Acta Biochim Biophys Sin 2012, 44: 197–206 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr127.
Advance Access Publication 26 January 2012

ABBS

Original Article

Low microRNA-199a expression in human amniotic epithelial cell feeder layers maintains human-induced pluripotent stem cell pluripotency via increased leukemia inhibitory factor expression

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Human-induced pluripotent stem (iPS) cells share the same key properties as embryonic stem cells, and may be generated from patient- or disease-specific sources, which makes them attractive for personalized medicine, drug screens, or cellular therapy. Long-term cultivation and maintenance of normal iPS cells in an undifferentiated self-renewing state is a major challenge. Our previous studies have shown that human amniotic epithelial cells (HuAECs) could provide a good source of feeder cells for mouse and human embryonic stem cells, or spermatogonial stem cells, as they express endogenous leukemia inhibitory factor (LIF) at high levels. Here, we examined the effect of exogenous microRNA-199a regulation on endogenous LIF expression in HuAECs, and in turn on human iPS cell pluripotency. We found that HuAECs feeder cells transfected with microRNA-199a mutant expressed LIF at high levels, allowing iPS to maintain a high level of alkaline phosphatase activity in long-term culture and form teratomas in severe combined immunodeficient mice. The expression of stem cell markers was increased in iPS cultured on HuAECs feeder cells transfected with the microRNA-199a mutant, compared with iPS cultured on HuAECs transfected with microRNA-199a or mouse embryo fibroblasts. Taken together, these results suggested that LIF expression might be regulated by microRNA-199a, and LIF was a crucial component in feeder cells, and also was required for maintenance of human iPS cells in an undifferentiated, proliferative state capable of self-renewal.

Keywords human amniotic epithelial cells; feeder layer; human-induced pluripotent stem cells; leukemia inhibitory factor; microRNA-199a; pluripotency

Received: September 18, 2011 Accepted: October 19, 2011

Introduction

Embryonic stem cells (ESCs) are regarded as the potential gold standard for cell-based regenerative medicine due to their pluripotent nature, the ability to self-renew indefinitely in culture while retaining the capacity to differentiate into any cell type [1]. Although recent research has greatly increased our fundamental understanding on the safe and effective use of ESCs, ethical considerations are the major impediment to the application of these cells in the clinic [1,2]. Recently, in order to avoid ethical obstacles, an alternative source of pluripotent cells was provided by direct reprogramming of murine somatic fibroblast cells into induced pluripotent stem (iPS) cells using the factors defined by Galach and Utikal [3], Takahashi *et al.* [4], Takahashi and Yamanaka [5], Maherali *et al.* [6], Okita *et al.* [7], Hanna *et al.* [8], Wernig *et al.* [9], Liu *et al.* [10], and Yu *et al.* [11]. Furthermore, shortly after these reports, human fibroblasts were successfully reprogrammed, using either the same combination of factors or with a combination of Oct3/4 and Sox2 supplemented with either Nanog and Lin28 or c-Myc and Klf4. These human iPS cells are highly similar to human ESCs in terms of morphology, proliferation, gene expression, and the epigenetic status of pluripotency-specific genes. To date, iPS cells that share the key properties of unlimited self-renewal and pluripotency as ESCs have been generated in different species by various methods [2,8,10,12–17]. In addition, iPS cells can be generated from patient- or disease-specific sources, which makes them attractive for personalized medicine, drug screening, and tissue engineering, as well as for gene and cellular therapy in a wide range of human diseases including Parkinson's and other neurodegenerative diseases, and for diabetes, cardiac, and vascular therapy [2,18].

Although human iPS is a better source of cells for clinical treatment, the question of maintenance of their cell self-

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renewal and pluripotency during *in vitro* culture remains. In our previous studies, we indicated that leukemia inhibitory factor (LIF) secreted by human amniotic epithelial cells (HuAECs) might be a crucial component, by which the feeder cells could maintain mouse and human ESCs, as well as mouse spermatogonial stem cells, in an undifferentiated, proliferative state capable of self-renewal [19–21].

LIF, a secreted glycoprotein, was first identified as a factor that induced mouse myeloid leukemia M1 cells to differentiate into macrophages [22,23]. LIF exerts pleiotropic effects in many physiological systems including proliferation, differentiation, and cell survival [24–28]. LIF is also involved in the regulation of early mouse embryonic development and is expressed in rat testis [29], and can inhibit the differentiation of pluripotent ESCs derived from the inner cell mass of the blastocyst [30]. In addition, LIF has an important role in the regulation of spermatogonial cell compartment [31]. The reports by Bauer and Patterson [32] and Wright *et al*. [33] showed that LIF could promote long-term growth of embryonic human neural stem cells in culture, and other reports indicate that LIF signaling could support the maintenance of self-renewal in cultured mouse neural stem cells [34–37].

Although we previously found that HuAECs can be effectively used as feeder cells, very little is known about how they maintain iPS cell self-renewal and inhibit their differentiation. Oskowitz *et al*. [38] reported that microRNA (miRNA)-199a can interfere with LIF expression in human multipotent stromal cells. miRNAs are 20–23 nucleotide non-coding RNAs which function as sequence-specific regulators of gene expression, through translational repression and/or transcript cleavage [39–42]. Since the roles and functions of endogenously expressed miRNAs in *Caenorhabditis elegans* were first described in 1993 [43], increasing number of studies have shown that miRNAs play key roles in development, especially the timing of morphogenesis and maintenance of undifferentiated or incompletely differentiated cell types such as stem cells [44–47].

In view of this evidence, we hypothesize that low endogenous expression of miRNA-199a in HuAECs feeder layers could lead to high LIF expression, which would maintain human iPS cells pluripotency and self-renewal. Therefore, in this study, we examined how LIF expression is regulated by endogenous miRNA-199a and the effect on HuAEC-induced maintenance of human iPS cells in a self-renewing and undifferentiated state.

Materials and Methods

Preparation of mouse embryo fibroblast and HuAECs

Mouse embryo fibroblast (MEF) cells were isolated from 13-day-old C57BL/6 mouse embryos. Cells were mitotically inactivated using mitomycin C (Sigma, St Louis, USA) as described previously [19]. MEF cells were mitotically inactivated by treatment with 10 μg/ml mitomycin C (Roche, Basel, Switzerland) for 2 h at 37°C. Cells are washed three times with phosphate-buffered saline, trypsinized (Invitrogen, Calsbad, USA), and plated at a density of 1×10^5/ml with 2.5 ml per well of a gelatin-coated six-well dish.

Human placentas were obtained from the pregnant women who were negative for HIV-I, hepatitis B, and hepatitis C with written and informed consent. We were recognized for the appropriate use of human amnion by the institutional ethics committee. Amnion membranes were mechanically peeled from chorines of placentas obtained from women with an uncomplicated Cesarean section. The epithelial layers with basement membrane attached were obtained and used to harvest HuAECs as previously described with some modification [19]. Briefly, the membrane was placed in a 250-ml flask containing Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen), and cut with a razor to yield 0.5–1.0 cm^2 segments. The segments were digested with 0.25% trypsin– ethylenediaminetetraacetic acid (EDTA) at 37°C for 45 min. The resulting cell suspensions were seeded in a six-well plate in DMEM medium supplemented with 10% fetal calf serum (PAA, Linz, Austria), penicillin (100 U/ml), and glutamine (0.3 mg/ml), and incubated in a humidified tissue culture incubator containing 5% CO_2 at 37°C. The HuAECs were used as the feeder layers for human iPS culture until they reached to a density of ∼100%.

Co-culture of human iPS cells with HuAECs and MEF

The human iPS cells were kindly provided by Dr Lei Xiao (The College of Animal Sciences at Zhejiang University, Hangzhou, China). iPS cultures were separated from the feeder cells by treatment with 0.125% trypsin–EDTA solution and plated onto and co-cultured with HuAECs (miR-199a transfected), HuAECs (miR-199a-mutant transfected), or MEF. The cells were cultured in DMEM : F12 (1 : 1) medium supplemented with 15% KnockOut™ serum replacement (GIBCO, Invitrogen Corporation, Carlsbad, CA), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and penicillin (25 U/ml)–streptomycin (925 mg/ml) without LIF. Those cells were incubated in a humidified tissue culture incubator containing 5% CO_2 at 37°C. All cells were cultured on the same feeder until the fourth passage (P4) before making ulterior experiments.

Recombinant adenovirus vector construction

An RNAi-Ready pSIREN-RetroQ-ZsGreen Retroviral system was used to create retroviral virus vectors (BD Biosciences Clontech, San Jose, USA). For vector RNAi-

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Ready pSIREN-RetroQ-ZsGreen-mir199a (pre-miRNA of mir199a expression element), an oligonucleotide pairs for pre-miRNA of miR-199a and linker sequences with *Bam*HI and *Eco*RI sites were chemically synthesized [46]. The sequences of the oligonucleotides were: top strand, 5′-GTggatccGCCAACCCAGTGTTCAGACTACCTGTTC AGGAGGCTCTCAATGTGTACAGTAGTCTGCACATTG GTTAGGCTTTTTTcctaagCC-3′, and bottom strand, 5′-GGcaatggAAAAAAGCCTAACCAATGTGCAGACTA CTGTACACATTGAGAGCCTCCTGAACAGGTAGTCT GAACACTGGGTTGGCggatccAC-3′ (sequences corresponding to miR-199a seed sequences in capitalized, underlined and bold, and restriction enzyme sites in lower case and underlined). To build the expression plasmid the pairs of oligos were annealed and inserted into the multiple cloning sites between *Bam*HI and *Eco*RI sites in the pSIREN-RetroQ-ZsGreen vector. The negative control plasmid pSIREN-RetroQ-ZsGreen-mir199a-Mut was similarly built, except that 23 nucleotides in sequences corresponding to miR-199a seed sequences were mutated (CCCAGTGTTCAGACTACCTGTTC changed to CttAc TGccCAtAgTAttTGgcC, mutations shown in lower case). Then, the pSIREN-RetroQ-ZsGreen-mir199a or pSIREN-RetroQ-ZsGreen-mir199a-Mut was recombined in the package cell lines PT67 to create retroviruses. Recombinant viruses were propagated in PT67 cells, purified, and titered by standard methods, as previously described by our laboratory [21]. The corresponding viruses were named Rdv-mir199a or Rdv-mir199a-Mut. Co-transfection of HuAECs was conducted to use 4×10^7 PFU/ml Rdv-mir199a or Rdv-mir199a-Mut retrovirus, respectively, according to the manufacturer's protocol. The cells were seeded in a six-well plate in DEME (Sigma) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone, Rockford, USA), at 37°C in a humidified atmosphere of air containing 5% CO_2, until 80% confluent.

RNA extraction and analysis by quantitative real-time polymerase chain reaction

Total RNA from each cell was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA samples were treated with Dnase I (Sigma-Aldrich), quantified, and reverse transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using a RealPlex4 real-time PCR detection system from Eppendorf Co. Ltd (Hamburg, Germany), with SyBR Green RealTime PCR Master MIX (Toyobo) used as the detection dye. qRT-PCR amplification was performed over 40 cycles with denaturation at 95°C for 15 s and annealing at 58°C for 45 s. Target cDNA was quantified using the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator) and steady-state mRNA levels are reported as an *n*-fold difference relative to the calibrator. For each sample, the maker genes Ct values were normalized using the formula $\Delta Ct = Ct_genes - Ct_18SRNA$. To determine relative expression levels, the following formula was used $\Delta\Delta Ct = \Delta Ct_HuAECs_group - \Delta Ct_MEF_group$. The values used to plot relative expressions of markers were calculated using the expression $2^{-\Delta\Delta Ct}$. The mRNA levels were calibrated based on levels of 18S rRNA. The cDNA of each gene was amplified using primers as follows (Table 1).

Alkaline phosphatase staining

Alkaline phosphatase (AP) activity of human iPS cells, which were cultured on HuAECs or MEF, or cultured solely without any feeds, were determined using the AP substrate kit (Sigma-Aldrich) according to the manufacturer's instructions [48].

Table 1 The sequences of human gene QRT-PCR primer [11]

Gene product	Primer sequence (5′→3′)	Size (bp)
LIF	F: GCATCTGAGGTTTCCTCCAA	99
	R: TGTTTCCAGTGCAGAACCAA	
NANOG	F: GATTTGTGGGCCTGAAGAAA	93
	R: ATGGAGGAGGGAAGAGGAGA	
OCT-3/4	F: CAGTGCCCGAAACCCACAC	161
	R: GGAGACCCAGCAGCCTCAAA	
SOX-2	F: AACCCCAAGATGCACAACTC	100
	R: GCTTAGCCTCGTCGATGAAC	
REX-1	F: GGTGGCATTGGAAATAGCAG	148
	R: TGCCTAGTGTGCTGGTGGT	
SOX-1	F: TCAAGGAAACACAATCGCTG	108
	R: ATTATTTTGCCCGTTTTCCC	
AFP	F: AGAGGAGATGTGCTGGATTG	110
	R: GTGGTCAGTTTGCAGCATTC	
ALB	F: TGCTGATGAGTCAGCTGAAAA	105
	R: TCAGCCATTTCACCATAGGTT	
PAX6	F: GTTGGTATCCGGGGACTTC	101
	R: TCCGTTGGAACTGATGGAGT	
CXCR4	F: GTTACCATGGAGGGGATCAG	104
	R: TTTTCTTCACGGAAACAGGG	
FLK-1	F: AGCATGGAAGAGGATTCTGG	143
	R: CGGCTCTTTCGCTTACTGTT	
HNF-4	F: CGTGGTGGACAAAGACAAGA	128
	R: CATAGCTTGACCTTCGAGTGC	
18S rRNA	F: CAGCCACCCGAGATTGAGCA	253
	R: TAGTAGCGACGGGCGGTGTG	

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Immunofluorescence staining

The cultured cells were washed three times with fetal calf serum and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min. After blocking, the cells were incubated first with rabbit anti-human Oct3/4 polyclonal antibody (1 : 200; Chemicon, Temecula, USA) and rabbit anti-human Nanog polyclonal antibody (1 : 200; Chemicon) overnight at 4°C, and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1 : 200; Abcam, Cambridge, UK) and 5 μg/ml 4′-6-diamidino-2-phenylindole (Sigma-Aldrich) at room temperature for 30 min. Then the cells were thoroughly washed with Tris-buffered saline containing Tween 20 (TBST, 25 mM Tris-HCl, pH 8.0, 125 mM NaCl, and 0.05% Tween 20) and viewed through a fluorescence microscope (DMI3000; Leica, Allendale, USA).

Western blot analysis

The HuAEC and MEF cells were lysed using a 2 × loading lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium clodecyl sulfate, 10% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue). The total amount of proteins from the cultured cells were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto hybrid-polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA). After blocking with 5% (w/v) non-fat dried milk in TBST, the PVDF membranes were washed four times (15 min each) with TBST at room temperature and incubated with primary antibody (rabbit anti-mouse/human LIF polyclonal antibody, 1 : 1000; Santa Cruz Technology, Santa Cruz, USA). Following extensive washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1 : 1000; Santa Cruz Technology) for 1 h. After washing for four times (15 min each) with TBST at room temperature, the immunoreactivity was visualized by enhanced chemiluminescence using ECL kit from Perkin-Elmer Life Science (Norwalk, USA).

Northern blot analysis

All steps of northern blotting were according to the previously described one [49]. For all groups, 20 μg of good quality total RNA was analyzed on a 7.5 M ureum 12% PAA denaturing gel and transferred to a Hybond N^+ nylon membrane (Amersham, Freiburg, Germany). Membranes were cross-linked using UV light for 30 s at 1200 mJ/cm^2. Hybridization was performed with the miR-199a antisense starfire probe, 5′-CCCAGTGTTCAGACTACCTGTTC-3′ (IDT, Coralville, USA), to detect the 22-nt miR-199a fragments according to the instruction of the manufacturer. After washing, membranes were exposed for 20–40 h to Kodak XAR-5 films (Sigma-Aldrich). As a positive control, the ethidium bromide-stained gels prior to transfer of tRNA were used as the control to show equal loading of RNA samples.

Teratoma formation

All animal procedures were carried out at Shanghai Jiaotong University with Institutional Animal Care and Use Committee approval in accordance with institutional guidelines. The 1×10^6 human iPS cells were inoculated into the hind leg of severe combined immunodeficient (SCID) mice. Teratomas were embedded in paraffin and histologically examined after hematoxylin and eosin staining. The procedure of teratoma formation experiment was performed as described in [19].

Statistical analysis

Each experiment was performed at least three times and data were shown as the mean ± SE. The differences were evaluated using Student's t-tests. $P < 0.05$ was considered to be statistically significant.

Results

Bioinformatic analysis of miR-199a

T15907130071he pre-miRNA sequences, mature miRNAs sequences, chromosomal location, and length of the miR-199a were determined and analyzed using bioinformatic analysis. The miRBase target database (http://www.mirbase.org) [38,45,46] demonstrates that miR-199a is completely conserved in human, mouse, and rat (**Fig. 1**).

HuAECs express mature miR-199a at low levels and LIF at high levels

Northern blot, qRT-PCR, and western blot were used to determine the expression of mature endogenous miR-199a and LIF in HuAECs and MEFs. Northern blot indicated the hybridization signal of mature miR-199a in HuAECs was weaker than in MEF [**Fig. 2(A)**]. QRT-PCR and western blot analysis indicated that the expression of

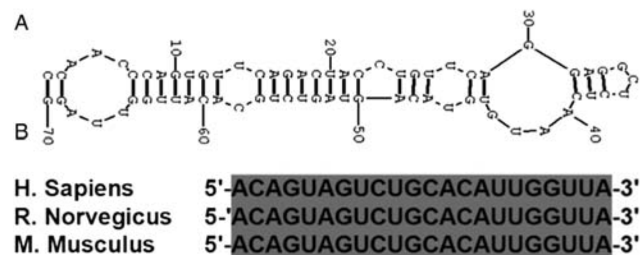

Figure 1 The secondary structure of human, mouse, and rat miR-199a (A) Typical secondary structure of hsa-miR-199a precursor miRNAs (pre-miRNAs), containing stem-loop and hairpin structures, with a binding site located in an unstable region with a multi-branching loop-like RNA structure. (B) Human, mouse, and rat miR-199a are 100% homologous.

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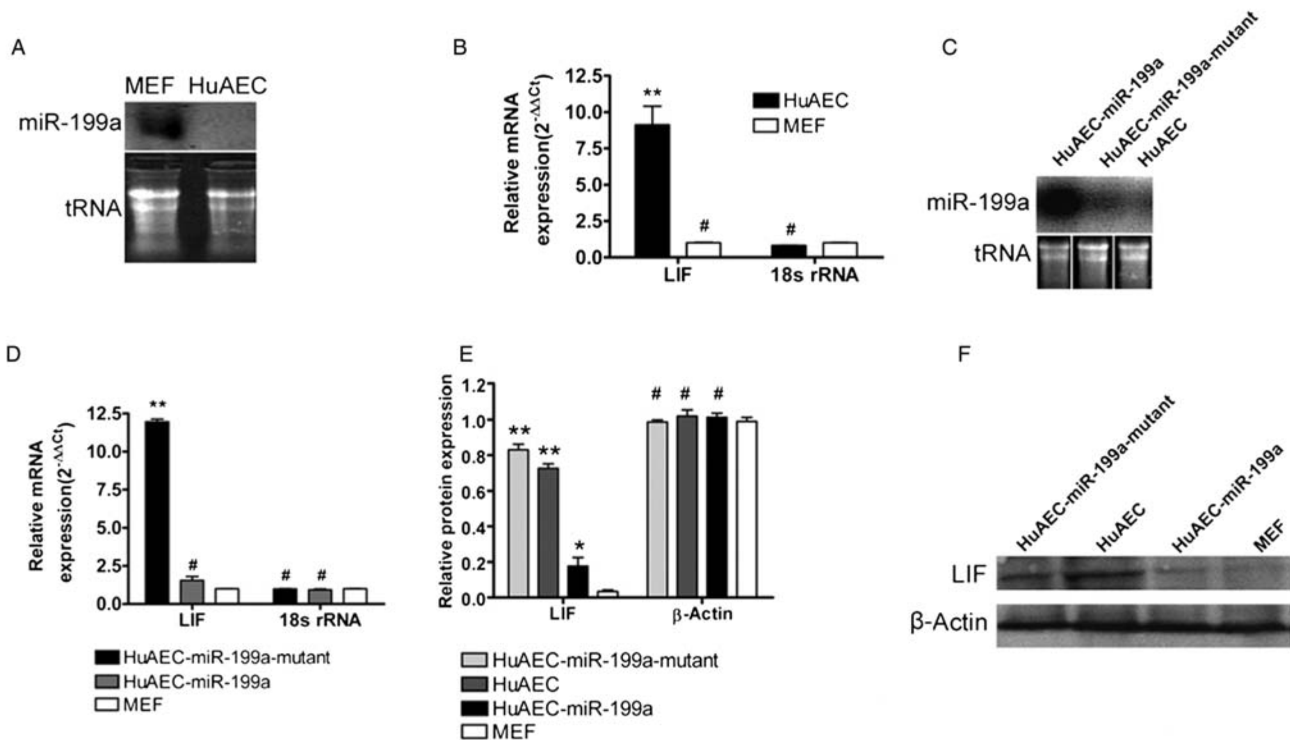

Figure 2 Mature miR-199a and endogenous LIF expression in MEFs and HuAECs (A) Northern blotting indicating a weaker mature miR-199a hybridization signal in HuAECs than in MEFs. tRNA was used as a loading control. (B) qRT-PCR indicating that the expression level of endogenous *LIF* mRNA in HuAECs is higher than that in MEFs. Relative mRNA expression is normalized to 18S rRNA as an internal control (**$P < 0.01$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$). (C) Northern blot showing strong hybridization signal in miR-199a-transfected HuAECs, compared with mutant miR-199a and untransfected HuAECs. tRNA was used as a loading control. (D) qRT-PCR showing the endogenous LIF mRNA expression in miR-199a-transfected HuAECs is lower than that in miR-199a-mutant-transfected HuAECs. Relative mRNA expression is normalized to 18S rRNA as an internal control (**$P < 0.01$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$). (E, F) Western blot and quantification showing expression of endogenous LIF in untransfected HuAECs or mutant-miR-199a-transfected HuAECs is significantly higher than that in miR-199a-transfected HuAECs or MEFs, indicating that exogenous miR-199a downregulates LIF expression (**$P < 0.01$ vs. MEF; *$P < 0.05$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$).

endogenous *LIF* mRNA (9.12 ± 1.29, $n = 3$, $P < 0.05$ vs. 'β-actin' levels) and protein was significantly high in HuAECs compared with that in MEFs [**Fig. 2(B,F)**]. These results revealed that mature miR-199a was expressed at low levels in HuAECs, compared with MEFs, which may account for high expression of LIF due to reduced interference by miR-199a in HuAECs.

MiR-199a is expressed in HuAECs and interferes with LIF expression

In order to validate our hypothesis, we constructed a retrovirus over-expressing human miR-199a, and detected the expression level of miR-199a and LIF in HuAECs transfected with the human miR-199a expressing retrovirus. Northern blot analysis revealed that mature miR-199a hybridization signal was only observed in the HuAECs transfected with miR-199a, and not in untransfected HuAECs or in the HuAECs-miR-199a-mutant-transfected group [**Fig. 2(C)**]. The results of qRT-PCR indicated that the miR-199a expressing retrovirus transfection led to decreased endogenous expression of *LIF* (1.54 ± 0.26) in HuAECs, compared with miR-199a mutant transfection (11.96 ± 0.17, $n = 3$, $P < 0.05$), using 18S rRNA as an internal control [**Fig. 2(D)**]. Western blot analysis demonstrated that LIF protein expression in the miR-199a-transfected group (0.177 ± 0.048, relative to β-actin) was significantly lower than the miR-199a-mutant-transfected group and -untransfected group (0.830 ± 0.032 and 0.727 ± 0.026, respectively, $n = 3$, $P < 0.05$) [**Fig. 2(E,F)**]. MEFs expressed low level of LIF protein (0.030 ± 0.009). These results demonstrated that the transfection of exogenous miR-199a can interfere with the expression of endogenous LIF mRNA and protein in HuAECs.

Transfection of HuAECs with exogenous miR-199a accelerates human iPS differentiation via reduced endogenous LIF expression

To examine whether exogenous miR-199a can influence LIF expression in HuAECs, and influence the self-renewal and pluripotency in human iPS cells, we transfected primary HuAECs with miR-199a or micR-199-mutant retrovirus, and used the HuAEC cells as a feeder layer to culture iPS. The iPS morphology was compared after the

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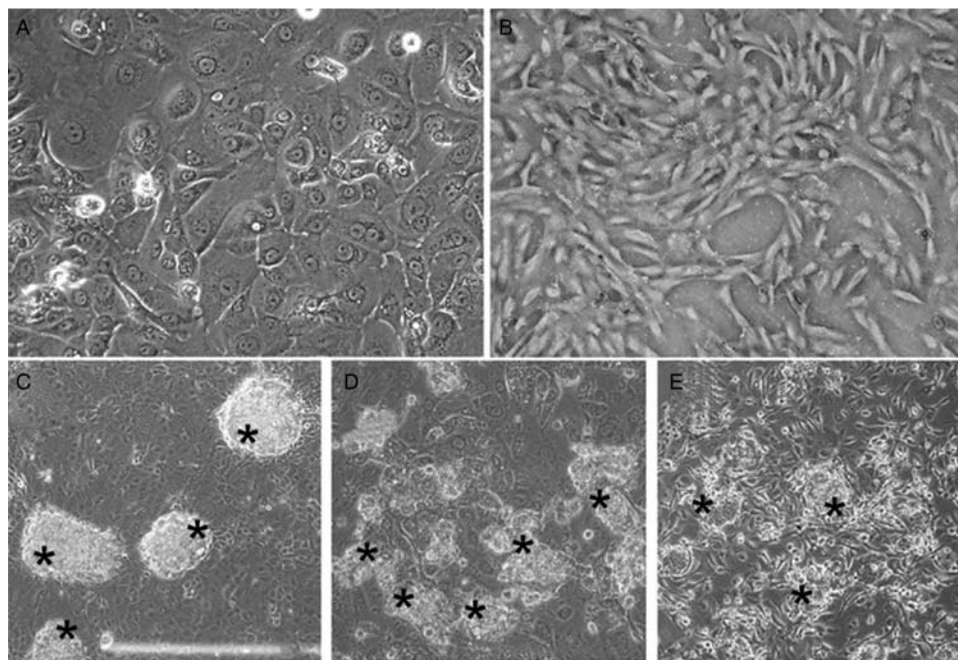

Figure 3 Morphology of human iPS cells cultured on different feeder layers Morphology of HuAECs (A) and MEFs (B). Magnification, ×200. (C) Morphology of iPS cells cultured on miR-199a-mutant-transfected HuAECs, iPS colonies (stars) appear more isolated and rounded with obvious boundaries with feeder cells, consistent with the appearance undifferentiated cells. Magnification, × 200. Morphology of human iPS cells cultured on (D) miR-199a-transfected HuAECs and (E) MEFs, iPS colonies (stars) appear to be migrating into the feeder layer with a blurry cellular boundary, consistent with the appearance of more differentiated cells. Magnification, × 200.

fourth passage on the same feeder layer (**Fig. 3**). Colonies of iPS cells cultured on HuAECs-miR-199a-mutant-transfected cells appeared more isolated and rounded, with an obvious boundary with feeder cells, consistent with undifferentiated cells. In comparison, human iPS cells cultured on HuAECs-miR-199a-transfected cells or MEFs appeared to be migrating into the feeder layer and a blurry cellular boundary between the two cell types was observed, which was consistent with more differentiated iPS cells. In addition, the mean number of human cells per well was significantly increased on HuAECs-miR-199a-mutant-transfected feeder layers, compared to HuAECs-miR-199a transfected or MEFs after 3 days, indicating a higher proliferation iPS rate on HuAECs-miR-199a-mutant-transfected HuAEC feeder layers [**Fig. 4(A)**].

HuAECs Maintain Human Pluripotency and Inhibit Differentiation of iPS via Low Endogenous miR-199a Expression

As AP levels decrease as stem cells lose their pluripotency and differentiate, we measured AP activity in human iPS cells cultured on HuAECs-miR-199a mutant, HuAECs-miR-199a, and MEF feeder layers. At the 6th day of P4, the AP activity of iPS cultured on HuAECs-miR-199a mutant was higher, compared with that cultured on HuAECs-miR-199a or MEFs [**Fig. 4(B)**]. We analyzed the expression of several stem cell markers using qRT-PCR to evaluate the degree of differentiation and stemness of human iPS grown on different feeder layers. The expression of stem cell markers *Oct-4, Nanog, Sox2*, and *Rex-1* in human iPS cells cultured on HuAECs-miR-199a mutant was ∼20–60 folds higher than that in iPS grown on HuAECs-miR-199a or MEFs [**Fig. 4(C)**]. The expression level of the differentiated markers *Pax-6, Sox-1, Flk-1, Cxcr-4, Afp, Alb*, and *Hnf-4* in human iPS cells grown on HuAECs-miR-199a transfected or MEFs was significantly reduced compared with iPS cells cultured on HuAECs-miR-199a mutant [**Fig. 4(D)**]. Immunofluorescent (IF) staining indicated increased Nanog and Oct-4 protein expression in human iPS cells cultured on HuAECs-miR-199a mutant, compared with iPS cultured on MEF or HuAECs-miR-199a (**Fig. 5**). These results demonstrated that human iPS cells cultured on HuAECs-miR-199a or MEF feeders were in differentiated state, whereas iPS cultured on HuAECs-miR-199a mutant had more stemness and pluripotency. To evaluate the pluripotent potential of human iPS cells cultured on different feeder layers, 5×10^6 human iPS cells were injected into the hind leg of SCID mice. After 4 weeks, there were no teratomas in mice-injected iPS cells cultured on MEFs or HuAECs-miR-199a; however, teratomas were observed on the legs of SCID mice injected with human iPS cultured on HuAECs-miR-199a-mutant cells [**Fig. 6(A)**]. The iPS-derived teratomas contained cellular representatives of all three germ layers, as HE staining demonstrated immature tissues with neurocoele and glands, muscle, lipocytes,

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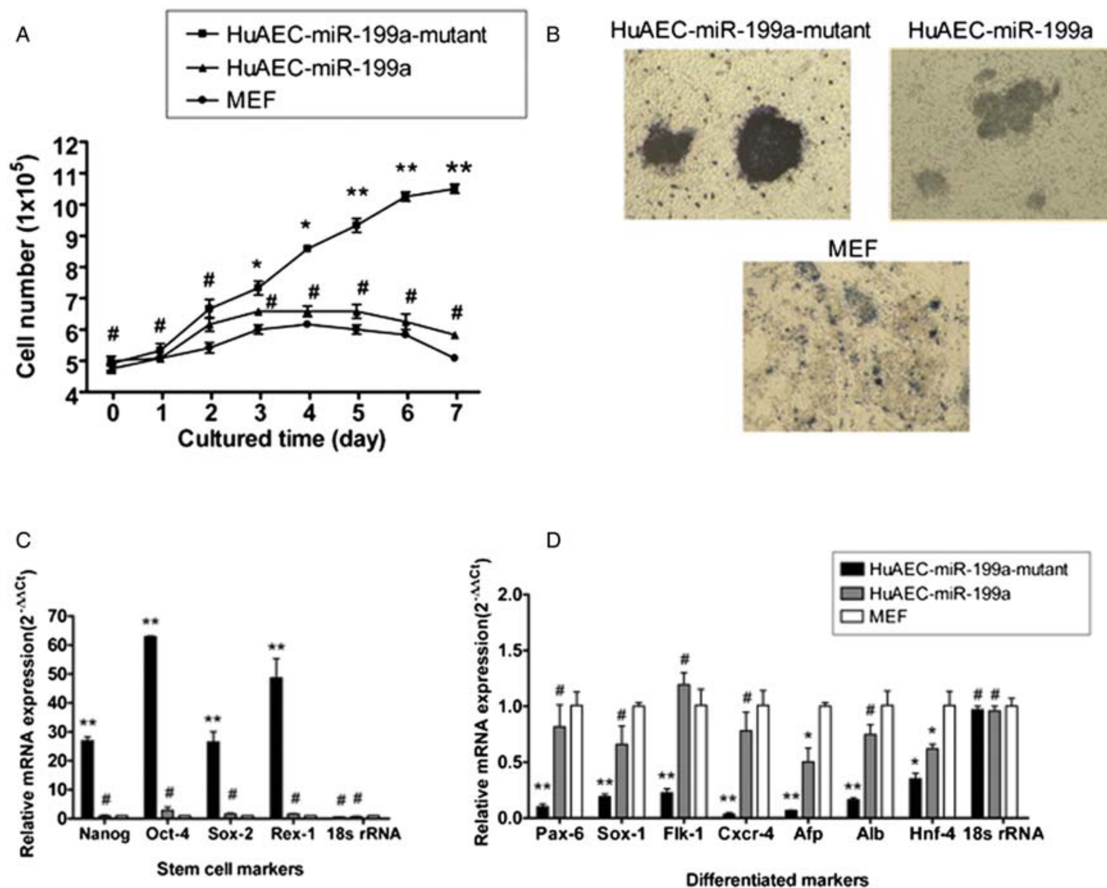

Figure 4 The pluripotency and self-renewal of human iPS cells cultured on different feeder layers (A) Cell number count indicating that the growth rate of human iPS cells on miR-199a-mutant-transfected HuAECs was significantly higher than that on miR-199a-transfected HuAECs or MEFs between day 3 and 7 ($^*P < 0.05$ vs. MEF; $^{**}P < 0.01$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$). (B) AP activity in human iPS cells cultured on transfected miR-199a HuAECs or MEFs was reduced compared with that cultured on miR-199a-mutant-transfected HuAECs. (C) qRT-PCR demonstrated the expression of the stem cell markers *Oct-4, Sox2, Nanog, Rex-1* was significantly higher in human iPS cells cultured on miR-199a-mutant-transfected HuAECs than cultured on miR-199a-transfected HuAECs or MEFs ($^{**}P < 0.01$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$). (D) qRT-PCR demonstrating that the expression of the differentiated stem cells markers *Pax-6, Sox-1, Flk-1, Cxcr-4, Afp, Alb, Hnf-4* mRNAs was significantly lower in human iPS cells cultured on miR-199a-mutant-transfected HuAECs compared with that cultured on miR-199a-transfected HuAECs and MEFs ($^{**}P < 0.01$ vs. MEF; $^*P < 0.05$ vs. MEF; $^{\#}P > 0.05$ vs. MEF; $n = 3$).

and bone cells [**Fig. 6(B)**]. These results indicated that human iPS cells cultured on HuAECs-miR-199a-mutant feeder layers maintained pluripotency.

Discussion

Understanding the cellular and molecular characteristics of human iPS cell *in vitro* culture has therapeutic potential for the treatment of many regenerative diseases. To date, many studies have indicated that proliferation and differentiation of human iPS is dependent on a specific microenvironment, including various cytokines, LIF, and other unknown factors *in vivo*. In order to maintain self-renewal, proliferation, and inhibit differentiation of iPS *in vitro*, we must provide a similar microenvironment and provide the essential ingredients for growth. Many experiments typically culture mouse ESCs on a feeder layer of MEFs, with addition of LIF and growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to maintain ESCs in an undifferentiated self-renewing state. These techniques require many animals for the time-consuming preparation of MEFs, and consume costly growth factors and LIF. The addition of growth factors can result in extraneous contamination, which may possibly induce iPS variation.

In order to avoid the disadvantages of using MEFs, we chose HuAECs as the feeder layer. HuAECs are temporary specialized fetal cells, derived from the placenta, which can maintain the pluripotency of early epiblast cells. Previous studies have indicated that HuAECs express many growth factors such as LIF, EGF, bFGF, transforming growth factor-α/β, and bone morphogenic protein-4 as well as stem cell markers including Nanog, Oct-4, and Nestin [21,50,51]. Grueterich *et al*. [52] reported that amniotic membrane culture conditions could promote limbal SC expansion. Chen *et al*. [53] reported that HuAECs could be

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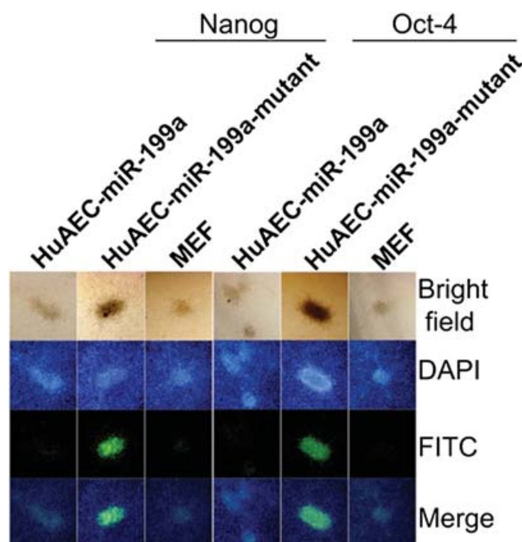

Figure 5 IF assay to determine Oct-4 and Nanog protein expression in human iPS cells cultured on different feeder layers The expression of Oct-4 and Nanog in iPS cells cultured on miR-199a-mutant-transfected HuAECs was more intense than that cultured on HuAECs-miR-199a or MEFs. Magnification, × 200.

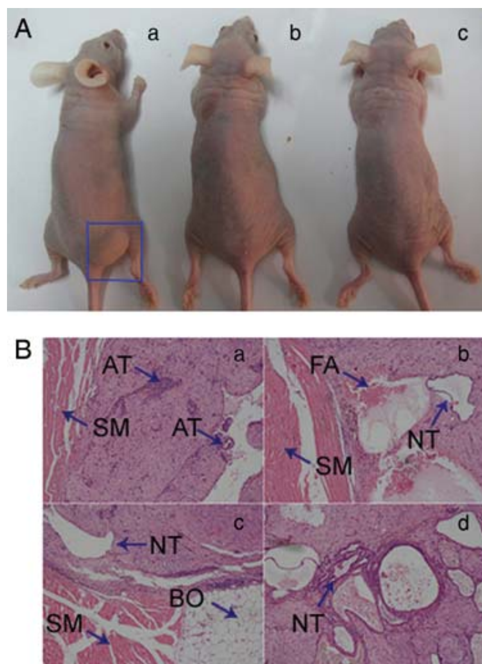

Figure 6 Histology of teratomas in SCID mice (A) Four weeks after injection into the hind leg of SCID mice, teratomas formed from human iPS cells cultured on mutant miR-199a-transfected HuAECs (blue pane), but not from MEFs or HuAECs-miR-199a. (B) Histology of a teratoma composed of ectodermal, endodermal, and mesodermal tissue. AT, archenteron; SM, striated muscle; FA, fat; NT, neural tube; BO, bone. Magnification, ×200.

used as a human feeder layer equivalent, effective for *ex vivo* expansion of adult epithelial stem cells from human limbus. Lekhanont *et al*. [54] developed a serum and feeder-free technique of culturing human corneal epithelial

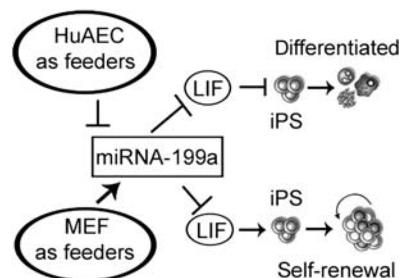

Figure 7 The pathway of endogenous miR-199a-regulated LIF expression in HuAECs and MEFs

stem cells on amniotic membrane. Mouse ESC culture procedures would be simplified if the human placental amnion, routinely discarded as medical waste, could be used as a feasible abundant source of feeder cells [50]. In our previous studies, we suggested that the expression of LIF by HuAECs could maintain mouse and human ESC and mouse spermatogonial stem cells in an undifferentiated, proliferative state capable of self-renewal [19,20,50]. These results indicated that the human placental amnion could also be used as an abundant source of feeder cells for human iPS culture procedures, with a low toxicity and a high safety due to the presence of few exogenous foreign proteins and without ethical constraints.

Recently, Oskowitz *et al*. [38] reported that miRNA-199a can interfere with expression of LIF in human multipotent stromal cells. Therefore, we hypothesized that HuAEC feeder layers maintain human iPS cells pluripotency and self-renewal via high levels of LIF expression, due to the low expression of endogenous miRNA-199a.

In this study, we examined how LIF expression, regulated by endogenous miRNA-199a, participated in HuAEC-induced maintenance of human iPS cells in a self-renewing undifferentiated state. We confirmed that HuAECs expressed low levels of endogenous miR-199a, and high levels of endogenous LIF, compared with MEFs. Exogenous miR-199a interfered with endogenous LIF mRNA and protein expression in HuAECs transfected with miR-199a expressing retrovirus. Additionally, human iPS cells seeded on HuAECs-miR-199a mutant expressed higher levels of LIF compared with those seeded on HuAECs-miR-199a or MEFs. HuAECs-miR-199a-mutant feeder cells allowed human iPS cells to maintain a higher level of AP activity in long-term cultures and express high levels of stem cell markers. The iPS cells cultured on HuAECs-miR-199a mutant were undifferentiated, proliferated, and maintained pluripotency, compared to those cultured on HuAECs-miR-199a and MEFs. One interesting observation from our studies was the presence of LIF in the medium of human iPS cells cultured on HuAECs, even though the medium was not supplemented with LIF, which

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makes the addition of commercially obtained LIF unnecessary. In summary, we found that the miR-199a mediated the regulation of LIF expression in HuAECs, which could maintain the self-renewal and pluripotency of human iPS cells. The mechanism is summarized in **Fig. 7**.

In conclusion, HuAECs, which are obtained from human amniotic membranes, are normally regarded as hospital waste. However, this study demonstrates that they can provide a novel, economical, and time-saving culture material for human iPS cell culture, which does not require the addition of growth factors or LIF. In addition, the use of HuAECs can prevent contamination with extraneous proteins that otherwise leads to cell variation.

Acknowledgements

We thank Dr Jun Liu (Rutgers University, Newark, USA) for discussion and proofreading the manuscript, and thank Dr Lei Xiao (The College of Animal Sciences at Zhejiang University, Hangzhou, China) for giving us the human iPS cells.

Funding

This work was supported by grants from the Shanghai Committee Medical Science Foundation of China (No. 10411967100) and the Natural Science Foundation of School of Medicine of Shanghai Jiaotong University (No. YZ1046).

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