Establishment of a feeder and serum-free culture system for human embryonic stem cells

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Summary
Stem cells are an immortal cell population capable of self-renewal; they are essential for human development and ageing and are a major focus of research in regenerative medicine. Despite considerable progress in differentiation of stem cells in vitro, culture conditions require further optimization to maximize the potential for multicellular differentiation during expansion. The aim of this study was to develop a feeder-free, serum-free culture method for human embryonic stem cells (hESCs), to establish optimal conditions for hESC proliferation, and to determine the biological characteristics of the resulting hESCs. The H9 hESC line was cultured using a homemade serum-free, feeder-free culture system, and growth was observed. The expression of pluripotency proteins (OCT4, NANOG, SOX2, LIN28, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) in hESCs was determined by immunofluorescence and western blotting. The mRNA expression levels of genes encoding nestin, brachyury and α-fetoprotein in differentiated H9 cells were determined by RT-PCR. The newly developed culture system resulted in classical hESC colonies that were round or elliptical in shape, with clear and neat boundaries. The expression of pluripotency proteins was increased, and the genes encoding nestin, brachyury, and α-fetoprotein were expressed in H9 cells, suggesting that the cells maintained in vitro differentiation capacity. Our culture system containing a unique set of components, with animal-derived substances, maintained the self-renewal potential and pluripotency of H9 cells for eight passages. Further optimization of this system may expand the clinical application of hESCs.

Introduction
Human embryonic stem cells (hESCs) are pluripotent cells isolated from early blastocysts or primordial germ cells with the ability to proliferate and differentiate into any type of cell in the human body (Pera et al., 2000; Lior, 2002). hESCs have become a unique tool in regenerative medicine and tissue engineering, with broad prospects for tissue repair, organ transplantation, and drug screening and development (Qin et al., 2016). Accordingly, the induction of hESCs differentiation has been a major research topic since their initial isolation and establishment from human embryos in 1998 (Cowan et al., 2004). Studies of the mechanisms underlying the self-renewal and differentiation of hESCs have indicated that skt40 (serine/threonine kinase domain 40) activates Ras, Mek1/2 and Erk1/2 in the Erk/MAPK pathway and induces extraembryonic endoderm differentiation (Li et al., 2010). The calcineurin-NFAT signalling pathway can activate hESCs transition from self-renewal to differentiation (Li et al., 2010). At present, there are three major methods for inducing differentiation of hESCs in vitro: cytokine induction (embryo bodies formation), selective marker and ectopic expression of specific transcription factors (Li and Wang, 2017). Ye et al. (2017) isolated hESCs from discarded embryos and found that they have an unlimited capacity for self-renewal and can maintain an undifferentiated state; in addition, the cells had potential to differentiate into all cell and tissue types in any of the three germ layers. Pan et al. (2017) described an in vitro culture technique that enabled the differentiation of hESCs into retinal vascular cells. Furthermore, Kim et al. (2017) replaced basic fibroblast growth factor (bFGF) in conventional hESC culture medium with prostaglandin E2 (PGE2), transforming growth factor β (TGF-β), and erythropoietin (EPO) and found that hESCs differentiated into hematopoietic stem cells. Under certain conditions, hESCs can also differentiate into neurons, oligodendrocytes, liver cells, and other cell types, indicating the considerable potential for the treatment of neurodegenerative diseases and liver transplantation (Matsuoka et al., 2017). A clinical trial in 2009 demonstrated that the intraspinal injection of hESC-derived oligodendrocyte progenitor cells has beneficial effects in patients with spinal cord injuries (Bretzner et al., 2011).
However, conventional hESC culture techniques require feeder cells and serum; these methods are labour intensive, and the cytokines secreted by feeder cells have not been determined. Moreover, serum may contain both known and unknown animal-derived pathogens (Dogan, 2018), which may result in xeno-contaminated hESCs and therefore hinder clinical applications (Kim et al., 2017). Therefore, multiple attempts have been made to develop new culture systems or to optimize existing culture systems to mitigate contamination of hESCs. For example, human placental fibroblasts outperformed mouse embryonic fibroblasts as feeder cells for hESC lines (Consortium et al., 2010). Genbacev et al. (2005) replaced mouse embryonic fibroblasts with human foreskin fibroblasts as feeder cells for culturing hESCs, resulting in stable hESC passage while preserving the pluripotency and differentiation potential. In addition, human bone marrow mesenchymal stem cells (Meng et al., 2008) and adult stem cells from the endometrium (Cheng et al., 2010) can also be used as human-derived feeder layers.

Feeder-free hESC culture systems, i.e. systems in which feeder layers are replaced with extracellular matrix (ECM), have been demonstrated to improve the adhesive capacity, morphogenesis, differentiation, and growth of hESCs. Ludwig et al. (2006) successfully isolated two hESC lines using TeSR1 medium in Petri dishes coated with laminins and fibronectin but were unable to ensure that the hESCs retained a normal karyotype. Xu et al. (2001) found that hESCs grow normally and maintained pluripotency in a feeder-free culture system involving Matrigel-coated Petri dishes. It is worth mentioning, however, that even if hESCs grow well in Matrigel-coated Petri dishes, Matrigel contains animal-derived ingredients, as it is a soluble extract of basement membrane proteins from mouse Engelbreth–Holm–Swarm tumours (Xu et al., 2001). Additionally, serum substitutes and/or xeno-free components can also be used in place of animal sera for culturing hESCs (Kohen et al., 2009; Lee et al., 2016).

Various hESC-specific medium, such as mTeSR1 (05850, Stemcell), StemPro hESC SFM (BG series, Thermo Fisher), and HEScGRO (SCM021, Millipore), have recently been developed. Each of these feeder-free, serum-free packaged culture systems has its own advantages, but they all exhibit poor reproducibility among cell lines and laboratories owing to differences in the culture requirements of different hESCs. Accordingly, the complexity and variability in the molecular mechanisms underlining the effects of each factor in the different culture systems have considerably restricted hESCs research (Kristiina et al., 2010). Establishing a safe, effective, and highly reproducible culture system that enables the large-scale cultivation of hESCs for clinical studies has been a long-standing goal. We aimed to culture hESCs derived from discarded embryos in a newly developed feeder-free, serum-free culture system and to determine their pluripotency and differentiation potential, providing a theoretical basis for further fundamental research and clinical applications.

**Materials and methods**

**hESC culture**

The hESC line H9 was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). After cells were revived according the conventional methods, they were centrifuged at 1500 rpm for 5 min. H9 cells were then resuspended and inoculated into a cell culture flask coated with laminin (10 mg/l) and containing hESC basal medium, which was composed of 80% Dulbecco’s modified Eagle’s medium (DMEM)/F12 (12660012, Thermo Fisher), 20% KnockOut Serum Replacement (A3181502, Thermo Fisher), 1% non-essential amino acids (11140076, Gibco), 1 mmol/l l-glutamine (A2916801, Thermo Fisher), 0.1 mmol/l 2-mercaptoethanol (21985023, Thermo Fisher), and DMSO (D8372, Solarbio). The cells were passaged when the colonies appeared enlarged with transparent centres and fused with adjacent colonies.

hESCs were divided into three experimental groups. In the first group, cells were cultured in KnockOut DMEM/F12 medium (DMEM). In the second group, cells were cultured in homemade serum-free medium (HSFM) supplemented with transferrin (T8010, Solarbio), TGF-β (14384862, Invitrogen), bFGF (13256029, Gibco), and insulin (Solarbio, 18830). In the third group, cells were cultured in modified serum-free medium (MSFM), consisting of HSFM supplemented with heparin (100 ng/ml, YZ-140818, Solarbio) and Y27632 (10 ng/ml, 72304, Stemcell). After thawing at 4°C for about 30 min, 2 ml of human recombinant laminin was transferred into a cell culture flask and swirled to ensure an even distribution on the surface. Following incubation at 37°C for 1 h, the corresponding medium was added to the cell culture flask and inoculated with hESCs, following by incubation at 37°C with 5% CO₂ for 5–7 days prior to being passaged at a split ratio of 1:3. hESCs were observed under an inverted microscope on the fourth day of culture to determine the number of colonies and the colony-formation rate.

**Immunofluorescence assay**

Cells with sufficient growth were inoculated into a 12-well plate coated with human recombinant laminin. After adhering to the surface of the well, the cells were fixed with 4% paraformaldehyde (P1110, Solarbio) for 15 min, blocked with 3% hydrogen peroxide for 15 min, and incubated with 5% bovine serum albumin (BSA) (Solarbio, SW3015) for 15 min. After rinsing with phosphate-buffered saline (PBS), the cells were incubated overnight with primary antibodies at 4°C, followed by incubation with a secondary antibody (goat anti-rabbit/mouse IgG H&L, Abcam) at 37°C for 1 h. The following primary antibodies were used: rabbit anti-Oct-4 (ab59545, Abcam), rabbit anti-Nanog (ab106465, Abcam), rabbit anti-Sox2 (ab97959, Abcam), mouse anti-SSEA-4 (ab16287, Abcam), mouse anti-TRA-1-60 (ab16288, Abcam), and mouse anti-TRA-1-81 (ab16289, Abcam). Subsequently, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for microscopic observation and imaging.

**Western blotting analysis**

H9 cells were harvested and lysed on ice using RIPA lysis buffer (P0013C, Beyotime), followed by centrifugation at 13,000 rpm for 10 min to harvest the supernatant for the measurement of the protein concentrations. Subsequently, the protein sample was denatured by boiling in 4× loading buffer for 5 min and stored at −20°C until use. Equal amounts of protein were loaded onto a gel consisting of 10% separating gel and 5% stacking gel for SDS-PAGE. After electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane, which was then blocked and incubated overnight with the primary antibodies mentioned in the previous section. Subsequently, the membrane was rinsed with PBS and incubated with the secondary antibody at 37°C for 1.5 h. After rinsing again with PBS, the membrane was developed using the enhanced chemiluminescence (ECL) reagent and photographed for analysis of D values.
RT-PCR analysis

Passaged hESCs were digested with 0.1% collagenase IV and cultured in suspension using inhibitor-free medium in a Petri dish for 4–6 days to form embryoid bodies. The embryoid bodies were subsequently cultured in a Petri dish coated with human recombinant laminin for further differentiation. After about 3 weeks, total RNA was extracted from hESCs after differentiation into various cell types using the RNasey Mini Kit (74104, Qiagen). The reverse transcription (RT) reaction was performed using a Reverse Transcription Kit (205411, Qiagen) following the manufacturer’s protocol. The quantification of mRNA was performed using the Quantnova SYBR Green PCR Kit (208054, Qiagen) according to the manufacturer’s instructions. The primers for nestin (ectoderm) were 5'-GAGGACCAGATTTGTGAC-3' and 5'-CACAGTTGGCTTGAGTTTC-3'; the primers for brachyury (mesoderm) were 5'-TGCTTCCCTGAGACCCAGTT-3' and 5'-GATCACTTCTTGGCATCAAAG-3'; and the primers for α-fetoprotein (endoderm) were 5'-ATGCCTTTAGTTGTTA-3' and 5'-TGTCACCTTACGTGCGTTTT-3'. The data were analyzed using the 2^ΔΔCt method. Quantitative results are presented as means ± standard error of the mean (SEM).

Evaluation of differentiation ability in vitro

hESCs were cultured in suspension to form embryoid bodies, and the cells were collected by centrifugation after 20 days. According to conventional immunofluorescence analyses, after cells were fixed with 4% paraformaldehyde (P1110, Solarbio), and blocked with 3% hydrogen peroxide, they were incubated with primary antibodies overnight at 4°C. Anti-rabbit IgG (Abcam) was used as a secondary antibody and incubated at 37°C for 1 h. Finally, the cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) for microscopic observation and imaging. The primary antibodies used were as follows: anti-nestin (1:200, ab6142, Abcam); anti-hBrachyury (1:200, ab20680, Abcam) and anti-α-fetoprotein (1:200, ab46799, Abcam).

Results

Morphological characteristics of hESCs

H9 cells grew normally in DMEM, exhibiting many large colonies, diverse morphological characteristics, tightly connected cells, a high nuclear-to-cytoplasmic ratio, and a tendency to differentiate during passage. H9 cells grown in HSFM formed smaller colonies with loosely arranged cells and a strong tendency to differentiate during passage; these cells also required a long time interval between passages. H9 cells grown in MSFM formed a greater number of larger colonies with tightly connected cells and clear boundaries compared with HSFM. These hESCs showed strong proliferation and formed classical nest-like compartments with clear boundaries and fewer differentiated cells (Fig. 1). We calculated the colony-formation rate and population doubling time of the cells grown in the three types of medium. We found that before the establishment of a stable culture, H9 cells grown in DMEM and HSFM showed relatively high differentiation rates, low colony-forming rates, and long population doubling times. In contrast, Cells grown in MSFM showed higher colony-forming rates and shorter population doubling times (Figs 1 and 2).

Expression of pluripotency proteins in H9 cells

Studies have demonstrated that the expression of proteins on hESCs is associated with the undifferentiated state. The results of immunofluorescence assays of hESCs showed that pluripotency-specific markers NANOG, OCT4, SOX2, SSEA-4, TRA-1-60, and TRA-1-81 were expressed in all three groups of H9 cells (Fig. 3). Western blot results were consistent with the immunofluorescence assay results (Fig. 4).

In vitro embryoid body formation

After culture in suspension, hESCs from all three groups formed embryoid bodies (Fig. 5), which continued to differentiate and grow after inoculation into a Petri dish coated with human recombinant laminin. After 20 days of cultivation, the mRNA expression levels of markers characteristic of each of the three germ layers were assessed by RT-PCR. The genes encoding nestin, brachyury, and α-fetoprotein were expressed in differentiated cells within the embryoid bodies in all three groups (Fig. 5).

Identification of differentiation ability in vitro by immunofluorescence

The results of immunofluorescence assay shown that nestin, hBrachyury and α-fetoprotein were positively expressed in ectoderm, mesoderm and endoderm, respectively (Fig. 6). The results indicated that hESCs have the potential to differentiate into all three germ layers.

Discussion

We successfully cultured hESCs in homemade serum-free, feeder-free culture medium supplemented with heparin and insulin. The feeder-free culture system has well defined ingredients and does not contain any animal-derived substances. Moreover, no major differences in culture outcomes were observed compared with those reported in previous studies.

Human embryonic stem cells (hESC) obtained from early embryos and primary gonads, are characterized as having infinite proliferation, self-renewal and totipotency (Pera et al., 2000; Lior, 2002; Jiang et al., 2014). hESCs can differentiate into all types of somatic cells in vitro and in vivo. Despite the ethical and legal constraints involved in the use of human early-stage human embryos (Amit et al., 2003), they have become an important source of cells for the treatment of a broad range of diseases (Jiang et al., 2014; Desai et al., 2015; Tolosa et al., 2015). So, the ultimate goal of hESCs research is to harness their differentiation potential for use in the treatment of various human diseases. To date, more than 1200 established hESC lines (Meng et al., 2008) are available for use as unique and novel research tools with broad prospects for clinical application (Thomson et al., 1998).

Importantly, an animal-free culture system is required for the clinical application of hESCs (Bretzner et al., 2011). hESC lines show different growth characteristics and require different feeder cells and culture conditions (Consortium et al., 2010).

The ability of hESCs to self-renew and to differentiate into different types of mature cells depends on both intrinsic genetic factors and exogenous signals, including cell growth factors, extracellular matrix and paracrine factors (Jin., 2007; Bendall et al., 2007, 2009). A number of studies have indicated that TGF-β1 is required for supporting self-renewing cultures of hESCs. For example, the addition of exogenous signalling molecules...
Y27632 is a Rho-associated kinase (ROCK) inhibitor that can affect actin–myosin interactions by regulating actin assembly, thereby regulating various cellular functions, such as cell growth, migration, apoptosis and cell adhesion to substrates (Riento and Ridley, 2003; Beattie et al., 2010; Yu et al., 2012; Thumkeo et al., 2013). In addition, Y27632 can also regulate the self-renewal of stem cells, promote clonal formation, and improve cell survival (Zhang et al., 2011; Harel et al., 2012). The differentiation of ESCs is an extremely complicated process, and studies have shown that Y27632 may inhibit cell differentiation by regulating the cytoskeleton. For instance, the addition of Y27632 to mouse ESC culture medium helps to maintain the membrane localization of E-cadherin, which may represent one of the mechanisms underlying Y27632-induced inhibition of cell differentiation (Shi and Wei, 2007). hESCs are arranged in tightly packed colonies, and resuspension as single cells may activate RhoA and enhance the contractile force of actin, leading to apoptosis (Li et al., 2011). ROCK is associated with the formation of cell–cell junctions and therefore the inhibition of ROCK activity may contribute to the maintenance of the self-renewal of ESCs by eliminating their dependence on intact cell–cell junctions (Ohad et al., 2013). In this study, the addition of Y27632 to the culture medium resulted in improved proliferation and maintenance of the undifferentiated state in hESCs.

Heparin, a soluble derivative of heparin sulfate, is a well known cofactor for FGF-2, with important roles in the growth, adherence and proliferation of HSCs; its effects are achieved by interactions with components in the medium (Furue et al., 2008; Young, 2011). The addition of IGF and heparin to the culture system helps to maintain the proliferation ability and pluripotency of mouse ESCs, as the binding of heparin to IGF activates the ERK pathway (Li et al., 1990; Bendall et al., 2007). Moreover, IGF and insulin activate the MAPK pathway and upregulate the expression of OCT4 (Furue et al., 2008; Bendall et al., 2007). The addition of heparin to the culture medium leads to bFGF aggregation, protecting bFGF from degradation by other physicochemical factors, and heparin binds tightly to the FGF receptor (FGFR) to activate various intracellular signalling pathways, such as the PI3K and ERK pathways (Li et al., 1990). Bendall et al. (2007) found that exogenous FGF maintains the self-renewal capacity of hESCs by stimulating the secretion of IGF and TGF-β1, which acts on IGF receptors. Therefore, we speculated that the addition of heparin, insulin, and bFGF to the serum-free, feeder-free culture system activated various pathways, such as the ERK, PI3K, and MAPK.
pathways (Lanner and Rossant, 2010), and this may have promoted the maintenance of an undifferentiated state in hESCs, improving their capacity for self-renewal and pluripotency. However, H9 cells showed an increasing tendency to differentiate as the passage number increased, indicating that the culture system is still incapable of completely maintaining an undifferentiated state.

**Figure 3.** Determination of pluripotency marker expression in H9 cells cultured in KnockOut DMEM/F12 (DM), homemade serum-free medium (HSFM), and modified serum-free medium (MSFM). Expression of OCT4 (A), SOX2 (B), NANOG (C), LIN28 (D), SSEA-3 (E), SSEA-4 (F), TRA-1-60 (G), and TRA-1-81 (H), as determined by immunofluorescence assays (×100 magnification). Red staining respects expression of OTC4, SOX2, NANOG, LIN28, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, blue staining is nuclei, and purple staining is a merger of red and blue staining.
Figure 4. Determination of the expression of OCT4, NANOG, SOX2, SSEA-4, TRA-1-60, and TRA-1-81 in H9 cells cultured in KnockOut DMEM/F12 (DMEM), homemade serum-free medium (HSFM), and modified serum-free medium (MSFM) by western blotting assay. (A) Western blotting strips. (B) Statistical analysis. Protein expression level is the ratio of optical density values of target protein and β-actin. *P < 0.05 compared with the DMEM group.

Figure 5. In vitro differentiation capacity of H9 cells in the serum-free, feeder-free culture system. (A) In vitro differentiation of H9 cells into embryoid bodies. (B) Determination of the expression of markers of the three germ layers in embryoid bodies by RT-PCR. (C) Expression of markers of the three germ layers in embryoid bodies by quantitative PCR. Values are means ± SEM. mRNA expression level is the ratio of optical density of target bands and GAPDH band. The means of these values 1.13, 0.96, 0.59, 0.75, 1.02, 0.80, 0.85, 1.10, 0.68 are in order, respectively.
Cell morphology, expression of specific pluripotency marker and differentiation capacity can be used as parameters for assessing hESCs (Gowan et al., 2004). In this study, the newly developed culture system can retained the in vitro culture characteristics of H9 cells, including growth patterns of cells, tight junctions and clear boundaries between cells. Previous reports have also indicated that nuclear transcription factors and cell surface markers, such as OCT4, NANOG, SOX2, TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4, are expressed in undifferentiated hESCs (Ye et al., 2017), consistent with our results.

In conclusion, we preliminarily established a serum-free, feeder-free culture system for hESCs. We found that the addition of TGF-β1, insulin, transferrin, heparin, and Y27632 improved the maintenance of the self-renewal capacity of H9 cells. Despite the elimination of contaminating proteins, colonies of differentiated hESCs were still observed in the serum-free, feeder-free culture system, indicating that the system requires further improvement but may serve as a basis for future studies of hESCs differentiation.

**Financial support.** This study was supported in part by Qinghai Province Science and Technology Project of China (2017-ZJ-740).

**Declaration of interest.** All authors declare that they have no conflict of interests in the article.

**Ethical standards.** Our work complies with ethical standards on the use of stem cells.

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