ARTICLE; MEDICAL BIOTECHNOLOGY

Early selection of human fibroblast-derived induced pluripotent stem cells

Elena Stoyanova*a, Milena Mourdjeva a and Stanimir Kyurkchiev b,c

aDepartment of Molecular Immunology, Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, Sofia, Bulgaria; bInstitute of Reproductive Health, Sofia, Bulgaria; cObstetrics/Gynecology hospital ‘Dr Shterev’, Tissue Bank Bulgen, Sofia, Bulgaria

(Received 27 January 2015; accepted 13 May 2015)

Induced pluripotent stem cells (iPSC) are ‘artificial’ stem cells that can be obtained by reprogramming of somatic cells. iPSC display properties, typical for embryonic stem cells (ESC) in terms of morphology, proliferation, differentiation capacity, genomic and epigenomic states. The generation of iPSC from somatic cells not only reduces some of the problems, connected with ESC, but also provides a potential human in vitro model for disease modelling and drug development. The current techniques for the production of iPSC are ineffective and often incomplete. Obtaining of extremely low amount of fully reprogrammed cells or iPSC is one of the current problems. The low efficiency of iPSC generation is a significant barrier for fast and accurate screening and makes the colony isolation time consuming and costly. In order to develop the full potential of iPSC technology, scientists are searching for new and more effective methods for generation and isolation of iPSC. Here, we report a method that could help for the isolation of iPSC cells in a short period after reprogramming. The method involves a selection of cells, based on the expression of OCT4 and a surface pluripotent marker TRA-1-60. Reprogrammed cells, isolated by this method, expressed pluripotent markers OCT4, SOX2, NANOG, KLF4 and TRA-1-60 and were able to differentiate into the three germ layers in vitro.

Keywords: cell reprogramming; human-induced pluripotent stem cells; human fibroblasts; selection of iPSC

Abbreviations
bFGF basic fibroblast growth factor
BSA Bovine serum albumin
DMEM Dulbecco’s modified Eagle’s medium
EB embryoid bodies
EDTA ethylenediaminetetraacetic acid
ESC embryonic stem cells
FCS fetal calf serum
iPSC induced pluripotent stem cells
MEF mouse embryonic stem cells
PBS phosphate buffered saline

Introduction
Since the initial report of Yamanaka and Takahashi in 2006, the generation of human-induced pluripotent stem cells (iPSC) has become an extensively used method.[1] This method makes possible the generation of pluripotent cells without the destruction of embryos and in this way, the difficulties of the broad ethical concerns are reduced. One of the most important advantages of iPSC, compared to the embryonic stem cells (ESC), is the opportunity to use mature somatic cells from patients, who suffer from various diseases. The generation of patient-specific iPSC lines and disease studying phenotype in vitro opens up new ways towards the personalized medicine. iPSC provide an invaluable resource for regenerative medicine application areas, not only for in vitro diseases modelling, but also for discovering and testing of new drugs.

Human iPSC have been generated from somatic cells by viral transfer of small number of defined factors.[1–3] The produced iPSC proliferate fast and form colonies with ESC-like morphology: flat monolayer colonies with distinctive cobbled cell morphology, prominent nuclei and large nuclear to cytoplasmic ratio. At the molecular level, iPSC gene expression and epigenetic profiles are similar to that of ESC. These cells display expression of markers, specific for pluripotent state (OCT4, SOX2, NANOG and others) and show wide ability for differentiation into various cell types.[4]

The current iPSC techniques involve stochastic, random events and the reprogramming process is indirect and non-specific. As a result, the generation of the iPSC is ineffective and often incomplete. This requires the development not only of new and more effective methods for iPSC generation, but also for the optimization of the techniques for early and correct selection of fully reprogrammed cells.

Therefore, in the present study we reported a method that could help for the selection of fully reprogrammed cells to a pluripotent state in a short period after reprogramming.
reprogramming. The method involved a selection of cells, based on the expression of OCT4 and the surface pluripotent marker TRA-1-60.

Materials and methods

Cell culture

Human neonatal foreskin fibroblasts – BJ cells (ATCC) were maintained in fibroblast medium – Dulbecco’s modified Eagle’s medium (DMEM) low glucose (PAA Laboratories) and penicillin/streptomycin/amphotericin B (PAA Laboratories), containing 10% fetal calf serum (FCS) (PAA Laboratories) and 15% FCS (PAA Laboratories), 5% knockout serum replacement (Invitrogen), 1% MEM non-essential amino acids (Invitrogen), 2.0 mmol/L L-alanyl-L-glutamine (Invitrogen), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich), 4 ng/mL bFGF-2 (Genaxxon) and penicillin/streptomycin/amphotericin B (PAA Laboratories).

The confluent monolayer of mouse embryonic fibroblasts (MEF) was subjected to mitotic inactivation by 5 μg/mL Mitomycin C (Sigma-Aldrich) for 1 h at 37 °C. After that, the cells were washed and used as feeder cells for the iPSC maintenance.

Human iPSC were cultured on amitotic MEF in ESC medium, containing DMEM/F12 (PAA Laboratories), 15% FCS (PAA Laboratories), 5% knockout serum replacement (Invitrogen), 1% MEM non-essential amino acids (Invitrogen), 2.0 mmol/L L-alanyl-L-glutamine (Invitrogen), 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich), 4 ng/mL bFGF-2 (Genaxxon) and penicillin/streptomycin/amphotericin B (PAA Laboratories).

Reprogramming protocol

Moloney-based retroviral vectors (pMXs), containing human complementary DNA for OCT4, SOX2, C-MYC and KLF4 (Addgene), were co-transfected into 293T/17 cells (fetal kidney cells, ATCC) together with packaging vectors (pUMVC and pCMV-VSV-G) by using Lipofectamine 2000 (Invitrogen). The four combined retrovirus-containing supernatants were harvested for 48 h and 72 h, pooled and concentrated by Amicon Ultra concentrators (Millipore).

On day zero, 1 × 10^5 human foreskin fibroblasts (BJ cells, ATCC) were infected with viruses, containing genes for human OCT4, SOX2, C-MYC and KLF4. The cells were cultured in fibroblast media seven days post-infection and then the media were changed with ESC medium.

Evaluation of alkaline phosphatase activity

For alkaline phosphatase staining, colonies were picked, seeded on MEF and cultured for a week. Cells were then washed with buffer (0.1 mol/L Tris, 0.1 mol/L NaCl, 0.005 mol/L MgCl2, pH 9.5) and incubated for 20 min at room temperature (RT) with 0.15 mg/L 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg nitro blue tetrazolium, diluted in wash buffer. Then, cells were washed with phosphate buffered saline (PBS) and images were taken by using microscope Leica DMI3000B (Leica Microsystems).

Positive selection of reprogrammed cells

For the reprogramming, vector hOCT4-GFP was used, which contained gene for OCT4 and puromycin resistance. Therefore, the OCT4+ cells were selected by culturing on ESC medium with 1 μg/mL puromycin for five days. The media were changed every second day.

Further selection of fully reprogrammed cells, OCT4+ cells were trypsinized 35 days after the infection and TRA-1-60+ cells were sorted over a magnetic column. The cell suspension was filtered through 45-μm cell strainer (BD), spun at 300 g for 10 min and washed in ice-cold column buffer – PBS (without Ca^{2+} and Mg^{2+}), 0.5% Bovine serum albumin (BSA), 2 mmol/L Ethylenediaminetetraacetic acid (EDTA). After the wash, the cell pellet was resuspended in 100 μL ice-cold column buffer containing 10 μL Anti-TRA-1-60-PE antibody (MiltenyiBiotec) and incubated in the dark at 4 °C for 15 min. After incubation, the cells were washed and the solution was spun at 300 g for 10 min. The cell pellet was resuspended in 80 μL ice-cold column buffer, containing 20 μL Anti-PE-Microbeads (MiltenyiBiotec) and incubated in the dark at 4 °C for 15 min. Next, the cell pellet was resuspended in 500 μL ice-cold column buffer and transferrred to the column. The cell suspension was allowed to drip through the column and the unlabelled cells were collected. The column was washed three times and was removed from the magnet. Cells were ejected by applying 1 mL column buffer and were spun at 300 g for 5 min, resuspended in ESC medium and transferred on MEF.

Embryoid bodies (EB) formation

iPSC colonies were scraped and gravity sedimented for 10 min at 37 °C. The cells were seeded on non-adherent surface (pre-coated with 0.8 mg/cm² polyhema (Sigma-Aldrich, St. Louis, MO, USA). Differentiation medium (80% DMEM/F12, 20% FCS, 1% MEM non-essential amino acids, 2 mmol/L L-alanyl-L-glutamine, 0.1 mmol/L β-mercaptoethanol and penicillin / streptomycin / amphotericin B) was changed every second day. At day 30, EB were seeded onto gelatin-coated cover slips and cultured for five days to allow attachment and monolayer spreading of EB’s cells.

Immunofluorescence staining of human iPSC

For the immunofluorescence assay, the cells were grown on cover slips, fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at RT, blocked with 1% BSA (Sigma-Aldrich) and together permeabilized in 0.1% Triton X-100 (Merck) in PBS for 1 h at RT.

The following antibodies were used: OCT4A (1:50; R&D Systems, Mineapolis, MN, USA), NANOG (1:20;
R&D Systems), SOX2 (1:50; R&D Systems), KLF4 (1:20; R&D Systems), TRA-1-60 (1:20; R&D Systems), VIMENTIN (mesenchyme, glial cells, pancreatic precursor cells) (1:500; eBioscience), NESTIN (neural stem cells, pancreatic islet progenitors, hematopoietic progenitors) (1:100; R&D Systems), α-ACTININ (skeletal, smooth muscle cells) (1:500; Abcam), DESMIN (skeletal, visceral, and certain vascular smooth muscle cells) (1:20; R&D Systems), GATA4 (primitive endodermal cells) (1:500; Abcam) and AFP (visceral endodermal cells) (1:500; Sigma-Aldrich). Appropriate secondary antibodies, conjugated to Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen), were used, cell nuclei were stained with 1 μg/mL Hoechst 33258 (Sigma-Aldrich) for 5 min at RT. In all experiments for the characterization of reprogrammed cells, we used embryonal stem cell line BG01V.

Figure 1. Schematic drawing, presenting the strategy used for human fibroblasts reprogramming and selection of iPSC.

Figure 2. Morphology of the reprogrammed cells. (A) AP staining of colonies, 20 days post-infection. (B) ESC-like colony, 24 days post-infection; flat and compact, with clear-cut round edges. (C) Non-ESC-like colony, 24 days post-infection; rough-cut edges and three-dimensional growth. (D) Colony after selection for OCT4 and TRA-1-60 positive cells. Note: Scale bar 200 μm.
as a positive control. Images were taken by using a confocal microscope Leica DM2500 (Leica Microsystems).

Results and discussion

Generation and isolation of iPSC from culture

For the generation of iPSC, we used human foreskin fibroblasts, because fibroblasts are one of the most accessible cellular sources in adult patients. Also, fibroblast cultivation and cryoconservation properties are uncomplicated with respect to nutritional requirements and viability in culture.[5] The results from the co-transfection of the fibroblasts with four plasmids, coding OCT4, SOX2, C-MYC and KLF4, produced in 293T/17 cells, are shown in Figure 1. Seven days after transfection with concentrated viruses-containing media, the cells were transferred to feeder layer. When treated cells were assessed for appearance of colonies within 14–16 days, the colonies were composed of tight, fast-growing cells and were positive for the pluripotency marker alkaline phosphatase (Figure 2(A)). However, even though alkaline phosphatase activity is considered to be a marker for pluripotent cells,[6] it is detected early in the reprogramming process and does not necessarily connect to fully reprogrammed cells.[7] Around the 20–25 d, we observed colonies with the typical morphology of human ESC (ESC-like morphology — flat colonies of cells with large nuclear to cytoplasmic ratio and prominent nucleoli, and with well distinctive edges) (Figure 2(B)) and non-ESC colonies with distinct morphology (colonies with rough-cut edges and three-dimensional growth) (Figure 2(C)). Similar morphological heterogeneity in reprogrammed human fibroblast has been already reported.[3,8] These morphological differences in the colonies were the first indication for the coexisting of not fully reprogrammed cells. One of the main problems in iPSC generation is how to select fully reprogrammed cells, since the selection of colonies is highly subjective, but critical for the production of high-quality iPS cells. To overcome subjective selection of iPSC colonies, we have developed a simple and time-saving method for the isolation of fully reprogrammed cells from the heterogeneous cell population. Our method combined antibiotic selection and magnet sorting.

Reprogrammed cells containing transgene OCT4 could have higher chance of giving rise to a fully reprogrammed cells (iPSC), because of the key role of OCT4 in the induction and maintenance of pluripotent state. For the selection, we used puromycin resistance of vector, coding OCT4. After the selection of the reprogrammed cells, they were seeded on MEF where they expanded.

![Figure 3. Expression of OCT4, NANOG and TRA-1-60 in OT-iBJ1 and OT-iBJ2 clones two months post-infection. Note: Positive control – Human ESCs from line BG01V; negative control – parental fibroblast; Cells’ nuclei stained with Hoechst (blue). Scale bar 100 μm.](image-url)
Figure 4. Expression of pluripotent markers in OT-iBJ2 clones four months post-infection. Note: Positive control — Human ESCs from line BG01V; negative control — parental fibroblast; Cells’ nuclei stained with Hoechst (blue). Scale bar 100 μm.
Next, cells were separated by the expression of the surface marker TRA-1-60. Similar method for the identification of iPSC, according to membrane molecule expression, has been reported.[9,10] TRA-1-60 is not directly connected to the maintenance of pluripotent state, but its expression is detected in pluripotent cells.[11,12] The appearance of this membrane protein on the reprogrammed cells is a signal for changing of somatic cells into pluripotent cells. Therefore, on the 30–35 d, reprogrammed cells were selected, based on Anti-TRA-1-60-PE antibody binding. The cells were run through a magnetic column, which retained the positive cells. TRA-1-60 negative cells were washed through the column and discarded. The positive cells were eluted from the column and seeded on fresh MEF. Three days later, colonies, composed of few cells, were detected. They grew rapidly and showed ESC-like morphology (Figure 2(D)). In contrast, the mechanical passaging of iPSC colonies to produce similar number of fully reprogrammed cells took several weeks.

**Characterization of human iPSC clones**

In order to assess whether the isolated cells presented ESC-like features, we performed immunofluorescence staining for the pluripotent markers OCT4, NANOG, KLF4, SOX2 and TRA-1-60 and in vitro differentiation. We focused on the expression of transcription factors OCT4 and NANOG, because they are key regulators of the embryo development. OCT4 is involved in maintaining of the pluripotent state.[13,14] In addition, NANOG is a central part of the pluripotency network, together with OCT4 and SOX2.[15–17] Although NANOG expression is not required for the maintenance of pluripotent stem cells, it has been reported to be essential for the establishment of both ESC from blastocysts and iPSC from somatic cells.[18,19] Two months after the infection (20–25 days post-selection), we tested two of the reprogrammed cell clones (OT-iBJ1 and OT-iBJ2) for OCT4, NANOG and TRA-1-60 (Figure 3). Most of the reprogrammed cells showed nuclear OCT4 and NANOG staining similar to BG01V cells. A few cells demonstrated cytoplasmic localization of these proteins. This is no new data since cytoplasmic localization of OCT4 and NANOG is described in neuroendocrine,[20] cervical [21] and other types of tumours.[22] Oka et al. [23] defined Oct4 as a shuttle protein between nucleus and cytoplasm. For the further experiments, clone OT-iBJ2 was preferred, since it showed more than three times higher mRNA levels for OCT4 and NANOG (data not shown).

Four months post-infection cells were maintained in culture and the pluripotent markers were tested again. We detected clear nuclear expression of OCT4, NANOG, KLF4 and SOX2 (Figure 4). The presence of these proteins after long-term culturing was one of the proofs that we had selected iPSC. We detected low expression of all of the markers, studied in fibroblast cells. This was in correspondence with other authors that established the presence of pluripotent state markers in adult body tissues, but in lower levels, when compared to the ESC.[24]

To determine whether the reprogrammed cells were pluripotent, we tested their ability to differentiate into cells of three germ layers in vitro. The differentiation potential was investigated by subjecting the cells to EB formation, described in Materials and methods section. Compact aggregates were formed 3–7 days after the initiation of EB formation. The spontaneously formed EB varied in form and size (Figure 5(A)). The differentiation capacity

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**Figure 5.** *In vitro* differentiation of iPSC. Images of 20 and 30 days old EB (A), generated from clone OT-iBJ2 in suspension culture. Immunofluorescent staining (B) showed expression of differentiation markers nestin, vimentin, α-actinin, desmin, GATA4 and AFP in OT-iBJ2 and BG01V. Notes: Cells’ nuclei stained with Hoechst (blue). Scale bar 200 μm for (A) and 100 μm for (B).
of the clone OT-iBJ2 is shown in Figure 5(B). This staining patterns showed the ability of selected cells to onset differentiation towards all three embryonic germ tissues (ectoderm, mesoderm and endoderm) (Figure 5(B)). Only pluripotent cells would have the ability to express pluripotent markers and to differentiate into various tissues.

Conclusions

The reported method for early selection of iPSC was based on the expression of pluripotent markers OCT4 and TRA-1-60. Colonies, selected by this protocol, displayed all of the features of state of pluripotency and ability to differentiate into cells, typical for the three germ layers in vitro. Our method should provide a valuable technique for the selection of fully reprogrammed cells, facilitating the isolation of iPSC in a short time after reprogramming.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Bulgarian Ministry of Education and Science [grant number D01-4787/ReProForce FP-7-REGPOT-2009-1]; the European Social Fund and Operational Programme Human Resources Development (2007–2013) with co-funding from the Bulgarian Ministry of Education and Science [grant number BG051PO001-3.3.06-0059].

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