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Expression level of pluripotent genes in incomplete reprogramming

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ABSTRACT

Objective: To compare the expression levels of pluripotent genes among incomplete reprogrammed colonies and induced pluripotent stem cells (iPSCs), to explore the relationship between the expression of pluripotent genes and incomplete reprogramming. **Methods:** Four genes (Oct4, Sox2, Klf4, C-Myc) were introduced into human foreskin fibroblasts (HFFs) by retroviruses. The HFFs were induced to reprogramming. Different forms of colonies were picked up, analyzed, and compared with iPSCs from different aspects, including the morphology of clones, alkaline phosphatase (AP) staining, immuno-fluorescence, and Q-PCR. **Results:** In the reprogramming process, different colonies were emerged, some of them exhibited typical human embryonic stem cell morphology (*eg*., compact colonies, high nucleus-to-cytoplasm ratios, and prominent nucleoli). However, these colonies couldn't maintain these characters after passage. There was an intermediate state, named partially reprogramming. Through analysis and identification, AP staining results were weakly positive, compared with iPSC colonies. The immuno-fluorescence staining demonstrated these colonies just expressed pluripotent protein Oct4. Q-PCR indicated that the expression of exogenous transcription factors was inappropriate, either at a high level or at a low level. Most of the endogenous pluripotency genes were expressed at a low level. **Conclusions:** It may be one of the causes of incomplete reprogramming that the exogenous pluripotent gene is low-expressed or over-expressed, and successful reprogramming may depend on a specific stoichiometric balance of Oct4, Sox2, Klf4 and c-Myc.

1. Introduction

Thalassemia is a monogenetic blood disease which injures the health of people in the tropical and subtropical regions, such as Southeast Asia, Southern China and a few African regions. Current treatments mainly depend on blood transfusion, which brings heavy burdens on these families. Allogeneic hematopoietic stem cell transplantation is the only radical cure. However, the donors are limited. Since Takahashi and Yamanaka[1] generated induced pluripotent stem cells (iPSCs) from mouse embryonic fibroblasts (MEFs) by introducing four factors (Oct4, Sox2, c-Myc and Klf4), iPSCs have brought up a startling development in the field of stem cell biology, because it could provide disease-specific and patient-specific stem cells for modeling human disease and personalized regenerative cell therapy[1-3]. This would provide a new therapeutic option for Thalassemia.

It is termed reprogramming when somatic cells are transformed to iPSCs using exogenous factors. At the late stage of reprogramming, some colonies appear, similar to embryonic stem (ES) colonies in morphology, but only subsets of these colonies have comparable molecular and functional features[2]. Most of colonies could not meet the gold standard of embryonic stem (ES) clones, which are in

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an intermediate state, termed as incomplete reprogramming. Chan *et al*[3] have documented that incompletely reprogrammed colonies could exhibit some features of reprogramming, including downregulation of fibroblast markers, upregulation of pluripotency markers, changes in the histone modification state, acquisition of ES cell-like morphology, and ability to form teratoma-like tumors. It is proposed that the incomplete reprogramming state may be relative to the expression of exogenous and endogenous plruipotent genes. In this study, human foreskin fibroblasts (HFFs) were reprogrammed by introducing hOct4, hSox2, hc-Myc and hKlf4, and then different colonies were picked up, to compare the difference of expression of pluripotent genes between incomplete reprogramming cells and iPSCs.

2. Materials and methods

2.1. Mice

The ICR mice were purchased from STA experimental animal company (Hunan, China), and kept in the SPF class condition. These mice were used for the isolation of MEFs.

2.2. Cell culture

HFFs were derived from the foreskin of an approved nine-year-old donor, who has no history of viral infection and special diseases, after informed consent. The isolation of HFFs was performed as described[4] by Panula *et al*. In brief, the foreskin was washed with PBS, finely minced, and digested by 0.15% trypsin (Gibco) for 20 min at 37 ℃. Trypsinization was stopped by adding HFF medium (DMEM containing 10% FBS, 1% NEAA). Cells were collected by centrifugation, resuspended in fresh HFF medium, and then seeded on culture dishes and cultured in the incubator. When the cells were 80%-90% confluent, HFFs were frozen or expanded. Mycoplasma-negative HFFs could be used for reprogramming.

MEFs were used as feeder for iPSCs derivation and prepared as described[5] by Jozefczuk *et al*. The pregnant mouse was sacrificed at 13.5 d.p.c.. Uteri were dissected off and washed with PBS. Embryos were separated from placenta and embryonic sac, and the head, limb, tail and red organs were removed. The remaining bodies were washed, minced, and then digested by adding 0.15% trypsin (Gibco) for 20 min at 37 ℃. After trypsinization, MEF medium (DMEM containing 10% FBS and 1% NEAA) was added and pipetted up and down to help with tissue dissociation. The suspension was centrifuged, and cells were resuspended with MEF medium and cultured on 100 mm dishes. When cells were 80%-90% confluent, MEFs were frozen or expanded. Generally, MEFs were used within three passages to avoid replicative senescence. When the test of mycoplasma was negative, MEFs were inactivated with 10 μg/mL of mitomycin C and used as feeder.

iPSCs, granted by the Chinese Academy of Sciences Guangzhou Institute of Biomedicine and Health and used as controls, were maintained in mTeSR medium (Stemcell) on Matrigel (Stemcell)-coated tissue culture plate[6]. Cells were passaged routinely with EDTA as described previously[7]. In Brief, cells were washed twice with F12, and then incubated with 0.5 mM EDTA for 5 min at 37℃. After EDTA was removed, stem cells were washed off swiftly with appropriate mTeSR, and transferred into new Matrigel-coated culture plates. iPSCs were passaged every 5-6 d.

293T packaging cells were used to produce retroviruses, were maintained in DMEM containing 10% FBS.

2.3. Retrovirus production

The plasmid pMXs (containing GFP, Oct4, Sox2, Klf4, and c-Myc) and pCL were purchased from Adgene. The 293T cells were transfected by calcium phosphate precipitation as described by Longan *et al*[8]. Two rounds (48 h and 72 h after transfection) of supernatants were collected, filtered (0.45 μm pore-size filter, Millipore). Polybrene (8 μg/mL, Sigma) was added to increase infection efficiency.

2.4. iPS cells derivation

HFFs were seeded in a 6-well plate at 3×10^4 cells per well one day before transduction, and were incubated with the virus-containing supernatant. On the sixth day of post-infection, the infected cells were split by using 0.25% trypsin, and plated at 1×10^4 cells on feeder in a 100mm dish. After 24 h, the medium was replaced with the reprogramming medium (DMEM/F12 containing 20% DFBS, 2 mM non-essential amino acid, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 80 ng/mL FGF-2 and 1% penicillin-streptomycin), and treated with 1 mM VPA and 50 μg/mL vitamin C[9]. The medium was changed every day until the ES-like colonies were emerged, and then the medium were replaced with mTeSR. Generally, these colonies were picked up on the 20th-23rd days of post-infection. The GFP ratio was determined by Flow Cytometer at 4-days after viral infection, monitoring the infection efficiency.

2.5. AP staining

AP staining was performed as previously[10]. Briefly, iPS, ES-like colonies (T1 and T2) were fixed with 4%

paraformaldehyde for 2 min, rinsed twice with TBST, and then stained with BCIP/NBT (Boling Kewei Biotechnology Co. Ltd, Beijing). Cells were rinsed with PBS, and observed under microscopy.

2.6. Immuno-fluorescent staining

iPS, ES-like colonies (T2) were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton in PBS for 30 min, blocked with 10% goat serum for 1h. Cells were incubated with primary antibodies for 1h, washed, and incubated with secondary antibodies for 1h. After washing, the nuclei were stained with DAPI (Invitrogen) for 5min. Coverslip was mounted on a slide using glycerol and sealed with nail polish, and cells were observed under the conventional fluorescence microscope (Olympus). The primary antibodies (anti-Oct4, anti-SSEA4) were purchased from Chemicon/Millipore (Billerica, MA), the secondary antibodies were purchased from GeneTex.

2.7. Extraction of RNA and RT-PCR

Total RNAs were extracted from iPSC, T1 clone and T2 clone using Rneasy® Plus micro kit (Qiagen, Valencia, CA). Total RNAs were reverse-transcribed into cDNAs with Reverse Transcription system (Promega). Q-PCR was performed using quantitative PCR instrument (Agilent) and Brilliant SYBRR Green QPCR Master Mix (Agilent), according to the manufacturer's instructions, GPDH was used as an internal control gene. The primers were shown in the Table 1.

Table 1

Primers of Q-PCR for the endogenous and exogenous gene.

Gene	Sequence of primer (5′to3′)	
GPDH	Forwards primer	CGAGATCCCTCCAAAATCAA
	Reverse primer	TGTGGTCATGAGTCCTTCCA
Oct4	Forwards primer	GAAGGATGTGGTCCGAGTGT
	Reverse primer	GTGAAGTGAGGGCTCCCATA
Sox2	Forwards primer	ACACTGCCCCTCACACAT
	Reverse primer	TGTCATTTGCTGTGGGTGAT
Nanog	Forwards primer	AAAGCTTGCCTTGCTTTGAA
	Reverse primer	CTTTGGGACTGGTGGAAGAA
TERT	Forwards primer	GCATCATCAAACCCCAGAAC
	Reverse primer	GAGGAGCTCTGCTCGATGAC

3. Results

3.1. Package of retrovirus and the transfection of HFF

The 293T cells were transfected with pMXs-based vector and pCL package vector by calcium phosphate precipitation. After 48 h, the 80%-90% of 293T showed expression of GFP, as shown in Figure 1A and 1B, which indicated the 293T could provide enough virus to infect HFFs. After two rounds of infection, over 80% of HFFs showed expression of GFP under the inverted Immuno-fluorescence microscope (Olympus IX 71), shown in Figure 1C and 1D. The infection efficiency of HFF was tely 85%, evaluated by analyzing GFP-infected cells with AriaII Flow Cytometer (BD Company), shown in Figure 1E and 1F.

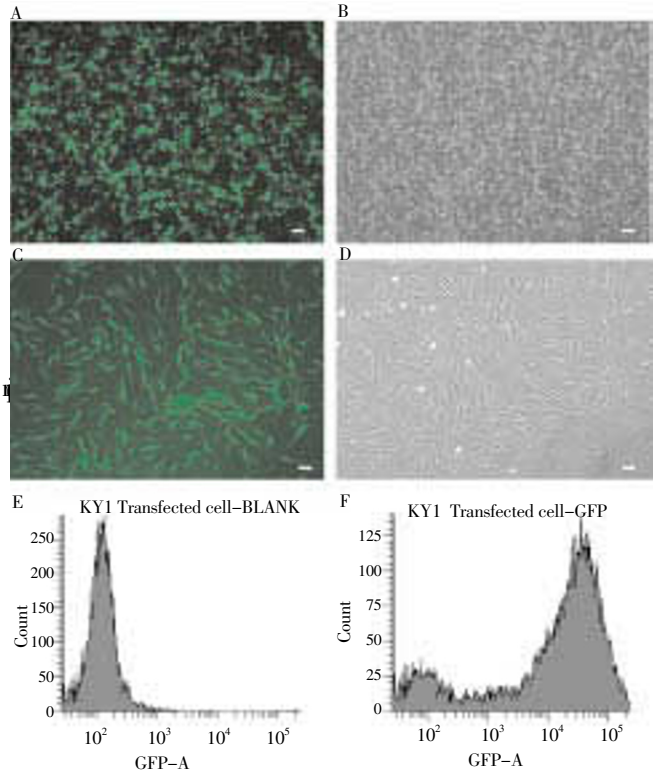

Figure 1. Package of virus and the infection of HFFs.
293T cells were transfected with pMXs-based vectors and PCl package vectors by calcium phosphate precipitation. After 48 hours, The supernatant containing virus were collected and were used to infect HFFs. The contrast group were infected by the virus carring GFP. The GFP rate could monitor the efficiency. A and B: the GFP-transfected 293T showed the efficiency of virus production. B and C: The GFP-transfected HFFs indicated the infection efficiency. E and F: On the forth day post-transfection, HFFs were collected and analyzed by flow cytometor, the rate of GFP-positive HFF was 86%. (Bar: 200 μm).

3.2. Derivation of iPS cells

HFFs were grown by adherence, and were uniformly spindle-shaped, shown as Figure 2A. Generally, HFFs were used in reprogramme at the early passage. When four genes (Oct4, Sox2, Klf4 and c-Myc) were introduced into HFFs, HFFs gradually changed in the morphology, including

transformation from the spindle shape to the circular or polygonal shape, increased nucleo-cytoplasmic ratio, and more obvious nucleolus, as shown in Figure 2B -2F.

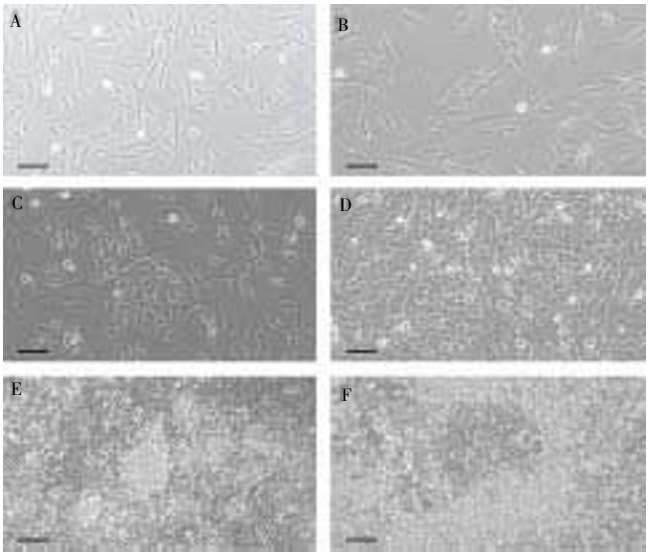

Figure 2. Change of morphology of HFFs after transfection.
After transfection, the HFFs were cultured in iPS medium, which was changed each day. A: HFFs at the third passage before transfection. B. The first day after transfection, very few HFFs varied in morphology. C. The second day after transfection, the spindle-shaped HFFs become elliptic and polygonal. D. The third day after transfection, the HFFs proliferated rapidly, more HFFs changed in morphology. E. The forth day after transfection. F. the sixth day after transfection. (Bar: 100 μm).

On the 6th day after viral infection, the infected cells were trypsinized and seeded on the feeder. These cells grew in a concentrated trend, shown in Figure 3A. Then, some colonies emerged, as shown in Figure 3B and 3C. However, cells would differentiate or die in most of colonies. These colonies could not maintain the characteristics of stem cells, as shown in Figure 3D-3H. Among these colonies, T1 clone and T2 clone were chosen to identify. A typical iPS clone would grow and maintain like ES colonies: compact colonies, high nucleus-to-cytoplasm ratios, and prominent nucleoli, shown in Figure 3I.

3.3. Expression of alkaline phosphatase

Alkaline phosphatase is related to pluripotency[11], expressed highly in undifferentiated iPSCs. AP staining can indirectly reflect the pluripotent state. It was shown in Figure 4 that the color was deep in iPS clone, but the color was light in T1 clone and T2 clone, which indicated the high expression of alkaline phosphatase in iPS clone and the low expression of alkaline phosphatase in T1 clone and T2 clone.

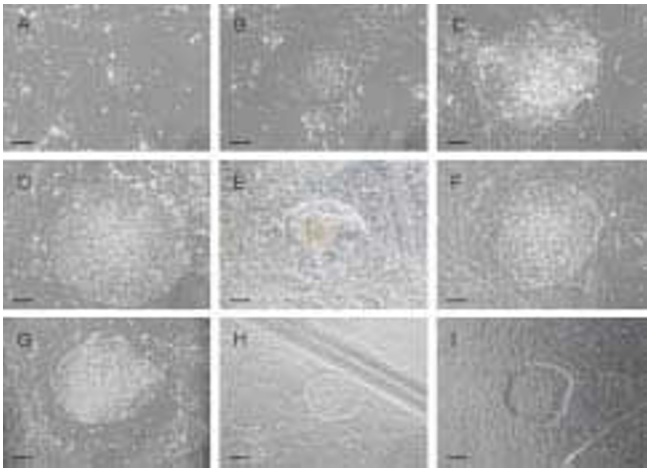

Figure 3. Evolution of HFFs after seeded on feeder.
On the sixth day after transfection, HFFs were trypsinized, seeded on feeder layer at 1×10^4/100 mm dish, and cultured in iPS medium supplemented with VPA and Vitamin C. A: On the second day after plating. B: the fourth day after plating. C: the sixth day after plating. D, E, F, G, H: Between 15th and 25th day after plating, some colonies were emerged, named by T1, T2, T3, T4, T5 clone respectively. F: The iPS clone. as the contrast group. (Bar: 100 μm).

Figure 4. Alkaline phosphatase staining of T1 clone, T2 clone and iPS clone.
On the forth day after passage, the colonies were detected by AP staining. A: T1 clone. B: T2 clone. C: iPS clone. (Bar: 200 μm).

3.4. Immuno-fluorescent staining of Oct4 and SSEA4 in T2 clone and iPS clone.

Oct4 and SSEA4 were the specific markers which reflected the pluripotency. Immuno-fluorescent staining showed that Oct4 and SSEA4 expression in iPSCs, Oct4 expression in T2 clone, shown in Figure 5.

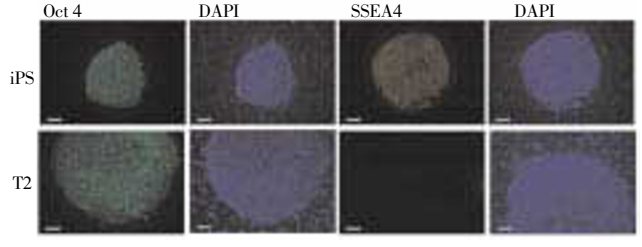

Figure 5. Immuno-fluorescent staining of iPS clone and T2 clone.
On the forth day after passage, the colonies were washed and stained for anti-Oct4 , anti-SSEA4, and DAPI. iPS clone: Oct4(+), SSEA4(+). T2 clone: Oct4(+), SSEA4(-). (Bar: 200 μm).

3.5. Expression of pluripotent genes in iPS colonies and incomplete reprogramming colonies.

For the expression of endogenous genes (Oct4, Sox2, Nanog, TERT), these genes were expressed at high level in the iPS clone. However, Nanog, TERT and Sox2 were expressed at low level in the T1 clone, and Oct4, Nanog and TERT were low-expressed in the T2 clone. The results were shown in Figure 6.

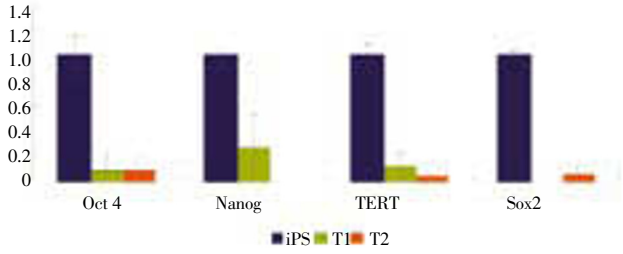

Figure 6. Expression of pluripotent genes in different colonies.
The colonies were cultured in Matrigel-coated OC dish with mTeSR, on the fifth day after passage, the expression of plruipotent genes was determined by Q-PCR. The expression of endogenous genes (Oct4, Sox2, Nanog and TERT) were detected in T1 clone, T2 clone, compared with iPS clone.

4. Discussion

The somatic cell can be induced to iPSCs by the ectopic co-expression of Oct4, Sox2, Klf4, c-myc[12]. The iPSCs exhibit the similar morphology as ESCs: compact colonies, high nucleus-to-cytoplasm ratios, and prominent nucleoli, and have the potential for unlimited expansion and the ability to generate cells of all three germ layers – endoderm, mesoderm and ectoderm[13]. In this paper, HFFs were reprogrammed by introducing four genes through the infection of virus.

The expression of reprogramming factors resulted in the nuclear reprogramming and the cytoplasm reprogramming, characterized by the variation of cellular morphology and growth characteristics. In this study, it was observed that, in the early reprogramming, the infected cells grew with the concentrated trend, keeping some characteristics of the fibroblasts. In the late reprogramming, some colonies appeared, but only a handful of colonies can reach the standards of ES cells, most of them could not maintain the features, followed by differentiation or apoptosis. It is assumed that the evolution in the reprogramming may be involved in the expression of pluripotent genes.

In this study, pluripotent genes (Oct4, Sox2, Nanog, TERT) were lower-expressed in T1 clone and T2 clone, compared with iPS. In the reprogramming, the expression of pluripotency genes is upregulated, and the expression of differentiation genes is downregulated[14]. But the expression levels of pluripotent genes need to reach a certain degree to maintain pluripotent stem cells. For example, the expression of Oct4 is strictly limited to stem cells in the development of body, which is necessary for the continuous proliferation of stem cells. This is the key factor for the start of reprogramming, when the expression of Oct4 is 50% -150% of the normal expression[15,16]. Therefore, the inappropriate expression of pluripotent genes may be the cause that those ES-like colonies couldn't maintain the features of ES cells. From another perspective, over-expression or low-expression of pluripotent genes is also a feature of partial reprogramming[17].

Orkin[18] proposes that there is an intermediate state in the reprogramming, namely a state of partial reprogramming[19]: including that chromatin structure is relaxed, and some pluripotent genes are activated. Cells in such state can be induced to iPSC or other types of cell under different culture conditions[20]. This state may be conducive to further explore the mechanism of reprogramming.

Conflict of interest statement

We declare that we have no conflict of interest.

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