The role of EmSOX2 in maintaining multipotency of pluripotent stem cells based on the technology of induced pluripotent stem cells

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Received: July 8, 2016; accepted: September 9, 2016.

This study aims to explore the role of EmSOX2 in maintaining the multipotency of pluripotent stem cells and provide a theoretical reference for the research and development of anti-*Echinococcus multilocularis Leuckart* drugs. In this paper, the potential homologous gene full-length sequence (EmSOX2) was obtained by SOX2 cloning on *Echinococcus multilocularis Leuckart*. The role of EmSOX2 was studied with the technology of induced pluripotent stem cells (iPS technology). In replacement of the exogenous SOX2 gene of mice, EmSOX2 could generate iPS cells along with the exogenous genes Oct4, c-Myc, and Klf. In the absence of exogenous gene Sox2 or gene EmSOX2, the exogenous genes (Oct4, Klf4, and c-Myc) could not generate iPS cells through inducing, which indicated that EmSOX2 gene played a key role in inducing pluripotent stem cells, and its function was similar to that of the exogenous gene Sox2 of mice. It can be concluded that the induced EmSOX2 gene can replace the exogenous gene Sox2 of mice; along with Oct4, c-Myc, and Klf genes in mice. EmSOX2 can infect the embryonic cells and induce iPS cells from them. The research has a certain effect on the multipotent endogenous factors of germinal layer of *Echinococcus multilocularis Leuckart* and provides a theoretical basis for the relevant study and drug development.

Keywords: pluripotency; stem cell technology; EmSOX2; multipotency; embryonic stem cells.

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Introduction

The growth stages (ovum, oncosphere, metacestode and imago) of an *Echinococcus multilocularis Leuckart* are completed with the aid of hosts [1, 2, 3]. Some animals (such as fox and dog) dispose *Echinococcus multilocularis Leuckart* ova through defecation. Once rodents (such as mouse and gray hamster) swallow the ova, they can be infected. The ova gradually develop into oncosphere and metacestode larvae, which proliferate and develop rapidly in hosts' livers [4]. The final hosts of *Echinococcus multilocularis Leuckart* are mainly fox, wolf, dog, and cat [5]. On the basis of the infection in their livers, *Echinococcus multilocularis Leuckart* can further develop into imagoes. Human is not the suitable intermediate host for *Echinococcus multilocularis Leuckart*. However, human infection is caused by the accidental intake of water and food contaminated by *Echinococcus multilocularis Leuckart* ova.

In 2006, Takahashi and Yamanaka [6] first introduced induced pluripotent stem (iPS) cells which were generated by importing Oct4, Sox2, and c-Myc exogenous genes into mouse embryonic fibroblasts by using retrovirus

vectors. Through that way, mouse embryo fibroblasts (MEF) could be turned into pluripotent stem cells (named as iPS cells), which were similar to embryonic stem cells (ESC) in performance and multidirectional differentiation ability [7]. By using iPS technology, the mouse exogenous Sox2 gene was replaced by EmSOX2 to see whether MEF cells could be turned into iPS cells. The generated iPS cells were selected and identified. Wernig et al. [8] found that in the process of MEF cells turning into iPS cells, the expression of exogenous genes was gradually reduced, while the endogenous ES marker genes (M4, Sox2, Nanog, Klf, Cripto, and Fgf4) were highly expressed. Maherali et al. [9] revealed that, on the basis of morphology, iPS cell line could be established and iPS cells induced from MEF cells could be isolated [10, 11].

In this study, we selected monoclonal cells for passage investigation and observed their morphological changes. By using alkaline phosphatase (ALP) dyeing method [12], we screened and identified the induced iPS cells in the experiment, and therefore, to determine the role of EmSOX2 gene in maintaining the multipotency of mouse stem cells.

Materials and Methods

All procedures related to experimental animal handling and managing in this study were approved by Huanghuai University Institutional Research Board (IRB).

Development of EmSOX2 gene

About 1,000 *Echinococcus multilocularis Leuckart* protoscolices (Research Institute of Inner Mongolia Grassland, China) were used for total RNA extraction according to QIAGEN operation method. All RNAs were subjected to 1.5% agarose gel electrophoresis for quality determination. The RNA integrity was determined by using an ultraviolet gel imager (Maisky High-tech Co., Ltd, Beijing City, China). Full length EmSOX2 cDNA was cloned by following the instructions of SMART™ RACE cDNA Amplification Kit (Takara Bio USA, Inc., Mountain View, CA, USA). According to the acquired potential homogenous segment sequence of Sox2, specific primers of 3'-RACE and 5'-RACE were designed by using Primer 5.0. The first-strand cDNA of *Echinococcus multilocularis Leuckart* was then synthetized through reverse transcription. The sequences and GenBank ID numbers of the primers are shown in table 1.

The polymerase chain reaction (PCR) products were then generated under the following experimental conditions: 95 °C, 5 min; 34 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 2 min; extension at 72 °C for 5 min. Additional PCR cycles were applied as: 95°C, 5 min; 34 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 2 min; extension at 72 °C for 5 min. At last, the PCR products were applied under the following conditions: 95°C for 5 min; 34 cycles of 95°C for 30 s, 62.5°C for 30 s, 72°C for 2 min; extension at 72 °C for 5 min.

MEF cells *in vitro* culture

All the pregnant wild type B6 mice (JRDUN Biotechnogy Co. Ltd., Shanghai City, China) were sacrificed and the embryos were separated from blastoderms. In brief, the embryo (with blastoderm) was first rinsed with PBS in a Petri dish (6 cm). Through aseptic separation, a separated embryo (without blastoderm) was transferred to a new dish. After removal of head, limbs, and viscus tissues, the trunk of embryo was rinsed with PBS again and was chopped up. 0.5 ml of 0.25% Trypsin-EDTA was then added into the Petri dish. The tissues were placed in the incubator for digestion for 15 min.

Subculture of MEF cells

When the confluence of MEF reached 80%-90%, the cells were subcultured. After washing cells with PBS, 2 ml of EDTA digestive juice was added to the Petri dish. The Petri dish was incubated at 37°C and then was shaken until a large number of round cells rose and hiked up. 3 ml of MEF nutrient solution was added to terminate the

Table 1. Sequences and GenBank ID numbers of the primers.

Primers	Sequences	GenBank ID numbers
3'- RACE-GPS2	5'-CGTTCATGGTGTGGTCAAGAGGTCAGCG-3'	CP012201
3'- RACE-NGPS2	5'-CCCACGCCAACCCGAAGATGCACAAC-3'	CP014129
5'-RACE- GPS1	5'-CGGGTGCTCCTTCATATGATTGGCTCTC-3'	CP015345
5'-RACE- NGPS1	5'-AATGTTTCCACTCCACGCCCAGACG -3'	CP014274

digestion. The cells were scattered and then were transferred to 8 new dishes with 10 ml of fresh MEF broth in each dish respectively. The Petri dishes were shaken to evenly scatter the cells. The cells were then cultured overnight at 37°C with 5% of CO_2 and saturated humidity.

Cultivation of the feeder layer cells

The second generation of cells were cultured until the density was up to 90-95%. Briefly, after digestion, the cells were subcultured in the proportion of 1:2 at 37°C and 5% of CO_2. When the density of the cells was up to 90-95%, they were irradiated with an X-ray apparatus. The petri dish (3.5 cm) that contained 2.5 ml of MEF broth and the irradiated cells was placed at 37°C for 30 min with the addition of 0.1% of Gelatin in the proportion to the cells of 1:9. Then the gelatin was removed.

Inducing iPS with MEF cells

In replacement of mouse exogenous gene Sox2, EmSOX2 gene (along with Oct4, c-Myc, and Klf4 mouse exogenous genes) was used to induce iPS cells from MEF cells. Since the proliferation activity of MEF primary cells with different times of passage can affect the iPS results, we chose MEF primary cells and the first-generation cells for the induction. There was no difference in the methods and inducing conditions; retrovirus mediation was applied to the inducing process.

Screening of iPS cells

In 37°C incubator, 80-90% of the cells at feeder layer were inoculated to a 24-well plate (coated with 0.1% gelatin) and were incubated for 16 to 24 h. MEF broth was removed followed by adding 600 µl of iPS inducing broth for monoclone of iPS cells. The wells were rinsed with PBS after removing the iPS inducing broth. And then, the cells were immersed in 1 ml of PBS. 15 µl of Trypsin-EDTA (0.25%) and 15 µl of PBS were added to a sterile PCR tube and mixed evenly. The feeder layer was punctured and the clone was transferred to the PCR tube. The clone was digested at 37°C for 5 to 8 min before 3 µl of MEF broth was applied to terminate the digestion. The cells were transferred to a new 24-well plate coated with feeder layer cells. The cells were fully mixed and cultured at 37°C for 3 to 5 days. When the density of iPS clone in the PCR tube increased to 70%, pancreatic enzymes (75 µl/well) and PBS (75 µl/well) were added for digestion at 37°C for 3 min. The cells were transferred to a fresh 24-well plate (1:4) coated with 0.1% of gelatin and inoculated with 80-90% of feeder layer cells one day ahead. Before inoculation and cloning, MEF broth was removed; 600 µl of iPS inducing broth was added and the cells were cultured at 37°C for 3 to 4 days. The differentiated clone was removed; the well-grown clones were subcultured and digested every 3 to 4 days. After five times of subculture, the clones were basically well-grown iPS clones; the clones were either subcultured or preserved.

Identification of iPS cells

The iPS inducing broth in the 24-well plate was removed. The plate was washed with 0.5 ml of PBS. 0.5 ml of paraformaldehyde (4%) was added and the plate was oscillated in a vortex mixer and then briefly centrifugation. Paraformaldehyde was removed from the plate followed by 0.5 ml of Rain Buffer washing twice. The cells were immersed by 0.5 ml of PBS. The fast red violet solution, napthol AS-BI phosphate solution, and ddH_20 were mixed at a ratio of 2:1:1. 0.5 ml of the mixed solution was applied for cell dyeing at the room temperature for 15 min. After dyeing,

the cells were washed with 0.5 ml of Rain Buffer solution twice and were observed under the microscope and photographed.

Results

MEF cells *in vitro* culture

After the incubation of MEF cells for 18 hours, the density of cells was increased to 30% while the cell size was relatively small. After incubation for 48 hours, the cell density was increased to 95% with fibrous and flamboyant appearance (figure 1). The cells were ready for digestion and passage.

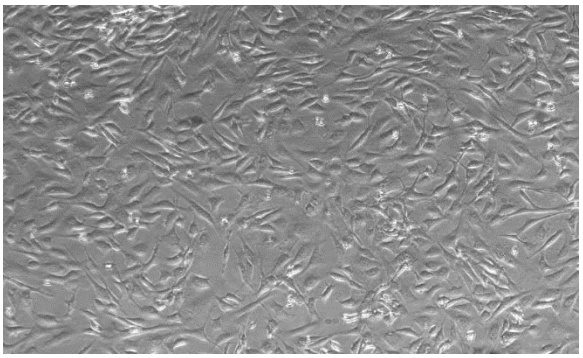

Figure 1. MEF primary cells (100X).

Cultivation of feeder layer cells

The MEF cells of the third generation were growth well with fibrous cell shape (figure 2). Through X-ray irradiation at the radiation dose of 30 Gry, the well-grown MEF cells turned to feeder layer cells (figure 3). The cells were cultivated in MEF broth for 5 more days. The feeder layer cells were cultured successfully if there were no changes observed in both cell shape and amount.

The iPS inducing of MEF cells

(1) The iPS inducing of MEF primary cells

The iPS inducing technology was applied to induce MEF primary cells on the ninth day after the cloning of OSMK group (coinfection of Oct4, Sox2. C-Myc, and Klf) or OSEMK group (coinfection of Oct4, c-Myc, Klf, and EmSOX2). The iPS cell numbers of each group on the 14th day were shown in table 2. There was no iPS cells in control group or OMK group, while iPS clone cells were generated in OSMK and OSEMK groups with the clone numbers of 48 and 33, respectively.

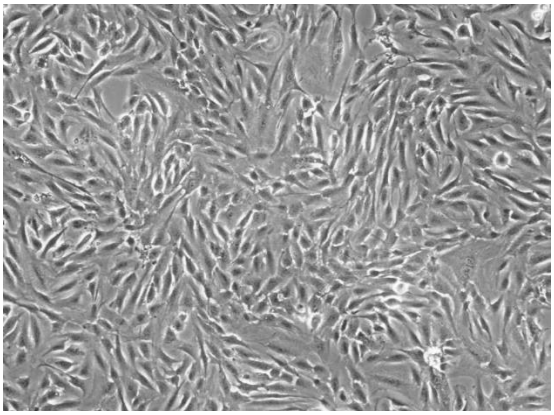

Figure 2. MEF cells of the third generation (100X).

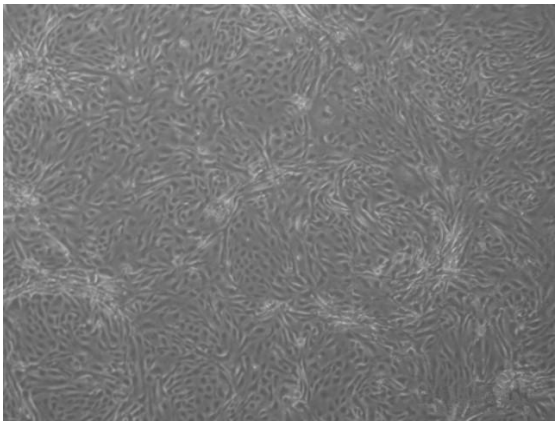

Figure 3. Feeder layer cells (10X).

(2) Changes of MEF cells

In OSMK group, MEF cells were fibrous or flamboyant on the 7th day. MEF cells transformed into small colonies in the shape of islands and scattered in the Petri dish on the 14th day. Most of the MEF cells had been induced into iPS cells with small colonies. The clones were mainly round or oval without solitary cell form in the center. There were clear boundaries between the clones and feeder layer cells. In OSEMK group, the changes of MEF cells were similar to that of OSMK group. The clones of both groups showed certain similarities eventually.

Table 2. The number of clone generated from iPS inducing.

Groups	Number of clones
Control	0
OMK	0
OSMK	48
OS_EMK	33

Morphological observation after passage

Fourteen days after inducing, iPS monoclonal cells were selected for digestion and passage. The cell shape was similar in both OSMK and OSEMK groups. Two days after the cultivation, round or oval colonies of embryonic stem cells formed rapidly. After the passage of four generations, the cells were cultured for additional five days. There were clear boundaries observed with no changes on cell shape. The results indicated that the induced iPS sample cell clones in OSEMK group were positive and were the same as that of OSMK group.

Alkaline phosphatase dyeing

After cultivation of monoclonal iPS cells for 3 days, the cells were dyed by using alkaline phosphatase (ALP). Almost all the clone cells were stained by modena evenly in OSMK group except feeder layer cells. The dyeing results of clone cells in OSEMK group was similar to that in OSMK group while the staining color was amaranth. The results indicated that ALP was highly expressed in iPS clones.

Expression of marker genes

RNA was extracted from iPS clone cells for RT-PCR test. With Natl as the internal reference, Cripto, Fgf4, Nanog, Oct4, and Sox2 were not expressed in MEF cells. However, the above five genes were expressed in OSEMK and OSMK groups, which confirmed that the induced clones in both groups were positive clones.

Discussion

Induced pluripotent stem cells are the cells which can get updated and differentiated continuously by inducing cellular reprogramming [13, 14]. In the study, the iPS inducing was performed under the culture conditions of embryonic stem cells. A previous study showed that the culture conditions of embryonic stem cells not only had a great promoting effect on the separation of embryonic stem cells, but also benefited the reprogramming of mouse fibroblasts [15]. Mouse embryonic stem cells can be isolated and cultured without feeder layers. However, it is apt to cause the differentiation of iPS cells, with negative effects on the growth and long-term culture of iPS cells [16]. In this study, we selected MEF feeder layer to cultivate the cells. MEF between the third and the fifth generations can be used for iPS feeder layers. However, with the increase of passage, there is an increase in the proliferation speed of cells, which is adverse to the growth of iPS cells. In this study, the third generation of MEF cells was used for the feeder layer. For cell screening, instead of using filter system, we analyzed the morphology and molecule of cells. After reprogramming, the colony of iPS cells was circular or elliptic. Cells were concentrated in the colony with good refraction. However, the cell size decreased while the nuclear-cytoplasmic ratio increased with clear boundary. The clones (non-iPS cells) with similar morphology were loose and granular and contained bright cells. In this experiment, we adopted RT-PCR immunofluorescence staining to analyze the genes of iPS cells to increase the accuracy of the experiment.

In combination with the exogenous genes Oct4, c-Myc, and Klf, EmSOX2 could replace SOX2 gene of mouse and induce iPS cells from the embryonic fibrocytes. Through inducing, circular or elliptical iPS clones were generated. The cell colonies formed without solitary cell form. After iPS monoclonal subculture, it was found that iPS cell form was circular and elliptic, and there were cell colonies of embryonic stem cells. ALP staining results showed that ALP was highly expressed in iPS clones. RT-PCR results showed that the expressions of Cripto, Fgf4, Nanog, Oct4, and Sox2 genes were positive in iPS clones. In summary, the role of EmSOX2 gene in inducing

pluripotent stem cells was similar to that of Sox2 exogenous gene in mouse.

Acknowledgement

This study was supported by Core Teachers Funding Scheme Project of Huanghuai University, Foundation Item: Science and Technology Key Project of Henan Province (142102110102), Scientific and Technological Research Project of Henan Science and Technology Agency (122102310264).

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