Generation of Induced Pluripotent Stem Cells from Human Amniotic Fluid Cells by Reprogramming with Two Factors in Feeder-free Conditions

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Abstract: The ectopic expression of transcription factors for reprogramming human somatic cells to a pluripotent state represents a valuable resource for the development of in vitro-based models for disease modeling, drug screening and cell therapy applications. iPSCs are usually obtained from fibroblasts after infection with viral constructs carrying the four transcription factors OCT4, SOX2, KLF4 and c-MYC [1]. However, one of the factors, c-MYC, is known as a proto-oncogene, and its reactivation could give rise to transgene-derived tumors [2]. Several studies have successfully produced iPSCs without using c-MYC and obtained viable chimera mice with reduced tumorigenicity [3–5]. Recently, Hester et al. [6] demonstrated that human fetal neural stem cells, which endogenously express SOX2 at a high level, can be reprogrammed by either of two factors (OCT4 and KLF4). This is a major advance toward identifying cells that can be reprogrammed more easily with less manipulation. However, in humans, neural stem cells that can be reprogrammed with only two factors, OCT4 and KLF4, are rare and difficult to obtain.

Human amniotic fluid represents a heterogeneous population that contains cells from the developing fetus. Various types of fetal cells have been detected in amniotic fluid (AF) [7]. Approximately 1% of amniotic fluid cells (AFCs) are categorized as amniotic fluid stem cells [8]. The remaining cells in the AF are terminally differentiated cells (approximately 99%) that have mainly been desquamated from the fetal skin. Recently, our group and other researchers reported that human amniotic fluid cells (hAFCs) could be reprogrammed more rapidly and efficiently than skin fibroblast cells using the four Yamanaka factors [5, 9, 10]. The hAFCs may therefore possess a different genetic and epigenetic landscape that is more ideal for reprogramming than that of fibroblast cells. Here, we succeeded in reprogramming hAFCs into iPSCs via the ectopic expression of OCT4 and KLF4. The hAFCs yielded two-factor iPSCs that fulfill all pluripotency criteria, as determined by their pluripotency gene expression, their capacity to differentiate into various somatic cell types in vitro and in vivo and their direct differentiation into beating cardiomyocytes after induction and differentiation. Furthermore, the two-factor iPSCs can be readily derived on feeder-free surfaces using Matrigel-coated tissue culture dishes, thereby reducing the variability of the reprogramming processes associated with mouse feeder cells. Our results indicate that hAFCs represent an accessible source of cells that can be reprogrammed into iPSCs with two Yamanaka factors. Therefore, hAFCs may become a preferred cell type in the future for safe reprogramming without any exogenous genetic material.

Key words: Feeder-free, Human amniotic fluid cells, Induced pluripotent stem cells

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Materials and Methods

Cell culture

All experiments were approved by the ethical committee of The
Third Affiliated Hospital of Guangzhou Medical College. Human amniotic fluid was obtained by ultrasound-guided amniocentesis performed on pregnant women for routine prenatal diagnosis purposes. This period, ES-like colonies were subjected to analyses of marker dispase. This passaging was repeated for up to 10 passages. During

### Derivation of induced pluripotent stem cells from amniotic fluid cells

Retroviruses were produced as previously described [1]. Briefly, 293T cells for retrovirus production were maintained in retrovirus infection medium [DMEM containing 10% FBS (HyClone, Logan, UT, USA), 2 mM L-glutamine, and 1 mM nonessential amino acids (Invitrogen)]. The cells were transfected with the pMX-based retroviral vectors (a gift from Dr Duanqing Pei of the Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences) encoding the human cDNA of OCT4, SOX2, KLF4 and c-MYC with Lipofectamine LTX & Plus Reagent (Invitrogen) according to the manufacturer’s instructions. To monitor the infection efficiency, a GFP-expressing plasmid, pMX-GFP, was used as a control. Virus-containing supernatants were collected at 48 h after transfection. For viral transduction, hAFCs were seeded at 1×10^5/well in a 6-well plate (Costar, Corning, NY, USA) and 6 h later, the medium was replaced with the virus-containing supernatants (OCT4, SOX2, KLF4 and c-MYC or OCT4 and KLF4) with 8 µg/ml polybrene (Sigma, St. Louis, MO, USA) for infection overnight. After 24 h, infected hAFCs were replated onto mitomycin C-treated MEF cells with human ESC culture medium supplemented with 20% KnockOut Serum Replacement, 1 mM nonessential amino acids, 2 mM GlutaMAX, 0.1 mM b-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen) and 4 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA).

### Alkaline phosphatase staining and immunostaining

To detect alkaline phosphatase (AP) activity, iPS colonies were fixed with PBS containing 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were treated with PBS containing 0.1% Triton X-100 for 15 min at room temperature and then incubated with PBS containing 5% normal goat serum (Chemicon, Billerica, CA, USA) for 1 h at room temperature. The primary antibodies included TRA-1-60 (1:200, Chemicon), NANOG (1:200, Abcam, Cambridge, MA, USA), OCT4 (1:200, Chemicon), α-fetoprotein (AFP) (1:100, Human Germ Layer Marker Kit, Chemicon), α-smooth muscle actin (SMA) (1:200, Human Germ Layer Marker Kit, Chemicon) and troponin T type 2 cardiac (TnTc) (1:200, a cardiomyocyte marker, Chemicon). The secondary antibodies Alexa Fluor 488-conjugated goat-anti-mouse IgG (1:500, Invitrogen) and Alexa Fluor 488-conjugated goat-anti-rabbit IgG (1:500, Invitrogen) were used. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

### Quantitative RT-PCR

Total RNA and cDNA from each sample were prepared using an RNAeasy Plus Mini kit (Qiagen) and QuantiTect Reverse Transcription Kit (Qiagen), respectively, according to the manufacturer’s instructions. Quantitative RT-PCR used to measure gene expression levels was performed using an Applied Biosystems 7500 instrument with SYBR Premix Ex Taq™II (TaKaRa, Otsu, Shiga, Japan). The primer sequences used for quantitative RT-PCR amplification were GACAGGGGGAGGGAGGAGGCTAGG, forward, and CTTCCTCTAACAACAGGTGGCCCAAAAC, reverse, for OCT4; CAGCCCGGATCTTCACAGCCTG, forward, and CGGAAGATTCCTCGGGGTTTACC, reverse, for NANOG; CCCAGGGCCCACTTGGTACT, forward, and TTATCGTCGACTGTGTGCTGCT, reverse for sg-OCT4: GGCACCCTTGCCATGGCCTTGTCCTG, forward, and TTATCGTCGACCACCTTGGTACT, reverse, for sg-NANOG; AAGCCGTTGGCCCTTACTGACCACCT, forward, and TTATCGTCGACCACCTTGGTACT, reverse, for sg-MYC.
additional 7 days. For cardiomyocyte differentiation, the medium was supplemented with 100 ng/ml human recombinant activin A (R & D Systems, Minneapolis, MN, USA) for 24 h, followed by 10 ng/ml human recombinant bone morphogenetic protein 4 (BMP4, R&D Systems) for 4 days. The medium was changed every other day.

**In vivo differentiation**

The iPSC colonies from passages 10 or beyond were treated with a 1 ng/ml dispase solution for 10–15 min and then dispensed into 300–400 small iPS colony suspensions. The colonies were collected and subcutaneously injected into the inguinal grooves of 6-week-old male severe combined immunodeficiency (SCID) mice (Vital River Laboratories, Beijing, China). Eight weeks later, the resultant tumors were removed, fixed for 4–8 h in 4% paraformaldehyde and embedded in paraffin. After staining with hematoxylin and eosin, the sections were examined under a light microscope for the presence of tissues derived from the three germ layers.

**Microarray analysis**

Affymetrix U133 2.0 GeneChips were used for this study. All experiments were performed and analyzed at CapitalBio (Beijing, China). Three biological repeat samples from human AFCs, human ES (FY-hES-1) [11], hAFC-iPS2f-1 (P15) and hAFC-iPS2f-1 (P21) cells were collected and analyzed as described in the Affymetrix Technical Manual.

**Results**

**Generation of iPSCs from hAFCs using two or four factors**

When the hAFCs were passaged 5 times in vitro, we compared expression levels of several marker genes among human ES cells, hAFCs and human fetal fibroblast cells (hFFCs). Data from quantitative real-time PCR (qPCR) revealed strong expression of C-MYC and KLF4 in hAFCs. Trace amounts of NANOG were detected, being similar in hAFCs and hFFCs, but much lower than that in human ES cells (Fig. 1).

The human reprogramming factors OCT4, KLF4, SOX2 and c-MYC or factors OCT4 and KLF4 were expressed in hAFCs as previously described. To monitor the infection efficiency, a GFP-expressing plasmid, pMX-GFP, was used as a control. The infection efficiency reached approximately 80% as determined by the GFP expression. Twenty-four hours after infection, virus-infected hAFCs were plated onto mitomycin C-treated MEF cells. One day later, the medium was changed to human ES cell culture medium. The typical human ES-like colonies appeared 5 days after replating and could be picked on day 9 after four-factor infection. The typical human ES-like colonies appeared 20 days after replating and could be picked on day 25 after two-factor infection. On day 21 after infection, AP staining was performed. We counted the number of human ES-like colonies and those that were positive for AP staining (Fig. 2A). The efficiency of the induction of pluripotency was approximately 0.46% in hAFCs infected with four factors and approximately 0.01% in hAFCs infected with two factors (data not shown). The efficiencies of reprogramming human dermal and neonatal foreskin fibroblasts to iPSC cells utilizing OCT4, KLF4, SOX2 and c-MYC have been shown to range between 0.01% and 0.02% [1, 3, 12], which is quite similar to our reprogramming efficiencies of hAFCs with OCT4 and KLF4. Eight colonies from 1 × 105 hAFCs infected with four factors were picked and transferred onto Matrigel for further expansion, and 5 iPS cell lines (named hAFC-iPS4f-1, 2, 3, 4 and 5) were established. Three colonies from 1 × 105 hAFCs infected with two factors were picked and transferred onto Matrigel for further expansion, and two iPS cell lines (named hAFC-iPS2f-1 and 2) were established. HAF-iPS4f-1 and hAFC-iPS2f-1 (Fig. 2B, C) were passaged more than 20 times. The two iPS lines showed normal karyotypes (46XX) (Fig. 2D).

**3.2 hAFC-iPS cells express hES markers**

To validate the characteristics of the hAFC-iPS4f and hAFC-iPS2f cells, the expression of undifferentiated ES marker genes was tested by quantitative RT-PCR and immunocytochemical analysis. Quantitative RT-PCR results revealed that the endogenous expression levels of OCT4 and NANOG were comparable to those in hES cells (Fig. 2E). The expression of OCT4, NANOG and the human ES cell-specific surface marker TRA-1-60 was detected in hAFC-iPS4f and hAFC-iPS2f cells. As shown in Fig. 2F, all selected colonies were positive for OCT4, NANOG and TRA-1-60. Quantitative RT-PCR using primers specific for retroviral transcripts confirmed efficient silencing of all the four retroviruses (Fig. 3).

**In vitro differentiation of hAFC-iPS cells**

Floating cultivation of EBs was used to determine the differentiation ability of hAFC-iPS cells in vitro. After 7 days in suspension culture, hAFC-iPS2f-1 cells formed EBs, which were transferred onto gelatin-coated plates and cultured for an additional 7 days. Immunocytochemistry results further showed that the differentiated cells were positive for α-fetoprotein (AFP, endoderm), α-smooth muscle actin (α-SMA, mesoderm) and Tuj1 (ectoderm) (Fig. 4 A–I). We then examined the directed cardiac differentiation of hAF-iPS2f-1 cells using a recently reported protocol, which utilizes activin A and bone morphogenetic protein (BMP) 4 [13]. Twelve days after the
Fig. 2. Characterization of iPSCs from hAFCs using two or four factors. A: hAFC-iPS<sup>2f</sup> cells were stained for alkaline phosphatase (AP). B: A typical hAFC-iPS<sup>2f</sup> cell colony growing on a feeder-free Matrigel surface. C: A typical hAFC-iPS<sup>2f</sup> cell colony growing on a feeder-free Matrigel surface. D: The karyotype of hAFC-iPS<sup>2f</sup> cells is 46XX. E: Quantitative RT-PCR results showing that the endogenous expression levels of OCT4 and NANOG in hAFC-iPS<sup>2f</sup> and hAFC-iPS<sup>4f</sup> cells are similar to those in hES cells. F: Immunostaining of hAFC-iPS<sup>2f</sup> cells and hAFC-iPS<sup>4f</sup> cells. hAFC-iPS<sup>2f</sup> cells and hAFC-iPS<sup>4f</sup> cells were positive for the pluripotency genes OCT4 and NANOG, and they expressed the cell surface marker TRA-1-60 (scale bars, 100 μm).

Fig. 3. Quantitative RT-PCR for expression of retroviral transgenes in hAFC-iPS<sup>2f</sup>-1, hAFC-iPS<sup>4f</sup>-1, hAFC and hAFC 6 days after transduction with the four retroviruses (hAFC/4f-6d). Shown are the averages and standard deviations of three independent experiments. The value of hAFC/4f-6d was set to 1 in each experiment.

Fig. 4. In vitro differentiation of hAFC-iPS<sup>2f</sup> cells. iPSCs were first cultured in a floating culture for 7 days and were then attached to gelatin-coated dishes for an additional 7 days for differentiation. Immunocytochemistry results show differentiated hAFC-iPS<sup>2f</sup> cells expressing (A) Tuj1, (D) α-smooth muscle actin and (G) α-fetoprotein. (J) Phase-contrast image of iPSCs differentiated into cardiomyocytes. (L) Immunocytochemical analysis of cardiomyocyte marker TnnTc (scale bars, 100 μm).
induction of differentiation, clumps of cells started beating (Suppl Movie S1: online only). Immunocytochemistry results further showed that these cells expressed cardiomyocyte markers, such as troponin T type 2 cardiac (TnTc) (Fig. 4 J–L). These data demonstrated that hAFC-iPS cells possess the potential to differentiate into all three germ layers in vitro.

In vivo differentiation of hAFC-iPS cells

To demonstrate the pluripotency of hAFC-iPS cells in vivo, hAFC-iPS cells were subcutaneously injected into immunodeficient (SCID) mice. Eight weeks after injection, we observed teratoma formation. Histological examinations showed that the teratomas contained various tissues comprising all three germ layers, such as neural cells (ectoderm), cartilaginous tissue (mesoderm) and glandular tissue (endoderm) (Fig. 5).

Gene expression of hAFC-iPS cells

To characterize the hAFC-iPS cells at the global gene expression level, we performed microarray analysis for the following cell lines: hAFCS, FY-hES-1, hAFC-iPS2f-1 and hAFC-iPS4f-1. Global gene expression patterns indicated that hAFC-iPS2f-1 and hAFC-iPS4f-1 had a high similarity with FY-hES-1, with correlation coefficients of 0.9750 and 0.9709, respectively (Fig. 6). In contrast, the hAFC-iPS2f-1 and hAFC-iPS4f-1 cells had low similarities with parental hAFCs, indicating that the hAFCs were successfully reprogrammed.

Discussion

In this study, we identified a new source of human cells that can be efficiently reprogrammed into human iPSCs by the ectopic exposure of only two factors, OCT4 and KLF4. The mechanism of this two-factor reprogramming is probably largely due to the intrinsic expression of C-MYC and KLF4 within hAFCs. This is an important step toward the goal of developing safe and efficient protocols for generating patient-specific iPSCs from accessible cells for directed cell differentiation and transplantation. Furthermore, we show that human iPSCs can be readily generated under feeder-free conditions using hAFCs, which reduces the variability of reprogramming associated with the use of mouse feeder cells. HAFC-iPS2f and hAFC-iPS4f cells express ES cell markers and genes associated with pluripotency at similar levels to hES cells and are morphologically indistinguishable from their hES cell counterparts. Global gene expression patterns are also similar to hES cell profiles. HAFC-iPS cells can differentiate into cells belonging to all three germ layer types both in vitro and in vivo and can also directly differentiate into beating cardiomyocyte after induction and differentiation, thereby indicating they are true pluripotent cells.

Using hAFCs as a potential source of for reprogramming to iPSCs offers multiple advantages. First, AFCs are routinely harvested in antenatal examinations to enable the early detection of diseases. In most cases, more cells are obtained than are actually needed, and these cells are eventually discarded. Because these cells have not aged, they have fewer environmentally induced mutations, making them genetically more stable. Thus, it is possible to reprogram these AFCs faster and more easily than other cell types, making hAFC-iPS cells an interesting complement to ES cells. Second, because the reprogramming process requires only two factors, the risk of changes due to insertional mutagenesis is minimized. Furthermore, obviating the requirement for c-MYC reduces the risk of inducing tumorigenicity [2, 3]. In addition, because two factors are sufficient for human iPSC induction from hAFCs, it may be possible to substitute one or both of the two factors with small molecules that activate OCT4 or KLF4 gene expression. For example, several recent reports have demonstrated that small molecule activators and inhibitors of epigenetic regulation and signaling pathways can replace the activity of individual reprogramming transcription factors [14-17]. These reports suggest that it may be possible to reprogram hAFCs into iPSCs by OCT4 and small molecule modifiers of epigenetic regulation, further minimizing the undesired effects of utilizing retroviral vectors. An additional advantage of utilizing hAFCs is that if the homozygous state of a genetic disease is diagnosed, reprogramming these cells into iPSCs can begin during the pregnancy immediately after diagnosis to offer early treatment in the neonatal period.

In summary, we demonstrated that hAFCs obtained during prenatal diagnosis can be reprogrammed into iPSCs by two transcription factors, OCT4 and KLF4, without the use of additional small molecules. The feeder-free derivation of iPSCs from hAFCs represents a more clinically applicable method for the derivation of iPSCs compared with other cell types and should enable the more efficient and rapid generation of disease-specific iPSCs, such as for β-thalassemia.
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