

RESEARCH ARTICLE

Rapid conversion of human ESCs into mouse ESC-like pluripotent state by optimizing culture conditions

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ABSTRACT

The pluripotent state between human and mouse embryonic stem cells is different. Pluripotent state of human embryonic stem cells (ESCs) is believed to be primed and is similar with that of mouse epiblast stem cells (EpiSCs), which is different from the naïve state of mouse ESCs. Human ESCs could be converted into a naïve state through exogenous expression of defined transcription factors (Hanna et al., 2010). Here we report a rapid conversion of human ESCs to mouse ESC-like naïve states only by modifying the culture conditions. These converted human ESCs, which we called mhESCs (mouse ESC-like human ESCs), have normal karyotype, allow single cell passage, exhibit domed morphology like mouse ESCs and express some pluripotent markers similar with mouse ESCs. Thus the rapid conversion established a naïve pluripotency in human ESCs like mouse ESCs, and provided a new model to study the regulation of pluripotency.

KEYWORDS human embryonic stem cells (hESCs), mouse ESCs, naïve, pluripotent state

INTRODUCTION

Embryonic stem cells are the cells derived from the inner cell mass (ICM) of preimplantation blastocysts. Mouse and human embryonic stem cell lines were established in 1981 and 1998, respectively, for the first time (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). In 2007, a new kind of pluripotent stem cell was established from post-implantation epiblasts and named Epiblast stem cells

(EpiSCs). Mouse EpiSCs share many features with human ESCs but differ from mouse ESCs (Brons et al., 2007; Tesar et al., 2007). Thus mouse ESCs and EpiSCs are believed to have different pluripotent states: naïve and primed, respectively (Nichols and Smith, 2009). The naïve pluripotent stem cells contribute to the chimera at a high efficiency when injected into blastocysts, but the primed pluripotent stem cells cannot. Naïve pluripotent stem cells' self-renewal is dependent on leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), but the primed pluripotent stem cells' self-renewal is dependent on basic fibroblast growth factor (bFGF) and Activin (Rossant, 2008). There are also many other different features between naïve and primed pluripotent stem cells. For example, one X chromosome in female mouse EpiSCs is inactivated while both X chromosomes in female mouse ESCs are activated. Naïve pluripotent stem cells will be also differentiated under Fgf/Erk signals which can promote the self-renewal of primed pluripotent stem cells. In addition, they have some different markers and pluripotent factors (Nichols and Smith, 2009). LIF was thought to be essential for mouse ESCs self-renewal (Smith et al., 1988; Williams et al., 1988; Matsuda et al., 1999) and bFGF could inhibit some correlated factors to sustain undifferentiated state of human ESCs (Dvorak et al., 2005). BMP4 plays opposite roles in human ESCs and mouse ESCs: it could activate the gene ID which is essential for mouse ESCs but its expression promotes human ESCs differentiation into trophoderm-like cells (Ying et al., 2003; Vallier et al., 2005). Besides relying on different pluripotent signaling pathways, the morphologies of human ESCs are also different from that of mouse ESCs. Mouse ESCs grow as three-dimensional domed colonies with well-rounded borders, and human ESCs colonies appear flatter, which are the same as mouse EpiSCs. Moreover, unlike mouse ESCs, passaging of mouse EpiSCs and human ESCs using single cell digestion

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would induce widespread cell death (Thomson et al., 1998; Tesar et al., 2007).

At present, mouse EpiSCs have been successfully converted into naïve pluripotent stem cells (Bao et al., 2009). EpiSCs have also been produced from ESCs by modifying culture agents (Guo et al., 2009). Moreover, mouse EpiSCs have been generated from preimplantation embryos (Najm et al., 2011). Human ESCs have reached a new pluripotent state with similar characters of mouse ESCs by exogenous expression of three pluripotent factors (*Klf4*, *Oct4*, *Sox2*), but the naïve pluripotent stem cell lines could not be maintained for longer than 20 passages without the expression of exogenes (Hanna et al., 2010). Small compounds (Erk inhibitor PD0325901, Gsk3 inhibitor CHIR99021, and adenylyl cyclase activator Forskolin) could convert human ESCs into naïve states, but domed colonies of naïve cells would form after 8–12 days of incubation in the medium containing small compounds and the naïve pluripotent stem cells could not be passaged stably (Hanna et al., 2010). Two compounds (Thiazovivin/Tzv and Tyrntegin/Ptn) were also reported to significantly increase single cell survival of human ESCs and promote domed colony formation; however, the authors did not further characterize the morphologically changed ESCs (Xu et al., 2010). In the current study, we report a rapid conversion of human ESCs into a naïve pluripotent state only by modifying the culture medium.

Culture media have profound effect on cell identity. Cell fate transition was shown to be induced or promoted by switching different culture media. A commercially available serum replacer (Knockout Serum Replacer, KSR) has been reported to promote the growth of pluripotent stem cells (Zhao et al., 2010). N2B27 medium was used to isolate rat embryonic stem cells (Buehr et al., 2008) and was successfully used in the establishment of rat naïve ESCs combined with Erk inhibitor PD0325901 (PD) and Gsk3 inhibitor CHIR99021 (CH) (Li et al., 2008). These two compounds both inhibit the pathways which promote differentiation and can help maintain self-renewal for the naïve pluripotent stem cells (Ying et al., 2008) and also have been indicated to promote mouse pluripotent state conversion (Guo et al., 2009; Hanna et al., 2009). LIF and bFGF respectively played important roles in maintaining mouse and human embryonic stem cell pluripotency (Williams et al., 1988; Bendall et al., 2007). The N2B27 medium containing bFGF was reported to be able to sustain human ESCs with the similar phenotype of that cultured in the traditional medium KOSR (Liu et al., 2006). Ascorbic acid (Buryanov and Shevchuk, 2005), a common nutrient for human health, could promote ESC proliferation (Chen et al., 2011) and was reported to promote reprogramming (Esteban et al., 2010). Human ESCs with two active X chromosomes have been generated under physiological oxygen concentrations which could prevent cell senescence (Lengner et al., 2010). All of these agents may contribute to the conversion of the pluripotent cells from primed states to naïve states. In our

study, we chose different combinations of basal media (KOSR, N2B27), factors (LIF, bFGF) and small compounds (PD, CH, and VC) to initially screen conditions that promote pluripotent state conversion. We revealed that the morphology and epigenetic markers of converted human ESCs (also called mhESC) were closer to naïve states. Our findings provided satisfactory host cells for future transgenic medicine as well as a new method for human ESCs culture.

RESULTS

Determining the optimized conversion condition

The H9 cells were first digested into single cells (we named the passage for these cells P0) and planted on feeder cells using different media and at a density of 1×10^6 cells per dish. On the third day (D3), the domed colony emerged. A large number of colonies in the media containing N2B27 looked like mouse ESCs (Fig. 1A). But the cell colonies looked explanate and were only a little prominent in the KOSR medium with LIF, LIF + bFGF, or bFGF. On D3, all cells in different dishes were digested into single cells and seeded in the new medium respectively. After passaging (the passage was called P1), the single N2B27 medium could not maintain human ESCs pluripotency. Rather, they might have been differentiated. The media containing N2B27 and KOSR, LIF and bFGF or bFGF could continually sustain the growth of human ESCs with dome morphology. We compared the conversion efficiencies of different media by the colony number per unit area (CNpUA) (Fig. 1C). The media LBH (KOSR + LIF + bFGF), BH (KOSR + bFGF), LBX (LIF + bFGF + KOSR + N2B27) and BX (bFGF + KOSR + N2B27) converted H9 cells into mhESCs with a higher efficiency (3.33 ± 0.58 , 9.00 ± 1.00 , 8.00 ± 1.00 , respectively) (Fig. 1C) and the cells could proliferate with mouse ESCs morphology.

All the basal media contained the same small compounds (CH, PD, SB431542, and VC). LBH, BH and LBX could support the converted H9 cells for more than 20 passages when digested with TrypLE™ (Invitrogen). The results also showed that all the media supporting the stable passage of mhESCs contained the factor bFGF. If the medium contained LIF, the colonies looked more domed than those in the media containing only bFGF (Fig. 1C). However, if the medium only contained the factor LIF, mhESCs would subsequently be differentiated in the following passages (Fig. 1B). The mhESCs in LBX medium had good phenotype (Fig. 1C), so we chose it to carry out the following experiments.

Characterization of mESC-like human ESCs (mhESCs)

There were many commonly used criteria to characterize and assess the developmental potential for the stem cells. Firstly, our karyotype analysis of mhESCs in LBX medium demonstrated that the converted human embryonic stem cells had a

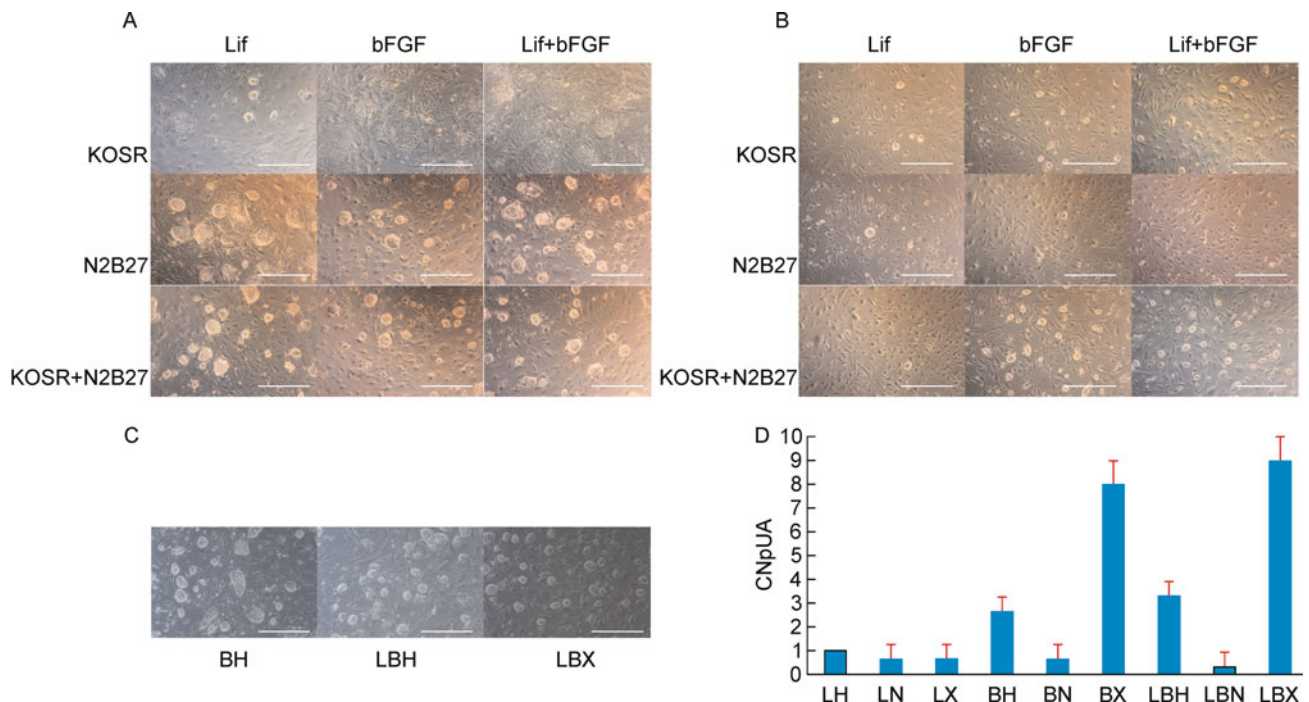


Figure 1. Determining the optimized conversion condition. (A) The P0 mhESCs in different media. H9 cells (P37) with flat colonies were digested to single cells using Tryple™ (Invitrogen). On the third day (3 days after incubation on feeders, named as D3), the colonies appeared as shown in the figure. (B) The P1 mhESCs on D2 in different media. The P0 mhESCs were digested on D3 of P0. On the second day, the colonies of P1 mhESCs appeared as shown in the figure. (C) Cell morphology. The mhESCs at P20 in BH, LBH and LBX media. The mhESCs in BH, LBH and LBX had been passaged for more than 20 generations. Bars = 500 μm. (D) Colony number per unit area (CNpUA) of mhESCs in different media, LN: LIF + N2B27; LH: LIF + KOSR; LBH: LIF + bFGF + KOSR; BN: bFGF + N2B27; LBN: LIF + bFGF + N2B27; LX: LIF + KOSR + N2B27; BX: bFGF + KOSR + N2B27; LBX: LIF + bFGF + KOSR + N2B27. The unit area was 1 mm²; the CNpUA of LN, LH, LBH, BN, LBN, LX, BX and LBX was 0.67 ± 0.58, 1.00 ± 0.00, 3.33 ± 0.58, 0.67 ± 0.58, 0.67 ± 0.58, 8.00 ± 1.00, 9.00 ± 1.00, respectively.

normal karyotype of 46(XX) (Fig. 2B). Next, we characterized the expression of some pluripotent markers in mhESCs. Specific markers for human ESCs (Tra-1-60, SSEA4) were still expressed (Fig. 2A). Moreover, the specific marker SSEA1 for mouse ESCs could also be stained in mhESCs (Fig. 2A). Later, we used RT-PCR and electrophoresis technology to detect some pluripotency genes expression, including *Oct4*, *Sox2*, *Nanog*, *Lin28*, *Rex1*, *Gdf3* and *Fgf4* and these genes all expressed in mhESCs as normally as the H9 cells (Fig. 2C). The assays of embryoid body (EB) formation *in vitro* were used to determine the differentiation ability of mhESCs. The mhESCs could form embryoid body-like structures through suspended growth (Fig. 2D) and RT-PCR analyses showed these bodies expressed differentiation markers for the three germ layers (Endoderm: *Amylase* and *Ncstn*, Mesoderm: *Enolase* and *Osteonectin*, Ectoderm: *Gad1* and *Gfap*).

The pluripotent state of mhESCs

Real-time PCR analyses for the *Xist* gene (less expressed in

naïve pluripotent stem cells (Nichols and Smith, 2009) were also performed to confirm that the converted mhESCs were similar to mouse ESCs. The expression of *Xist* (the first and second exons of *Xist* gene *Xist12*; the fifth and sixth exons of *Xist* gene *Xist56* (Lengner et al., 2010)) was downregulated in the converted mhESCs cultured in LBH and LBX medium (Fig. 3A). SSEA4's expression was also found to be downregulated in the converted mhESCs (Fig. 3A). Furthermore, the staining of histone H3 lysine 27 trimethylation (H3K27me3), which is associated with the inactive X chromosome (Plath et al., 2003), indicated that the accumulation of H3K27me3 of H9 was more than that of mhESCs because there was no H3K27me3 'spot' in mhESCs (Fig. 3B). The copy of X chromosomes which was inactivated in H9 cells might have been activated during the conversion. Thus, H9 cells might have been converted to naïve pluripotent stem cells.

Conversion of other human ESC lines

We tried to convert human parthenogenetic embryonic stem

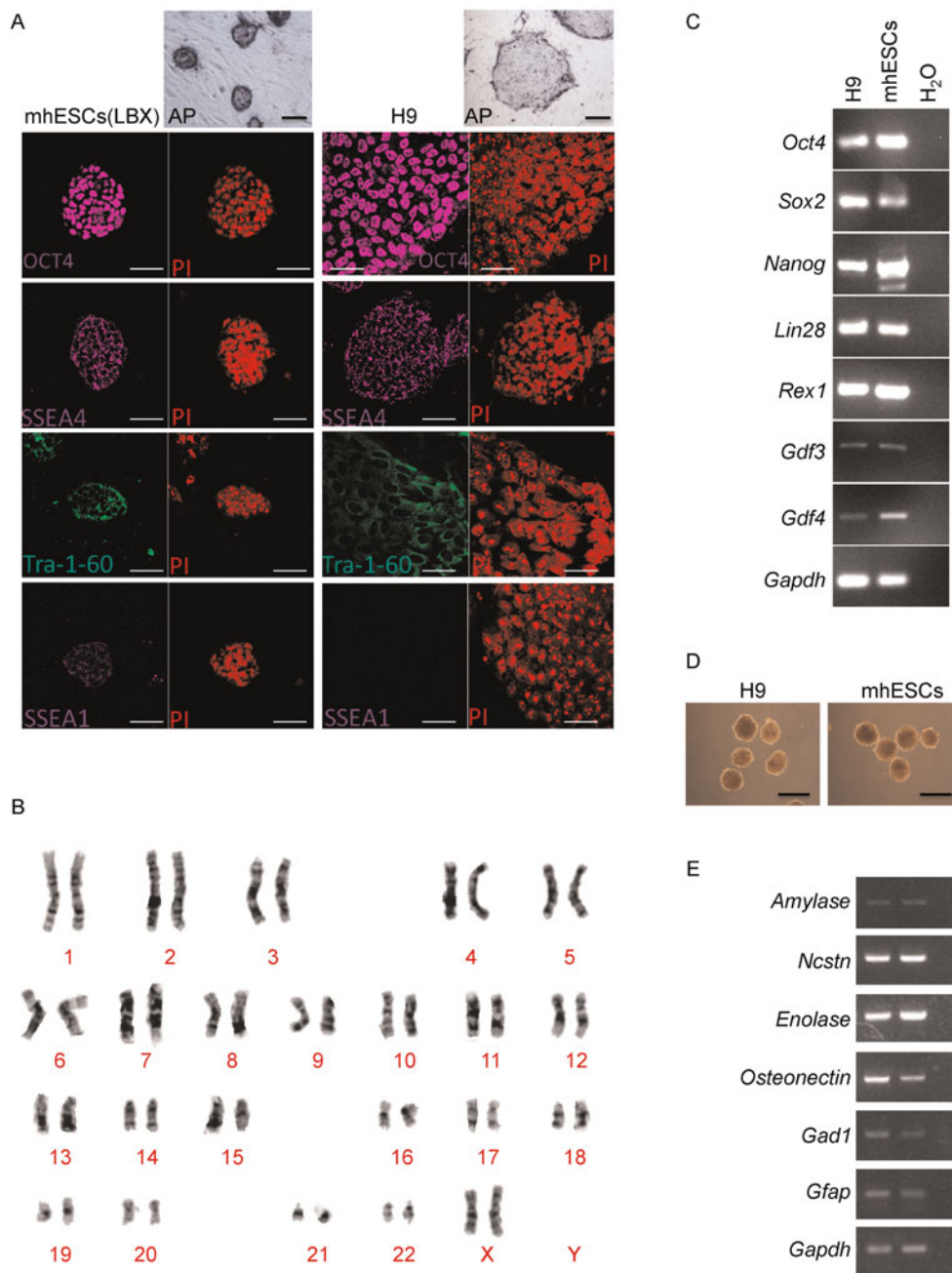


Figure 2. Characterization of mESC-like human ESCs (mhESCs). (A) Immunostaining for pluripotency markers (OCT4, SSEA1, SSEA4, and TRA-1-60) of mhESCs and H9 cells. AP, alkaline phosphatase. mhESCs in LBX medium and H9 cells in KOSR were stained with the same antibodies. Positive AP, Oct4, SSEA4 and Tra-1-60 were observed in H9 and mhESCs and SSEA1 is only positive in mhESCs. PI was used to stain the nucleus. Bars = 150 μ m (AP), Bars = 25 μ m (Immunostaining). (B) Karyotype of mhESCs. mhESCs in LBX medium were supplied for karyotype analysis. More than 75% of the cells showed normal human karyotype of 46 chromosomes. (C) RT-PCR for pluripotency genes (*Oct4*, *Sox2*, *Nanog*, *Lin28*, *Gdf3* and *Gdf4*) of H9 and mhESCs. Seven pluripotency genes primers were supplied for PCR of H9, mhESCs and H₂O. (D) Embryoid body (EB) formation of H9 cells and mhESCs. Bars = 500 μ m. (E) RT-PCR for three layers markers. The first column was the EB of H9 and the second and third columns were mhESCs' EBs and H₂O, respectively.

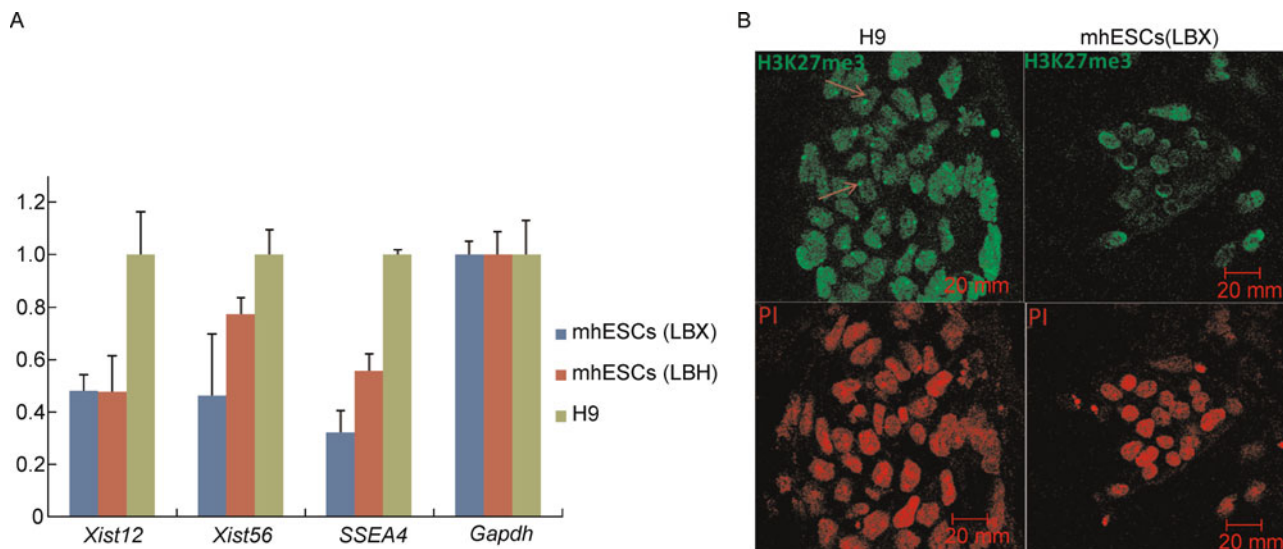


Figure 3. The pluripotent state of mhESCs. (A) Quantitative RT-PCR for *Xist* gene (*Xist12* (*Xist* exons 1, 2), *Xist56* (*Xist* exons 5, 6)) and *SSEA4*. The expression of *Xist* and *SSEA4* was relative to the expression of *Gapdh*. (B) H9 cells and mhESCs converted from H9 cells were stained with polyclonal antibodies against H3K27me3. Red arrow points to the H3K27me3 'spot'. PI was used to stain the nucleus. Bars = 20 μ m.

cells (P-TJ cells (Lu et al., 2010)) into mouse ESC-like cells which we called m_P-TJ cells. The P-TJ cells displayed

similar morphology as compared with H9 cells (Fig. 4A). P-TJ cells were also digested to single cells using Tryple™ and

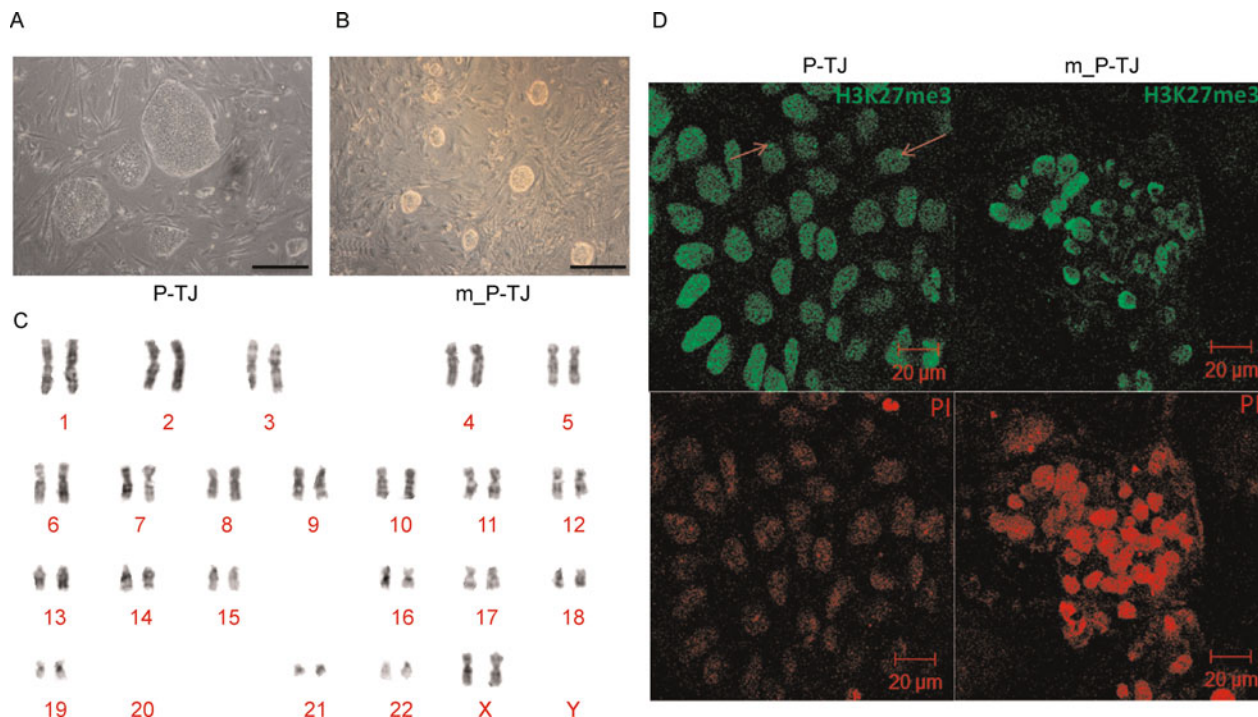


Figure 4. Conversion of other human ESC lines. (A) Cell morphology. P-TJ cells that were digested by collagenase IV. Bars = 500 μ m. (B) Cell morphology. The mhESCs converted from P-TJ cells. Bars = 500 μ m. (C) Karyotype analysis of m_P-TJ cells. More than 75% of the cells showed normal human karyotype of 46 chromosomes. (D) P-TJ cells and m_P-TJ cells were stained with polyclonal antibodies against H3K27me3. Red arrow points to the H3K27me3 'spot'. PI was used to stain the nucleus. Bars = 20 μ m.

grew on feeders in LBX medium. On the third day after being digested, the colony grew bigger and could be digested for the second time and the converted cells had been digested for fifteen passages (Fig. 4B). The m_P-TJ cells had normal karyotype (Fig. 4C) and the H3K27me3 'spot' could be detected in P-TJ cells but its fluorescence intensity was weaker in m_P-TJ cells (Fig. 4D). So P-TJ cells might also have been converted to naïve pluripotent stem cells.

DISCUSSION

The H9 cells were quickly converted to naïve states with an alternative culture medium. Basal media played important roles in the fast conversion process because on D3 of P0, it was observed that colonies in the media containing N2B27 looked more domed than colonies in the media only containing KOSR, and the number of the colonies in the media containing N2B27 was also larger than that in the media only containing KOSR. Small compounds were also important to complete the conversion. At the early passages, the small compounds chosen were CH, PD and VC. They could only support mhESCs for less than 3 passages and then the cells would prefer to differentiate and some went to apoptosis. The compound VC may accelerate the conversion process because it can promote cell proliferation (Chen et al., 2011). Another compound (activin receptor inhibitor SB431542, SB) was found by screening other small compounds (data not shown) and finally the small compounds array (CH, PD, SB, and VC) was determined. Activin receptor inhibitor SB was reported to increase BMP signaling activity (Xu et al., 2008) and induce rapid differentiation of EpiSCs (Brons et al., 2007). Therefore, all small compounds (CH, PD, SB, and VC) could support mhESCs in naïve states by promoting self-renewal and proliferation of mhESCs without differentiation.

We converted human ESCs into mhESCs at a high efficiency and the converted cells could be passaged stably (Fig. 1). The mhESCs karyotype did not change during conversion (Fig. 2B and 4). The mhESCs still expressed some human ESCs specific markers but they could also express mouse ESCs specific markers (Fig. 2A). The expression of *Xist* gene can induce X chromosome inactivation (XCI) and the region of XCI is accumulated by H3-K27 methylation (Plath et al., 2003). In our research, the expression of the *Xist* gene in mhESCs was downregulated and the fluorescence intensity of H3K27me3 'spot' in mhESCs was weaker than that in H9 and P-TJ cells (Fig. 3B and 4D). So the mhESCs may have two active X chromosomes (XaXa) and the pluripotent state of mhESCs may be naïve.

Human ESC-based gene modifications would benefit much from the mhESCs. Homologous recombination works poor in human ESCs (Eiges et al., 2001) because of low colony formation efficiency of human ESCs which makes it difficult to screen complete positive colonies (Zwaka and

Thomson, 2003). When mhESCs were digested to single cells, the colonies would be formed from single cells, and the screening could also be performed by selecting the positive colonies generated from single cells. The positive cells could also be quickly screened by flow cytometry, which was useful for gene targeting and some other gene modification experiments. Furthermore, genetically modified human ESCs have particular application in establishing disease-specific models (Li and Zhou, 2010) and the mhESCs will be the desirable materials.

Although the induced pluripotent stem cells of some species (mouse, human, monkey, rat, pig, rabbit, sheep, cow and dog) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Liu et al., 2008; Ezashi et al., 2009; Liao et al., 2009; Honda et al., 2010; Bao et al., 2011; Han et al., 2011; Luo et al., 2011) and embryonic stem cells of some species (mouse, human, rat, monkey and rabbit) (Evans and Kaufman, 1981; Thomson et al., 1995; Thomson et al., 1998; Wang et al., 2007; Buehr et al., 2008) were established, most of these pluripotent stem cells were prone to be a primed pluripotent state rather than a naïve state. The aim of the present experiment was to explore a medium suitable for obtaining naïve pluripotent stem cells. Our subsequent study is to obtain a general medium for as more species as possible. Further identification of the core transcriptional regulatory circuitry in mhESCs will provide us a comprehensive understanding of pluripotent state of human ESCs.

MATERIALS AND METHODS

Reagents and media

All the reagents and media used for this study were purchased from Life Technologies Inc. unless otherwise mentioned. H9 cells were from the Capital Medical University (CMU), Beijing; P-TJ cells were established in our labs (Lu et al., 2010). ICR mice for mouse embryonic stem cells (MEF) were purchased from Beijing Vital River Company. All animal operations followed the *Guidelines for the Care and Use of Laboratory Animals* established by the Beijing Association for Laboratory Animal Science, Beijing.

Cell culture

The KOSR medium consisted of 80% knockout Dulbecco's modified eagle medium (DMEM), 20% knockout serum replacement, 2 mmol/L L-glutamine, 0.1 mmol/L β -mercaptoethanol (Sigma), 1% MEM nonessential amino acids, 1% penicillin-streptomycin. N2B27 medium consisted of 48% DMEM/F12, 48% Neurobasal, 1% N2, 2% B27, 0.25 mg/mL BSA (Sigma), 5 μ g/mL insulin (Folch et al., 2009) and 1% penicillin-streptomycin.

Human ESCs were cultured as previously described (Hao et al., 2009). Feeder layer that was from the mouse embryonic fibroblast cells (MEF) treated with 10 μ g/mL mitomycin and MEF were both cultured in 10% FBS DMEM medium and the human ESCs culture medium was KOSR of 8 ng/mL basic fibroblast growth factor (bFGF, R&D). After the clumps were dissociated mechanically for ten

passages, they were passaged routinely using 1 mg/mL type IV collagenase (Sigma) every 6 days. All of the stem cells were cultured at 37°C in a 5% CO₂ incubator. The mhESCs were cultured in a different medium containing 2000 U/mL leucocyte inhibitory factor (LIF, Millipore) and 16 ng/mL basic fibroblast growth factor (bFGF, R&D). The mhESCs were passaged routinely using TrypLE™ (TrypLE™ Express & TrypLE™ Select, Invitrogen) every 3 days. All the mhESCs media contained small compounds (Erk inhibitor PD0325901 (1 μmol/L), Gsk3 inhibitor CHIR99021 (3 μmol/L), activin receptor inhibitor SB431542 (2 μmol/L) and ascorbic acid (50 ng/mL)).

Colony number per unit area (CNpUA)

mhESCs were planted on feeders at the same density when passaged from P0. On the second day, the colony number of the same area (1 mm²) for different media was counted and at least three different regions of the same area were selected. Variance analysis was performed. The mean value of the colony number for different regions in the same medium was named medium CNpUA.

Immunofluorescence analysis and alkaline phosphatase

Stained cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.5% Triton X-100 for 10 min followed by blocking with 5% donkey serum (Jakson) in PBS. The cells were incubated with primary antibodies against SSEA1 (Chemicon), OCT4 (Santa Cruz), TRA-1-60 (Chemicon), SSEA-4 (Chemicon) and H3K27me3 (Chemicon) overnight at 4°C, followed by the use of secondary antibodies at room temperature for 1 h. Finally, DNA was stained with propidium iodide (10 μg/mL; Molecular Probes, OR) for 5 min. For each separate sample, the experimental procedure was repeated for three times for one antibody, and the experiments were

replicated for at least three times. Observations were made using a confocal microscope (Zeiss, LSM 510 META). Alkaline phosphatase (AP) staining was performed with BCIP/NBT Alkaline Phosphatase Colour Development Kit (Beyotime) following the manufacturer's instructions.

Karyotype analysis

Karyotype analysis was performed as previously described following the standard instructions (Zhao et al., 2009); G-binding was carried out by Peking Union Medical College, Beijing.

Embryoid body formation

For EB formation, the mhESCs were digested into single cells and were transferred to gelatin-coated plates and incubated in DMEM containing 10% FBS for 10 min to discard the feeder cells. Then the suspensions were harvested and transferred to poly(2-hydroxyethyl methacrylate)-coated dish in KOSR medium lacking bFGF. The medium was changed every other day and after 6 days, the EBs were collected.

RT-PCR and quantitative PCR analysis

Total RNA was isolated from freshly obtained cells using TRIzol reagent. First strand cDNA was synthesized using MMLV reverse transcriptase (Promega) and oligo-dT (Promega) according to the manufacturer's instructions. RT-PCR was performed according to standard procedures and the products were electrophoresed on a 2% agarose gel. QPCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo) on Agilent Mx3005P. All primers used in the research are listed in Table 1.

Table 1 Primer sequence for PCR

Gene name	Forward primers	Reverse primers
<i>Xist12</i>	GAAGAGTCTCTGGCTCTTTAGAATACTGA	CAGCGTGGTATCTTCAATGGG (Lengner et al., 2010)
<i>Xist56</i>	GCCTGGCACTCTAGCACTTGA	AGGAGACAAAGAAATACACATTCATTC (Lengner et al., 2010)
<i>SSEA4</i>	TGGACGGGCACAACCTTCATC	GGGCAGGTTCTTGGCACTCT
<i>Oct4</i>	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCAAAAC
<i>Sox2</i>	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
<i>Nanog</i>	CAGCCCCGATTCTTCCACCACTCCC	CGGAAGATCCCAGTCGGGTTCCACC
<i>Lin28</i>	GCAGAAGATCACTCCGTTCCA	CGCACATTGAACCACTTACAGT
<i>Rex1</i>	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAGTCCAGA
<i>Gdf3</i>	CTTATGCTACGTAAAGGAGCTGGG	GTGCCAACCCAGTCCCGGAAGTT
<i>Fgf4</i>	CTACAACGCCTACGAGTCCTACA	GTTGCACCAGAAAAGTCAGAGTTG
<i>Amylase</i>	AATGATGCTACTCAGGTCAGAGATTGTC	TGTCCTCGTTGATTGTCATGGTTATCC
<i>Ncstn</i>	CGAGGATGGTCTACGATATGGAGAAGG	TCAGCCAGAACAACGCCAGAGAT
<i>Enolase</i>	GCTCCGTGACCGAGTCTCTT	TAGCCAACAGGTGACCGAAGG
<i>Osteonectin</i>	CCAGGTGGAAGTAGGAGAATT	CTCAGTCAGAAGGTTGTTGTC
<i>Gad1</i>	GGAAGTACGCGAGAACGAGGAAG	AGGAGGTTGCGGACGAAGAT
<i>Gfap</i>	TGAGTCGCTGGAGGAGGAGAT	GTCGTTGGCTTCGTGCTTGG
<i>Gapdh</i>	CGCTTCGCTCTCTGCTCCTCTGTT	CGCTTCGCTCTCTGCTCCTCTGTT

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ABBREVIATIONS

bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; EpiSCs, mouse epiblast stem cells; ESCs, embryonic stem cells; mhESC, mouse ESC-like human ESCs; ICM, inner cell mass; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast cells; XCI, X chromosome inactivation

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