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Current Technology for the Derivation of Pluripotent Stem Cell Lines from Human Embryos

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Technology for the derivation, propagation, and characterization of pluripotent stem cell lines from the human embryo has undergone considerable refinement and improvement since the first published description of human embryonic stem cells in 1998. In particular, there has been extensive effort to optimize protocols and develop defined culture systems with a view toward future clinical applications of embryonic stem cell-derived products. Here, we review the current status of methodology for human embryonic stem cell derivation and culture, and we highlight the challenges that remain for workers in the field.

Introduction

Today the derivation of new pluripotent stem cell lines from human embryos continues to be a highly active area of research, for several reasons. First, the technology for derivation and propagation of pluripotent human stem cell lines is constantly being refined and improved, providing opportunities to optimize development of cell lines for clinical use. Second, cell therapies based on human embryonic stem cells (hESCs) are likely to enter clinical trial very soon. It is predictable that the lessons learned from the first trials will drive further refinement of stem cell technology, and that as clinical work progresses, wider panels of cell lines will be required for particular applications and for tissue matching. Third, changes to policy for US Federal funding of embryonic stem cell research now allow workers there to study a much larger range of cell lines than was previously possible. Finally, despite the remarkable discovery of adult cell reprogramming to the pluripotent state (induced pluripotent stem cells [iPSCs]), continued uncertainty about the bioequivelance of human ESCs and hiPSCs suggests that hESCs will remain the gold standard for research and therapy for the near to mid-term future.

Here, we review the current status of human ESC line derivation. We consider the rationale for generation of new cell lines, the technologies available now for their derivation and propagation, and the current standards for their characterization (Figure 1).

Scientific Justification and Ethical Criteria for New Derivations

Any efforts to derive new hESC lines should be justified scientifically. A recent analysis of the literature indicated that there are now over 1000 hESC lines in existence and that ~70% of these have been characterized to some degree in peer-reviewed publications (Löser et al., 2010). However, much of the scientific literature on hESC is based on only a handful of cell lines (McCormick et al., 2009; Scott et al., 2009). Given that there are over a thousand hESC lines in existence, there is certainly a case for more work on a wider range of these established cell lines (assuming that the majority are in fact available for distribution). However, what scientific rationale justify generation of additional cell lines?

First, genetic or epigenetic variation in the ability of hESC lines to undergo differentiation into particular lineages remains a largely unknown factor. Thus it is uncertain how many cell lines might be required to provide a panel with, for example, high competency for beta islet cell formation. Similarly, little is known about how genetic or epigenetic stability varies between different hESC lines. Second, estimates of the number of hESC lines that will be required to provide adequate coverage for tissue matching in transplantation vary. Limited studies suggest that existing cell lines are not representative of a wide range of ethnic diversity (Laurent et al., 2010 ; Mosher et al., 2010), and the problem of matching populations of mixed racial origin has not really been addressed. Third, only a small minority of the published cell lines have been derived under conditions that are optimal for future clinical use. Technology for hESC derivation, maintenance, and expansion are constantly evolving, and there is a strong argument for deriving cell lines with improved methodology.

Although the advent of iPSC technology may circumvent ethical roadblocks around the use of embryos in research, there are still questions over the biological equivalence and safety of iPSC lines compared to hESC (Lee et al., 2009). Some concerns include limitations or reductions in developmental potential (Hu et al., 2010; Kim et al., 2010), variability relating to the starting cell type used for reprogramming (Kulkeaw et al., 2010), and an epigenetic/gene expression profile that may suggest an independent pluripotent state for iPSCs when compared to ESCs (Doi et al., 2009; Chin et al., 2009). hESCs, as such, remain the standard by which all other pluripotent cell lines are judged (Hyun et al., 2007; Smith et al., 2009).

The research community has been moving toward uniform international ethical standards for the derivation of new cell lines. The European Union started the process in 2004 with Directive 2004/23/EC, the International Society for Stem Cell Research (ISSCR) followed in 2007, and the National Institutes of Health (NIH) followed in 2009. Each of these sets of guidelines, subjected to public comment and extensive refinement, adheres

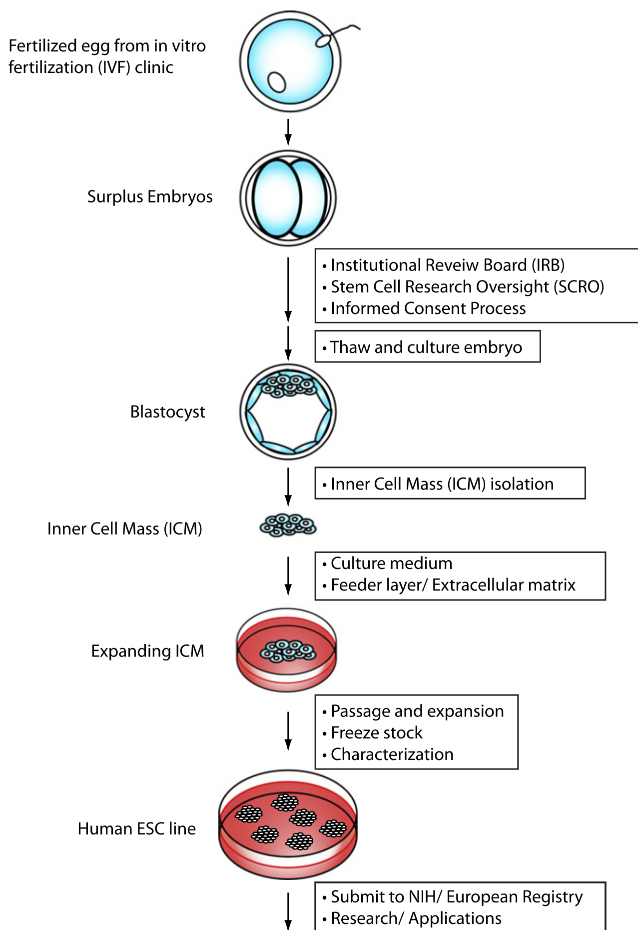

Figure 1. Schematic of the Process of Human Embryonic Stem Cell Line Establishment

to some very basic principles to ensure the highest level of ethical standards in the study of hESCs (Table 1). The push to implement these standards is reflected in the European Human Embryonic Stem Cell Registry (hESCreg) and the NIH Embryonic Stem Cell Registry. The NIH registry currently comprises 64 lines that adhere to US federal guidelines with 12 more in submission as of May 2010. Although hESCreg has over 650 lines listed, the provenance of only a small fraction has been validated to meet European/international guidelines.

As hESC technology moves into the translational and clinical stage, standards for derivation of cell lines will become more stringent. The first step toward the clinic involves the derivation and characterization of Good Manufacturing Practice (GMP) quality cell lines (Ährlund-Richter et al., 2009). In order to qualify for GMP, the cell lines must be derived and cultured in: (1) defined and (2) controlled conditions (3) by trained staff (4) with full documentation. Some proprietary lines have been derived under GMP conditions (ESI, WiCell), and only one cell line, H1, has been maintained under GMP conditions and approved for clinical trials (Geron Corporation). It should be noted that GMP does not preclude the use of products derived from animal sources, such as fetal calf serum, so long as the product meets the GMP standards defined above and no other suitable products

Table 1. Common Principles Shared across hESC Derivation Guidelines

Only unused embryos created for the purpose of in vitro fertilization (IVF) should be used for the derivation of ESCs.
Donors should voluntarily consent to the donation of embryos for research without influence from those participating in the study.
The standard of IVF care should be unaffected by the decision to donate.
No financial compensation was made for the donation.
Donors should be informed of alternatives to donation, that embryos would be used for the derivation of ESCs, that no direct medical benefit was intended, that the ESCs may have commercial potential to which they would not be entitled, that identifying information would remain confidential, and that they may withdraw from the study until the embryos are actually used.

are available. Future clinical acceptance of any new hESC line, however, can only be improved by the combination of xeno-free GMP methods for derivation and maintenance.

Human Embryo Culture, Assessment, and Establishment of hESCs

Embryo Culture and Assessment

Figure 1 illustrates some key phases in the life history of a hESC line, beginning with embryo culture. Human embryo culture has been refined over the 30+ years since the first successful in vitro fertilization (IVF) procedure. In general, procedures for culture of the fertilized egg from the two pronuclear (2PN) stage of fertilization through initial cleavage and transfer to the uterus for implantation have sought to mimic the conditions that a zygote would experience while traveling through the fallopian tube. Human tubal fluid nearest the ovary is high in pyruvate and lactate, whereas the concentration of glucose increases as the zygote nears the uterus. Therefore, it is common to culture human embryos in sequential media wherein a human tubal fluid analog medium is used from fertilization to the eight-cell stage at day 3, and this is followed by a switch to a high glucose, complete medium for compaction and blastocyst formation on days 5–6 (Bongso and Tan 2005; Mercader et al., 2006; Ilic et al., 2007; Sathananthan and Osianlis, 2010). However, some workers argue that a single-media system yields equivalent results (Biggers and Summers, 2008). Many embryo culture protocols have employed coculture systems using fibroblasts, endometrial cells, or other cell types to support development, and a recent meta-analysis indicated that coculture does indeed improve embryo quality (Kattal et al., 2008). However, coculture of embryos has the same drawbacks as the use of feeder cells during establishment and maintenance.

Embryo quality is a critical factor in hESC derivation. For the most part, assessment of embryo quality continues to rely on morphological criteria (Bongso and Tan, 2005), although metabolomics (Botros et al., 2008) and proteomics (Katz-Jaffe et al., 2009) may ultimately provide more objective and accurate evaluation. The highest level of success comes from implantation of high-quality, expanded blastocysts on day 5/6 (Figure 2 A), and reported success in hESC derivation is also greatest under these parameters. Because the best available embryos are of course used for transfer to the uterus,

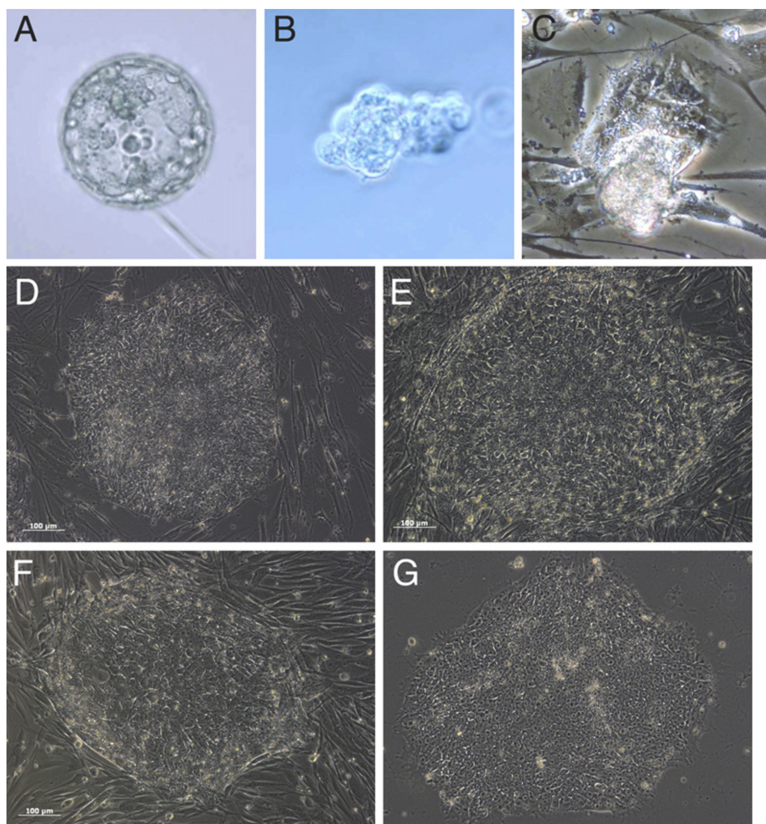

Figure 2. hESC Colonies Derived and Cultured under Various Conditions
Full grown day 6 human blastocyst (A) and isolated ICM (B). (C) shows an attached ICM with growing pluripotent cells, 1 day after seeding on feeder layer. Morphologies of hESC colonies growing in KSR medium on conventional MEF feeder-layer (D), in KSR medium on human dermal fibroblast feeder-layer (E), in KSR medium on xeno-free prepared human dermal fibroblast feeder-layer (F), in xeno-and feeder-free medium mTesR2 (G). (A)–(C) are courtesy of Dr Suemori and were generated according to the methods published in Suemori et al. (2006).

surplus embryos for hESC derivation may not always be of the highest quality. Several groups (Mitalipova et al., 2003) have reported derivation of ESC lines from poor-quality embryos, and a recent study confirmed the potential of this approach (Lerou et al., 2008), although success rates were quite low unless the embryos were able to reach the blastocyst stage. Timing of inner cell mass (ICM) isolation is another critical factor determining the outcome of derivation. Most of hESC lines have been derived from blastocysts at day 5/6 of culture. A recent study indicated up to 50% efficiency when blastocysts were allowed to develop until day 6 (Chen et al., 2009). At this stage, the isolated ICM attaches to the feeder layer with relative ease and starts proliferating (Figures 2B and 2C). These results are reflective of the higher rates of successful implantation during IVF treatment when blastocyst stage embryos are used.

Avoiding Immunosurgery and Exposure to Animal Products

Although some workers have derived hESCs from explanted blastocysts, most have relied on immunosurgery for isolation of the ICM (Figure 2B) (Solter and Knowles, 1975; Bongso et al., 1994). Because trophectoderm cells show rapid rates of growth and may inhibit the expansion of the ICM in culture, their early removal is considered beneficial by most workers. Immunosurgery requires the use of xenomaterials in the form of animal-sourced antibodies and complement. Whole and partial-embryo culture methods (Kim et al., 2005) can eliminate the need for immunosurgery, but do not enrich for ICM during initial derivation. Two alternate approaches avoid the pitfalls of immunosurgery with animal components and whole-embryo culture. First, the ICM can be isolated mechanically by dissection with sharpened metal needles (Ström et al., 2007). The second possibility is to perform the isolation with infrared lasers, which are widely used for drilling holes in the zona pelucida of eggs and early embryos for preimplantation genetic diagnosis (PGD) testing. The infrared laser can be used to isolate the ICM through ablation of the zona pelucida and trophectoderm (Turetsky et al., 2008). Proof of concept of this technique was demonstrated on genetically abnormal embryos identified during PGD, with three out of eight ICMs producing disease-specific hESC lines. A more recent study using laser-assisted derivation in human embryos reported derivation efficiency as high as 52% when isolating ICMs from day 6 blastocysts (Chen et al., 2009).

Blastomere Culture

Several groups have examined the potential for hESC derivation from single blastomeres. Given that PGD entails biopsy of a single blastomere and allows for the normal development of the remaining cleavage-stage embryo, blastomere biopsies were undertaken to create new hESCs without the destruction of embryos, to avoid ethical concerns. The technique was first reported in the mouse in 2006 (Chung et al., 2006), and this report was closely followed by the derivation of hESCs from human blastomeres (Klimanskaya et al., 2006). Coculture of the blastomere with existing hESC cell lines was a necessity in these studies, a potential limitation to the use of the technique for deriving clinical grade lines. Another study employed coculture with the parent embryo with some success (Chung et al., 2008). This method of coculture with the parent embryo also produced blastomere derived hESCs in the presence of human feeders and minimal xenomaterials (Ilic et al., 2009). It is possible that maintaining the parent embryo in culture with the biopsied blastomere may restrict its future use in IVF treatment, so elimination of this step is important. In 2009 a group used four-cell stage embryos and isolated individual blastomeres (Geens et al., 2009). Each blastomere was allowed to develop in sequential medium until day 3 or 4 when they were transferred to inactivated MEFs. Two new cell lines were established, only one of which was karyotypically normal.

Derivation of hESCs from blastomeres represents an interesting technical achievement, and the technique has the

potential to provide insight into possible differences in cell lines derived from presumed totipotent blastomeres of early stages versus pluripotent cells derived from the later-stage ICM of the blastocyst. Although it has been argued that such an approach can leave a viable embryo intact (on the basis of experience with preimplantation genetic diagnosis) and thus circumvent ethical issues around embryo destruction, it seems unlikely that clinicians would chose to implant an embryo that had undergone biopsy in preference to one that had not (unless there were a clinical indication for carrying out the biopsy). Therefore, it is unclear whether such procedures would ultimately impact on the long-term viability of the embryos, which would likely be discarded anyway.

Culture Methodology for hESCs

This section, and the following one on cell characterization, focuses on methodologies developed for hESCs. Although a detailed discussion of the generation, maintenance and characterization of iPSCs is beyond the scope of this review, it is worth noting that the same culture systems used to propagate hESCs have been used in the establishment and expansion of human iPSCs. There is as yet no evidence that hESCs and iPSCs differ in terms of the extrinsic signaling mechanisms that control their growth and differentiation, although the question has not been systematically investigated. Arguably, human iPSC development would not have been possible without previous studies of hESC culture methodology, the requirements of which are of course different to those of mouse ESC culture.

Culture Media

Originally, hESCs were established and cultured in medium supplemented with 10%–20% fetal calf serum (FCS) on mouse embryonic fibroblast (MEF) feeder layers (Reubinoff et al., 2000; Thomson et al., 1998). Currently, the most common culture system in research use of hESCs is based on supplementation with knockout serum replacement (KSR) and 4–10 ng/ml FGF2, using either MEF feeder cell layers or feeder cell-conditioned medium (Amit et al., 2000) (Figure 2D). This platform includes xenomaterials, a significant drawback for clinical use because of the potential for transmission of pathogens. Therefore, several media containing human serum (or serum components) instead of FCS and human feeder layers instead of MEF have been developed for establishment and maintenance of clinical-grade hESCs (Crook et al., 2007; Ellerström et al., 2006; Rajala et al., 2007) (Figure 2E). Recently, xeno-free serum replacements, such as xeno-free KSR (Invitrogen), or xeno-free culture medium, such as HESGRO (Millipore) have become commercially available and enable the maintenance of hESCs in an undifferentiated state in feeder cell culture systems (Figure 2F). Notably, undifferentiated hESCs retain slightly different morphologies in different culture systems.

hESC culture on a feeder cell layer introduces another source of possible contamination by adventitious agents. Feeder cell layers can produce proteins and small molecules that interfere with studies of stem cell maintenance and differentiation factors in unpredictable ways. In addition, a human feeder layer may introduce ambiguity in any subsequent genetic analysis. Therefore, the development of xeno-free and feeder-free, fully defined systems for establishment and culture of hESCs had been a goal in this field for some time. The first attempts to eliminate the feeder cell component from hESC culture used MEF-conditioned medium (Xu et al., 2001), which of course still carries the same risk of contamination with adventitious agents as feeder cell culture. The elucidation of the key signaling pathways for hESC self-renewal has enabled replacement of any requirement for feeder cells or their secreted products. In contrast to mESCs, hESCs do not appear to require LIF/STAT3 or BMP signaling for self-renewal (Dahéron et al., 2004; Humphrey et al., 2004; Xu et al., 2002). However, activation of signaling by receptor tyrosine kinases, in particular by FGF2 (Eiselleova et al., 2009; Gonzalez et al., 2010), IGF/insulin (Bendall et al., 2007; Levenstein et al., 2008; Li et al., 2007), and sphingosine-1-phosphate/ PDGF (Wong et al., 2007), upstream of ERK and PI3K/AKT (Armstrong et al., 2006; Li et al., 2007; Soh et al., 2007) and in combination with TGF-ß/Activin/ Nodal/activation of SMAD 2/3 (Amit et al., 2004; Vallier et al., 2005; Vallier et al., 2009; Xiao et al., 2006), appears to be critical for hESC maintenance. Other studies have implicated WNT/ β-catenin (Dravid et al., 2005; Lu et al., 2006; Sato et al., 2004), and TNF receptor superfamilies in hESC survival (Lu et al., 2006). Inhibition of BMP signaling can prevent spontaneous differentiation (Pera et al., 2004; Xu et al., 2005). On the basis of these findings, many feeder-free, xeno-free defined culture media consisting of combinations of recombinant growth factors activating stem cell maintenance pathways or inhibiting differentiation have been reported (Li et al., 2005; Liu et al., 2006; Ludwig et al., 2006; Peiffer et al., 2010; Swistowski et al., 2009; Wagner and Vemuri, 2010; Yao et al., 2006). Notably, the use of these defined media often requires a process of culture adaptation; hESC may become unstable for a few passages after transfer from feeder cell culture conditions (Akopian et al., 2010). Some of the xeno-free and feeder-free defined culture media are now commercially available, such as mTeSR2 (StemCell Technologies), StemPro (Invitrogen), SBX (AxCell), NutriStem (Stemgent), and VitroHES (Vitrolife). hESCs can be expanded and maintained in the undifferentiated state in these media (Figure 2G). However, only TeSR1, a noncommercially available analog of mTeSR2, has been reported to support establishment of hESC lines from blastocysts (Ludwig et al., 2006).

Given that all recombinant or purified growth factors are costly, many groups have used hESCs in high-throughput screening system to identify small molecules capable of supporting stem cell maintenance (Damoiseaux et al., 2009; Desbordes et al., 2008; Lin et al., 2009). It was reported that a combination of a histone deacetylase (HDAC) inhibitor and an inhibitor of GSK3β could maintain hESCs in the undifferentiated state (Sato et al., 2004; Ware et al., 2009). Recently, a small-molecule BMP receptor type 1 (BMPR-I)-specific inhibitor LDN-193189 that might inhibit spontaneous differentiation in hESC culture was identified through chemical screening (Yu et al., 2008). It was also recently reported that the combined use of a MEK/ERK inhibitor and Activin inhibitor could improve human iPSC induction (Lin et al., 2009), although given the widely recognized role for these pathways in hESC maintenance, the mechanism of this effect requires further study.

One report has suggested that feeder-free culture is associated with more chromosomal instability than conventional feeder cell based systems (Catalina et al., 2008). Therefore, feeder-free culture systems may require more frequent karyotypic

examination, and it is important to discover what culture conditions favor maintenance of a normal karyotype. Several reports have demonstrated that low oxygen tension could reduce chromosomal abnormalities and also prevent hESC differentiation (Ezashi et al., 2005; Forsyth et al., 2006). A very recent study showed that a small molecule selectively inhibits growth of chromosomally abnormal hESCs and cancer cells, but not normal hESCs (Gauthaman et al., 2009). This report requires further confirmation, but this or related strategies might be necessary to reduce the risk of generating and propagating abnormal cells in defined culture systems.

Given the limited published success with establishment of hESC lines in xeno-free/feeder-free medium, it seems realistic that at present the most reliable strategies for establishment of clinical grade hESC lines include use of a human feeder cell layer in xeno-free medium, followed by expansion in a feeder-free culture system. In general, hESC cultures show a significant amount of cell death and spontaneous differentiation, which can be minimized by daily medium exchange and passage before the culture becomes over grown. Growth rates of hESC cell lines vary (Cowan et al., 2004) and careful adjustment of the starting size of the clumps used to seed new dishes at subculture, colony density, split ratio, and passage interval are essential. Suboptimal conditions may necessitate removal of differentiated cells during passage (Ludwig and Thomson, 2007).

Passage Methodology

Initially, hESCs were passaged by mechanical dissociation of mature colonies into cell clumps with sharpened glass or steel needles (Reubinoff et al., 2000; Thomson et al., 1998). In the process of establishment of hESC lines from ICM, differentiated cells, which may represent extra embryonic lineages, frequently appear within a colony of undifferentiated cells. Mechanical dissection produces clumps of an appropriate size for passage and enables elimination of differentiated cells. The simplest mechanical passage method is dissection of colonies into uniform size clumps with a sharp needle (Cowan et al., 2004).

After establishment of hESC lines, large numbers of cells are required for quality assurance and establishment of cell banks. Despite considerable efforts to identify factors that improve cell viability, hESCs survival remains very low after complete dissociation into single cells, and continuous complete dissociation may lead to selection of chromosomal abnormal cells that are capable of survival under these conditions (Hasegawa et al., 2006). Therefore mechanical, enzymatic, or chemical dissociation of colonies into 50- to 1000-cell clumps remains the most common passage method for expansion of hESC culture. Mechanical dissection is not amenable to scale up or rapid expansion. Recently, a specialized roller cutting device for mechanical hESC passage has become commercially available (EZ passage tool, Invitrogen) (Wagner and Vemuri, 2010), and such a device might facilitate rapid dissection of a large number of colonies into uniform size clumps. To further speed and standardize the process and minimize operator contact, some workers have used laser dissection of hESC colonies (Terstegge et al., 2009).

For enzymatic dissociation, collagenase IV, dispase, or combinations of these and other proteolytic enzymes are commonly used to detach and dissociate hESCs colonies into clumps. This procedure requires some experience to produce clumps of the appropriate size for subculture. Many of these enzymes are derived from animal products. More recently commercially available xeno-free enzymes (Accutase, Innovative Cell Technologies, or TrypLE, Invitrogen) have been used to dissociate hESCs to single cells and are reported to provide higher cell survival after passage (Bajpai et al., 2008; Ellerström et al., 2010). These products may prove to be useful in expansion of cell lines. The small molecule Rho-associated kinase (ROCK) inhibitor Y-27632 or a combination of ROCK inhibitor and protein kinase C inhibitor have been shown to enhance hESC survival after passage as single cells (Damoiseaux et al., 2009; Watanabe et al., 2007). Another report suggested the ROCK inhibitor-mediated cell-cell interaction was associated with cell surface E-cadherin stabilization (Xu et al., 2010). In this report, it was also suggested that enhanced integrin signaling synergizes with growth factors to enhance hESC survival (Xu et al., 2010).

Nonenzymatic dissociation of hESC can also be achieved by chelation of calcium and magnesium with EDTA (Ludwig and Thomson, 2007). Either enzymatic or chemical dissociation may carry a higher risk of induction of chromosomal abnormalities than mechanical colony dissection (Mitalipova et al., 2005) (Catalina et al., 2008), possibly because either technique can release single cells. Many of the common chromosomal abnormalities seen in cultured hESCs provide a survival advantage, and dissociation to single cells is a selective pressure, favoring expansion of abnormal clones.

To date, no approach has provided for high survival of single hESCs after subculture. Current best practice would probably utilize mechanical dissection for establishment and maintenance of hESCs and either enzymatic or chemical harvest, with care to avoid dissociation to single cells, for expansion of hESCs over a limited number of passages.

Several studies describe scale-up of hESC cultures in small bioreactors (Fong et al., 2005; Krawetz et al., 2009), microcarrier-based suspension culture (Oh et al., 2009; Phillips et al., 2008), or an automated culture system (Terstegge et al., 2007; Thomas et al., 2009). Steiner et al. (2010) recently described the derivation of hESCs in a suspension culture system using basal medium extensively supplemented with a serum replacement including beta D-xylopyranose, growth factors (Activin A, FGF2 and neurotrophic factors), and extracellular matrix molecules (laminin, fibronectin, and gelatin). hESCs could be expanded and maintained in this system, although cell production was lower relative to monolayer culture on feeder cells due to increased cell loss, and questions remain over long-term genetic stability in cultures grown with this technique. Although experience to date with these scaled-up culture systems is limited, they may provide a future option for rapid expansion of hESCs.

Feeder Cells

Although several commercial, defined media are now available for feeder-free propagation of hESCs, there is only limited experience with the use of these preparations in derivation of cell lines. Therefore, many groups continue to use feeder cells in establishment of hESC lines. The original methodology for hESC derivation used MEF feeder cells. Subsequently, several groups reported the use of human fibroblasts as feeder cells, including cells derived from fetal, neonatal, and adult tissues (Unger et al., 2008). Cell lines have been derived under GMP

conditions with human feeder cells (Crook et al., 2007). Also, fibroblast-like cells derived from hESC culture have been shown to support hESC growth (Stojkovic et al., 2005), through a paracrine mechanism (Bendall et al., 2007).

More recently, several groups have shown that extracellular matrix from human fibroblasts can support hESC maintenance, either with the use of conditioned medium from the feeder cells or without it (Escobedo-Lucea and Stojkovic, 2010; Meng et al., 2010a). In both studies, the fibroblasts were grown in medium containing human serum. These and related culture systems, though xeno free, nevertheless are still undefined.

Extracellular Matrix

Derivation of new cell lines in fully defined conditions will require provision of a natural or synthetic extracellular matrix capable of supporting hESC attachment survival and growth. As discussed above, single hESC do not survive in suspension, and even suspension cultures of cell clumps require supplementation with extracellular matrix or extracellular matrix addition to microcarriers. Most studies have employed serum, Matrigel (BD Biosciences), or extracellular matrix deposited by the feeder cell layer to support adhesion, spreading, and growth of hESCs. None of these systems provides for fully defined culture conditions and many contain animal-derived products. The definition of the active components in these complex biological preparations might help in designing a more defined system. The major attachment factors in serum are fibronectin and vitronectin. Matrigel is a commercial extract of natural basement membrane that contains laminin, collagen type IV (Braam et al., 2008), entactin, and heparan sulfate proteoglycans. Feeder cells secrete fibronectin, collagen types I and IV, and laminin. Finally, hESCs themselves synthesize laminin 511 and nidogen 1 (Evseenko et al., 2009), and differentiated derivatives in the cultures are likely to synthesize other matrix components. This information, alongside analysis of integrin expression in stem cell cultures, provides a rational basis for the use of defined natural or recombinant extracellular matrix components to support hESC growth and derivation. hESCs have been reported to express the integrin subunits α-2,-5,-6,-v and β1,-3,-5 (Braam et al., 2008; Evseenko et al., 2009; Meng et al., 2010b; Xu et al., 2001) . Functionally, the α6β1 integrin has been defined as a laminin receptor in hESC, the αVβ5 and αVβ3 integrins have been identified as vitronectin receptors, and the α5β1 receptor shown to be a receptor for fibronectin (Braam et al., 2008; Meng et al., 2010b).

The first report of purified extracellular matrix components in a defined culture system used a combination of fibronectin, laminin, collagen IV, and vitronectin (Ludwig and Thomson, 2007). These proteins, purified from natural sources, may contain various contaminants and generally are not xeno free. Recombinant laminins 332, 511, and 111 were shown to support hESC maintenance for up to ten passages under defined conditions (Miyazaki et al., 2008). However, in one study of hESC establishment from explanted blastocysts, it was necessary to combine a laminin substrate with the use of a feeder cell layer to derive cell lines (Fletcher et al., 2006). Vitronectin was identified some years ago as a key attachment factor for pluripotent human embryonal carcinoma cells (Cooper and Pera, 1988). More recently, three groups have reported maintenance of hESCs or human iPSCs in defined conditions on natural or recombinant vitronectin (Braam et al., 2008; Manton et al., 2010; Rowland et al., 2009). One study has employed short peptide ligands for the αVβ3 α6β1 and α2β1 integrins to support hESC adhesion and growth (Meng et al., 2010b). The peptides were only able to support hESC maintenance for short periods, which suggests that the full-length integrin ligands might contain other domains that are important for longer-term propagation of hESCs. One study showed that laminin 511 and nidogen together enabled reassembly of single hESCs into clumps of cells (Evseenko et al., 2009). Although the authors of this study focused on embryoid body formation, it is possible that reaggregation and adhesion mediated by these factors would enable survival of hESCs after dissociation.

In addition to these extracellular matrix molecules, hyaluronan is a candidate factor for promotion of hESC survival. Hylaruronan may be particularly relevant to hESC derivation because of its presence in the female reproductive tract and its known roles in early embryo growth. hESC express the hyaluronan receptor RHAMM on their surface (Choudhary et al., 2007). Knockdown of this receptor resulted in death and differentiation. One report has described long-term maintenance of hESCs in hyaluronan containing hydrogels, although the study was carried out in the presence of conditioned medium from mouse embryo fibroblasts (Gerecht et al., 2007).

Cryopreservation

In general, the recovery of hESCs after cryopreservation is very low after use of conventional slow cooling and rapid thawing protocols widely employed for cultured cells (cryopreservation medium containing 5%–10% DMSO and culture medium supplemented with serum or serum replacement and freezing at ~1°C/min) (Fujioka et al., 2004; Reubinoff et al., 2001). Several groups reported marked improvement in cell recovery by using vitrification in straws or vials (Fujioka et al., 2004; Hunt and Timmons, 2007; Reubinoff et al., 2001; Richards et al., 2004). Given that these vitrification media consist of simple culture medium and chemical regents only, they provide a means to cryopreserve cells without animal components. However, these vitrification protocols often require contact with liquid nitrogen, posing contamination risks. There are several reports demonstrating liquid nitrogen contact-free cryopreservation with controlled cooling systems (Lee et al., 2010; Morris et al., 2006; Ware and Baran, 2007). In addition, there are several reports of methods to enhance survival of hESCs after cryopreservation, such as the use of ROCK inhibitor (Li et al., 2009; Martin-Ibañez et al., 2008), caspase inhibitors (Heng et al., 2007), or microcarriers (Nie et al., 2009). In general, for cryopreservation, hESC colonies need to be dissociated into clumps, just as in routine passage, and concentrated to high density for freezing. Closed straws or vials should be used for freezing, and the vials or straws should be stored in either ultra-low temperature freezer (−150°C) or vapor phase of the liquid nitrogen tank.

Characterization

Standard methods for the characterization of hESC lines typically include cell-surface marker profiling, gene expression analysis, and biological assay of differentiation potential, as well as examination of genetic integrity. The International Stem Cell Initiative study of 59 cell lines from 17 laboratories established a panel of cell-surface markers and pluripotency genes that

were consistently expressed across this diverse group of cell lines (Adewumi et al., 2007). Flow cytometry using surface markers and some of the key pluripotency transcription factors yields quantitative information about the proportion of cells in the culture that are positive for these indicators and is an important addition to immunofluorescence, which defines cellular localization of the markers. Likewise, quantitative RT-PCR (qRT-PCR) should be employed for measuring pluripotency markers and comparing them with established hESC cultures. These parameters should be examined at early passage levels and then at regular intervals.

Characterization of cell lines should include in vitro tests of differentiation potential. The most commonly used paradigm is embryoid body formation, with assessment of gene expression in differentiated cells in the embryoid body or in adherent cultures derived from it, by immunoflourescence and qRT-PCR, for lineage-specific markers representative of the three embryonic germ layers. The reaggregation technique (Ng et al., 2005) provides for uniform input of cell numbers and avoids heterogeneity that results from incorporating only selected regions of colonies into the embryoid body.

The advent and widespread adoption of reprogramming technology for the derivation of pluripotent cell lines has engendered a certain amount of debate over the vexed issue of how best to evaluate pluripotency of cultured human cells (Ellis et al., 2009; Maherali and Hochedlinger, 2008). The increasing availability of robust protocols with quantitative endpoints for the neural, mesodermal, and endodermal differentiation of hESC has led some to suggest that a panel of such assays could supplant the need for in vivo teratoma assays. The International Stem Cell Initiative is convening a series of expert discussion groups to assess currently available assays and make recommendations concerning their use.

In the absence of an agreed and proven set of surrogate in vitro assays, the gold standard for pluripotency in the human is the formation of teratomas in immunodeprived animals (Przyborski, 2005). Protocols for formation of teratomas and their histological assessment have been published (Gertow et al., 2007), but they may not always be followed and/or reported in detail (Müller et al., 2010). Some groups have combined standard histological examination with immunochemistry to validate the human origin of various differentiated tissues in the graft and to better define the cell lineages represented. However, quantitative assessment of differentiation, and thus definitive evaluation of the ability to give rise to a wide variety of tissue lineages, remains problematic with this assay. Recent studies have suggested that certain sites and modes of injection might provide improved yields of tumors (Cooke et al., 2006; Prokhorova et al., 2008). It is important to remember that in vivo teratoma formation can provide information not only about differentiation potential but also about the propensity of cell lines to form malignant growths (teratocarcinomas) (Blum and Benvenisty, 2008; Przyborski, 2005). hESCs with a normal karyotype generally give rise only to teratomas, which do not contain undifferentiated cells. By contrast, chromosomally abnormal cells, or hESC lines with small genetic lesions, can yield teratocarcinomas that contain undifferentiated cells resembling embyonal carcinoma cells. The presence of undifferentiated cells within a teratoma is thus a cause for concern.

Genetic stability of hESC cell lines is routinely assessed by G-banded karyotype or, less frequently, spectral karyotyping, at regular intervals during the development and growth of cell lines in vitro. More recently, it has become apparent that hESCs can acquire submicroscopic genetic alterations, including small amplifications and deletions, that are not detected by karyotype analysis. It appears that there are hotpsots for such changes (Närvä et al., 2010) and that small changes may be associated with alterations in cell behavior such as reduced growth factor requirements, reduced ability to undergo differentiation, and formation of teratocarcinomas (Werbowetski-Ogilvie et al., 2009). In one recent study, copy-number variants (CNVs) were reported to occur frequently in regions containing genes associated with cancer, but the biological significance of this observation remains unclear (Närvä et al., 2010). Assessment of CNV is thus becoming more important to the assessment of genetic integrity of cell lines. None of the current methodologies has addressed the possibility that hESCs may acquire point mutations in oncogenes or tumor suppressor genes. As costs of genomic sequencing decline this may become a realistic option. Preservation of DNA from the blastocyst at hESC derivation would enable unambiguous determination of whether CNVs are constitutional or acquired during culture.

Conclusions

Our understanding of the basis of the extrinsic regulation of pluripotency in hESC (Pera and Tam, 2010) has progressed considerably and this knowledge has been put to practical implementation in the design of new culture systems. There is now a variety of technological options for workers wishing to derive new hESC lines. Nevertheless significant challenges to the field remain. Among these are the development of defined culture systems that function robustly in hESC derivation, the development of defined extracellular matrix components for support of hESC cultures, the improvement of single-cell survival of hESCs, and the definition of conditions that provide for long-term genetic and epigenetic stability. In the areas of hESC characterization, the establishment of a standard panel of in vitro assays for the assessment of pluripotency should be a priority for the field. Finally, the cost of many defined culture systems is a major roadblock to their wider use in expansion of hESC cultures. It can be anticipated that given the current pace of human pluripotent stem cell research, platforms that address these challenges will emerge and come into widespread use within the next few years.

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