to be associated with a selective growth advantage for hESCs, because this transcription factor affects cell proliferation via cell cycle progression (Secombe *et al*., 2004). Overexpression of *MYC* has also been observed in the spontaneous transformation of murine and human adult mesenchymal stem cells *in vitro* (Miura *et al*., 2005; Rubio *et al*., 2005). Mitochondrial DNA mutations have also been observed in 2/10 cell lines in the Maitra *et al*. (2005) study, and it is of interest that these are a common feature of ageing and cancer (Singh, 2004; Czarnecka *et al*., 2006). Because the alteration in the balance between self-renewal/proliferation and spontaneous differentiation is considered a hallmark of cancerous transformation (Hanahan and Weinberg, 2000; Reya *et al*., 2001; Pardal *et al*., 2003; Gudjonsson and Magnusson, 2005; Pathak and Multani, 2006), careful evaluation of the phenomena reviewed above pertaining to hESC 'adaptation to culture' must be addressed to ensure safety and efficacy in downstream applications.

Epigenetic stability

Although the genetic stability of hESCs has received general attention, little is known about their epigenome. The complex range of epigenetic modifications to DNA and associated histones determine whether and when a particular gene is expressed (Jenuwein and Allis, 2001; Jaenisch and Bird, 2003; Fuks, 2005). Epigenetic changes during culture may have profound implications for the use of hESCs in regenerative medicine by affecting differentiation capacity and tumorigenic potential (Allegrucci *et al*., 2004).

DNA methylation is a heritable epigenetic modification that contributes to gene silencing, and many cell types accumulate methylation defects during *in vitro* culture (Antequera *et al*., 1990; Zhu *et al*., 1999; Hannula *et al*., 2001; Smiraglia *et al*., 2001; Paz

et al., 2003). DNA methylation defects can affect different components of the genome, including CpG islands in promoter regions, repetitive sequences and imprinted genes (Robertson, 2005). Methylation alterations in imprinted genes can be induced by cellular stress (Pantoja *et al*., 2005) and specific culture conditions (Allegrucci *et al*., 2004). Disrupted imprinting was found in cultures of mESCs (Dean *et al*., 1998; Humpherys *et al*., 2001) and monkey ESCs (Fujimoto *et al*., 2005), but only one line of four examined showed evidence of imprinting instability in undifferentiated hESCs, and then only at high passage number (Rugg-Gunn *et al*., 2005; Sun *et al*., 2006). Similarly, no significant sign of imprinting disruption has been found in differentiated human embryonic germ cells (hEGCs; Onyango *et al*., 2002). Monoallelic expression of the imprinted genes *H19*, *KCNQ1*, *PEG10* and *NDNL1* has been observed in SHhES1 and HUES-7 cell lines at both early and late passage (Sun *et al*., 2006). Similarly, the aneuploid BG01V cell line shows the expected monoallelic methylation of *SNRPN*, *H19* and *DLK1/MEG3* (Plaia *et al*., 2005). Expected imprinting status of *KCNQ1*, *IGF2*, *SCL22A18*, *NESP55* and *SNRPN* has also been shown in early and late passage H9, H7, HUES-3 and HSF6 hESC lines (Rugg-Gunn *et al*., 2005). Because the disruption of imprinted genes is associated with disease and carcinogenesis (Lucifero *et al*., 2004; Holm *et al*., 2005), more extensive studies examining a larger panel of imprinted genes in additional hESC lines would be still prudent to conclude the widespread stability of genomic imprinting in these cells.

DNA methylation alteration at non-imprinted genomic loci can induce genomic instability and is associated with cancer (Gaudet *et al*., 2003; Herman and Baylin, 2003; Baylin, 2005; Esteller, 2005). Both DNA hypomethylation (activating oncogenes) and hypermethylation (inactivating tumour suppressors) at diverse genomic sequences are found in tumours, often associated with altered expression of the DNA methyltransferase enzymes (Jones and Baylin, 2002). A high incidence hypermethylation of CpG islands in the promoter region of the tumour suppressor genes, *RASSF1* and/or *PTPN6*, has been associated with long-term culture of the BG01, BG02, BG03, HUES-2, HUES-3, H7, H9, SA001 and SA002 cell lines. Methylation of *TNFRSF10C* was observed but only in HUES-2 and SA002 and not in the other seven lines examined (Maitra *et al*., 2005). Differential methylation in 5′-flanking regions of pluripotency-associated genes *DPPA3* and *DPPA5* between ESM01, ESM02 and ESM03 hESC lines upon differentiation is also indicative of variation in the epigenetic status of hESCs (Lagarkova *et al*., 2006).

The de-novo DNA methyltransferase, DNMT3B, plays a fundamental role in the methylation of CpG islands in mESCs (Hattori *et al*., 2004). High expression of this enzyme has been demonstrated in several hESC lines, although expression levels between lines are variable (Sperger *et al*., 2003; Bhattacharya *et al*., 2004; Brandenberger *et al*., 2004a; Rao *et al*., 2004; Richards *et al*., 2004; Skottman *et al*., 2005a). Expression of *DNMT3B* varies over 2-fold between undifferentiated hESC lines (Rao and Stice, 2004; Skottman *et al*., 2005a), and its expression can be influenced by the presence of serum in the culture medium (Skottman *et al*., 2005b). DNMT3B is generally down-regulated in hESCs during differentiation (Richards *et al*., 2004; Bhattacharya *et al*., 2005; Enver *et al*., 2005), but this is not observed when hESC lines are cultured in feeder-free conditions compared with cultures on mouse embryonic fibroblast feeders (Noaksson *et al*., 2005). It is possible that variations in the level of this key enzyme could account for the epigenetic diversity of hESCs as de-novo methylation of target sequences may be regulated by an instructive mechanism depending on the levels on the DNA methyltransferase enzymes (Keshet *et al*., 2006). However, because the family of DNA methyltransferases is known to co-operate to methylate DNA (Kim *et al*., 2002), studies of other methyltransferases are also likely to be informative. Some nutrients that regulate the availability of methyl group donors for cellular methylation reactions are also present in high and variable levels in hESC culture media, and in the range of media used for human embryo culture (Steele *et al*., 2005). Interaction between methyl metabolism and methyltransferases provides another potential route to altering the epigenetic status of cultured hESC over time (Allegrucci *et al*., 2005).

Epigenetic variation between hESCs may also perturb X chromosome inactivation. In order that female embryos express similar levels of X-linked genes to males, epigenetic regulation of the X chromosome in mammalian females constitutes an important mechanism for gene dosage compensation. Inactivation of one of the two female chromosomes is achieved via expression of the non-coding *XIST* mRNA, by histone modifications and by DNA methylation (Chang *et al*., 2006). Discordant data report variable failures of X chromosome inactivation in different hESC lines. Hoffman *et al*. (2005) observed the expected *XIST* expression indicative of X-inactivation in both undifferentiated and differentiated cells of H9 and CyT25, but not H7. The cell lines, H7 and H13, also expressed *XIST* in the undifferentiated state, whereas H9 did not (Sperger *et al*., 2003). Variations have also been reported within a line; undifferentiated H9 cells in an independent study exhibited only active X chromosomes, although X-inactivation was established after differentiation (Dhara and Benvenisty, 2004). Furthermore, undifferentiated and euploid H7 cells demonstrated *XIST* expression that was lost in high passage, aneuploid cells from the same line even after their differentiation (Enver *et al*., 2005). Failure to silence one of the X chromosomes is also observed frequently in female mESC lines (Sado *et al*., 1996). As a consequence, complete loss of one of the two active X chromosomes is observed together with global hypomethylation of the genome (Zvetkova *et al*., 2005). For this reason, establishment of stable male ESC lines may be more successful in the mouse. In contrast, the frequency of derivation and maintenance of female and male hESCs is almost equivalent (http://stemcells.nih.gov), and it is not clear why differences in X chromosome inactivation between hESC lines are observed, nor is the functional significance defined.

One possibility is that these variations may depend on the status of X-inactivation at the blastocyst stage at the time of hESC derivation (Enver *et al*., 2005), because X-inactivation is thought to occur in the human embryo around this time (Reik and Lewis, 2005). However, this would not account for the disruption of X chromosome inactivation observed in the H7 cell line after prolonged culture, and it is likely instead that the occurrence is a consequence of *in vitro* culture conditions. This hypothesis agrees with the observation that the same hESC line can behave differently in different independent experiments where varying culture conditions are used, like for instance H7 and H9 hESCs cultured on feeders cells or feeder-free conditions (Dhara and Benvenisty, 2004; Enver *et al*., 2005; Hoffman *et al*., 2005). However, until

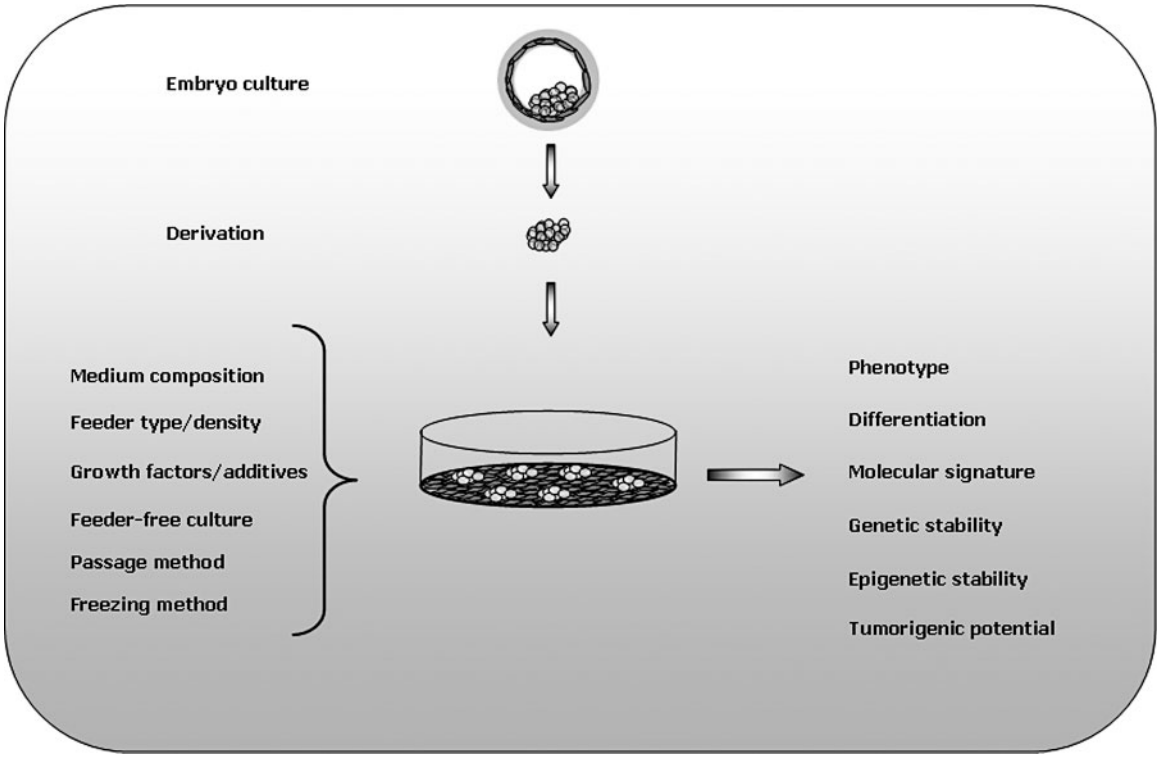

Figure 2. Possible sources of environmental influence and potential effects on human embryonic stem cells (hESCs) upon long-term culture.

the degree of replication is increased in more hESC experiments, the possibility of variations even between culture dishes within an experiment cannot be ruled out.

Implications for therapy

Collectively, all of the observations reviewed above regarding differences between hESC lines point to a degree of additive inherited variation between the lines that depends on the environment(s) to which the cells have been exposed since the time of embryo culture and derivation (Figure 2). Only by applying genome-wide analyses of hESC epigenotype, genotype, transcriptome, proteome and metabolome across many cell lines derived and cultured in a range of comparable means can future studies resolve the impact of environmentally induced effects. With the current cost, material transfer agreement and intensive manual labour limitations of current hESC culture, this type of comprehensive analysis is only likely to occur through large-scale, collaborative efforts and with the development of automated culture systems. Without high-throughput, multi-line hESC culture, many of the developments currently being made by individual laboratories on their own lines are unlikely to be generically applicable—a pitiful waste of our scientific and financial resources and a strategy that can only lengthen developmental timelines for transferring hESC-based therapies to the clinic.

In this context, it is important to consider that an optimal protocol for obtaining cells that will be suitable for producing a particular therapeutic cell type or cell lines that represent key alleles for drug screening or immune haplotype may not be ideal for all applications. For basic science applications of these cells, such as studying early human development and modelling disease, lines that resemble as closely as possible the embryo *in vivo* are the ideal goal (Rossant, 2001; Smith, 2001; Zwaka and Thomson, 2005). Considering that no available line has been made to the good manufacturing practice (GMP) and clinical grade standards required for transplantation (Rodriguez *et al*., 2006), the effort required to ensure that new lines are derived in conditions optimal for applications, in addition to being GMP-compliant, must surely be a high international priority.

Acknowledgements

The authors thank Chris Denning for helpful discussion. This work was funded by BBSRC (grant number BBS/B/06164) and the University of Nottingham.

References

Abeyta MJ, Clark AT, Rodriguez RT, Bodnar MS, Pera RA and Firpo MT (2004) Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum Mol Genet 13,601–608.

Aldhous P (2006) Stem-cells: Miracle postponed? New Scientist 2542,42–46.

Allegrucci C, Denning C, Priddle H and Young L (2004) Stem-cell consequences of embryo epigenetic defects. Lancet 364,206–208.

Allegrucci C, Denning CN, Burridge P, Steele W, Sinclair KD and Young LE (2005) Human embryonic stem cells as a model for nutritional programming: an evaluation. Reprod Toxicol 20,353–367.

Amit M and Itskovitz-Eldor J (2002) Derivation and spontaneous differentiation of human embryonic stem cells. J Anat 200,225–232.

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J and Thomson JA (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227,271–278.

Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R and Itskovitz-Eldor J (2003) Human feeder layers for human embryonic stem cells. Biol Reprod 68,2150–2156.

Amit M, Shariki C, Margulets V and Itskovitz-Eldor J (2004) Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod 70,837–845.

Andrews PW, Benvenisty N, McKay R, Pera MF, Rossant J, Semb H and Stacey GN (2005) The international stem cell Initiative: toward benchmarks for human embryonic stem cell research. Nat Biotechnol 23,795–797.

Antequera F, Boyes J and Bird A (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 62,503–514.

Baharvand H, Ashtiani SK, Valojerdi MR, Shahverdi A, Taee A and Sabour D (2004) Establishment and in vitro differentiation of a new embryonic stem cell line from human blastocyst. Differentiation 72,224–229.

Baharvand H, Ashtiani SK, Taee A, Massumi M, Valojerdi MR, Yazdi PE, Moradi SZ and Farrokhi A (2006) Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. Dev Growth Differ 48,117–128.

Baylin SB (2005) DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2,S4–S11.

Beqqali A, Kloots J, Ward-van Oostwaard D, Mummery C and Passier R (2006) Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24,1956–1967.

Bhattacharya B, Miura T, Brandenberger R, Mejido J, Luo Y, Yang AX, Joshi BH, Ginis I, Thies RS, Amit M *et al*. (2004) Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 103,2956–2964.

Bhattacharya B, Cai J, Luo Y, Miura T, Mejido J, Brimble SN, Zeng X, Schulz TC, Rao MS and Puri RK (2005) Comparison of the gene expression profile of undifferentiated human embryonic stem cell lines and differentiating embryoid bodies. BMC Dev Biol 5,22.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG *et al*. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122,947–956.

Brandenberger R, Khrebtukova I, Thies RS, Miura T, Jingli C, Puri R, Vasicek T, Lebkowski J and Rao M (2004a) MPSS profiling of human embryonic stem cells. BMC Dev Biol 4,10.

Brandenberger R, Wei H, Zhang S, Lei S, Murage J, Fisk GJ, Li Y, Xu C, Fang R, Guegler K *et al*. (2004b) Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. Nat Biotechnol 22,707–716.

Brimble SN, Zeng X, Weiler DA, Luo Y, Liu Y, Lyons IG, Freed WJ, Robins AJ, Rao MS and Schulz TC (2004) Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev 13,585–597.

Burdon T, Smith A and Savatier P (2002) Signalling, cell cycle and pluripotency in embryonic stem cells. Trends Cell Biol 12,432–438.

Buzzard JJ, Gough NM, Crook JM and Colman A (2004) Karyotype of human ES cells during extended culture. Nat Biotechnol 22,381–382; author reply 382.

Cai J, Chen J, Liu Y, Miura T, Luo Y, Loring JF, Freed WJ, Rao MS and Zeng X (2005) Assessing self-renewal and differentiation in hESC lines. Stem Cells 24,516–530.

Caisander G, Park H, Frej K, Lindqvist J, Bergh C, Lundin K and Hanson C (2006) Chromosomal integrity maintained in five human embryonic stem cell lines after prolonged in vitro culture. Chromosome Res 14,131–137.

Carpenter MK, Rosler E and Rao MS (2003) Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells 5,79–88.

Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, Lucero M and Rao MS (2004) Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229,243–258.

Cervantes RB, Stringer JR, Shao C, Tischfield JA and Stambrook PJ (2002) Embryonic stem cells and somatic cells differ in mutation frequency and type. Proc Natl Acad Sci USA 99,3586–3590.

Chang SC, Tucker T, Thorogood NP and Brown CJ (2006) Mechanisms of X-chromosome inactivation. Front Biosci 11,852–866.

Chen RZ, Pettersson U, Beard C, Jackson-Grusby L and Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395,89–93.

Chiou SK, Jones MK and Tarnawski AS (2003) Survivin – an anti-apoptosis protein: its biological roles and implications for cancer and beyond. Med Sci Monit 9,PI25–29.

Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, Firpo MT and Reijo Pera RA (2004) Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. Stem Cells 22,169–179.

Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D *et al*. (2004) Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350,1353–1356.

Czarnecka AM, Golik P and Bartnik E (2006) Mitochondrial DNA mutations in human neoplasia. J Appl Genet 47,67–78.

Damelin M and Bestor TH (2006) Decatenation checkpoint deficiency destabilizes the stem cell genome. Cell Cycle 5,345–346.

Damelin M, Sun YE, Sodja VB and Bestor TH (2005) Decatenation checkpoint deficiency in stem and progenitor cells. Cancer Cell 8,479–484.

Darnfors C, Flodin A, Andersson K, Caisander G, Lindqvist J, Hyllner J, Wahlstrom J and Sartipy P (2005) High-resolution analysis of the subtelomeric regions of human embryonic stem cells. Stem Cells 23,483–488.

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W and Feil R (1998) Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. Development 125,2273–2282.

Denning C, Allegrucci C, Priddle H, Barbadillo-Munoz MD, Anderson D, Self T, Smith NM, Parkin CT and Young LE (2006) Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7. Int J Dev Biol 50,27–37.

Dhara SK and Benvenisty N (2004) Gene trap as a tool for genome annotation and analysis of X chromosome inactivation in human embryonic stem cells. Nucleic Acids Res 32,3995–4002.

Dodge JE, Okano M, Dick F, Tsujimoto N, Chen T, Wang S, Ueda Y, Dyson N and Li E (2005) Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. J Biol Chem 280,17986–17991.

Draper JS, Pigott C, Thomson JA and Andrews PW (2002) Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat 200,249–258.

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA and Andrews PW (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22,53–54.

Dvash T, Mayshar Y, Darr H, McElhaney M, Barker D, Yanuka O, Kotkow KJ, Rubin LL, Benvenisty N and Eiges R (2004) Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. Hum Reprod 19,2875–2883.

Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, Thykjaer T, Maltby E, Smith K, Dawud RA *et al*. (2005) Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Hum Mol Genet 14,3129–3140.

Esteller M (2005) Aberrant DNA methylation as a cancer-inducing mechanism. Annu Rev Pharmacol Toxicol 45,629–656.

Faden RR, Dawson L, Bateman-House AS, Agnew DM, Bok H, Brock DW, Chakravarti A, Gao XJ, Greene M, Hansen JA *et al*. (2003) Public stem cell banks: considerations of justice in stem cell research and therapy. Hastings Cent Rep 33,13–27.

Fang D, Leishear K, Nguyen TK, Finko R, Cai K, Fukunaga M, Li L, Brafford PA, Kulp AN, Xu X *et al*. (in press) Defining the conditions for the generation of melanocytes from human embryonic stem cells. Stem Cells 24,1668–1677.

Fortunel NO, Otu HH, Ng HH, Chen J, Mu X, Chevassut T, Li X, Joseph M, Bailey C, Hatzfeld JA *et al*. (2003) Comment on ‘ “Stemness”: transcriptional profiling of embryonic and adult stem cells’ and ‘a stem cell molecular signature’. Science 302,393.

Fujimoto A, Mitalipov SM, Kuo HC and Wolf DP (2005) Aberrant genomic imprinting in rhesus monkey ES cells. Stem Cells 24,595–603.

Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev 15,490–495.

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H and Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300,489–492.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J *et al*. (2004) Differences between human and mouse embryonic stem cells. Dev Biol 269,360–380.

Goh G, Self T, Barbadillo Munoz MD, Hall IP, Young L and Denning C (2005) Molecular and phenotypic analyses of human embryonic stem cell-derived cardiomyocytes: opportunities and challenges for clinical translation. Thromb Haemost 94,728–737.

Golan-Mashiach M, Dazard JE, Gerecht-Nir S, Amariglio N, Fisher T, Jacob-Hirsch J, Bielorai B, Osenberg S, Barad O, Getz G *et al*. (2005)

Design principle of gene expression used by human stem cells: implication for pluripotency. Faseb J 19,147–149.
Gudjonsson T and Magnusson MK (2005) Stem cell biology and the cellular pathways of carcinogenesis. Apmis 113,922–929.
Hanahan D and Weinberg RA (2000) The hallmarks of cancer. Cell 100,57–70.
Hannula K, Lipsanen-Nyman M, Scherer SW, Holmberg C, Hoglund P and Kere J (2001) Maternal and paternal chromosomes 7 show differential methylation of many genes in lymphoblast DNA. Genomics 73,1–9.
Hanson C and Caisander G (2005) Human embryonic stem cells and chromosome stability. Apmis 113,751–755.
Hardarson T, Caisander G, Sjogren A, Hanson C, Hamberger L and Lundin K (2003) A morphological and chromosomal study of blastocysts developing from morphologically suboptimal human pre-embryos compared with control blastocysts. Hum Reprod 18,399–407.
Hart AH, Hartley L, Ibrahim M and Robb L (2004) Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev Dyn 230,187–198.
Hattori N, Abe T, Hattori N, Suzuki M, Matsuyama T, Yoshida S, Li E and Shiota K (2004) Preference of DNA methyltransferases for CpG islands in mouse embryonic stem cells. Genome Res 14,1733–1740.
Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C and Semb H (2004) Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22,367–376.
Heins N, Lindahl A, Karlsson U, Rehnstrom M, Caisander G, Emanuelsson K, Hanson C, Semb H, Bjorquist P, Sartipy P *et al.* (2006) Clonal derivation and characterization of human embryonic stem cell lines. J Biotechnol 122,2867–2874.
Herman JG and Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349,2042–2054.
Herszfeld D, Wolvetang E, Langton-Bunker E, Chung TL, Filipczyk AA, Houssami S, Jamshidi P, Koh K, Laslett AL, Michalska A *et al.* (2006) CD30 is a survival factor and a biomarker for transformed human pluripotent stem cells. Nat Biotechnol 24,351–357.
Hoffman LM and Carpenter MK (2005a) Characterization and culture of human embryonic stem cells. Nat Biotechnol 23,699–708.
Hoffman LM and Carpenter MK (2005b) Human embryonic stem cell stability. Stem Cell Rev 1,139–144.
Hoffman LM, Hall L, Batten JL, Young H, Pardasani D, Baetge EE, Lawrence J and Carpenter MK (2005) X-inactivation status varies in human embryonic stem cell lines. Stem Cells 23,1468–1478.
Holm TM, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout WM III and Jaenisch R (2005) Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell 8,275–285.
Hook EB (1977) Exclusion of chromosomal mosaicism: tables of 90%, 95% and 99% confidence limits and comments on use. Am J Hum Genet 29,94–97.
Hovatta O, Mikkola M, Gertow K, Stromberg AM, Inzunza J, Hreinsson J, Rozell B, Blennow E, Andang M and Ahrlund-Richter L (2003) A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 18,1404–1409.
Hsiao LL, Dangond F, Yoshida T, Hong R, Jensen RV, Misra J, Dillon W, Lee KF, Clark KE, Haverty P *et al.* (2001) A compendium of gene expression in normal human tissues. Physiol Genomics 7,97–104.
Huettner JE, Lu A, Qu Y, Wu Y, Kim M and McDonald Iii JW (2006) Gap junctions and connexon hemichannels in human embryonic stem cells. Stem Cells 24,1654–1667.
Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM III, Biniszkiewicz D, Yanagimachi R and Jaenisch R (2001) Epigenetic instability in ES cells and cloned mice. Science 293,95–97.
Hyslop LA, Armstrong L, Stojkovic M and Lako M (2005) Human embryonic stem cells: biology and clinical implications. Expert Rev Mol Med 7,1–21.
Ingelman-Sundberg M and Rodriguez-Antona C (2005) Pharmacogenetics of drug-metabolizing enzymes: implications for a safer and more effective drug therapy. Philos Trans R Soc Lond B Biol Sci 360,1563–1570.
Inzunza J, Sahlen S, Holmberg K, Stromberg AM, Teerijoki H, Blennow E, Hovatta O and Malmgren H (2004) Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod 10,461–466.
Inzunza J, Gertow K, Stromberg MA, Matilainen E, Blennow E, Skottman H, Wolbank S, Ahrlund-Richter L and Hovatta O (2005) Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23,544–549.
Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA and Lemischka IR (2002) A stem cell molecular signature. Science 298,601–604.
Jaenisch R and Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33,245–254.
James D, Levine AJ, Besser D and Hemmati-Brivanlou A (2005) TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132,1273–1282.
Jenuwein T and Allis CD (2001) Translating the histone code. Science 293,1074–1080.
Jones PA and Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet 3,415–428.
Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H *et al.* (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 38,149–153.
Kim GD, Ni J, Kelesoglu N, Roberts RJ and Pradhan S (2002) Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. Embo J 21,4183–4195.
Kim SJ, Lee JE, Park JH, Lee JB, Kim JM, Yoon BS, Song JM, Roh SI, Kim CG and Yoon HS (2005) Efficient derivation of new human embryonic stem cell lines. Mol Cells 19,46–53.
Klimanskaya I, Chung Y, Meisner L, Johnson J, West MD and Lanza R (2005) Human embryonic stem cells derived without feeder cells. Lancet 365,1636–1641.
Kothapalli R, Yoder SJ, Mane S and Loughran TP Jr (2002) Microarray results: how accurate are they? BMC Bioinformatics 3,22.
Lagarkova MA, Volchkov PY, Lyakisheva AV, Philonenko ES and Kiselev SL (2006) Diverse epigenetic profile of novel human embryonic stem cell lines. Cell Cycle 5,416–420.
Lakshmipathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS and Verfaillie CM (2004) Efficient transfection of embryonic and adult stem cells. Stem Cells 22,531–543.
Lee JB, Kim JM, Kim SJ, Park JH, Hong SH, Roh SI, Kim MK and Yoon HS (2005a) Comparative characteristics of three human embryonic stem cell lines. Mol Cells 19,31–38.
Lee JB, Lee JE, Park JH, Kim SJ, Kim MK, Roh SI and Yoon HS (2005b) Establishment and maintenance of human embryonic stem cell lines on human feeder cells derived from uterine endometrium under serum-free condition. Biol Reprod 72,42–49.
Li T, Zhou CQ, Mai QY and Zhuang GL (2005) Establishment of human embryonic stem cell line from gamete donors. Chin Med J (Engl) 118,116–122.
Liu X, Wu H, Loring J, Hormuzdi S, Disteche CM, Bornstein P and Jaenisch R (1997) Trisomy eight in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. Dev Dyn 209,85–91.
Lucifero D, Chaillet JR and Trasler JM (2004) Potential significance of genomic imprinting defects for reproduction and assisted reproductive technology. Hum Reprod Update 10,3–18.
Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS *et al.* (2006a) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24,185–187.
Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS *et al.* (2006b) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24,185–187.
Maitra A, Arking DE, Shivapurkar N, Ikeda M, Stastny V, Kassauei K, Sui G, Cutler DJ, Liu Y, Brimble SN *et al.* (2005) Genomic alterations in cultured human embryonic stem cells. Nat Genet 37,1099–1103.
Mandal A, Tipnis S, Pal R, Ravindran G, Bose B, Patki A, Rao MS and Khanna A (2006) Characterization and in vitro differentiation potential of a new human embryonic stem cell line, ReliCellhES1. Differentiation 74,81–90.
Mecham BH, Klus GT, Strovel J, Augustus M, Byrne D, Bozso P, Wetmore DZ, Mariani TJ, Kohane IS and Szallasi Z (2004) Sequence-matched probes produce increased cross-platform consistency and more reproducible biological results in microarray-based gene expression measurements. Nucleic Acids Res 32, e74.
Mitalipova M, Calhoun J, Shin S, Wininger D, Schulz T, Noggle S, Venable A, Lyons I, Robins A and Stice S (2003) Human embryonic stem cell lines derived from discarded embryos. Stem Cells 21,521–526.
Mitalipova MM, Rao RR, Hoyer DM, Johnson JA, Meisner LF, Jones KL, Dalton S and Stice SL (2005) Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 23,19–20.

Miura M, Miura Y, Padilla-Nash HM, Molinolo AA, Fu B, Patel V, Seo BM, Sonoyama W, Zheng JJ, Baker CC *et al*. (2005) Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells 24,1095–1103.

Niwa H, Miyazaki J and Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24,372–376.

Noaksson K, Zoric N, Zeng X, Rao MS, Hyllner J, Semb H, Kubista M and Sartipy P (2005) Monitoring differentiation of human embryonic stem cells using real-time PCR. Stem Cells 23,1460–1467.

Oh SK, Kim HS, Ahn HJ, Seol HW, Kim YY, Park YB, Yoon CJ, Kim DW, Kim SH and Moon SY (2005) Derivation and characterization of new human embryonic stem cell lines: SNUhES1, SNUhES2, and SNUhES3. Stem Cells 23,211–219.

Onyango P, Jiang S, Uejima H, Shamblott MJ, Gearhart JD, Cui H and Feinberg AP (2002) Monoallelic expression and methylation of imprinted genes in human and mouse embryonic germ cell lineages. Proc Natl Acad Sci USA 99,10599–10604.

Owen-Smith J and McCormick J (2006) An international gap in human ES cell research. Nat Biotechnol 24,391–392.

Pain D, Chirn GW, Strassel C and Kemp DM (2005) Multiple retropseudogenes from pluripotent cell-specific gene expression indicates a potential signature for novel gene identification. J Biol Chem 280,6265–6268.

Pantoja C, de Los Rios L, Matheu A, Antequera F and Serrano M (2005) Inactivation of imprinted genes induced by cellular stress and tumorigenesis. Cancer Res 65,26–33.

Pardal R, Clarke MF and Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3,895–902.

Park SP, Lee YJ, Lee KS, Ah Shin H, Cho HY, Chung KS, Kim EY and Lim JH (2004) Establishment of human embryonic stem cell lines from frozen-thawed blastocysts using STO cell feeder layers. Hum Reprod 19,676–684.

Pathak S and Multani AS (2006) Aneuploidy, stem cells and cancer. EXS 96,49–64.

Paz MF, Fraga MF, Avila S, Guo M, Pollan M, Herman JG and Esteller M (2003) A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 63,1114–1121.

Pera MF (2004) Unnatural selection of cultured human ES cells? Nat Biotechnol 22,42–43.

Pickering SJ, Braude PR, Patel M, Burns CJ, Trussler J, Bolton V and Minger S (2003) Preimplantation genetic diagnosis as a novel source of embryos for stem cell research. Reprod Biomed Online 7,353–364.

Plaia TW, Josephson R, Liu Y, Zeng X, Ording C, Toumadje A, Brimble SN, Sherrer ES, Uhl EW, Freed WJ *et al*. (2005) Characterization of a new NIH registered variant human embryonic stem cell line BG01V: a tool for human embryonic stem cell research. Stem Cells 24,531–546.

Pyle AD, Lock LF and Donovan PJ (2006) Neurotrophins mediate human embryonic stem cell survival. Nat Biotechnol 24,344–350.

Rajagopalan H and Lengauer C (2004) Aneuploidy and cancer. Nature 432,338–341.

Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC and Melton DA (2002) 'Stemness': transcriptional profiling of embryonic and adult stem cells. Science 298,597–600.

Rao M (2004) Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells. Dev Biol 275,269–286.

Rao RR and Stice SL (2004) Gene expression profiling of embryonic stem cells leads to greater understanding of pluripotency and early developmental events. Biol Reprod 71,1772–1778.

Rao MS and Civin CI (2005) Translational research: toward better characterization of human embryonic stem cell lines. Stem Cells 23,1453.

Rao MS and Auerbach JM (2006) Estimating human embryonic stem-cell numbers. Lancet 367,650.

Rao MS and Civin CI (2006) How many human embryonic stem cell lines are sufficient? A US Perspective. Stem Cells 24,800–803.

Rao RR, Calhoun JD, Qin X, Rekaya R, Clark JK and Stice SL (2004) Comparative transcriptional profiling of two human embryonic stem cell lines. Biotechnol Bioeng 88,273–286.

Reik W and Lewis A (2005) Co-evolution of X-chromosome inactivation and imprinting in mammals. Nat Rev Genet 6,403–410.

Reubinoff BE, Pera MF, Fong CY, Trounson A and Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18,399–404.

Reubinoff BE, Pera MF, Vajta G and Trounson AO (2001) Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod 16,2187–2194.

Reya T, Morrison SJ, Clarke MF and Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414,105–111.

Rho JY, Yu K, Han JS, Chae JI, Koo DB, Yoon HS, Moon SY, Lee KK and Han YM (2006) Transcriptional profiling of the developmentally important signalling pathways in human embryonic stem cells. Hum Reprod 21,405–412.

Richards M, Tan SP, Tan JH, Chan WK and Bongso A (2004) The transcriptome profile of human embryonic stem cells as defined by SAGE. Stem Cells 22,51–64.

Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6,597–610.

Rodriguez CI, Galan A, Valbuena D and Simon C (2006) Derivation of clinical-grade human embryonic stem cells. Reprod Biomed Online 12,112–118.

Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS and Carpenter MK (2004) Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn 229,259–274.

Rossant J (2001) Stem cells from the Mammalian blastocyst. Stem Cells 19,477–482.

Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC and Bernad A (2005) Spontaneous human adult stem cell transformation. Cancer Res 65,3035–3039.

Rugg-Gunn PJ, Ferguson-Smith AC and Pedersen RA (2005) Epigenetic status of human embryonic stem cells. Nat Genet 37,585–587.

Sado T, Tada T and Takagi N (1996) Mosaic methylation of Xist gene before chromosome inactivation in undifferentiated female mouse embryonic stem and embryonic germ cells. Dev Dyn 205,421–434.

Sato N, Sanjuan IM, Heke M, Uchida M, Naef F and Brivanlou AH (2003) Molecular signature of human embryonic stem cells and its comparison with the mouse. Dev Biol 260,404–413.

Sato N, Meijer L, Skaltsounis L, Greengard P and Brivanlou AH (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 10,55–63.

Secombe J, Pierce SB and Eisenman RN (2004) Myc: a weapon of mass destruction. Cell 117,153–156.

Semb H (2005) Human embryonic stem cells: origin, properties and applications. Apmis 113,743–750.

Simon C, Escobedo C, Valbuena D, Genbacev O, Galan A, Krtolica A, Asensi A, Sanchez E, Esplugues J, Fisher S *et al*. (2005) First derivation in Spain of human embryonic stem cell lines: use of long-term cryopreserved embryos and animal-free conditions. Fertil Steril 83,246–249.

Singh KK (2004) Mitochondrial dysfunction is a common phenotype in aging and cancer. Ann N Y Acad Sci 1019,260–264.

Sjogren-Jansson E, Zetterstrom M, Moya K, Lindqvist J, Strehl R and Eriksson PS (2005) Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system. Dev Dyn 233,1304–1314.

Skotheim RI, Monni O, Mousses S, Fossa SD, Kallioniemi OP, Lothe RA and Kallioniemi A (2002) New insights into testicular germ cell tumorigenesis from gene expression profiling. Cancer Res 62,2359–2364.

Skottman H, Mikkola M, Lundin K, Olsson C, Stromberg AM, Tuuri T, Otonkoski T, Hovatta O and Lahesmaa R (2005a) Gene expression signatures of seven individual human embryonic stem cell lines. Stem Cells 23,1343–1356.

Skottman H, Stromberg AM, Matilainen E, Inzunza J, Hovatta O and Lahesmaa R (2005b) Unique gene expression signature by human embryonic stem cells cultured under serum free conditions correlates with their enhanced and prolonged growth in an undifferentiated stage. Stem Cells 24,151–167.

Smiraglia DJ, Rush LJ, Fruhwald MC, Dai Z, Held WA, Costello JF, Lang JC, Eng C, Li B, Wright FA *et al*. (2001) Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies. Hum Mol Genet 10,1413–1419.

Smith AG (2001) Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol 17,435–462.

Speicher MR and Carter NP (2005) The new cytogenetics: blurring the boundaries with molecular biology. Nat Rev Genet 6,782–792.

Sperger JM, Chen X, Draper JS, Antosiewicz JE, Chon CH, Jones SB, Brooks JD, Andrews PW, Brown PO and Thomson JA (2003) Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci USA 100,13350–13355.

Steele W, Allegrucci C, Singh R, Lucas E, Priddle H, Denning C, Sinclair K and Young L (2005) Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction? Reprod Biomed Online 10,755–766.

Stojkovic M, Lako M, Stojkovic P, Stewart R, Przyborski S, Armstrong L, Evans J, Herbert M, Hyslop L, Ahmad S *et al*. (2004) Derivation of

human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture. Stem Cells 22,790–797.
Storchova Z and Pellman D (2004) From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol 5,45–54.
Suarez-Farinas M, Noggle S, Heke M, Hemmati-Brivanlou A and Magnasco MO (2005) Comparing independent microarray studies: the case of human embryonic stem cells. BMC Genomics 6,99.
Sun BW, Yang AC, Feng Y, Sun YJ, Zhu Y, Zhang Y, Jiang H, Li CL, Gao FR, Zhang ZH *et al*. (2006) Temporal and parental-specific expression of imprinted genes in a newly derived Chinese human embryonic stem cell line and embryoid bodies. Hum Mol Genet 15,65–75.
Suss-Toby E, Gerecht-Nir S, Amit M, Manor D and Itskovitz-Eldor J (2004) Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. Hum Reprod 19,670–675.
Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA and Bradley JA (2005) Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet 366,2019–2025.
Taylor RA, Cowin PA, Cunha GR, Pera M, Trounson AO, Pedersen J and Risbridger GP (2006) Formation of human prostate tissue from embryonic stem cells. Nat Methods 3,179–181.
Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS and Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282,1145–1147.
Trounson A (2006) The production and directed differentiation of human embryonic stem cells. Endocr Rev 27,208–219.
Vallier L, Alexander M and Pedersen RA (2005) Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118,4495–4509.
Wei CL, Miura T, Robson P, Lim SK, Xu XQ, Lee MY, Gupta S, Stanton L, Luo Y, Schmitt J *et al*. (2005) Transcriptome profiling of human and murine ESCs identifies divergent paths required to maintain the stem cell state. Stem Cells 23,166–185.
Westermann F and Schwab M (2002) Genetic parameters of neuroblastomas. Cancer Lett 184,127–147.
Wobus AM and Boheler KR (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 85,635–678.
Wong RC, Pebay A, Nguyen LT, Koh KL and Pera MF (2004) Presence of functional gap junctions in human embryonic stem cells. Stem Cells 22,883–889.
Xiao L, Yuan X and Sharkis SJ (2006) Activin A maintains self-renewal and regulates FGF, Wnt and BMP pathways in human embryonic stem cells. Stem Cells 24,1476–1486.
Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD and Carpenter MK (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19,971–974.
Xu RH, Peck RM, Li DS, Feng X, Ludwig T and Thomson JA (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Meth 2,185–190.
Yauk CL, Berndt ML, Williams A and Douglas GR (2004) Comprehensive comparison of six microarray technologies. Nucleic Acids Res 32, e124.
Zeng X, Miura T, Luo Y, Bhattacharya B, Condie B, Chen J, Ginis I, Lyons I, Mejido J, Puri RK *et al*. (2004) Properties of pluripotent human embryonic stem cells BG01 and BG02. Stem Cells 22,292–312.
Zhan M, Miura T, Xu X and Rao MS (2005) Conservation and variation of gene regulation in embryonic stem cells assessed by comparative genomics. Cell Biochem Biophys 43,379–405.
Zhu X, Deng C, Kuick R, Yung R, Lamb B, Neel JV, Richardson B and Hanash S (1999) Analysis of human peripheral blood T cells and single-cell-derived T cell clones uncovers extensive clonal CpG island methylation heterogeneity throughout the genome. Proc Natl Acad Sci USA 96,8058–8063.
Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R and Brockdorff N (2005) Global hypomethylation of the genome in XX embryonic stem cells. Nat Genet 37,1274–1279.
Zwaka TP and Thomson JA (2005) A germ cell origin of embryonic stem cells? Development 132,227–233.

Submitted on April 18, 2006; resubmitted on July 12, 2006; accepted on July 29, 2006