REPRODUCTION
REVIEW

Focus on Stem Cells

Culture conditions for human embryonic stem cells

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Abstract

Human embryonic stem cell (hESC) lines have been derived and cultured in variable conditions. The idea behind derivation of hESC lines is to use them in human cell transplantation after differentiation, but already now these cells are widely used for research purposes. Despite similarities among the established lines, important differences have been reported between them, and it has been difficult to compare the results obtained using different lines. Recent optimization of hESC culture conditions has moved from cultures on mouse embryonic fibroblasts (MEFs) in fetal bovine serum-containing medium towards feeder-free culture methods using more defined animal substance-free cultures. The aim has been to establish robust and cost-effective systems for culturing these cells and eliminate the risk of infection transmitted by animal pathogens and immunoreactions caused by animal substances in cell cultures before clinical treatment. It is important to take these modifications into account when carrying out research using these cells. It is known that culture conditions influence gene expression and, hence, probably many properties of the cells. Optimization and standardization of culture methods is needed for research as well as for clinical purposes.

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Introduction

Human embryonic stem cell (hESC) lines can be derived from the inner cell mass (ICM) of preimplantation blastocysts (Thomson *et al.* 1998) which have been donated for research and would otherwise be discarded. Frozen good quality embryos not used in the infertility treatment, but have been used for the establishment of most of the existing lines. (hESC) Lines have been derived also form morula stage embryos (Strelchenko *et al.* 2004), and there is a recent report stating that two hESC lines were obtained from single isolated blastomeres (Klimanskaya *et al.* 2006), even though these blastomeres had to be cultured in groups in order to survive.

Since the establishment of the first hESC lines by Thomson *et al.* (1998), rapid progress has been made and several groups have described the derivation and culture of new hESC lines in various culture conditions (Tables 1–3). Human ESCs are cable of unlimited self-renewal, can be propagated in culture for extended periods and has the ability to differentiate to multiple cell types representing all primitive embryonic germ layers. Their differentiation potential has raised hope that these cells could provide new opportunities as a renewable source for cell transplantation for severe degenerative diseases. The successful differentiation of hESC to multiple cell lineages is not the topic of this review and are described elsewhere (see for example, Hoffman & Carpenter 2005). To enable the use of hESC in cell transplantation in human, it is necessary to eliminate the risk of infection transmitted by retroviruses and other animal pathogens, and immunoreactions caused by animal substances in cell cultures (Martin *et al.* 2005) and to establish robust and cost-effective systems for culturing hESC. This is important not only for clinical use of the cells, but also for reliable research carried out using these cells. It is difficult to evaluate the influence of various culture conditions on cells. Hence, a defined culture system with known composition of culture medium is desirable to minimize the variability that affects the reproducibility of research results. Standardized and optimized culture systems are needed, and the normality of the cells has to be followed up regularly.

Derivation of human embryonic stem cell lines

The first described establishment of hESC lines (Thomson *et al.* 1998) included the use of pronase to remove zona pellucida, and immunosurgery for isolation of the ICM

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Table 1 Derivation methods for removal of zona pellucida and isolation of inner cell mass of blastocyst for the establishment of human embryonic stem cell line.

Pronase	Tyrode's acid	Immunosurgery	Mechanical	Reference
Yes	–	Yes	–	Thomson *et al.* (1998); Reubinoff *et al.* (2000); Richards *et al.* (2002); Hovatta *et al.* (2003); Pickering *et al.* (2003); Mitalipova *et al.* (2003); Strelchenko *et al.* (2004); Stojkovic *et al.* (2004); Heins *et al.* (2004); Draper *et al.* (2004); Klimanskaya *et al.* (2005); Lee *et al.* (2005); Oh *et al.* (2005); Chen *et al.* (2005); Inzunza *et al.* (2005); Kim *et al.* (2005*b*); Hong-mei & Gui-an (2006); Mateizel *et al.* (2006); Ludwig *et al.* (2006)
–	Yes	Yes	–	Lanzendorf *et al.* (2001); Cowan *et al.* (2004)
Yes	–	–	Yes	Strelchenko *et al.* (2004); Kim *et al.* (2005*b*); Wang *et al.* (2005*c*)
–	Yes	–	Yes	Genbacev *et al.* (2005)

from the blastocyst. Several research groups have since published the derivation of new hESC lines using similar ICM isolation protocols (Table 1). However, immunosurgery may not be the optimal derivation method when poor quality discarded embryos with hardly visible ICM is used (Kim *et al.* 2005*a*). Immunosurgery also involves animal-derived substances, mouse antibodies, and guinea pig complement, which are not desirable thinking of cell transplantation. For standardization of derivation methods, more simplified protocols would be appreciated. Tyrode's acid for the removal of zona pellucida and mechanical isolation of ICM is probably advantageous thinking of the quality of the cells since there is no contact of blastocyst with animal-derived pronase, antibodies, and complement factors. The latter procedure has been recently adapted for the establishment of new hESC line by Genbacev *et al.* (2005). We have also derived our latest five hESC lines mechanically (Fig. 1).

Feeder cells in human embryonic stem cell cultures

The establishment of hESC lines was first described by using mouse embryonic fibroblasts (MEFs) as feeder cells and fetal bovine serum (FBS)-containing culture medium for both feeder cells and hESC (Thomson *et al.* 1998). For replacing animal cells, various alternative human cell lines have been successfully tested as feeder cells for hESC. Table 2 summarizes the sources of human feeders that have been validated for culture of undifferentiated hESC.

Richards and co-workers have studied different kinds of human cells, such as human fetal muscle and skin, human adult Fallopian tube epithelial feeders, and adult muscle cells as feeders for hESC (Richards *et al.* 2002, 2003). A finding from their results was that for some reason MEF cells seemed to support hESC growth better than human feeders. Our group has derived and cultured all our 25 h ESC lines using commercially available human foreskin fibroblasts (hFF) as feeder cells (Hovatta *et al.* 2003, Inzunza *et al.* 2005). In our cultures, hFF seem to support the derivation and undifferentiated growth of hESC well. On the other hand, we have not compared the growth rates of hESC cultured with hFF or MEFs as feeder cells. The commercially available hFF lines (and other reported lines) have been derived and cultured in FBS-containing medium which often has batch-to-batch variation causing variation to culture conditions. Also, human adult marrow stroma cells for hESC maintenance (Cheng *et al.* 2003), and uterine endometrium cells (Lee *et al.* 2005) as well as human placenta fibroblasts (Genbacev *et al.* 2005) for derivation and maintenance of hESC have been used, but all these feeder cells have been cultured using FBS in culture medium.

Table 2 Human cells used as feeder cells for human embryonic stem cell cultures.

Feeder cell source	Feeder cell medium composition	Derivation (D)/maintenance (M)	Reference
Human foreskin	FBS/HS	M	Amit *et al.* (2003)
	FBS	D/M	Hovatta *et al.* (2003)
	FBS	D/M	Inzunza *et al.* (2005)
Placenta	FBS	D/M	Genbacev *et al.* (2005)
Uterine endometrium	FBS	D/M	Lee *et al.* (2005)
Adult marrow stroma	FBS	M	Cheng *et al.* (2003)
hESC-derived fibroblasts	FBS	M	Xu *et al.* (2004)
		M	Stojkovic *et al.* (2005*b*)
	HS	D/M	Wang *et al.* (2005*c*)
Fetal muscle	HS	D/M	Richards *et al.* (2002)
Fetal skin		D/M	Richards *et al.* (2003)
Adult fallopian tube		D/M	
Adult skin		M	
Adult muscle		M	

FBS, fetal bovine serum; HS, human serum; hESC, human embryonic stem cell.

Table 3 Feeder-free culture methods established for human embryonic stem cells.

Coating material	Medium supplements	Derivation (D)/ maintenance (M)	Reference
Matrigel/laminin	CM (MEFs), SR, bFGF	M	Xu *et al.* (2001)
Matrigel/laminin	CM (MEFs), SR, bFGF	M	Carpenter *et al.* (2004)
Matrigel	CM (hESCd-F), SR, bFGF	M	Xu *et al.* (2004)
Matrigel	SR, bFGF, GSK3 inhibitor	M	Sato *et al.* (2004)
Matrigel	CM (MEFs/HES), S1P, PDGF, bFGF	M	Pebay *et al.* (2005)
Matrigel/laminin	X-VIVO 10, bFGF	M	Li *et al.* (2005)
Matrigel/collagen IV+ fibronectin+laminin+vitronectin	Defined, animal-free medium	D/M	Ludwig *et al.* (2006)
FBS coating	CDM, activin A, noudal, bFGF	M	Vallier *et al.* (2005)
Laminin	SR, activin A, KGF, NIC	M	Beattie *et al.* (2005)
Matrigel	SR, bFGF+noggin or high bFGF	M	Xu *et al.* (2005)
Matrigel	SR, bFGF, noggin, high bFGF	M	Wang *et al.* (2005*c*)
Fibronectin	SR, TGF-β, LIF, bFGF	M	Amit *et al.* (2004)
Fibronectin	CM (MEFs), SR, bFGF	M	Noaksson *et al.* (2005)
ECM from MEFs	SR, Plasmanate, bFGF	D/M	Klimanskaya *et al.* (2005)
HS matrix	CM (hESCd-F)	M	Stojkovic *et al.* (2005*a*)
Laminin	X-VIVO10, high bFGF	M	Genbacev *et al.* (2005)

CM, conditioned medium; MEFs, mouse embryonic fibroblasts; SR, serum replacement; bFGF, basic fibroblast growth factor; hESC-dF, human embryonic stem cell derived fibroblasts; GSK3 inhibitor, glycogen synthase kinase-3 inhibitor; HES, human embryonic stem cell; S1P, sphingosine-1-phosphate; PDGF, platelet derived growth factor; CDM, chemically defined medium; KGF, keratinocyte growth factor; NIC, nicotinamide; TGF-β, transforming growth factor β; LIF, leukaemia inhibitor factor; HS, human serum.

The use of hESC-derived fibroblasts or fibroblast-like cells, and hESC-derived immortalized fibroblasts as a feeder cells has recently been described (Stojkovic *et al.* 2005*b*, Wang *et al.* 2005*b*). Xu *et al.* (2004) established a culture system where conditioned medium from hESC-derived fibroblasts were used. Immortalized cell lines might provide feeder cells for extended time as compared with primary cell lines, such as adult fibroblast cell lines. Human ESC-derived feeders provide interesting opportunity where autogenic feeder cells could be derived from hESC and used for certain hESC lines. On the other hand, differentiation of fibroblast-like cells and using those for maintenance is very labor intensive and hardly the best way to standardize and produce mass cultures of undifferentiated hESC for research purposes.

Systematic comparisons of the properties of hESC derived and grown on different feeders are needed in order to decide which feeder cells are optimal. In contrast, it may be possible that the hESC get adapted to the feeders on which they have been derived, which makes such comparison difficult.

Culture medium composition for human embryonic stem cells

Traditionally, hESC culture medium contained FBS (Thomson *et al.* 1998, Reubinoff *et al.* 2000). It has, by most of the teams, been replaced by alternatives. The use of human serum instead of FBS in hESC culture medium has been reported (Richards *et al.* 2002). The problem is that FBS as well as human serum are complex mixtures containing unknown compounds, and serum batches vary in capability of maintaining hESC at an undifferentiated stage. Serum may also contain factors inducing hESC differentiation.

To avoid these problems, we and other researchers have optimized serum-free culture conditions for hESC lines using a basic fibroblast growth factors (bFGF) and a more defined serum replacement (KnockOut Serum Replacement, Invitrogen), which still contains animal proteins, which are not fully defined (see for example, Amit *et al.* 2000, Koivisto *et al.* 2004, Stojkovic *et al.* 2004, Strelchenko *et al.* 2004). During optimization of our hESC culture conditions by replacing FBS with serum replacement (SR) in hESC medium, we made the interesting observation that hESC were proliferating faster in SR-containing medium than they did in FBS-containing medium (Koivisto *et al.* 2004). To further study this, we

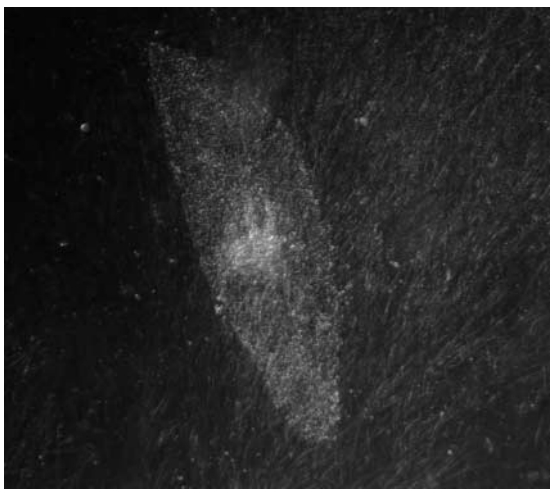

Figure 1 A colony of human embryonic stem cell (hESC) line HS420 (passage 1) derived by mechanical isolation of inner cell mass. The hESC line was derived and grown on human foreskin fibroblasts in SR-containing medium. This line has been fully characterized. Magnification ×100.

made gene expression profiles using DNA microarrays to compare whole genome gene expression changes between hESC cultured in FBS- and SR-containing media (Skottman *et al.* 2005). Although the stem cells characteristics of these cells (the expression of many known hESC markers) and their differentiation capacity in embryoid bodies were similar, surprisingly, over 100 genes were found to be significantly differentially expressed when hESC cultured in serum-containing medium were compared with those cultured in SR medium. As a conclusion, we suggested that such changes may have fundamental importance for hESC. The gene expression changes should be monitored as a part of cell culture optimization aiming at establishment of robust and cost-effective culture system of hESC. We have also established SR-containing derivation conditions for our recent hESC lines (Inzunza *et al.* 2005), but even though the use of SR in derivation and maintenance of hESC is a step forward it is not an entirely defined procedure.

Feeder-free culture methods for human embryonic stem cells

The contribution of feeder cells in hESC cultures is not entirely understood, but it has been suggested that feeder cells provide both a suitable attachment substrate and important soluble factors for the maintenance of undifferentiated hESC. However, such factors remain the most poorly defined component of hESC cultures. Feeder-free cultures would be free of many difficulties of the standardization and optimization procedures, and they would make analyses of the hESC properties easier.

Substrate

Feeder-free propagation of hESC has been feasible on Matrigel-coated plates and SR-containing conditioned medium (medium conditioned overnight incubation with mouse or human feeder cells) (Xu *et al.* 2001, 2004). Matrigel is a complex mixture of mouse sarcoma origin which contains extracellular molecules, such as laminin, collagen IV, and also growth factors and other substances. Also other research groups have successfully used Matrigel as substrate for hESC (Sato *et al.* 2004, Pebay *et al.* 2005, Wang *et al.* 2005*a*, Xu *et al.* 2005). Matrigel is not an optimal matrix for cells because it contains many unknown components and is of non-human origin. To replace Matrigel with alternatives, several research groups have used laminin (Xu *et al.* 2001, Beattie *et al.* 2005, Genbacev *et al.* 2005), fibronectin (Amit *et al.* 2004, Noaksson *et al.* 2005) and human serum (Stojkovic *et al.* 2005*a*) -coated plates for hESC. Table 3 summarizes different coating materials which have been used as replacement of feeder cells in hESC cultures. A synthetic human matrix, such as recombinant human laminin (Nikolova *et al.* 2006), would be optimal for hESC cultures.

Culture medium supplements

Unlike in the mouse (Smith *et al.* 1988), leukemia inhibitory factor (LIF) is not able to maintain the non-differentiated growth of hESC without feeder cells (Thomson *et al.* 1998) and so far no such component having an important effect on hESC as LIF has for mouse ESC has been identified. To replace feeder cells and conditioned medium in hESC cultures, identification of conditioned medium components facilitating non-differentiated growth would be crucial. Conditioned medium most likely contains variable amounts of growth factors with batch-to-batch variation. There are also differences between conditioned media from different feeder cells in supporting the growth of undifferentiated hESC (Xu *et al.* 2001). In our laboratory, we have not succeeded in culturing non-differentiated hESC in feeder-free cultures for longer than a few passages using conditioned medium from human foreskin fibroblasts (unpublished).

Several research groups have reported successful use of supplements in hESC culture medium, such as bFGF and hyaluronic acid (Heins *et al.* 2004), transforming growth factor-β (TGF-β), bFGF and LIF (Amit *et al.* 2004), bFGF and GSK3 inhibitor (Sato *et al.* 2004), activin A and bFGF (Xiao *et al.* 2006), and spingosine-1-phosphate with platelet-derived growth factor (Pebay *et al.* 2005). Recently, two groups found that a combination of the BMP signaling antagonist Noggin and high concentration of bFGF was sufficient to maintain undifferentiated hESC in feeder-free culture conditions (Wang *et al.* 2005*a*, Xu *et al.* 2005). In our laboratory, these factors in similar high concentrations proved unsuitable to our hESC (unpublished). Beattie *et al.* (2005) showed that hESC cultured in the presence of activin A, nicotinamide (NIC), and keratinocyte growth factor (KGF) remained undifferentiated. They suggested that activin A may block the differentiation and NIC and KGF may have function in hESC proliferation. In addition to this, Vallier *et al.* (2005) have demonstrated the role of activin/Nodal/TGF-β pathway in the maintenance of undifferentiated stage of hESC. Very recent results have demonstrate that addition of bFGF, litidium chloride, γ-aminobutyric acid (GABA), pipecolic acid, and TGF-β supplements into defined culture medium support undifferentiated growth of hESC (Ludwig *et al.* 2006), and that neurotrophins may behave as survival factors for hESC (Pyle *et al.* 2006). Although karyotype analyses have been made after several passages, real long-term studies are missing and the genetic and epigenetic stability of hESC in all these conditions has still to be confirmed.

Feeder-free derivation of human embryonic stem cell lines

Feeder-free derivation of hESC lines has been successful as described by Klimanskaya *et al.* (2005) using a mouse-derived matrix, and more recently by Ludwig *et al.* (2006) on Matrigel and human laminin. The problem

with the later feeder-free derivation was that one of the lines had karyotype 47, XXY, which may have come from the embryo. On the contrary, the other line gained an extra chromosome at passage level 40. It has been suggested that feeder-free cultures may be so demanding for the hESC that they become more prone to abnormalities (Draper *et al.* 2004). The use of extracellular matrix eliminates the risk of transmitting animal pathogens with feeder cells to the hESC and decrease variability in culture conditions. Enzymes (especially trypsin) used in passaging may, on the other hand, push cells to too demanding culture conditions which may induce abnormalities (Draper *et al.* 2004).

Defined culture methods

Conditioned medium from feeder cells or a medium with animal components (such as the widely used Invitrogen SR) is not optimal when we think of pathogens and possible immunoreactions as well as batch-to-batch variation of culture media. Very recently, important work has been carried out by replacing animal components in SR-containing medium with more defined media and supplements. Genbacev *et al.* (2005) and Li *et al.* (2005) used X-VIVO 10 with bFGF, Vallier *et al.* (2005) used chemically defined medium with activin A, nodal and bFGF, and Ludwig *et al.* (2006) used defined culture medium with bFGF, LiCl, GABA, TGF-β, and pipeolic acid.

The next step towards entirely defined derivation and maintenance of hESC would be the combination of mechanical isolation of ICM, use of human feeder cells derived and cultured in defined medium, and use of defined culture medium in the hESC culture (Fig. 2). In addition to this, even better and more defined culture system with feeder-free conditions (Ludwig *et al.* 2006) is desirable to minimize the variability in culture conditions. In contrast, deriving and culturing hESC in such demanding conditions would need continuous controls of genetic and epigenetic normality of the cells.

Defined quality cells also needed for research purposes

To enable the use of hESC derivates for transplantation in humans, it would be crucial to eliminate the potential contamination by pathogens and animal proteins/substances from the hESC culture methods. Elimination of these variable factors is also important for constant and standardized culture conditions for research purposes. Human ESC for therapeutic as well as for research purposes have to be genetically and epigenetically normal. There is clear indication that hESC cultured in feeder-free conditions may gain chromosomal changes (Draper *et al.* 2004). Many reviewed studies have not detected any chromosomal abnormalities, but even though karyotypes have been analyzed after several passages, real long-term studies are missing. A number

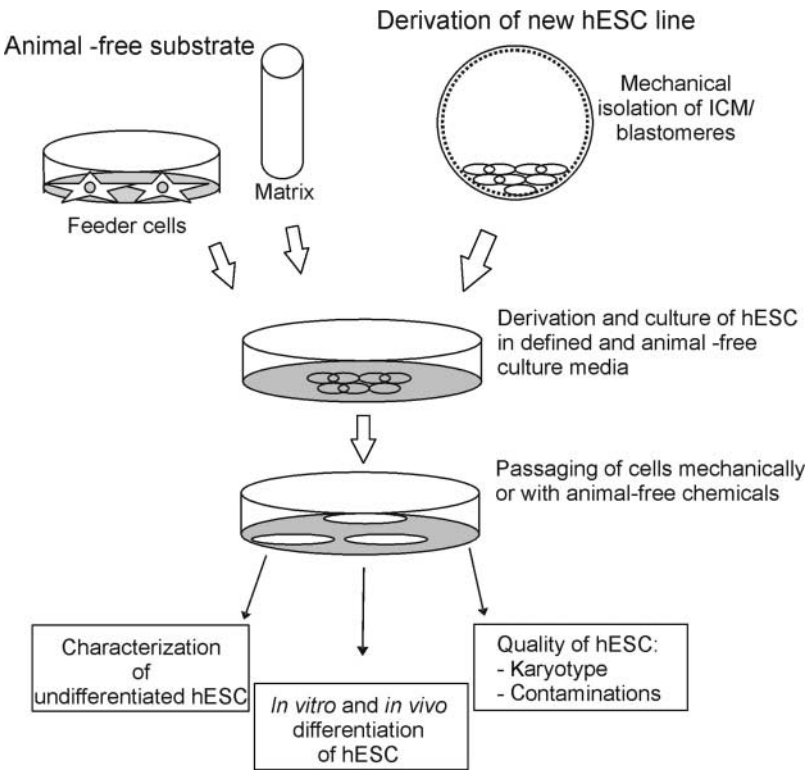

Figure 2 Key features of human embryonic stem cell line culture conditions which need to be optimized.

of karyotypic abnormalities have been identified in cultured hESC (Allegrucci & Young 2006). Human ESC is also epigenetically sensitive (Steele *et al.* 2005). In feeder-free culture of hESC, new gently passaging methods without dissociation of hESC for single cells might enable preventing abnormalities caused by selective pressure in long-term cultures of hESC.

If the cells are cultured only for research purposes, we need not consider so much the immunoreactions caused by animal substances in culture medium, but pathogens may change the properties of the cells. The current culture methods are by no means perfect, and they need optimization also to gain high efficiency for the production of sufficient amounts of hESC for research purposes. The use of feeder cells sets limitations for research, since experimental data might result from a combination of hESC and feeder cells (Klimanskaya *et al.* 2005). The variation in culture protocols may also affect the ability to differentiate hESC with standard differentiation protocols or to provide standard systems for developmental studies, disease modeling, drug discovery, and toxicology applications. Recently established culture methods with high concentration of growth factors and use of human matrix components are very expensive for research purposes. Understanding the mechanisms of hESC self-renewal and proliferation as non-differentiated cells would greatly facilitate the establishment of defined derivation and culture conditions for hESC (Vallier *et al.* 2005, Wang *et al.* 2005*a*, Armstrong *et al.* 2006, Rho *et al.* 2006, Stewart *et al.* 2006). Recent advantages in the use of biomaterials (Anderson *et al.* 2004) and perfusion culture systems (Fong *et al.* 2005) for the culture of hESC suggest that large-scale culture of hESC in bioreactors under controlled environment using defined culture medium and biomaterial substrates may be feasible in future.

International stem cell initiative (Andrews *et al.* 2005) has been established by several organizations which fund hESC research. It is an important attempt to study the properties of several hESC lines in similar conditions. A large range of hESC lines will be evaluated using the same antibodies and same laboratory systems in selected laboratories. However, so far it has not been possible to choose the most optimal conditions for these comparisons and further optimization and standardization of culture methods is needed for research as well as for clinical purposes.

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