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Establishment and identification of induced pluripotent stem cells in liver cancer patients

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ABSTRACT

Objective: To induce pluripotent stem (IPS) cells from fibrocytes that are separated from liver cancer patients. **Methods:** The fibrocytes were reprogrammed to IPS cells by lentiviral vector, stained and identified by immunohistochemistry. **Results:** The IPS cells were successfully established from fibrocytes after infection, and IPS cell clones formed in round shape under a microscopy. The induction rate was $0.013\% \pm 0.007\%$. No tumor formed at the back of nude mice within 8 weeks after the inoculation of cell clones. However, tetatoma appeared in nude mice within 1 week after IPS inoculation. A few tumors formed in nude mice within 4 weeks after the inoculation of cell clones. However, subcutaneous tumors formed within 1 week after IPS inoculation. The induced IPS cells showed three germ layers in tetatoma. Nanog and OCT4 in the induced IPS cells showed hypomethylation. SSEA-A, TRA-1-6-, TRA-1-81 and Nanog were highly expressed in the induced IPS cells, indicating the IPS cells possessed the similar ability as the stem cells. **Conclusion:** The IPS cells of liver cancer patients can be established effectively from fibrocytes and can be cultured stably *in vitro*, which provides an approach for the treatment of intermediate or advanced stage liver cancer.

1. Introduction

Liver cancer is a malignant cancer in liver, including primary and metastasized cancer. China is a country where hepatitis B outbreaks frequently, and a large number of patients develop liver cancer based on liver cirrhosis. Besides, there is usually no obvious symptom in the early stage. The patients are already in the intermediate or advanced stage when being diagnosed, and liver transplantation is the only treatment[1]. The problem is that there are too limited liver donors to meet the needs of most patients. Since 1990s, researchers have established induced pluripotent stem (IPS) cells from fibrocytes to provide a novel approach for the treatment of liver cancer[2]. We established and identified pluripotent stem cells induced from fibrocytes in liver cancer patients.

2. Materials and methods

2.1. Materials

Liver tissues were excised from liver cancer patients during April, 2009–April, 2011 in People's Hospital of Zhengzhou, China. The pMXs-IRES-Sox2, pMXs-IRES-Klf4 and pMXs-IRES-Oct4 virus vectors were obtained from Addgene Company. The plasmid extraction kit was purchased from Omega Company. Lipofectamine™ 2000 was purchased from Invitrogen. Dulbecco's minimum essential medium (DMEM) with high glucose and DMEM/F12 were purchased from the Hyclone Corporation. LIF factors, SSEA-1, Oct4 and AKP staining reagents were purchased from CHEMICON International, Inc.

2.2. Culture of fibrocytes

The liver tissue was cut into pieces, digested and separated by 2.5 g/L pancreatin. DMEM with high glucose and DMEM/F12 were used for cell culture and resuspension. The cells were then inoculated into common culture bottles and thermostatically cultured at 37 °C. After 1 day, the medium

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was changed or subcultured according to the density of cells[3–5]. The fibrocytes of the 1st and 2nd generation were selected and cryopreserved for further use.

2.3. Induction and packaging of vectors

Retroviruses plasmid vectors of Sox2, Klf4, Oct4 and C-MYC were extracted using plasmid DNA extraction kit. The above fibrocytes were virus-packaged by Lipofectamine™ 2000 and then dyed by green fluorescent protein to observe the efficiency of transfection[6].

2.4. Induction of IPS cells

The fibrocytes of the 1st and 2nd generation were thawed and cultured by DMEM with high glucose and DMEM/F12. The medium was exchanged once every day. The cells were inoculated into six well plates at a density of 3×10^4 cells/well after 3 days. Then, the cells were culture for 6 hours in DMEM containing 10% FBS. The cells were transfected until the confluence area was up to 60%[7]. The transfection continued for 18 hours. The cells were transfected again after 6-hour culture, followed by further culture. Then the cells were passaged and identified after cloning.

2.5. Identification of IPS cells

The IPS cells were fixed by 4% paraformaldehyde and dyed according to the introduction of AKP staining kit after 20 minutes post fixation[8,9]. After 20-minute incubation, the cells were washed by PBS and sealed by glycerin to be observed under a microscope.

2.5.1. RT-PCR

The RT-PCR primers were designed referring to the relevant references. The primer sequences are shown in Table 1.

Table 1

Sequences of RT-PCR primers.

Primer	Sequence (5'–3')
hOct4-R	GGA GAC AGG GGG AGG GGA GGA GCT AG
hOct4-F	GTT CCC TCC AAC CAG TTG CCC CAA AG
hSox2-R	GGG AAA TGG GAG GGG TGC AAA AGA GG
hSox2-F	TTG GGT GAG TGT GGA TGG GAT TGG TG
hKlf4-R	ACG ATC GTG GCC CCG GAA AAG GAC C
hKlf4-F	TGA TTG TAG TGC TTT CTG GGG CTC C

2.5.2. Identification of stem cell marker proteins

SSEA-4, TRA-1-60, TRA-1-81, and specific proteins (OCT4 and Nanog) related to stem cell expression were detected by immunohistochemistry. Nanog protein was detected by Western blotting. Methylation level of *Nanog* gene promoter region was detected by bisulfite-genome

sequencing method. All kits were purchased from Shanghai Stan (stem) Biological Technology Co., Ltd.

2.5.3. Teratoma assay

First, 1×10^6 – 5×10^6 IPS cells were subcutaneously injected into SCID Beige mouse. The formation of subcutaneous teratoma was observed after 1 month. The tumor tissue was sliced and observed to analyze each germ layer.

2.5.4. Methylation assay

G9a protein methylation transferase inhibitor was used to treat receptor cells to induce reprogramming.

2.5.5. In vivo identification assay

The successfully established IPS cells were inoculated into the back of nude mice to observe the formation of teratoma and subcutaneous tumor[10,11].

3. Results

3.1. Infection of fibrocytes and establishment of IPS cells

The fibrocytes were infected effectively, and the expression of green fluorescence was high, showing high infection ability. The IPS cells were successfully established after fibrocytes were infected, and round IPS cell clones were observed under the microscopy (Figure 1). The induction rate was $0.013\% \pm 0.007\%$.

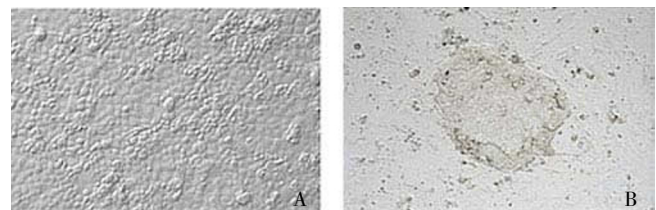


Figure 1. Microscopic observation of normal fibrocytes (A) and pluripotent stem cells induced from fibrocytes (B) (x400).

3.2. Detection of stem cell marker proteins and chromosome in established IPS cells

Obvious expression of SSEA-4, TRA-1-60, TRA-1-81 and Nanog proteins was observed (Figure 2). The IPS cells had normal 46xy chromosome, which was the same as that in the fibrocytes.

3.3. Teratoma formation in nude mice

No tumor formation was found at the back of nude mice within 8 weeks after the inoculation of cell clones. However, teratoma formed in all nude mice within 1 week post the inoculation of IPS cells. The diameter of tumor was around 2 cm after 8 weeks. The nude mouse was killed and the tumor

was obtained for observation. After subcutaneous inoculation of IPS cells into the nude mouse, HE staining was used to observe the teratoma. Three germ layers were found. There were gland cells in endoderm, cartilage tissue in mesoderm, and hair follicle structure in ectoderm (Figure 3).

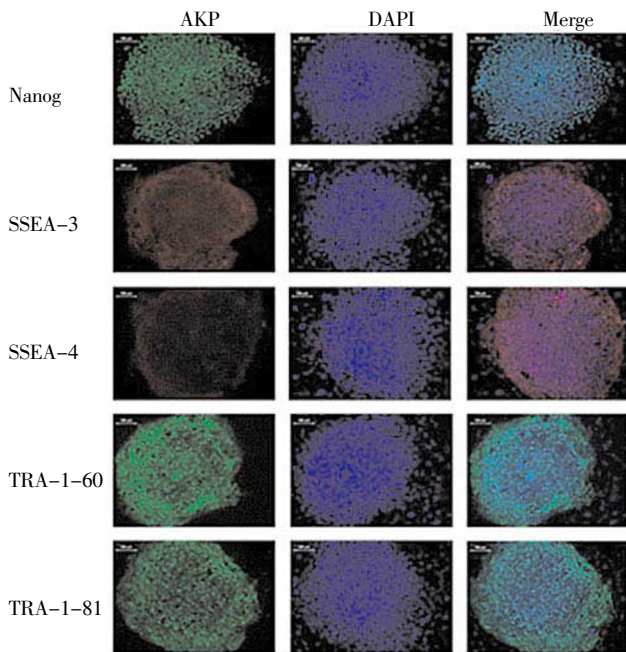


Figure 2. Expression of stem cell marker proteins in the induced pluripotent stem cells.

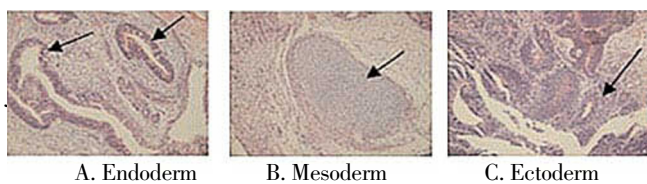


Figure 3. HE staining of teratoma in nude mouse after inoculation of induced pluripotent stem cells.

3.4. Subcutaneous tumor formation in nude mice

No tumor formed subcutaneously in the nude mice within 2 weeks post the inoculation of fibrocyte cell clones, and only one of the five mice had tumors during the last two weeks. In contrast, subcutaneous tumors appeared within 1 week after the inoculation of IPS cells in all nude mice. The diameter of tumor was less than 1 cm, and the tumor was not excised for the observation.

3.5. Methylation of fibrocytes and IPS cells

Figure 4 shows the methylation of fibrocytes and IPS cells. The Nanog and OCT4 showed high methylation status in the fibrocytes but low methylation status in the IPS cells, indicating that the IPS cells had the same ability as stem cells.

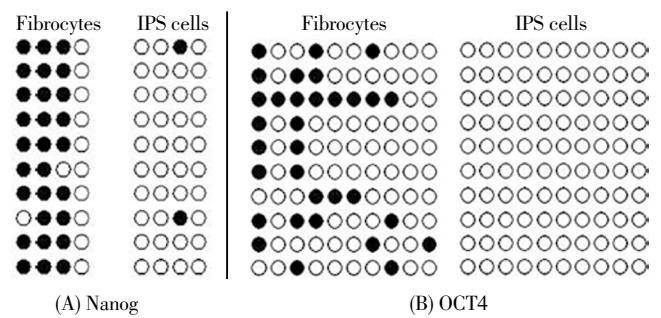


Figure 4. Comparison of methylation of Nanog and OCT4 between fibrocytes and induced pluripotent stem (IPS) cells.

4. Discussion

IPS cells is high self-renewal and has high ability of multidirectional differentiation *in vitro*, which may have wide applicable prospects in the treatment of various diseases, establishment of disease models and research of pathogenic mechanisms[12]. Due to the few obvious symptoms of early stage liver cancer, most of patients have entered intermediate or advance stage when being diagnosed. The liver cancer patients may be too late to receive surgical treatment. The patients can only receive conservative treatment or wait for liver donors. However, compared to the large group of liver cancer patients, the amount of liver donors is comparative less, and thus the life quality of liver cancer patients cannot be guaranteed[13-15]. Subsequently, to establish IPS cells by using fibrocytes has become a focal point to promote the regeneration of liver and has attracted more attention in clinic.

IPS cells have been successfully induced *in vitro* in the study of mouse cell sequencing, which made the establishment of individual specific IPS cell possible[16-18]. The methods using transposable genes and lentiviral vector show good effects on the establishment of IPS cells and help the obtainment of therapeutic IPS cells[19,20]. We established the IPS cells of human liver cancer by using this method, and the induction rate was acceptable. The carcinogenicity of IPS cells was also decreased to avoid the risk of regenerative cells induced tumor emergence[21,22]. Nonetheless, the related mechanisms are still uncertain[23]. By observing the formation of teratoma and subcutaneous tumor, we found the IPS cells had high potency of differentiation *in vivo*. The induced IPS cells had similar pluripotency as embryonic stem cells. (i) The fibrocytes had a similar strong infection capability as pluripotency stem factors. (ii) The stem cell marker proteins including SSEA-4, TRA-1-60, TRA-1-81 and Nanog were highly expressed in both the IPS cells and pluripotency stem cells, as evidenced by RT-PCR. (iii) Teratoma appeared in nude mouse within 1 week after the inoculation of IPS cells and there were three germ layers of tissue cells, showing a strong teratoma formation ability. (iv) According to the methylation results, the methylation level of Nanog and OCT4 was low in the IPS cells, indicating potency of differentiation. Thus, future studies should be focused on controlling the differentiation direction of IPS cells so as to provide cells of artificial liver. The key points

to establish IPS cells are as follows: control on the induction level of programming factor; selection of transcription factor activity time; selection of the fibrocyte resource; and control on induction level^[24–26]. In the establishing process of IPS cells, the selection of small molecular substances should be controlled according to the transcription factor level in fibrocytes, in order to increase the induction rate and decrease the carcinogenicity of cells^[27].

In conclusion, IPS cells of live cancer patient can be effectively established from fibrocytes. The IPS cells can be stably subcultured *in vitro*, which provides possibility for the treatment of liver cancer patients in the intermediate or advanced stage. In future study, more attention should be paid to the control of differentiation direction of IPS cells so that safe and clinically applicable IPS cells can be developed to improve survival rate.

Conflict of interest statement

We declare that we have no conflict of interest.

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