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Differentiation Peripheral Blood Mononuclear Cells into Functional Hematic-Like Cells by Hematopoietic Remodeling

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Abstract: The shortage of bone marrow donors and the high risk of allogenic transplant rejection have prompted investigation into alternative therapeutic approaches for patients with aplastic anemia. Reconstitution of hematopoietic function using stem cells derived from cord blood is a promising alternative which however, the limited number of stem cells obtained restricts clinical application. In the earlier study, researchers demonstrated the epigenetic reprogramming of mouse skin fibroblasts into multipotent cells using a transient *in vitro* method. After transient reprogramming, cells expressed reprogramming factors (Oct-3/4, Nanog and Sox-2) and formed teratomas in nude mice. In this study, PBMCs from aplastic anemia patients were reprogrammed using the transient *in vitro* method. Reprogrammed cells (iMS cells) were capable of differentiating into hematopoietic cells; iMS cells transplanted into lethally irradiated mice differentiated into bone marrow and splenic cells. Results indicate that reprogrammed PBMCs are multipotent and can restore hematopoietic function.

Key words: Peripheral blood mononuclear cells, reprogramming, fish oocyte extracts, multipotent stem cells, hematopoietic stem cells

INTRODUCTION

Stem cells are capable of self-renewal, infinite proliferation and multi or totipotent differentiation. Stem cells are required for development, play a role in the mechanisms of aging and certain hereditary diseases and may hold the key for treatment of clinical refractory disease and reconstruction of functional organs (Bischoff and Holtzer, 1969; Ogawa, 1993; Decheva and Tomova, 1972; Lalle *et al*., 2000; Quesenberry *et al*., 2003; Yi *et al*., 2012; Stork *et al*., 2012; Zhou *et al*., 2011).

Aplastic anemia is caused by a defect in the number and quality of hematopoietic stem cells Allogenic bone marrow transplantation is the current treatment for aplastic anemia resulting in restored hematopoietic function and normal hematopoietic stem cells. Treatment with hematopoietic stimulating factors was widely used in the clinic in the 1990s because treatment results in strong hematopoietic mobilization with few side effects (To *et al*., 1997). Therefore, acquisition of healthy donor hematopoietic stem cells for transplantation into aplastic anemia is a possible task (Hernandez-Rivera, 2005; Mun *et al*., 2012). However, there are few hematopoietic stem cells in the peripheral blood and a lack of bone marrow donors (Jansen *et al*., 2005). Cord blood is richer in stem cells than the bone marrow. Compared with peripheral blood stem cell transplantation, umbilical cord blood stem cell transplantation techniques have been slow to develop: the stem cell quantity is limited with only enough stem cells for children and it is not as easy to obtain HLA-matched umbilical cord blood as it is for adult peripheral blood (Kosmacheva *et al*., 2011; Hansen *et al*., 2000; Ishikawa *et al*., 2003). In both stem cell research and in clinical application, the biggest obstacle to technological development and effective clinical application is the limited source of stem cells.

Recent studies have demonstrated that fish oocyte extracts induce mouse fibroblasts to differentiate into multipotent stem cells (Zhu *et al*., 2010). The induced multipotent stem cells behave like mesenchymal stem cells in morphology and are able to differentiate into multiple functional cell types including insulin-secreting islets, beating myoblasts, skeletal muscle, adipocytes and neural cells (Zhu *et al*., 2010). Therefore, researchers used this approach to generate hematopoietic-like stem cells from the peripheral blood of aplastic anemia patients and investigated the therapeutic potential of these induced cells *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Preparation of fish oocyte extracts: Crucian carp were acquired from local fish production farms. Fish oocytes, collected under sterile conditions were ground using a mortar and pestle and extracted with an equal volume of 0.9% sodium chloride. Oocyte extracts were centrifuged at a speed of 500×g for 10 min, followed by 1500×g for another 10 min. Supernatants were collected and filtered using 0.22 mm filters. Protein levels of the extracts were determined as earlierly described (Zhu *et al*., 2010; Yao *et al*., 2003). The extracts were then diluted to 10 mg protein/mL using DMEM/F12 medium and stored at -80°C.

***In vitro* cell reprogramming of Peripheral Blood Mononuclear Cells (PBMCs):** PBMCs from patients with aplastic anemia were used for cell treatment studies. Cells (4×10^5) were inoculated in new flasks for cell reprogramming using 10-20 μg protein/mL fish oocyte extracts for 72 h as earlierly described (Zhu *et al*., 2010).

***In vitro* differentiation of iMS cells:** The reprogrammed cells were differentiated into hematopoietic-like stem cells using an *in vitro* approach modified from the earlier published method (Wang *et al*., 2012). Human PBMCs were induced with oocyte extracts for 72 h as described in the study. Cell differentiation was induced by co-culturing iMS cells with PBMCs from aplastic anemia patients. Following differentiation for 5 days, FACs analysis was performed using anti-human-CD34 (Santa Cruz Biotechnologies, Santa Cruz, CA) and RT-PCR analysis gene CD34.

Inducing hematopoietic failure in mice: Ten healthy pregnant BALB/c mice were purchased from Kunming Animal Institute (Yunnan, China) and were housed in Clinical Animal Center of Kunming Arm General Hospital. Healthy pregnant mice were exposed to a lethal dose of radiation (6.5 Gy) to induce hematopoietic failure. All animal studies were approved by the Animal use Committee of Kunming Arm General Hospital.

Immunohistochemical staining: Cells were fixed using 4% paraformaldehyde and washed with PBS three times for 5 min each. Cells were incubated with 0.3% Triton X-100 for 5 min, washed three times with PBS and blocked for endogenous peroxidase activity using 3.0% H_2O_2 for 5 min. Cells were washed three times with PBS. Anti-human-Oct-3/4 or SSEA-4 antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the fixed cells in both induced and control groups and incubated at 4°C for 12 h then washed three times with PBS. Cells were incubated with Horseradish Peroxidase (HRP)-labeled anti-human antibody (Gene Tech. Company Limited, Shanghai) was added and incubated at 37°C for 20 min, followed by three washes with PBS. Cells were then stained with DAB working solution according to the manufacturer's instructions (MaiXin Bio-Company, Fuzhou, China) and observed using an optical microscope.

Flow cytometric analysis: Cells (1×10^6) were fixed with 100 μL of 4.0% paraformaldehyde solution for 10-15 min. Cells were then incubated with 0.5% saponin solution (100 μL) at room temperature for 15 min and then washed twice with PBS/BSA buffer containing 0.5% saponin. Cells were incubated with the following antibodies: Oct-3/4-PE, Nanog-PE and SSEA-4-PE (BD, Franklin Lakes, NJ). Antibodies were diluted to a concentration of 1:100 and incubated at room temperature for 30 min. Cells were washed twice with 2 mL of PBS/BSA, centrifuged at 400×g for 5 min and the resulting supernatants discarded. Isotype controls were stained using an isotype-matched antibody.

PTM voltage and compensation were set using cell surface staining controls. Quadrant markers were set based on isotype controls and unstained cells. Data were acquired using a FACSCalibur flow cytometer and analyzed with CELLQuest Software (Becton Dickinson, Franklin Lakes, NJ).

Stem cell gene expression analysis: Total RNA was extracted from reprogrammed cells using TRI-REAGENT (Sigma, St. Louis, MO), according to the manufacturer's protocol and cDNA synthesized using RNA reverse transcriptase. Genes expression was examined in cDNA samples by RT-PCR (Hu *et al*., 1995, 1996) and quantitative PCR (qPCR) (Chen *et al*., 2006) analyses as earlier described. PCR products were resolved by 2.0% agarose gel electrophoresis and stained using SYBR Green I (Invitrogen, Carlsbad, CA). CD34 RT-PCR primers were as follows: (forward) GCGCTTTGCTTGCTGAGTTT and (reverse) GCCATG TTGAGACACAGGGT. The internal control 18S rRNA primers were as follows: (forward) GCGGCT TTGGTGACTCTA and (reverse) CTGCCTCC TTGGATGTG. Oct4 real time PCR primers were as follows: (forward) GAGAACCGAG TGAGAGGCAACC and (reverse) CATAGTCGCTG CTTGATCGCTTG. Nanog real time PCR primers were as follows: (forward) AATACCTCAGCCTCCAGCAGATG and (reverse) TGCG TCACACCATTGCTATTCTTC. GAPDH control gene real time PCR primers were as follows: (forward) ACGGATTTGGTCGTATTGGG and (reverse) ATCT CGCTCCTGGAAGATGG.

Chromosome analysis of peripheral blood: Blood (20 mL) from aplastic anemia patients was layered on ficoll lymphocyte separation medium (Rongsheng Biotech Com., Ltd. Shanghai, China) and PBMCs were isolated according to the manufacturer's protocol. Samples were divided into a control group (no treatment) and the reprogrammed group (incubated with oocyte extract for 72 h). Cells (1×10^6) were cultured for 72 h in media with Fetal Bovine Serum (FBS), L-glutamine and Phytohemagglutanin (PHA) at 5.0% CO_2 and 37°C and shaken twice a day in the morning and evening. Colchicine (0.1 μg mL^{-1}) was added to the culture 2-4 h before analysis. Cultured cells were pelleted and centrifuged at 1000×g for 10 min, supernatant removed and 6-8 mL of 0.075 mol L^{-1} KCl prewarmed to 37°C and incubated with mixing at 37°C for 15 min. Cells were fixed in 1 mL of methanol:acetic acid solution (3:1) and mixed by vortexing. This process was repeated three times; fixing for 30 min for the first round and 15 min for the second and third rounds. Cells were centrifuged at 500×g for 10 min, supernatant discarded, fixative added and samples were stored at -20°C. At the time of analysis, Acetic-Saline-Giemsa (ASG) banding technique was performed; briefly, 2-3 drops of cell suspension was added drop by drop from 10 cm above a clean grease-free slide with a 15°C tilt and dried. Samples were stained with 10% Giemsa for 20-30 min, washed with water, dried and microscopic analysis performed (Sutiakova *et al*., 2012; Bishun, 1967; Lawler *et al*., 1968).

Fish staining: Denatured probes (2.5 μL) were added drop by drop onto the slide with fixed samples and covered with a coverslip. Bubbles were removed and the gap between the coverslip and slide was sealed using rubber glue. Slides were denatured at 75°C for 5 min then put into a humidified box protected from light for 8-16 h at 37°C. After hybridization, coverslips were removed and slides were rinsed using solution I (0.4×SSC/0.3% NP-40) for 2 min at 73±1°C and solution II (2×SSC/0.1% NP-40) for 30 sec at room temperature. Nuclei were detected using DAPI staining. Signal was observed using fluorescence microscopy with UV filter combinations (Carl Zeiss) (Wauters *et al*., 2007).

RESULTS AND DISCUSSION

Expression of stem cell marker genes: PBMCs from patients with aplastic anemia were reprogrammed into iMS cells using fish oocyte extracts as described in Materials and Methods. Immunohistochemical staining was used to detect expression of 2 genes *Oct4* and *SSEA-4* related to cell pluripotency. As expected, the untreated PBMCs were negative for staining with human-specific Oct4 and SSEA-4 antibody, respectively (Fig. 1a and c). However,

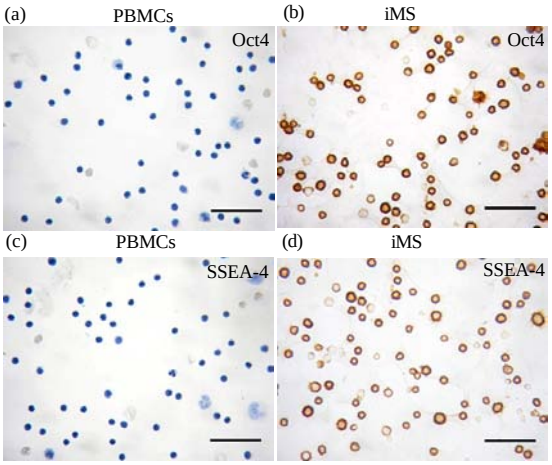

Fig. 1: Expression of pluripotent stem cell marker genes in treated PBMCs

the treated PBMCs for 72 h stained positive with human-specific Oct4 and SSEA-4 antibody, respectively. (Fig. 1b and d). Flow cytometric analysis was used to detect the expression of Oct4 and SSEA-4 in PBMCs treated with fish oocyte extracts. The results showed that the expression of pluripotent cell surface marker Oct4 and of SSEA-4 increased in treated PBMCs (Fig. 2).

Real time PCR results of stem cell genes showed that there was a dramatic increase in *Oct4* gene expression and *Nanog* was also, sharply upregulated in treated PBMCs (Fig. 3).

Karyotype analysis of reprogrammed cells: Researchers used karyotype analysis to examin the chromosomal structure of reprogrammed PBMCs. The results showed chromosomes of untreated PBMCs were not exhibited. However, chromosom number of reprogrammed cells was normal (Fig. 4). The results demonstrated treated PBMCs possessed proliferative ability whereas untreated PBMCs did not. In addition there were no change in the chromosome number and karyotype of treated PBMCs by short exposure of fish oocyte extracts.

Differentiation of human iMS cells into hematic-like cells: Human PBMCs were treated with oocyte extracts for 72 h. Then, the resulting iMS cells were co-cultured for 5 days with PBMCs from aplastic anemia patients. Expression of the hematopoietic stem cell marker *CD34* gene was detected by FACS analysis and RT-PCR, separately. Researchers found that the expression of hematopoietic stem cell marker CD34 increased in treated iMS cells (Fig. 5a). The RT-PCR showed there was a increase in *CD34* gene expression in treated iMS cells (Fig. 5 b).

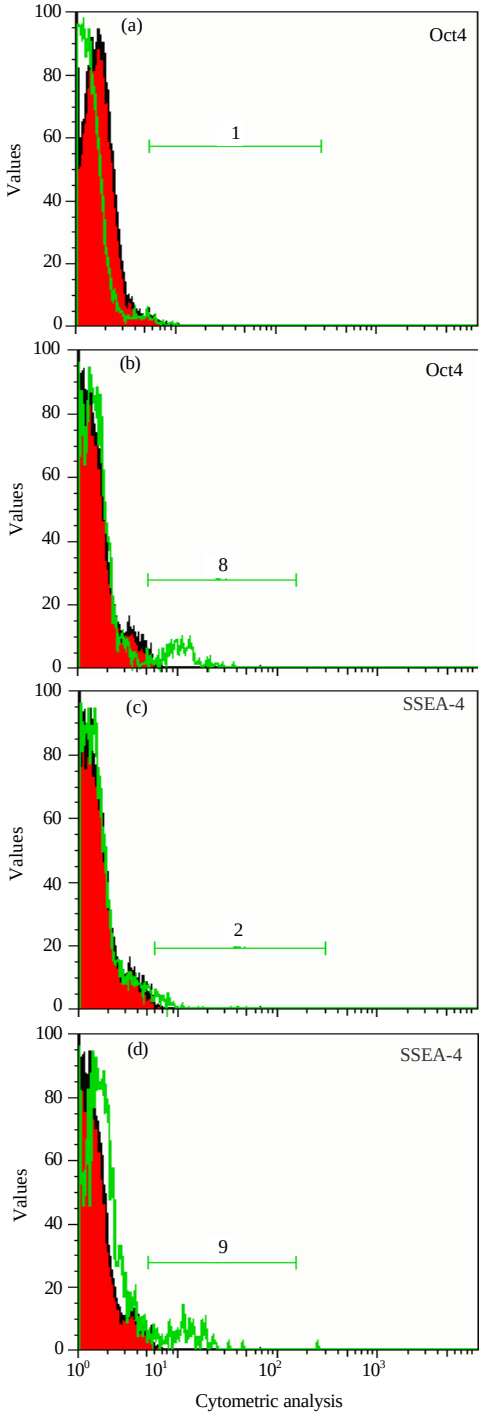

Fig. 2: Flow cytometric analysis of Oct4 and SSEA-4 in treated PBMCs; a) PBMCs; b) iMs; c) PBMCs and d) iMs

Establishment of reprogrammed stem cells in a mouse model of hematopoietic failure: PBMCs from male BALB/c mice were reprogrammed using fish oocyte extracts. Reprogrammed cells (~1×10^6) were injected into 4 h

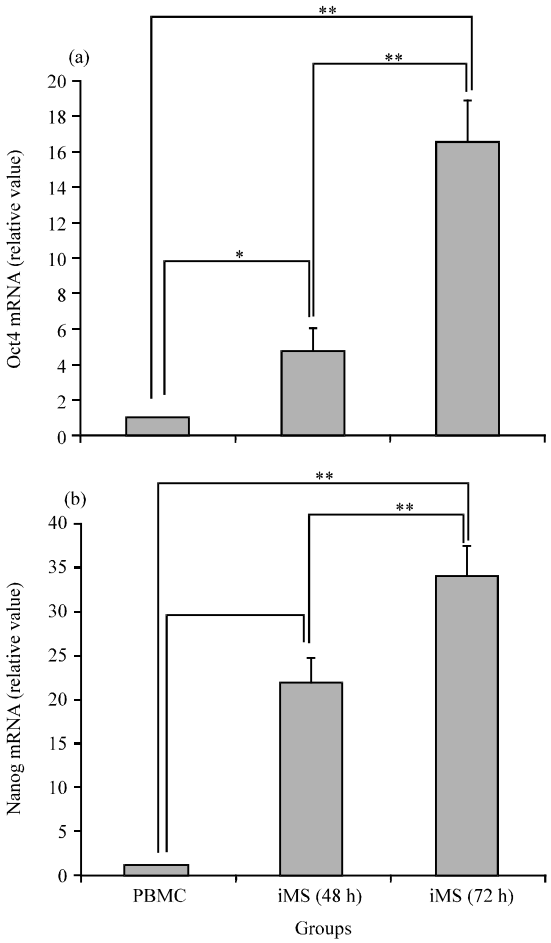

Fig. 3: Activation of pluripotent stem cell marker genes. a) Oct 4 mRNA and b) Nanong mRNA

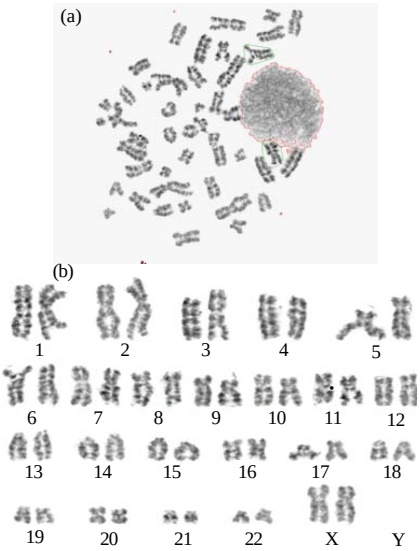

Fig. 4a, b): Karyotype analysis of treated PBMCs from female aplastic anemia patient

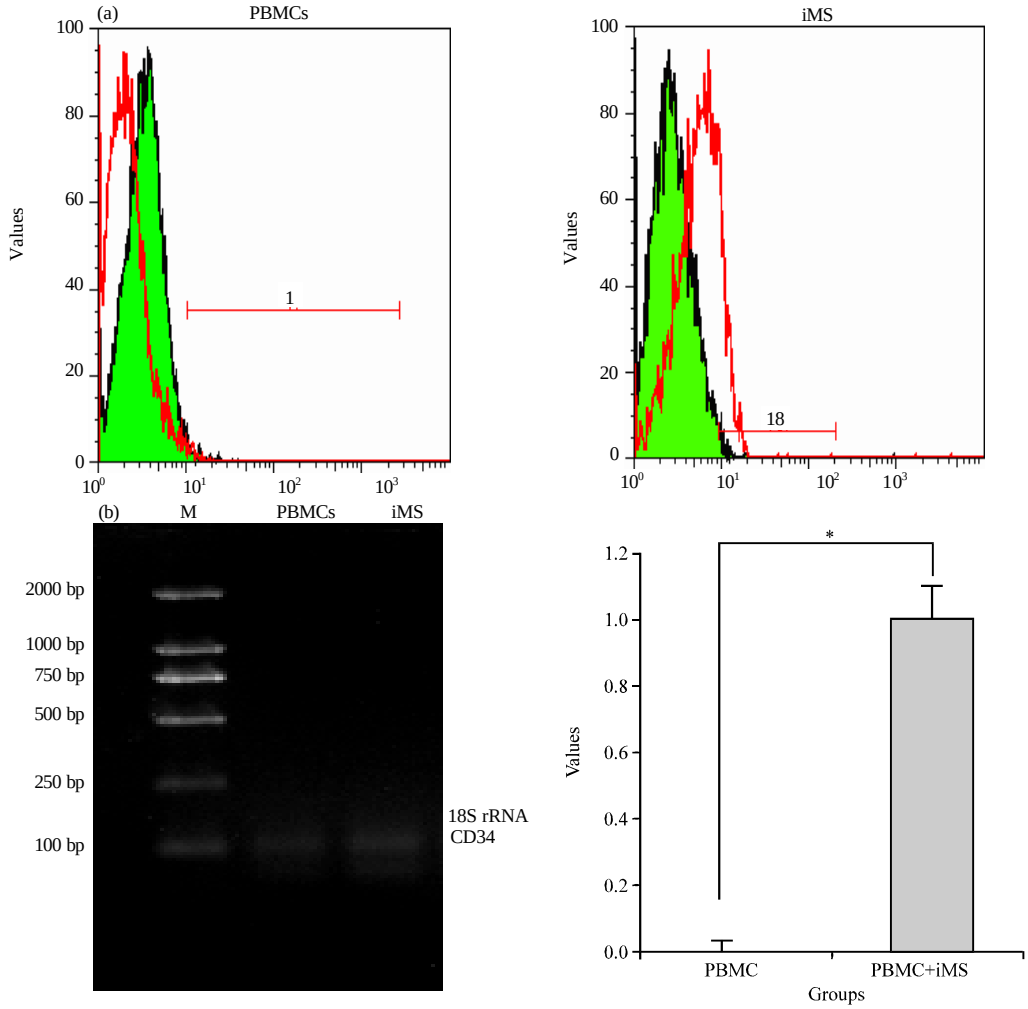

Fig. 5: Activation of hematopoietic stem cell marker *CD34* gene

lethally irradiated female BALB/c mice by the tail vein. Control mice were injected with PBS. The results showed that weight and White Blood Cell count (WBC) of reconstituted mice injected into reprogrammed cells had a decrease in the initially 8 days then began to increase (Fig. 6). However, weight and WBC of control mice straightly decreased and all of the control mice died within 8 days after lethally irradiated. In order to verify the bio-function of reprogrammed PBMCs *in vivo*, researchers used FISH to detect Y chromosome in the half-lethally irradiated BALB/c female mice injected reprogrammed PBMCs derived from male BALB/c mice. Researchers found that the Y chromosome was detected in bone marrow cells of the reconstituted female mice (Fig. 7). The long-term survival rate of the female mice with Y chromosome (karyotype, (40, XY)) was 63%.

In this study, researchers demonstrated the feasibility of reprogramming PBMCs as an economical and efficient approach to generate multipotent stem cells for the treatment of aplastic anemia. Instead of bone marrow stem cells, researchers transiently reprogrammed PBMCs by briefly incubating with fish oocyte extracts. Alterations in the gene expression profile and cell plasticity implicate global remodeling of chromatin during this short period of cell reprogramming. Notably, *Oct4* and *SSEA-4* genes which are expressed in embryonic stem cells and during the reprogramming phase were upregulated in reprogrammed PBMC (Villa *et al*., 2012; Liu *et al*., 2012; Modder *et al*., 2012; Varga *et al*., 2011).

Both embryonic stem cells and induced pluripotent stem cells can be differentiated into hematopoietic cells Sakamoto *et al*. (2010). At present, bone marrow reconstitution using stem cells is the best way for treating patients with aplastic anemia yet obtaining these cells are different because of ethics and security. Studies showed that transiently reprogrammed PBMCs appear to be similar in cell morphology to mesenchymal stem cells which are able to differentiate into multiple functional cell types

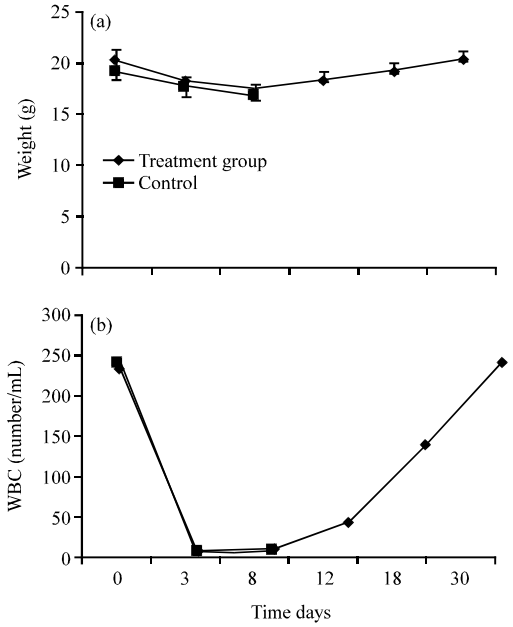

Fig. 6a, b): Reconstitution of female BALB/c mice with reprogrammed male BALB/c PBMCs

including hematopoietic cells, skeletal muscle cells, adipocytes and neurons suggesting the potential for multi-lineage differentiation and acquisition (Wang *et al*., 2012; Xu, 2012; Zhao *et al*., 2012).

In this study, researchers found that reprogrammed PBMCs by short exposure of fish oocyte extracts differentiated into multipotential stem cells. Activation of stem cell marker *Oct4* and *SSEA-4* genes in treated PBMCs showed that cells were reprogrammed and cell type was changed.

Treated PBMCs regained the proliferative ability and karyotype was normal which suggested that reprogramming process did not involve the variation of chromosom number and might only unlock multipotent genes and lock specific genes. After reprogrammed PBMCs were continuously co-cultued with PBMCs from aplastic anemia patients for 5 days, the expression of CD34 in reprogrammed PBMCs showed that reprogrammed PBMCs have potential to differentiate into hematopoietic stem cell.

In addition, the result suggested reprogrammed PBMCs were multipotent cells to differentiate into other type cells. Furthermore, Y chromosome existed in reconstituted lethally irradiated female mice with reprogrammed PBMCs and more than half of the reconstituted female mice were able to survive that demonstrated reconstituted female mice at least partly restored hematopoietic function. The possible mechanism underlying genomic reprogramming by short exposure of fish oocyte extracts were as followed:

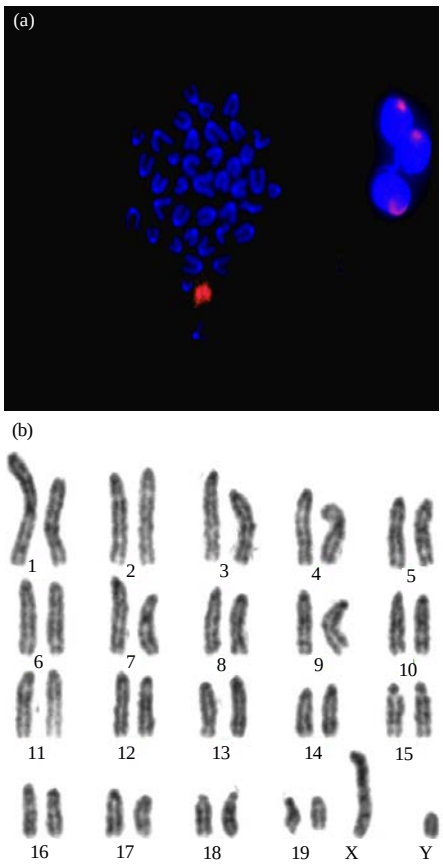

Fig. 7a, b): FISH analysis of bone marrow cells from female mice injected with reprogrammed PBMCs

Small molecules regulating: These small molecules mainly included small peptides, non-peptide such as small interfering RNA and other soluble factors. One of the regulating mechanisms was that these small molecules can freely penetrate into the cell to regulate the multiple target genes that stirred up cell nuclear reprogramming (Brennecke *et al*., 2005). On the other hand, some small molecules perhaps interact with cellular surface receptors that activate one or more signal pathways involved in cell reprogramming.

Macromolecules function: Theoretically these macromolecules, mainly polypeptide, e.g., Nanog, Oct4, SSEA-4 and Sox2 can not freely pass through cell surface. They perhaps penetrate into the cell to participate cell reprogramming by passive transport.

Cell microenvironment: Many studies have showed that cell microenvironment can alter the genes expression and cell phenotypes (Zhang *et al*., 2009; Grinnell and

Bickenbach, 2007). In this study, fish oocyte extracts may provide a reprogramming environment that unlock multipotent genes in PBMCs. Further studies are needed to explor the underlying mechanisms of cell reprogramming by short exposure of fish oocyte extracts.

Results showed that treated iMS cells expressed hematopoietic stem cell marker CD34 by co-cultured PBMCs from aplastic anemia patient and iMS for 5 days which indicate PBMCs with loss of bio-function can release one or more key factors (signals) to irritate the differentiation of iMS cells into hematopoietic-like stem cells. Key factors and underlying mechanism need in-depth study.

CONCLUSION

This study demonstrates multipotent stem cells can be induced from PBMCs of aplastic anemia patients and the reprogrammed cells can differentiate into functional multipotent stem cells *in vitro*. Preliminary study indicates that the reprogrammed cells have the potential ability to restore hematopoietic function *in vivo*. Further studies are needed to explain the underlying mechanism and establish animal model and explore how to safely utilize the iMS for treating diseases.

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