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SPECIAL ISSUE

Human Embryonic Stem Cells: Derivation, Maintenance and Cryopreservation

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Human embryonic stem cells (hESCs) are the most powerful candidate for the treatment of incurable diseases through the replacement of damaged cells and/or tissues in patients, although there are some obstacles to overcome for the clinical application of hESCs such as the assurance of guided differentiation and control of the immune response following cell therapy or tissue grafting. To obtain genetically stable hESCs and use them clinically, it is important to develop appropriate culture conditions. Additionally, the establishment of a hESC bank with a large number of hESC lines will be required for their clinical application because each hESC line is directed to have a different differentiation ability and immune characteristics such as HLA type. In this review, we describe the derivation and culture conditions of hESCs based on recent advances. Then, we will introduce several cryopreservation methods for hESCs, which is important for the development of cell bank.

Keywords: Human embryonic stem cell, Establishment, Culture, Cryopreservation

Introduction

Human embryonic stem cells (hESCs) are derived from inner cell mass of blastocyst-stage embryos, and exhibit unlimited proliferation ability and pluripotency to differentiate into various cell types originated from the three-germ layers. Their unique properties are expected to improve investigations in the field of human development and provide new materials for cell/tissue transplantation therapies and drug screening. However, there are prerequisites for clinical application of hESCs. In gerneral, all processes related to the derivation, maintenance and differentiation of hESCs should be accomplished under xeno-free culture condition using good manufacturing practice (GMP) systems (1, 2). Guided-differentiation protocols of hESCs into a given functional cell type must be established so that the resulting cells are homogeneous and do not form teratomas or cause cancer (3), and immune responses/rejection caused by the transplantation of hESCs or their differentiated derivatives should be prevented.

Since the first report of hESC establishment, approximately 1,000 hESC lines have been established worldwide (International stem cell registry, University of Massachusetts medical school; http://www.umassmed.edu/iscr/index.aspx). Efforts on the part of several stem cell registries to share information regarding various hESC lines, and requests for the development of large banks of hESC lines have been put forth.

In this review, we describe the derivation and culture conditions of hESCs based on recent advances. Then, we will introduce several cryopreservation methods for hESCs, which is important for the production of a cell bank.

Derivation conditions for human embryonic stem cells

In mammalian embryonic development, the blastocyst

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consists of an inner cell layer called the inner cell mass (ICM) and an outer cell layer called the trophoectoderm (TE). The successful derivation of a mouse embryonic stem cell line from ICM was first reported in 1981, and subsequently, advances in mESC culture techniques were applied to generate ESC lines in non-human primates and humans. The most important process involved in hESC derivation might be the isolation of the ICM from the TE. In the initial stages of hESC research, immunosurgery procedure employing anti-human serum and guinea pig complement was used for isolation of the ICM (4-7). However, these reagents may include animal pathogens and molecules, and the exposure of animal-derived products to the ICM and its derivatives (8), hESCs, during immunosurgery may bring about immune responses in patients after transplantation. To avoid the contamination of animal-derived components during the derivation procedure, several methods have been used instead of immunosurgery. Mechanical dissection (9) and mechanical isolation of the ICM using flexible metal needles with sharpened tips (10) has been introduced as a method for hESC derivation. The derivation of hESC lines from abnormal human pre-implantation genetic diagnosis (PGD) embryos using laser dissection for the isolation of ICMs has been reported (11). Human ESC lines can be derived from outgrowths of the ICM after plating intact blastocysts on feeder cells, without isolating the ICM (12). It is unclear which approach is more efficient, but it is certain that derivation of xeno-free hESC lines will be suitable for future clinical application of hESCs.

With regard to ethical and political concerns, hESCs derived from the ICM at the blastocyst stage have been criticized because destruction of the embryo results from this process. To address this issue, many attempts have been made to generate hESCs from earlier stages of embryonic development without destruction of the embryo, and the derivation of hESCs from a single blastomere at the 8-cell stage has been reported through co-culturing of isolated blastomeres with hESCs (13, 14). Development of an ICM from isolated blastomeres was found to be inefficient because the blastomere-derived aggregates mostly gave rise to trophectoderm-like vesicles. To increase the efficiency of hESC derivation using blastomere-derived blastocysts, a modified approach using culture medium supplemented with laminin was employed, which resulted in increasing the efficiency of this process to levels similar to hESC derivation from whole blastocysts (15). It was suggested that laminin added to blastomere culture might simulate the natural ICM niche and thus prevent polarization of the blastomeres into ICM and trophectoderm. Recently, other research groups reported the derivation of hESC lines from blastomeres of 4-cell stage and 8-cell stage embryos (16, 17). If optimization of culture conditions for blastomere development and xeno-free culture conditions for hES derivation are accomplished, blastomere-derived hESCs will become very useful resources for stem cell research and clinical applications, without ethical and political controversy.

Culture conditions for human embryonic stem cells

In the first report of hESC derivation in 1998 (4), mouse embryonic fibroblast (MEF) cells were used as feeder cells to support the undifferentiated proliferation of hESCs, and leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) were used as major components of the culture medium. However, the bovine serum generally used in cell cultures is a complex mixture of proteins of unknown composition and, is sometimes associated with batch-to-batch variation. Serum may also contain factors inducing hESC differentiation. To obtain stable and reproducible results, studies have been performed to identify essential serum-provided components in various stem cell cultures. KnockOut Serum Replacement (KO-SR), which is a commercially available serum substitute (Invitrogen), has been developed and contains components such as bovine albumin, transferrin and insulin (18). The proliferation of hESCs cultured in KO-SR containing media can be successfully maintained in undifferentiated state, and soon after this substance was developed, animal serum was replaced by KO-SR for hESC culture. LIF, which is a key regulatory factor involved in mESC self-renewal via the JAK/STAT3 signaling pathway in feeder-free systems, does not have the same effect in hESCs. It was found that hESCs could be grown in feeder-free conditions using MEF-conditioned medium on extracellular matrix-coated dishes (19, 20), and thus, essential factors for hESC maintenance were characterized and defined. Based on the studies for hESC culture conditions, basic fibroblast growth factor (bFGF) is the most important factor for maintaining hESC pluripotency and, is now commonly used in hESC culture medium, both in the presence and in the absence of feeder cells (21).

For clinical application of hESCs, xeno-free culture conditions, in which all-animal derived components are eliminated from the derivation, maintenance and differentiation procedures for hESCs, are needed. With the aim of achieving xeno-free culture of hESCs, human feeder culture systems and feeder-free culture systems have been

preferentially studied. In hESC cultures, feeder cells provide both a suitable attachment substrate and important soluble factors for the maintenance of undifferentiated state. Various human feeder cells that originate from fetal skin, foreskin, fallopian tubes, uterine endometrium, bone marrow, amniotic epithelium and other tissues have replaced MEFs in hESC culture (22-26). Based on the supportive ability of hESCs as fibroblast-like cells for maintaining the undifferentiated growth of hESCs in feeder-free conditions, several studies have shown that hESC-derived fibroblast cells can be used as feeder cells for hESC culture (27, 28). It was recently reported that immortalized human foreskin fibroblasts induced to secrete bFGF by lentiviral transfection could be used for reproducible and cost-effective culture of hESCs (29).

Although feeder cells have an important role in hESC maintenance, the preparation of feeder cells for hESC culture is a labor and cost-intensive process associated with batch-to-batch variation. To eliminate the need for feeder cells and establish defined culture conditions for hESC growth, various coating materials to support the attachment of hESCs and supplementary factors in hESC culture medium have been studied. Matrigel, a complex mixture including extracellular matrix molecules, several growth factors and other substances, has been commonly used as a coating material for hESC culture (19, 30-32). To replace Matrigel with more defined substrates, several research groups have tested laminin, fibronectin, collagen I, collagen IV, vitronectin and human serum as alternative substrates for feeder cells both alone and in combination for hESC cultures (19, 23, 33-37).

Many studies have been performed to find appropriate supplementary factors for culture medium for the maintenance of hESCs. Various proteins, cytokines and/or growth factor(s) have been tested for their ability to support undifferentiated growth of hESCs together with bFGF under feeder-free culture conditions (30-32, 34, 38-46). According to stimulation experiments followed by microarray gene expression profiling on both MEFs and undifferentiated hESCs, stimulation of MEFs with bFGF ultimately results in the regulation of key members of the transforming growth factor beta (TGFβ) pathway, including up-regulation of Inhba, TGFβ1 and Grem1 and down-regulation of BMP4. Basic FGF restimulation of feeder-free hESCs also revealed that the downstream targets of FGF signaling were a similar group of genes as seen in MEFs. Additionally, a gene ontology analysis of genes upregulated in hESCs in response to bFGF revealed an overrepresentation of genes involved in cell cycle regulation (i.e. CDKN2B and GADD45A) and transcription factors (i.e. Nanog and FoxD1) (47). Therefore, it is suggested that regulation of TGFβ signaling is one of the important roles played by bFGF in hESC self-renewal, and the addition of TGFβ1, Activin A, or Nodal, or the repression of BMPs (e.g. addition of the BMP4 antagonist Noggin), is useful in expanding embryonic cells (42, 48). Other factors expected to play a role in maintaining pluripotency are the insulin-like growth factors (IGFs), based on a report that inhibition of the IGF1 receptor represses hESC self-renewal, while the addition of an IGF1 analog facilitates the expansion of undifferentiated cells (49). The Wnt signaling pathway is also thought to affect hESC self-renewal, but its role is currently unclear. It was reported that the addition of Wnt1 to MEF-conditioned medium represses spontaneous differentiation of hESCs (50), whereas Wnt3a has been reported to induce hESC differentiation (51). Additionally, it was shown that low concentrations of glycogen synthase kinase-3 (GSK-3) inhibitor induce self-renewal, but higher concentrations promote mesoderm differentiation (52).

In spite of progress associated with human feeder cells or feeder-free culture systems, animal-derived components, such as FBS for feeder cell culturing or bovine serum albumin, still exist in hESC cultures. It was observed that hESCs cultured in KSR-containing medium and/or with MEFs incorporated and expressed substantial amounts of N-glycolylneuraminic acid (Neu5Gc), which is a common form of sialic acid found in non-human mammalian cells (8). Because humans are genetically unable to produce Neu5Gc, hESCs expressing Neu5Gc would be recognized as a foreign antigen and cause an immune response following transplantation into humans, although Neu5Gc contamination of human cells was found to be significantly reduced after culture in medium with human serum (53). When nine types of xeno-free culture media using commercial medium and serum substitutes were compared with conventional culture media (KO-SR+ bFGF) for hESCs in the presence of human feeder cells, xeno-free components were insufficient to sustain undifferentiated hESC proliferation (54). Therefore, with regard to clinical applications, a defined feeder-free culture medium with only human-originated proteins/components should be developed for hESC culture, and furthur efforts to determine the factors responsible for hESC self-renewal and pluripotency are required.

In 2006, a defined feeder-free culture medium for hESCs (TeSR1) composed of a DMEM/F12 base supplemented with human serum albumin, vitamins, antioxidants, trace minerals, specific lipids and cloned growth factors was developed, and new hESC lines were estab-

lished in these culture conditions (55). The finding of this study suggested that the addition of bFGF, LiCl, γ-aminobutyric acid (GABA), pipecolic acid, and TGF-β into a defined culture medium supported undifferentiated growth of hESCs. Because the cost of this defined medium makes it difficult to use routinely, a modified version (mTeSR1) that includes the use of animal source proteins (bovine serum albumin and Matrigel) and cloned zebrafish bFGF has been developed (56). Serum-free and feeder-free culture media combined with extracellular matrix are now commercially available for hESC culture (StemPro®hESC SFM provided by Invitrogen; mTeSR™1 provided by Stem Cell Technologies).

Another issue related to hESC culture that should be resolved for future clinical applications is large-scale propagation of hESCs to generate a more reproducible product at a lower cost. The transfer of hESCs to a fresh feeder layer or new substrate-coated dishes is performed by mechanical dissection or enzymatic treatment. Compared to mechanical passaging, enzymatic passaging is less labor intensive and can easily be applied to large scale culture, although enzymatic passaging is difficult to remove differentiated parts of hESC colonies. Collagenase IV and dispase are commonly used for enzymatic passaging. With respect to future clinical applications, these enzymes are animal derived, so recombinant animal protein-free enzymes and human collagenase might be more suitable for hESC culture. Regarding tissue engineering, sufficient numbers of cells, from a few tens of millions to a few billion, are required for cell therapy. Various scalable culture systems such as microfluidic systems, rotary cell culture systems and stirred culture systems have been developed for the expansion and differentiation of hESCs (57, 58). In these culture systems, hESCs are cultured as mono-layers or aggregates, or on scaffolds depending on the type of cells ultimately desired.

Cryopreservation conditions for human embryonic stem cells

The efficient cryopreservation of hESCs is crucial to preserve early-passage stocks and establish cell banks for future clinical application. However, cryopreservation of hESCs is quite difficult, typically resulting in high rates of cell death and spontaneous differentiation after freezing and thawing. In contrast to other cell types, hESCs should be cryopreserved in small aggregates of a few hundred cells to prevent cell loss from apoptosis when they are detached/dissociated (59). It is not easy to quantify how many cells are viable within a clump during freezing and thawing, and it is very important that attached cells remain undifferentiated after thawing.

Two cryopreservation methods are commonly used for hESCs: vitrification and slow freezing with rapid thawing. Vitrification using a high concentration of cryoprotectants is often used for cryopreservation of oocytes and embryos in certain species. Studies on hESC cryopreservation efficiencies using various vitrification procedures have reported 70～90% survival rates using open pulled straws (OPS), straws or EM grids (60-62). However, vitrification has several limitations preventing its widespread use: 1) it introduces the possibility of contaminating hESCs with infectious agents via contact with liquid nitrogen (LN_2) if sealed containers are not used; and 2) the process is very labor intensive, as colonies have to be physically moved from one solution to the next solution and it requires strict time and very small volume handling at a time to obtain good results.

Cryopreservation of hESCs through a slow-freezing method with dimethylsulfoxide (DMSO) as a cryoprotectant is commonly used and yields poor results compared with vitrification. It is suggested that a number of stresses during slow freezing, including osmotic stress and, stress to cell-junction and cell-transport systems (63, 64), can contribute to the loss of pluripotency. Using straws as a container, programmed cell freezer and/or a high concentration of FBS/KO-SR, the survival rate of hESCs after slow-freezing and thawing was found to increase nearly 80%, with no apparent increase in differentiation (65, 66). Based on these reports, adjusting the protocol by altering the seeding temperature, cooling rate, and final temperature before plunging into LN_2 may optimize the recovery of cryopreserved hESCs. Recently, it was suggested that step-wise equilibration of serum and cryoprotectant during freezing and thawing results in higher survival rates by reducing mechanical and osmotic damage (67).

Various attempts have been applied to improve the efficiency of post-thaw recovery and attachment of hESCs. The addition of extracellular matrix such as human collagen IV or laminin into freezing medium was found to improve post thaw recovery and reduced the differentiation of colonies during subsequent culturing, and the addition of trehalose into freezing medium was observed to increase the recovery of colonies after thawing (68, 69). The addition of Rho-associated kinase (ROCK) inhibitor during freezing and thawing after dissociation of hESCs into single cells was shown to improve the recovery of hESCs and cloning efficiency, although only 5～7% of the isolated cells formed colonies (70). As hESCs are usually attached and grown on feeder cells or substrate-coated

Table 1. Cryopreservation protocols for human embryonic stem cells

Cell lines	Culture condition	Cryo-method	Cryo-medium	Cryo-container	Results after thawing	Reference
hES-1,2	MEF, Dispase DMEM+20%FBS	Vitrification Slow freezing	20% DMSO+20% EG+0.5M S 10% DMSO+90% FBS	OPS CV+Container	100%[a] 16%	Reubinoff et al. 2001 (60)
hES-2,3,4	Fetal skin fibroblasts, M DMEM+20%FCS+ITS	Vitrification	20%DMSO+20%EG+0.3M S+12% HSA	CS	~80%[b]	Richards et al. 2004 (61)
		Vitrification Slow freezing	20% DMSO+20% EG+0.5M S 10% DMSO+90% FCS	OPS CV+Freezer	~80% ~10%	
hES-1	MEF, M KO-DMEM+20% SR or FBS	Vitrification	10% DMSO+90% SR+0.2M trehalose	CV+Container	~48%[c]	Wu et al. 2005 (69)
SNUhES-3	STO, M DMEM/F12+20% SR+bFGF	Slow freezing	5% DMSO+10% EG+50% FBS	CV+Container	~30%[d]	Ha et al. 2005 (74)
H1	MEF, Dispase KO-DMEM+15% SR	Slow freezing	10% DMSO+25% FBS	CS+Freezer+ seeding	~70%[e]	Ware et al. 2005 (65)
HS-207,401	Foreskin fibroblast, M KO-DMEM+20% SR+bFGF	Slow freezing (single cell)	10% DMSO+culture media (ROCK inhibitor pre-incubation)	CV+Container	6~7%[f]	Martin-Ibanez et al. 2008 (70)
CHA-hES 3,4	STO, M DMEM/F12+20% SR+bFGF	Vitrification	5.5M EG+1M S+10% FBS (pre-incubation on feeder/CM)	EM grid	~80%[b]	Cha et al. 2008 (62)
VAL-3 VAL-5	Foreskin fibroblast, M /Coll IV KO-DMEM+20% SR+bFGF	Slow freezing	Culture media+addition of increased concentration of DMSO	CV+Container/ Feezer	41~68%[d] 8~15%	Valbuena et al. 2008 (75)
α-ES-C	MEF, Coll IV KO-DMEM+20% SR+bFGF	Bulk vitrification	20%DMSO+20%EG+0.5M S	Cell strainer (nylon)	~94%[g]	Li et al. 2008 (76)
Royan H5,6 hiPSC1,4	Feeder-free, Coll IV/Dispase DMEM/F12+20% SR+ITS+bFGF	Slow freezing (single cell)	10% DMSO+90% FCS+ROCK inhibitor (ROCK inhibitor pre-incubation)	CV+Container	~90%[h]	Mollamohammadi et al. 2009 (77)
H1, H9	MEF/Matrigel coated Microcarrier Coll IV/Dispase, CM+bFGF	Slow freezing	10% DMSO+30% FCS+60% CM/bFGF (adherent on microcarriers)	CV+Container	1.5~1.9 times[i]	Nie et al. 2009 (73)
CHA-hES 3 H9	MEF, Dispase DMEM/F12+20% SR+bFGF	Slow freezing	10% DMSO+ 90% SR (stepwise addition)	CV+Container/ Feezer	42~51%[j] 20~30%	Lee et al. 2010 (67)
Shef-4,5,6,7	MEF, Coll IV/Accutase KO-DMEM+20% SR+bFGF	Slow freezing	10% DMSO+ 90% FCS (attached on culture cassette)	Culture cassette /Container	8~200 folds[k]	Amps et al. 2010 (72)

MEF: mouse embryonic fibroblasts; DMEM: Dulbeco's modified Eagle medium with high glucose; FBS: fetal bovine serum; FCS: fetal calf serum; SR: Knockout serum replacement; ITS: insulin transferrin selenite; M: mechanical passaging; Coll IV: collagenase type IV; CM: feeder conditioned media; DMSO: dimethylsulfoxide; EG: ethylene glycol; S: sucrose; HSA: human serum albumin; OPS: open pulled straw; CS: closed straw; CV: cryovial; Freezer: programmable cell freezer.

[a] count colonies including completely differentiated colonies, [b] count colonies with >50% undifferentiated, [c] count AP positive colonies after alkaline phosphatase (AP) staining at day 7 after thawing, [d] count developing colonies at 10 days after thawing, [e] calculated relative to the control using the formula (see the reference in detail), [f] colony formation efficiency of dissociated hES cells, [g] number of adhered clumps/number of thawed clumps after 48 hour of plating, [h] cell viability by the Trypan Blue exclusion, [i] recovery of hESCs cryopreserved on microcarriers/recovery of hESCs cryopreserved as suspended clumps (recovery by the Trypan Blue exclusion), [j] count AP positive colonies after alkaline phosphatase (AP) staining, [k] relative proliferation efficiency compared to cryovial-culture flask control (see the reference in detail).

dishes as colonies, some studies have improved post-thaw recovery rates by cryopreservation of hESC colonies attached to Matrigel-coated plates, gas-permeable membrane culture cassettes or microcarriers for culture/expansion (71-73). Different cryopreservation protocols for hESCs reported from various research groups are summarized in Table 1. To determine the most effective protocol for hESC cryopreservation, researchers should evaluate the quality of hESC colonies after freezing and thawing: frozen and thawed hESC colonies should remain undifferentiated and maintain stem cell characteristics and genetic stability.

In spite of improvements in cryopreservation protocols for hESCs, appropriate freezing protocols using xeno-free conditions to obtain higher cell survival rates with little or no differentiation and to handle large amounts of hESCs are needed for future clinical application.

Conclusion

Human ESCs present the most powerful resource for regenerative medicine, but several aspects remain to be developed before they will be ready for clinical application. It is essential that all processes related to the derivation, maintenance and differentiation of hESCs should be accomplished under xeno-free culture conditions using good manufacturing practice (GMP) system, and that large volumes of hESCs can be manipulated at the same time to transplant their derivatives into a patient. Furthermore, to find suitable cell lines for each patient, the establishment of worldwide hESC bank(s) that provide qualified hESCs to researchers and patients is required.

Since hESCs were first derived, much progress has been reported related to their derivation and culture conditions. Based on current understanding of the regulation of hESC self-renewal and pluripotency, defined culture conditions have been developed, but these procedures still need to be improved to achieve the optimal conditions for maintaining the undifferentiated state of hESCs with maximized proliferation rates using xeno-free materials. Additionally, it must be verified that genetic stability of hESCs is maintained during all processes of hESC culture and differentiation.

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Potential conflict of interest

The authors have no conflicting financial interest.

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