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

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Research Article

Cite this article: Wang LY *et al*. (2020) Establishment of a feeder-and serum-free culture system for human embryonic stem cells. *Zygote*. **28**: 175–182. doi: 10.1017/S0967199419000625

Received: 18 April 2019
Revised: 3 September 2019
Accepted: 12 September 2019
First published online: 22 January 2020

Keywords:
Basic fibroblast growth factor; Feeder-free; Human embryonic stem cells; Pluripotency; Self-renewal

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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 haematopoietic 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).

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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 their 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 underlying 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 (14834862, 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% CO_2 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, 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.

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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 RNeasy 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 Quantinova SYBR Green PCR Kit (208054, Qiagen) according to the manufacturer's instructions. The primers for nestin (ectoderm) were 5′-GAGGACCAGAGTATTGTGAGAC-3′ and 5′-CACAGTGGTGCTTGAGTTTC-3′; the primers for brachyury (mesoderm) were 5′-TGCTTCCCTGAGACCCAGTT-3′ and 5′-GATCACTTCTTTCCTTTGCATCAAG-3′; and the primers for α-fetoprotein (endoderm) were 5′-ATGCGCTATTAGTTCGTTA-3′ and 5′-TGTCACTTACTGGCGTTTT-3′. The data were analyzed using the $2^{-\Delta\Delta Ct}$ 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 specific 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

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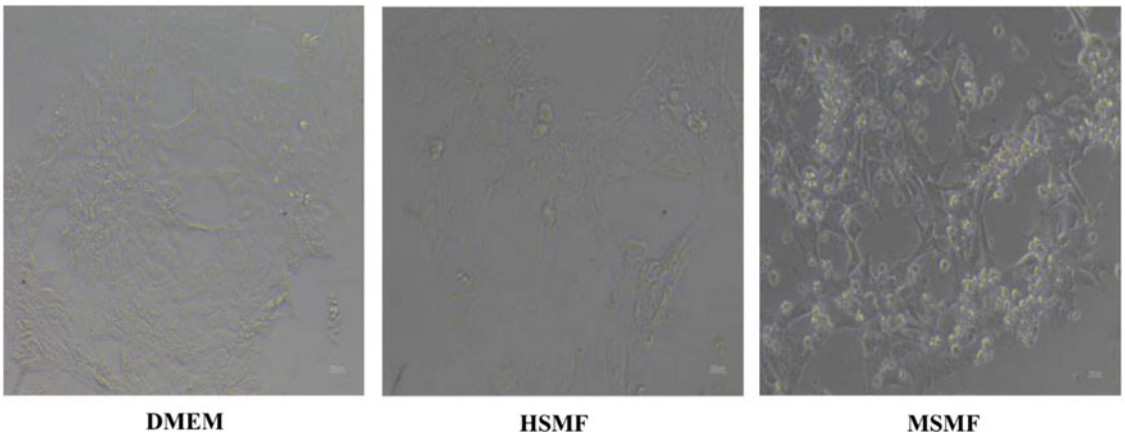

Figure 1. Cell morphology of H9 cells cultured under different conditions. DMEM, Dulbecco's modified Eagles's medium; HSFM, homemade serum-free medium; MSFM, modified serum-free medium.

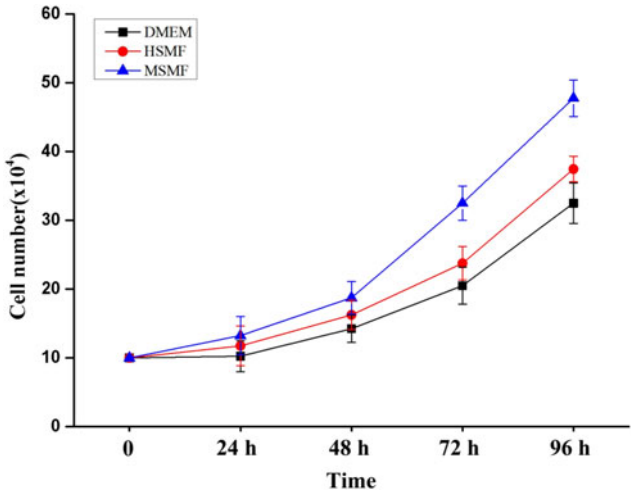

Figure 2. Growth curves for H9 cells cultured in KnockOut DMEM/F12 (DMEM), homemade serum-free medium (HSFM), and modified serum-free medium (MSFM).

(TGF-β1 and bFGF) to the culture system could help to maintain the proliferation potential and undifferentiated state of hESC lines (de Wert and Mummery, 2003; Teotia *et al.*, 2016). The inhibition of TGF receptors with a compound resulted in rapid differentiation of human ESC (James *et al.*, 2005). The addition of activin A, nicotinamide, and keratinocyte growth factors to the culture medium facilitates the maintenance of the undifferentiated state of ESCs (Wang *et al.*, 2014). Despite numerous studies on growth factors, their large-scale application has been limited owing to their high cost, inability maintain undifferentiated hESCs during prolonged culture, and potential to induce epigenetic changes in hESCs (Teotia *et al.*, 2016). Previous studies confirmed that bFGF and TGF-β help to maintain the self-renewal and proliferation capacities of hESCs. Transferrin eliminates oxygen free radicals and peroxidase in the medium, while insulin promotes cell proliferation by activating the insulin-like growth factor (IGF) signalling pathway (Wang *et al.*, 2007; Song *et al.*, 2008; Xi *et al.*, 2010). Based on these previous findings, we successfully cultured hESCs using a homemade serum-free culture system, mainly consisting of bFGF, TGF-β, transferrin, and insulin. However, hESCs cultured in this system showed poor adherence and a high differentiation rate.

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 binds 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

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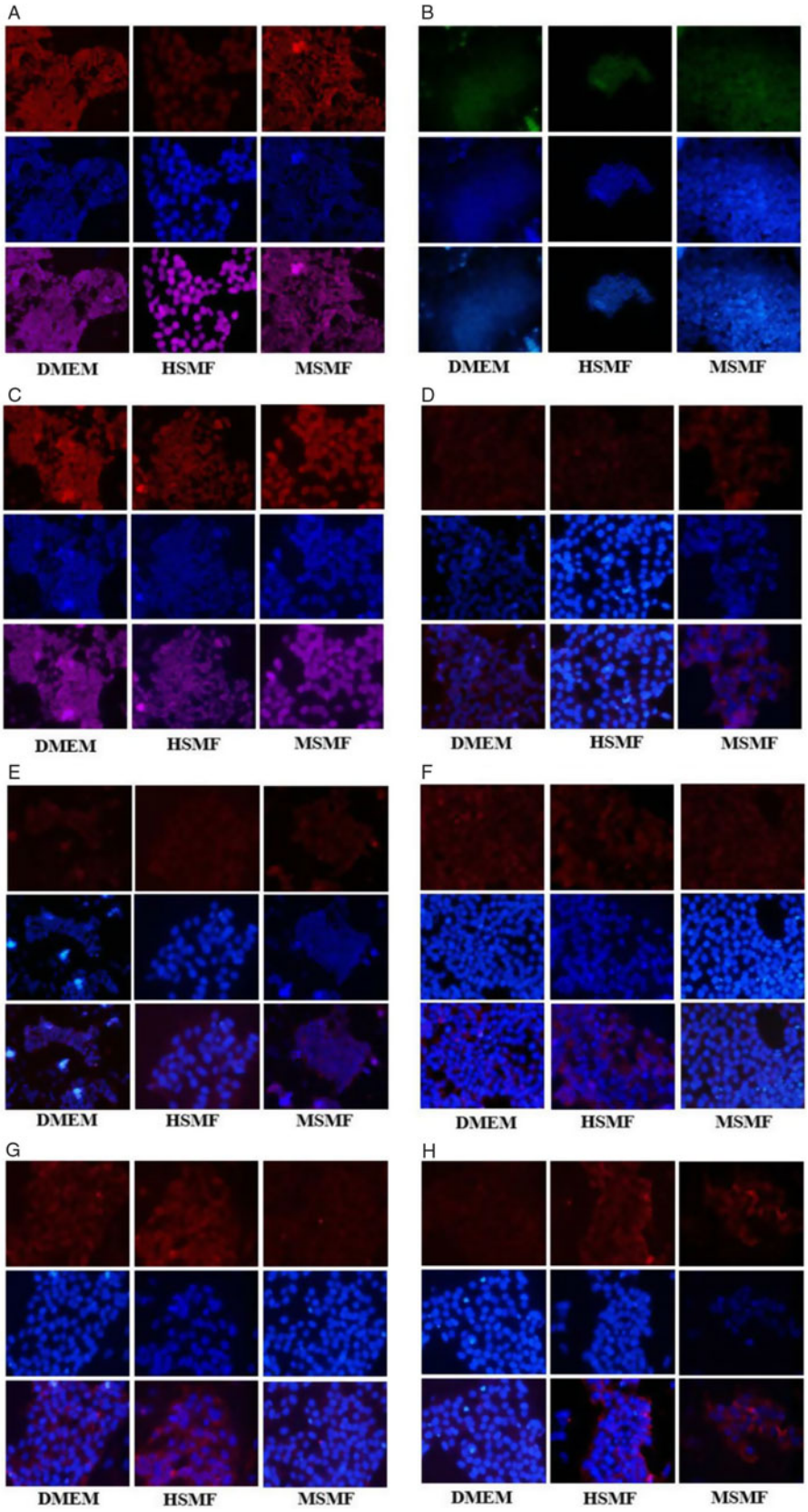

Figure 3. Determination of pluripotency marker expression in H9 cells cultured in KnockOut DMEM/F12 (DM), homemade serum-free medium (HSFM), and modified serum-free medium (MSFM). Expression of OCT4 (*A*), SOX2 (*B*), NANOG (*C*), LIN28 (*D*), SSEA-3 (*E*), SSEA-4 (*F*), TRA-1-60 (*G*), and TRA-1-81 (*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.

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.

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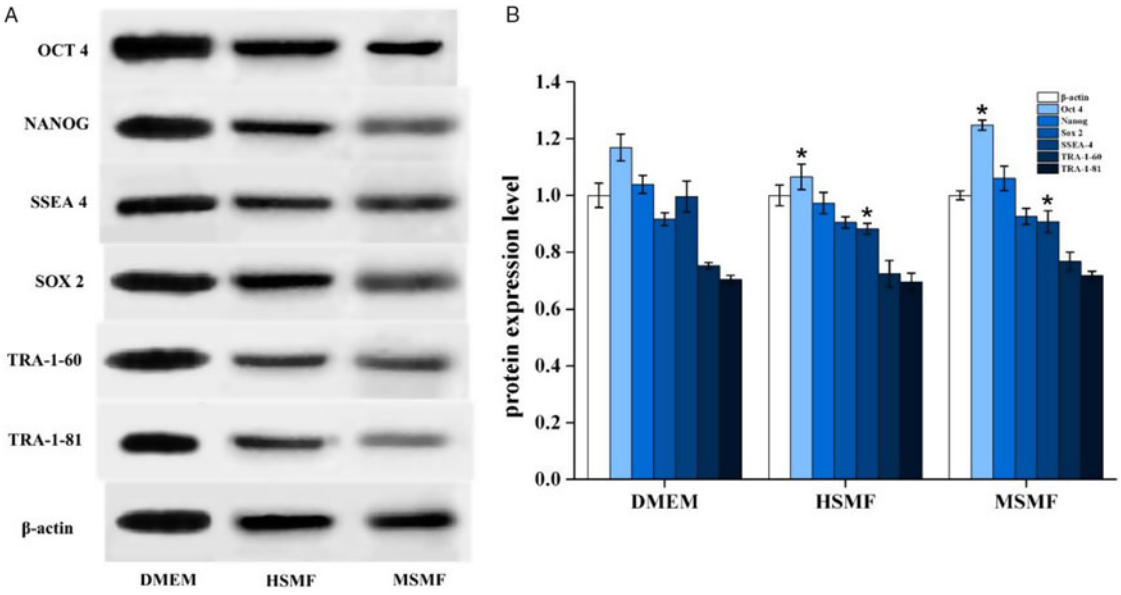

Figure 4. Determination of the expression of OCT4, NANOG, SOX2, SSEA-4, TRA-1-60, and TRA-1-81 in H9 cells cultured in KnockOut DMEM/F12 (DMEM), homemade serum-free medium (HSFM), and modified serum-free medium (MSFM) by western blotting assay. (*A*) Western blotting strips. (*B*) Statistical analysis. Protein expression level is the ratio of optical density values of target protein and β-actin. $^{*}P < 0.05$ compared with the DMEM group.

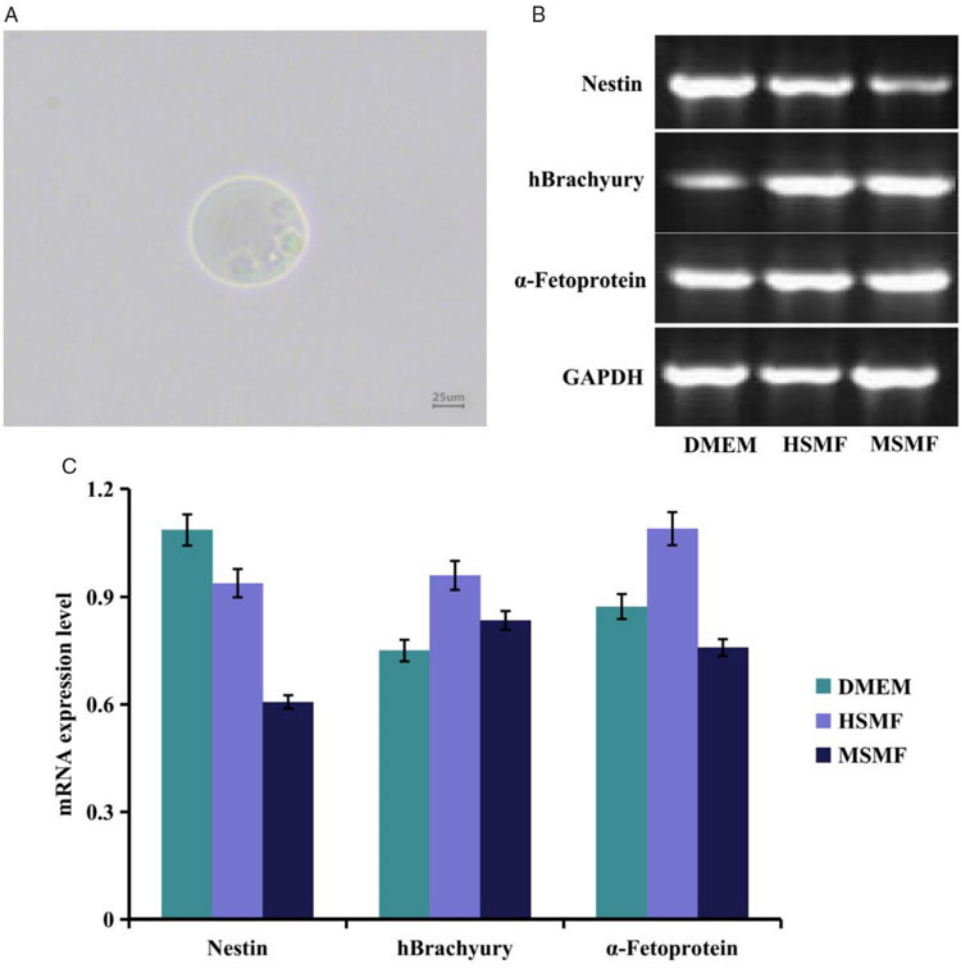

Figure 5. *In vitro* differentiation capacity of H9 cells in the serum-free, feeder-free culture system. (*A*) *In vitro* differentiation of H9 cells into embryoid bodies. (*B*) Determination of the expression of markers of the three germ layers in embryoid bodies by RT-PCR. (*C*) Expression of markers 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.

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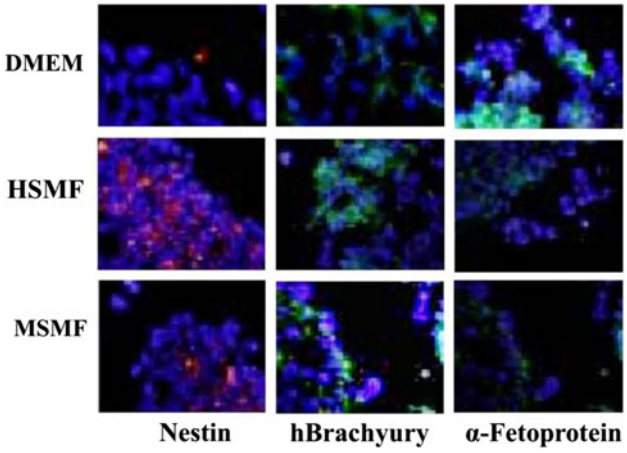

Figure 6. Immunofluorescence staining of cells in three germ layers differentiated from the embryoid bodies of H9 cells. Red staining respects nestin expression, green staining respects hBrachyury and α-Fetoprotein expression, and blue staining respects nuclei.

Cell morphology, expression of specific pluripotency marker and differentiation capacity can be used as parameters for assessing hESCs (Cowan *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-β, 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.

References

Amit M, Margulets V and Segev H (2003) Human feeder layers for human embryonic stem cells. *Biol Reprod* **68**, 2150–6.

Beattie GM, López AD and Bucay N (2010) Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cell* **23**, 489–95.

Bendall SC, Stewart MH, Pablo AM, George D, Vijayaragavan K, Werbowetski T, Ramos-Oglivie T, Rouleau A, Yang JB, Bosse M, Lajoie G and Bhatia M (2007) IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells *in vitro*. *Nature* **448**, 1015–21.

Bendall SC, Hughes C, Campbell JL, Stewart MH, Pittock P, Liu S, Bonnell E, Thibault P, Bhaita M and Lajoie GA (2009) An enhanced mass spectrometry approach reveals human embryonic stem cell growth factors in culture. *Mol Cell Proteomics* **8**, 421–32.

Bretzner F, Gilbert F, Baylis F and Brownstone RM (2011) Target populations for first-in-human embryonic stem cell research in spinal cord injury. *Cell Stem Cell* **8**, 468–75.

Cheng L, Hammond H, Ye Z, Zhan X, Dravid G (2010) Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cell* **21**, 131–42.

Consortium TISCI, Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, Christie M, Ford A, Fox V, Gokhale PJ, Healy L, Holm F and Hovatta O (2010) Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *In Vitro Cell Dev Biol Anim* **46**, 247–58.

Cowan CA, Irina K and Jill MM (2004) Derivation of embryonic stem-cell lines from human blastocysts. *New Engl J Med* **350**, 1353–6.

de Wert G and Mummery C (2003). Human embryonic stem cells: research, ethics and policy. *Hum Reprod* **18**, 672–82.

Desai N, Rambhia P and Gishto A (2015) Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. *Reprod Biol Endocrinol* **13**, 9–13.

Dogan A (2018) Embryonic stem cells in development and regenerative medicine. *Adv Exp Med Biol* **175**, 1–15.

Furue MK, Jie N, Jackson JP, Okamoto T, Jones M, Baker D, Hata RL, Moore HD, Sato JD and Andrews PW (2008) Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA* **105**, 13409–14.

Genbacev O, Krtolica A, Zdravkovic T, Brunette E, Powell S, Nath A, Caceres E, McMaster M, McDonagh S, Li Y, Mandalam R, Lebkowski J and Fisher SJ (2005) Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. *Fertil Steril* **83**, 1517–29.

Harel S, Tu EY, Weisberg S, Esquilin M, Chanbers SM, Liu B, Carson CT, Studer L, Reizis B and Tomishima MJ (2012) ZFX controls the self-renewal of human embryonic stem cells. *PLoS One* **7**, e42302.

James D, Levine AJ, Besser D and Hemmati-Brivanlou, A (2005) TGFbeta/activin/nodal signalling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* **132**, 1273–82.

Jiang WM, Huang YH and Ma YL (2014) The optimization of generating and culturing human embryonic stem cells. *Life Sci Res* **18**, 349–52.

Jin, Y (2007) Human embryonic stem cells create their own niche. *Cell Res* **17**, 744–5.

Kim, SJ, Jung, JW and Ha, HY (2017) Generation of hematopoietic stem cells from human embryonic stem cells using a defined, stepwise, serum-free, and serum replacement-free monolayer culture method. *Blood Res* **52**, 37–43.

Kohen, NT, Little ILE and Healy, KE (2009) Characterization of Matrigel interfaces during defined human embryonic stem cell culture. *Biointerphases* **4**, 69–79.

Kristiina, R, Bettina, L and Hussein, SM (2010) A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. *PLoS One* **5**, 10246.

Lanner F and Rossant J (2010). The role of FGF/Erk signalling in pluripotent cells. *Development* **137**, 3351.

Lee KH, Lee WY, Kim JH, Park CK, Do JT, Kim JH, Choi YS, Kim NH and Song HY (2016). Subculture of germ cell-derived colonies with GATA4-positive feeder cells from neonatal pig testes. *Stem Cell Int* **9**, 6029271.

Li LJ, Sun L, Gao FR, Jiang J, Yang Y, Li CL, Gu JJ, Wei Z, Yang A, Lu R, Ma Y, Tang F, Kwon SW, Zhao YM, Li JS and Jin Y (2010) Stk40 links the pluripotency factor Oct4 to the Erk /MAPK pathway and controls extraembryonic endoderm differentiation. *PNAS* **107**, 1402–7.

Li QG, Blacher R and Esch F (1990) A heparin-binding erythroid cell stimulating factor from fetal bovine serum has the N-terminal sequence of insulin-like growth factor II. *Biochem Biophys Res* **166**, 557–61.

Li XD and Wang XL (2017) Self-renewal and differentiation of embryonic stem cells. *J Dalian Medical University* **39**, 417–22.

Li X, Zhu L, Yang A, Yang AC, Lin JW, Tang F, Jin SB, Wei Z, Li JS and Jin Y (2011) Calcineurin-NFAT signaling critically regulates early lineage specification in mouse embryonic stem cells and embryos. *Cell Stem Cell* **8**, 46–58.

Lior G (2002) Derivation and potential applications of human embryonic stem cells. *Circ Res* **91**, 866–76.

Ludwig TE, Levenstein ME and Jones JM (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* **24**, 185–97.

https://doi.org/10.1017/S0967199419000625 Published online by Cambridge University Press

Matsuoka AJ, Morrissey ZD, Zhang CY, Homma K, Belmadani A, Miller CH, Chadly DM, Kobayashi S, Edelbrock AN, Matakatsu MT, Whitlon DS, Lyass L, McGuire TL, Stupp SI and Kessler JA (2017) Directed differentiation of human embryonic stem cells toward placode-derived spiral ganglion-like sensory neurons. *Stem Cell Transl Med* **6**, 923–6.

Meng GL, Liu SH, Krawerz R, Chan M, Chernos J and Rancourt DE (2008) A novel method for generating xeno-free human feeder cells for human embryonic stem cell culture. *Stem Cell Dev* **17**, 413–22.

Ohad G, Leehee W, Abed Alfatah M, Manor YS, Chomsky E, Yosef DB, Kalma Y, Viukov S, Maza I, Zviran A, Rais Y, Shipony Z, Mukarnel Z, Krupalnik V, Zerbib M, Geula S, Caspi I, Schneir D, Shwartz T, Gilad S, Zalcenstein DA, Benjamin S, Amit I, Tanay A, Massarwa R, Novershtern N and Hanna JH (2013) Derivation of novel human ground state naive pluripotent stem cells. *Nature* **504**, 282–6.

Pan YZ, Wang H and Gao F (2017) Culture conditions of human embryonic stem cells for differentiation into retinal vascular structure. *Eur Rev Med Pharmacol* **21**, 62–6.

Pera MF, Reubinoff B and Trounson A (2000) Human embryonic stem cells. *J Cell Sci* **113**, 5–10.

Qin J, Chang M, Wang S, Liu ZB, Zhu W, Wang Y, Yan F, Li J, Zhang BW, Dou GF, Liu J, Pei XT and Wang YF (2016) Connexin 32-mediated cell–cell communication is essential for hepatic differentiation from human embryonic stem cells. *Sci Rep* **6**, 1–16.

Riento K and Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4**, 446–56.

Shi J and Wei L (2007) Rho kinase in the regulation of cell death and survival. *Arch Immunol Ther Ex* **55**, 1–24.

Song T, Chen G and Bai HY (2008) Chemically defined sequential culture medium for TH+ cell derivation from human embryonic stem cells. *Mol Hum Reprod* **14**, 619.

Teotia P, Sharma S, Airan B and Mohanty S (2016) Feeder and basic fibroblast growth factor-free culture of human embryonic stem cells: role of conditioned medium from immortalized human feeders. *Indian Med Res* **144**, 838–51.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS and Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–47.

Thumkeo D, Watanabe S and Narumiya S (2013) Physiological roles of Rho and Rho effectors in mammals. *Eur J Cell Biol* **92**, 303–15.

Tolosa L, Caron J, Hannoun Z, Antoni M, López S, Burks D, Castell JV, Weber A, Gomez-Lechon M and Dubart-Kupperschmitt A (2015) Transplantation of hESC-derived hepatocytes protects mice from liver injury. *Stem Cell Res Ther* **6**, 246–63.

Wang L, Schulz TC, Sherrer ES, Dauphin DS, Shin SJ, Nelson AM, Ware CB, Zhan M, Song CZ, Chen XJ, Brimble SN, McLean A, Galeano MJ, Uhl EW, Amour KAD, Chesnut JD, Rao MS, Blau CA and Robins AJ (2007). Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signalling. *Blood* **110**, 4111–9.

Wang Y, Qian DJ and Zhong WY (2014) TGF-β1 induces the formation of vascular-like structures in embryoid bodies derived from human embryonic stem cells. *Exp Ther Med* **8**, 52–8.

Xi J, Wang Y, Zhang P, He LJ, Nan X, Yue W and Pei XT (2010) Human fetal liver stromal cells that overexpress bFGF support growth and maintenance of human embryonic stem cells. *PLoS One* **5**, 1–12.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD and Carpenter MK (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* **19**, 971–4.

Ye J, Bates N, Soteriou D, Grady L, Edmond C, Ross A, Kerby A, Lewis PA, Adeniyi T, Wright R, Poulton KV, Lowse M, Kimber SJ and Brison DR (2017) High quality clinical grade human embryonic stem cell lines derived from fresh discarded embryos. *Stem cell Res Ther* **8**, 128–40.

Young RA (2011) Control of the embryonic stem cell state. *Cell* **144**, 940–54.

Yu Z, Liu M, Fu P, Xie M, Wang W and Luo X (2012) ROCK inhibition with Y27632 promotes the proliferation and cell cycle progression of cultured astrocyte from spinal cord. *Neurochem Int* **61**, 1114–20.

Zhang L, Valdez JM, Zhang BY, Wei L, Chang J and Xin L (2011) ROCK inhibitor Y-27632 suppresses dissociation-induced apoptosis of murine prostate stem/progenitor cells and increases their cloning efficiency. *PLoS One* **3**, e18271.

https://doi.org/10.1017/S0967199419000625 Published online by Cambridge University Press