Transient Epigenetic Reprogramming Human Skin Fibroblasts Differentiates into Cells with Multipotentiality *In Vitro* and *In Vivo*

Xiang-Qing Zhu^{1,2}, Guang-Ping Ruan¹, Qing-Hua Chen², Rong-Qing Pang¹,
Xue-Ming Cai¹, Zi-An Li¹, Cui-Zhu Chen¹, Li Lei¹, Qiang Wang¹,
Jie He¹, Jing Zhao¹ and Xing-Hua Pan^{1*}

¹ The Research Center of Stem Cell, Tissue and Organ Engineering, Kunming General
Hospital of PLA, Kunming 650032, P. R. China

² Kunming University of Science and Technology, Kunming 650093, P. R. China

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Summary Stem cells are multipotent and have the potential to play an important role in the treatment of many diseases. However, limited sources of stem cells restrict their clinical application. To solve this problem, a simple *in vitro* epigenetic reprogramming technique to differentiate skin fibroblasts into multipotent cells has been established in our center. In the present study, human skin fibroblasts were induced into multipotent stem cells *in vitro* by epigenetic reprogramming. The differentiation potential of the induced multipotent stem (iMS) cells *in vitro* and *in vivo* was also explored. After transient reprogramming, stem cell markers, including Oct-3/4, Nanog, Sox-2, SSEA-4 and C-myc, were activated in the reprogrammed cells. When these cells were treated with β -mercaptoethanol (β -ME) and all-*trans* retinoic acid (RA), they were differentiated into neural cells *in vitro*. These reprogrammed cells were multipotent as demonstrated by their ability to differentiate and form teratomas. Neural cells were found in the teratomas. The results indicated that the iMS established in our laboratory are multipotent and can grow into neural tissues. iMS may be effective in regenerative medicine in order to treat tissue damage, genetic disorders, and neuronal degenerative diseases.

Key words Multipotent stem cells, Skin fibroblasts, Neural cells, Teratomas.

Induced pluripotent stem cells have great potential for treating many diseases; however, viral vectors are necessary for stem cell induction leading to very low efficiency. We previously used fish oocyte extracts to induce human skin fibroblasts into multipotent stem cells, an idea that led us to this study (Zhu *et al.* 2010). Fibroblast cells are terminal cells derived from mesenchymal stem cells (MSCs), which normally cannot differentiate into other types of cells *in vivo*. However, recent studies have shown that human skin and mouse skin fibroblasts can be induced into pluripotent stem cells *in vitro* using viral vectors, and they can differentiate into neurons, blood vessels and other types of cells under the appropriate conditions (Tweedell 2008, Yang *et al.* 2007). Thus, we proposed a simple approach in order to generate induced multipotent stem (iMS) cells from human skin fibroblasts. In the present study, we first explored a very simple approach to generate induced multipotent cells *in vitro* by epigenetic reprogramming. Second, we aimed to investigate whether neural cells can be produced from iMS cells *in vitro*. Third, to examine whether a teratoma could be formed and neural cells generated, we injected iMS cells into nude mice. Here we showed that transient epigenetic reprogramming of fibroblasts *in vitro* is effective to differentiated neural cells

* Corresponding author, e-mail: panxhynkm@yahoo.cn

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both *in vivo* and *in vitro*. Thus, this study provides the foundation for the treatment of neural degenerative disease.

Materials and methods

Experiment reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco. Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA, USA). The mouse anti-human *Oct-3/4* monoclonal antibody, used in immunohistochemical analysis, was from SANTA CRUZ Biotechnology (CA, USA). Mouse anti-human *Oct-3/4*, *Nanog*, *SSEA-4*, used in flow cytometric analysis, was from BD (USA). 3,3'-diaminobenzidine (DAB) chromogenic liquid was purchased from MaiXin Bio-Company (Fuzhou, P. R. China).

Culture of human fibroblasts

All experimental protocols and procedures were approved by the Institutional Care and Use Committee at the Kunming General Hospital of PLA (Permit Number: SYXK2007-041). (The patients were asked to sign the a form indicating informed consent). Human fibroblasts were cultured from the skin obtained after adult circumcision (from Kunming General Hospital of PLA) using methods previously described (Hu *et al.* 1998, Hu *et al.* 1997). Briefly, the skin tissues (1×1 cm²) were collected under sterile conditions. The skin was cut with scissors into small pieces (1 mm²) in phosphate buffered solution (PBS) containing 100 U/ml ampicillin and 100 mg/ml streptomycin, and was digested with DMEM containing 200 U/ml collagenase and 300 U/ml hyaluronidase for 24 h at 4°C. After centrifugation, the tissues were treated with PBS containing 0.25% trypsinogen and 0.02% ethylenediaminetetraacetic acid (EDTA) for 20 min in a shaking waterbath at 37°C. The reaction was terminated by adding 10 ml DMEM/F12 medium containing 10% fetal bovine serum (FBS). The cells were collected by centrifugation for 10 min at 1000 rpm. After rinsing twice with PBS, they (2×10⁵/ml) were seeded in 6 well plates in DMEM/F12 medium containing 10% FBS at 37°C, with 5% CO₂ and 90% humidity. The medium was changed every 2 d. After reaching confluency, the purity of cells were verified by anti-CK19 and anti-Vimentin antibodies (at 1 : 100 dilution) and then subsequently used for followed experiments.

Preparation of fish oocytes extracts

Fish oocytes were obtained from local fish production farms. They were collected under sterile conditions and were ground by mortar and extracted with equal volumes of 0.9% sodium chloride. Oocytes extracts were centrifuged at a speed of 1000 rpm for 10 min and followed by 4000 rpm for another 30 min. Supernatants were collected and filtrated using 0.22 μm filters. Protein levels of the extracts were determined as described previously (Chen *et al.* 2006). The extracts were diluted using DMEM/F12 medium into 40 mg protein/ml and preserved at -80°C until the experiment was conducted.

In vitro cell reprogramming

Third passage primary cultured fibroblasts were used for cell treatment experiments. Exponentially growing fibroblasts were digested with trypsin-EDTA (Invitrogen, CA, USA) and 4×10⁵ cells were inoculated in new 6-well plates for cell reprogramming.

Cell membrane permeabilization was not performed for cell reprogramming procedure, instead they were directly reprogramed using fish oocytes extract. In this approach, cells were directly treated for 48 h and 72 h (Zhu *et al.* 2010) with fish oocyte extract at a concentration of 1.2 mg protein/ml.

In vitro differentiation assay

After induction using the fish oocyte extract, the reprogrammed fibroblasts were incubated to test whether they could be differentiate into neural cells (Woodbury *et al.* 2000). Initially we used 100 μ mol/L β -mercaptoethanol (β -ME) with 20% newborn serum L-DMEM culture for 24 h, the culture medium was then replaced with culture medium containing 1 μ mol/L all-*trans* retinoic acid (RA), 100 μ mol/L β -ME, and 10% newborn serum in L-DMEM. The total time of induction was 10 d. Cells not undergoing induction by fish oocyte extract and with direct differentiation were used as negative controls.

Immunohistochemical staining

Cells were fixed using 4% paraformaldehyde and were washed with PBS 3 times for 5 min each. They were incubated with 0.3% Triton-100 for 5 min, washed 3 times with PBS, and blocked with endogenous peroxidase with 3% H₂O₂ for 5 min. Cells were washed 3 times with PBS. Anti-human antibody CK19, Vimentin and Oct-3/4 at a dilution of 1 : 100 was added to the fixed cells in both induced and control groups and incubated at 4°C for 12 h, after which time they were washed 3 times with PBS. Horseradish peroxidase (HRP)-labeled second antibody (Gene Tech Company, Ltd., Shanghai, China) was added and was incubated at 37°C for 20 min, followed by the PBS washing process. After staining with DAB working solution, cells were observed under an optical microscope.

Flow cytometric analysis

The cell suspension was adjusted to a concentration of 1×10^6 cells/ml, then they were fixed with 100 μ L of 4% paraformaldehyde solution for 10–15 min. The cells were incubated with saponin solution (0.5%, 100 μ L) at room temperature for 15 min. They were then washed twice with PBS/BSA buffer containing 0.5% saponin. The cells were incubated with the following antibodies: Oct-3/4-PE, Nanog-PE, and SSEA-4-PE. These 3 antibodies were used at 20 μ L per test at room temperature for 30 min. They were washed with 2 ml of PBS/BSA twice, centrifuged at 400 *g* for 5 min and the resulting supernatants were discarded. Isotype controls were stained with an isotype-matched control of irrelevant specificity.

PTM voltage and compensation were set using cell surface staining controls. Quadrant markers based on isotype control and unstained cells were set. The sample cells were analyzed. Data were acquired with a FACSCalibur flow cytometer and analyzed with CELLQuest software (both from Becton Dickinson).

Quantitation of stem cell marker gene expressions

Total RNA was extracted from cells with Tri-Reagent (Sigma, St. Louis, MO, USA) and treated with DNase I to eliminate contamination of genomic DNA. cDNA was synthesized with RNA reverse transcriptase and gene expression was examined by Polymerase Chain Reaction (PCR). The cDNAs and primers were heated to 95°C for 5 min, then amplified by 26 cycles at 95°C for 30 s (s), 57°C for 45 s and 72°C for 45 s. PCR primers used for mRNA quantitation included CD34-sense: 5'-GCG CTT TGC TTG CTG AGT TT-3' and antisense: 5'-GCC ATG TTG AGA CAC AGG GT-3'; Sox2-sense: 5'-AAA AAG CAG GCT TGT ATA ACA TGA TGG AGA CGG-3' and antisense 5'-AGA AAG CTG GGT TTC ACA TGT GCG ACA GGG GCA GT-3'; c-Myc-sense: 5'-CAC CAT GCC CCT CAA CGT GAA CTT CAC C-3' and antisense 5'-TTA TGC ACC AGA GTT TCG AAG CTG TTC G-3'; and internal control 18S RNA-sense: 5'-GCG GCT TTG GTG ACT CTA and antisense: 5'-CTG CCT CCT TGG-3' ATG TG.

Teratoma formation in nude mice

We further examined the ability of the induced cells to generate teratomas *in vivo*. Induced

cells were suspended at a concentration of 2.5×10^7 cells/ml in 0.9% normal saline. Two nude mice (6 to 8 weeks old) were used for this study. They were anesthetized with diethyl ether (Dongguan, P. R. China). We injected $200 \mu\text{L}$ of cell suspension (5.0×10^6 cells) subcutaneously into the dorsal flank. Four weeks after the injection, tumors were surgically dissected. Samples were fixed in 4% paraformaldehyde, and were embedded in paraffin. Sections were stained with hematoxylin and eosin. Neural tissues were immunostained by antibodies of the neuron specific enolase (NSE), neurofilament (NF), the glial cell marker S-100, and neural stem cell marker Nestin all at 1 : 100 dilution. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Results

Morphology and purification of skin fibroblast

We found that primary cultured skin fibroblasts adhered to culture dishes after 2 d of incubation. Fibroblasts appeared spindle shaped, and initially grew slowly and then became integrated after 4 to 5 d of incubation (Figs. 1A, 1B). The results showed that CK19 was not expressed but vimentin was expressed in the fibroblasts (Figs. 1C, 1D).

Stem cell marker expression

After induction using fish oocyte extract, we collected the reprogrammed cells and used RT-PCR to examine expressions of several marker genes commonly expressed in multipotent cells. The results showed that CD34, SOX2 and C-myc were not expressed in the cells after incubation in PBS. However, all of these multipotent markers were positive as early as 48 and 72 h following reprogramming (Figs. 2A–2C).

Next, to detect Oct-3/4 protein expression in fibroblasts treated with fish oocyte extract we

Fig. 1. Primary culture of human fibroblast cells ($\times 200$). A: Primary human skin fibroblast cells. B: Cells that were transferred and continually cultured. C: Human skin fibroblast cells were negative expressed for CK19. D: human skin fibroblast cells were positive expressed for Vimentin.

Fig. 2. Activation of pluripotent cell marker genes following cell reprogramming. Detection of A: CD34, B: C-myc, and C: Sox-2 expression by RT-PCR. Treated cells were collected at 48 and 72 h following extracts treatment and were used for PCR quantitation of CD34, C-myc, and Sox2 mRNA. CT: PBS control. 18S RNA was used as a PCR internal control. mRNA ratio: PCR band density of A: CD34, B: C-myc, and C: Sox-2 over that of 18S RNA. Note: all pluripotent genes were activated in treated cells. D: Immunohistochemical staining of Oct-3/4. Skin fibroblasts were treated with PBS control ($\times 100$). Different time points after treatment with 1.2 mg/ml fish oocyte extracts for 48 h ($\times 200$) or 72 h ($\times 200$). E–J: Flow cytometry analysis showing population of cells positive for Oct-3/4, Nanog and SSEA-4. E, F, and G: skin fibroblasts were treated with PBS control. H–J: skin fibroblasts were treated with 1.2 mg/ml fish oocyte extracts for 72 h.

performed immunohistochemical staining after different periods of incubation. Untreated fibroblasts did not stain for Oct-3/4; however, Oct-3/4 was expressed in the reprogrammed cells treated with 1.2 mg protein/ml extracts. Cells induced with fish oocyte extracts for 72 h expressed the highest level of Oct-3/4 protein; however, cell death occurred in these cells (Fig. 2D).

In addition, we used flow cytometry to analyze the expressions of Oct-3/4, Nanog, and SSEA-4 protein in fibroblasts after induction using oocyte extracts. We found that fibroblasts were not positive for Oct-3/4, Nanog, and SSEA-4 in PBS control cells. However, fibroblasts treated with fish oocytes extracts were positive for Oct-3/4, Nanog, and SSEA-4 (Figs. 2E–2J). The results of flow cytometry revealed that before and after induction, the percentage of Oct-3/4-positive fibroblasts were $2\pm 1\%$ and $12\pm 2\%$ ($n=3$), respectively; the percentage of Nanog-positive cells were $3\pm 1\%$ and $6\pm 2\%$ ($n=3$), respectively; and the percentage of SSEA-4-positive cells were $2\pm 1\%$ and $5\pm 1\%$ ($n=3$), respectively. The positive expression of Oct-3/4, Nanog and SSEA-4 increased significantly ($p<0.05$) indicating that our induction method was effective.

β -ME and RA treatment induced expression of nestin and NSE in iMS cells

To detect whether nestin and NSE protein are expressed in iMS cells treated with β -ME and RA, we performed immunohistochemical staining. We found that Nestin and NSE protein were both expressed in 80% of iMS cells and MSC cells treated with β -ME and RA (Figs. 3A–3D). The skin cells without induction were not expressed NSE and Nestin (Figs. 3E, 3F).

Teratoma formation induced by reprogrammed cells

To examine whether the cells we induced had pluripotent characteristics, cells were inoculated subcutaneously into nude mice and we observed them for teratoma formation. Four weeks after injection, we observed tumor formation in the 2 mice we injected. Histological examination showed that the tumor included various tissues containing the blood vessels, nerve, muscle and adipose tis-

Fig. 3. Immunohistochemical staining of NSE and Nestin. A: Expression of NSE and B: Nestin proteins in reprogrammed skin cells. The cells were induced into nerve cells 72 h after treatment of fish oocyte extracts and 10 d of incubation with β -ME and RA. C: NSE and D: Nestin proteins were expressed in MSC for 10 d after treatment of β -ME and RA ($\times 200$). E: Skin cells without induction did not express NSE. F: Skin cells without induction did not express Nestin.

Fig. 4. A representative histological section showing the tumor included various tissues containing blood vessels. A: H and E staining and immunohistochemical staining showed the presence of neural tissues that were positive for B: neuron specific enolase (NSE) and C: neurofilament (NF), and D: the glial cell marker S-100. E: Expression of the neural stem cell marker nestin in the middle of the original structure.

sues (Fig. 4A). Immunohistochemical staining showed the cells in the tumor were positive for NSE and NF (Figs. 4B, 4C), and the glial cell marker S-100 (Fig. 4D). The neural stem cell marker nestin was found to be expressed in the middle of the original structure (Fig. 4E).

Discussion

Stem cells are important in the early development of mesoderm and ectoderm. Mesenchymal stem cells (MSCs) are originally derived from bone marrow. Their multipotent nature and other characteristics make them intriguing for use in stem cell research. MSCs can be differentiated into fat, bone, cartilage, muscle, tendon, ligament, nerve, liver, heart, endothelial and other cells *in vivo* or *in vitro* using specific conditions (Barry and Murphy, 2004); therefore, MSCs can be used as ideal seed cells for studying the aging and diseased tissues and organs.

Although the number of cells obtained was limited by the method we used, we believe that we have developed a simple and efficient approach to generate effective cells for cell replacement therapy. After a brief exposure to fish oocyte extracts, skin fibroblasts became partially reprogrammed multipotent cells. The induced multipotent cells were morphologically similar to MSCs. Notably, Oct-3/4, Nanog, C-myc, SSEA-4, and Sox2 were expressed in our reprogramed cells, and these factors have been shown to help maintain pluripotency in embryonic stem (ES) cells (Cavaleri and scholar 2003, Chambers *et al.* 2007, Mitsui *et al.* 2003, Nichols *et al.* 1998, Niwa *et al.* 2000). It is possible that Oct-3/4 and Nanog regulate the ES potency network by acting on promoters of thousands of ES genes (Boyer *et al.* 2005, Kim *et al.* 2008, Loh *et al.* 2006).

When the induced cells were treated with β -ME and all-*trans* RA, these cells could be differ-

entiated into neural cells *in vitro*. At the same time, when these treated cells were subcutaneously injected into the dorsal flank of nude mice, teratomas were formed and were found to contain neural cells. Finally, we showed that the neuronal markers NSE and NF, the glial cell marker S-100, and the neural stem cell marker nestin were all expressed in the teratoma, indicating that skin fibroblasts induced by the fish oocyte extract could be differentiated into neurons, glial cells, and neural stem cells.

The direct conversion of a differentiated cell type into another differentiated cell type or into a pluripotent cell would provide technological, medical and societal benefits (Barry and Murphy 2004, Boquest *et al.* 2006, Pittenger *et al.* 1999, Woodbury *et al.* 2002, Zuk *et al.* 2001).

Our results imply that fish oocyte extracts contain reprogramming factors; however further investigation is necessary to elucidate the exact molecular mechanisms leading to this reprogramming process. Taken together, other results and findings from others indicate that fish oocyte extracts are able to partially reprogram somatic cells and may be useful for analyzing the mechanisms of nuclear reprogramming and dedifferentiation of mammalian somatic cells *in vitro*.

We found that after reprogramming, nearly all of the treated cells stained positive for Oct-3/4. In contrast, the existing strategies using defined factors are inefficient and generation of iPS cells are usually generated from millions of starting cells (Mali *et al.* 2008, Park *et al.* 2008, Takahashi *et al.* 2007). Based on the results of our study, it appears that fish oocyte extracts have distinct and efficient reprogramming factors that can reprogram somatic cells. It is likely that the transcription factors responsible for reprogramming in our study are different from those seen in reprogramming of iPS cells. Our proof-of-concept study opens up the possibility of producing patient-specific neural cells in a relatively simple and straightforward manner with high efficiency. We believe that such cells could be greatly helpful for investigating the mechanisms underlying neural degenerative diseases.

Conclusion

We demonstrated that a simple method of *in vitro* reprogramming with fish oocyte extracts may be sufficient to epigenetically remodel somatic cells to a state that resembles multipotent stem cells. In our study we were able to differentiate cells into neuronal, glial, and stem cells both *in vitro* and *in vivo*. Thus, *in vitro* reprogramming with fish oocyte extracts may be promising for regenerative medicine that is effective in treating tissue damage, genetic disorders, and neural degenerative diseases.

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