Generation of Induced Pluripotent Stem Cells by Reprogramming Human Fibroblasts with the Stemgent™ Human TF Lentivirus Set

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SUMMARY

We demonstrate the generation of induced pluripotent stem (iPS) cells from human somatic cells using lentivirus-mediated delivery of the human factors Oct4, Sox2, Nanog, and Lin28. Viral vectors have been widely used to deliver transcription factors into mammalian somatic cells. The Stemgent™ Human TF (transcription factor) Lentivirus Set utilizes the VSV-G pseudotyped lentivirus system that is capable of transducing both dividing and non-dividing cells from many mammalian species, including mouse and human. Here we show that co-transduction of the viruses from this set induces reprogramming in human foreskin fibroblast BJ cells grown on a mouse embryonic fibroblast (MEF) feeder layer. Pluripotency of the reprogrammed cells was confirmed by the presence of ES cell-specific markers. Our study demonstrates the utility of the Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) for the generation of iPS cells from human somatic cells.

INTRODUCTION

Reprogramming is the process by which cells are converted from a differentiated state to a pluripotent state, or converted directly from one differentiated state to another. In 2006, using a defined set of transcription factors and cell culture conditions, Shinya Yamanaka *et al.* first demonstrated that retrovirus-mediated delivery and expression of Oct4, Sox2, c-Myc, and Klf4 is capable of inducing the pluripotent state in mouse fibroblasts[1]. The same group reported the successful reprogramming of human somatic cells into induced pluripotent stem (iPS) cells using human versions of the same transcription factors delivered by retroviral vectors[2]. Additionally, James Thomson *et al.* reported that the lentivirus-mediated co-expression of another set of factors (Oct4, Sox2, Nanog, and Lin28) was capable of reprogramming human somatic cells into iPS cells[3].

iPS cells are similar to ES cells in morphology, proliferation, and ability to differentiate into all the tissue types of the body. In mice, pluripotency of iPS cells has been fully demonstrated through the generation of germline chimeras. Pluripotency of human iPS cells has been proven by formation of mature teratomas in mice[2,3]. Human iPS cells have a distinct advantage over ES cells as they exhibit key properties of ES cells without the ethical dilemma of destroying an embryo to obtain the cells.

Additionally, iPS cells generated from patients will provide a powerful research tool to investigate the mechanisms underlying the specific disease. The generation of patient-specific iPS cells circumvents an important roadblock to personalized regenerative medicine therapies by eliminating the potential for immune rejection of non-autologous transplanted cells.

Here we demonstrate that the Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) is capable of expressing each of the following four factors: Oct4, Sox2, Nanog, and Lin28. Also, co-transduction of the four factors can reprogram human somatic cells into iPS cells that display the pluripotency markers characteristic of ES cells.

RESULTS

Viral Infectious Unit Titer Determination

Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) contains four viruses carrying human Oct4, Sox2, Nanog, and Lin28. Viral titer was determined by p24 (lentivirus capsid protein) concentration. The p24 values for the each of the viruses used in this reprogramming experiment are listed in Table 1. To calculate the infectious titer for each virus, we first determined the transduction unit (TU) titer of the GFP lentivirus that was used as a manufacturing process control for the human TF virus production. For this batch of production, the TU titer of the control GFP virus was 2.72 x 10^7 TU/ml. The co-efficiency is 1.49 x 10^5 TU/ng. Therefore, the TU titer for each virus is p24 concentration times the co-efficiency value (Table 1).

Table1. p24 and infectious titers for each virus.

Lentivirus	p24 (ng/ml)	TU Titer (TU/ml)
hOct4	6.64	0.99×10^6
hSox2	68.8	1.02×10^7
hNanog	82.8	1.23×10^7
hLin28	68.8	1.02×10^7

Protein Expression Validation

To demonstrate that each virus expressed the transcription factor it encoded, we infected HEK293-AD cells with each of the four human TF viruses and the ectopically expressed proteins were detected by immunocytochemistry (ICC). In this experiment, 2 x 10^4 HEK293-AD cells were seeded for transduction. For hOct4, hSox2, hNanog, and hLin28 viruses, 200 µl, 20 µl, 20 µl and 20 µl were added to the cells, respectively, to reach an M.O.I. (multiplicity of infection) of 10. Seventy two hours after the transduction, the ectopically expressed proteins were detected using their corresponding antibodies (Figure 1). ICC staining for hOct4, hSox2, and hNanog was localized in the nucleus as indicated by the overlap with the DAPI stain for the cell nuclei, while the signal for hLin28 was present primarily in the cytoplasm (Figure 1). This showed the correct cellular compartment localization for each ectopically expressed protein.

Reprogramming of BJ Cells

To demonstrate the ability of Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) to induce reprogramming in somatic cells, human foreskin fibroblast cells (BJ cells) were co-transduced with the set of viruses. The transduction was performed in one well of a 6-well plate seeded with 1 x 10^5 BJ cells. In consideration of the low TU titer for hOct4 virus, the viral transduction was carried out at M.O.I. = 5. Therefore, 500 µl of hOct4 virus and 50 µl of the other viruses were used. The next day, the cells were passaged into 3 wells of a 6-well plate with pre-seeded CF-1 MEF feeder cells. Morphological changes were observed as early as Day 4 post-transduction. The elongated cells started to round up and form clusters sporadically in the well (Figure 2). The cluster of cells became more tightly packed at day 17 (Figure 2). The colonies were manually picked at day 25 and cultured on CF-1 MEF feeder cells. Recent studies have shown that Rho-associated kinase (ROCK)

RESULTS <CONT>

inhibitors can enhance the survival and cloning efficiency of dissociated human ES cells[4,5]. To facilitate the iPS cell colony formation after reprogramming, we used Stemolecule™ Y27632 (Cat. No. 04-0012) at 10 mM for the initial overnight seeding during each passage. iPS cell colonies with good morphology (i.e. tightly packed, flat and with clear edges) were observed after three sequential rounds of colony picking and passaging (Figure 3).

iPS Cell Characterization

To further characterize the isolated iPS cell colonies, we looked for the presence of common pluripotency markers expressed in ES cells. The colonies exhibited strong alkaline phosphatase activity as detected by the Stemgent™ Alkaline Phosphatase Staining Kit (Cat. No. 00-0013) (Figure 3). Additionally, immunocytochemistry (ICC) was performed on the iPS cell colonies with a panel of pluripotency marker-specific antibodies, including the surface markers TRA-1-81 (Cat. No. 09-0012), TRA-1-60 (Cat. No. 09-0009), SSEA-4 (Cat. No. 09-0003), SSEA-3 (Cat. No. 09-0014), and SSEA-1 (Cat. No. 09-0005) as well as the nuclear markers Oct4, Sox2, and Nanog. The isolated iPS cell colonies were positive for all markers except SSEA-1, which is not present in undifferentiated human ES cells (Figure 4). The ICC results showed that the iPS cells exhibited the appropriate pluripotency marker expression pattern, demonstrating that these iPS cells closely resemble undifferentiated human ES cells.

Conclusion and Discussion

We demonstrated that the Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) is able to reprogram human fibroblast cells into iPS cells. Since different cell types may have different reprogramming efficiencies, several factors should be taken into consideration when planning your reprogramming experiment. First and foremost, you may need to modify the active virus-to-target cell ratio (multiplicity of infection or M.O.I.) during the primary transduction step to achieve optimum transduction efficiency. Second, the growth condition of the target cells can impact reprogramming. Healthy and proliferative cells are more amenable to reprogramming. Third, when modifying the protocol for different cell numbers, it is recommended that target cell numbers are adjusted proportionally to the surface area of the culture dish (please see below for protocol). Lastly, applying ROCK inhibitors, such as Y27632 (Cat. No. 04-0012), should be considered to help ensure successful reprogramming as recent studies have demonstrated its utility in enhancing hES colony survival[4,5]. While no reprogramming method can guarantee reprogramming of any and all cell types, the Stemgent™ Human TF Lentivirus Set (Cat. No. 00-0005) was functionally validated in BJ cells to ensure that all researchers will be able to generate human iPS cells.

EXPERIMENTAL PROCEDURES

A. Materials

Lentivirus: Human TF (Transcription Factor) Lentivirus Set (Cat. No. 00-0005) contains hOct4-Lentivirus (Cat. No. 07-0013), hSox2-Lentivirus (Cat. No. 07-0012), hNanog Lentivirus (Cat. No. 07-0017) and hLin28-Lentivirus (Cat. No. 07-0016).

Cells: BJ human foreskin fibroblasts.

BJ Cell Growth Medium: 450 ml EMEM, 50ml ES-qualified FBS, 5ml 10mM Non-Essential Amino Acids, 5 ml penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml), 5 ml 200 mM L-glutamine, and 0.9 ml 55 mM β-mercaptoethanol.

Human ES/iPS Cell Medium: 400 ml DMEM/F12, 100 ml Knockout™ Serum Replacement, 5 ml 10mM non-essential amino acids, 5 ml 200 mM L-glutamine, 0.9 ml 55 mM β-mercaptoethanol, and 10ng/ml human recombinant bFGF (Stemgent™ Cat. No. 03-0002).

Feeder Cell Culture Medium: 450 ml DMEM, 50ml ES-qualified FBS, 5 ml 10mM non-essential amino acids.

MEF Conditioned Medium: CF-1 MEF feeders were seeded at 2×10^5 cells per well of a 6-well plate in feeder cell culture medium. After overnight incubation, the medium was changed to human ES/iPS cell medium. The supernatant was collected every 24 hours for 4 days. The supernatant was centrifuged and filtered through a 0.22 µm filter. Before adding the supernatant to the cells, the supernatant was supplemented with 50 ng/ml bFGF (Cat. No. 03-0002).

Fixative: 4% paraformaldehyde

Blocking Buffer: 10% goat serum and 0.5% Triton™ X-100 in PBS.

B. Reprogramming

1. **Seeding BJ cells:** P6 BJ cells were seeded at a density of 1 x 10^5 cells in one well of a 6-well plate. The cells were cultured in 2.0 ml of BJ medium overnight at 37°C and 5% CO_2.

2. **Viral Transduction:** BJ medium was removed, and 1.35 ml of BJ medium supplied with 6 µg/ml of polybreen, 500 µl hOct4-lentivirus, 50 µl hSox2-lentivirus, 50 µl hNanog-lentivirus, and 50 µl hLin28-lentivirus was added. After ensuring that the medium was distributed evenly by gentle rocking of the cell culture dish, the cells were incubated overnight at 37°C and 5% CO_2.

3. **Passaging of Transduced BJ Cells:** Twenty four hours post-transduction, the cells were trypsinized, centrifuged at 200 x *g* for 5 minutes, re-suspended in BJ medium, and re-plated in 3 wells of a 6-well plate with CF-1 MEF feeder cells seeded the previous day. These cells were incubated overnight at 37°C and 5% CO_2.

4. **Media Changes:** Twenty four hours after re-seeding, BJ medium was replaced with human ES/iPS cell culture medium. The cell culture medium was changed every day for the first seven days. After seven days, medium was transitioned to MEF conditioned medium.

EXPERIMENTAL PROCEDURES <CONT>

5. **iPS Colony Selection and Passaging:** All ES-like colonies were selected and re-seeded in human ES culture medium with 10 µM Stemolecule™ Y27632 (Cat. No. 04-0012) on cell culture dishes pre-seeded with CF-1 MEF feeder cells. Cell culture medium was changed every day for the first seven days and transitioned to MEF conditioned medium thereafter. Passaging of cells continued until they showed typical human ES morphology.

C. iPS cell characterization

1. **Immunocytochemistry (ICC):** Cells were washed gently with PBS and fixed with 500 µl of fixative for 20 minutes at room temperature. After 3 washes with PBS , the cells were blocked with blocking buffer for 1 hour at room temperature and incubated with target-specific primary antibodies at the specified dilution (see below) overnight at 4°C. After 3 washes with PBS, the appropriate conjugated secondary antibody at the specified dilution (see below) was added to the cells and incubated for 2 hours at room temperature. Plates were kept in the dark to avoid light. The cells were washed with 3 times with PBS. During the last wash, DAPI stain was added at a final concentration at 1 µg/ml for 5 minutes. After a final wash with PBS, the cells were analyzed under a fluorescent microscope.

   Primary Antibodies:
   Anti-Tra-1-81, mouse monoclonal IgM antibody (Cat. No. 09-0011) (1:100).
   Anti-Tra-1-60, mouse monoclonal IgM antibody (Cat. No. 09-0010) (1:100).
   Anti-SSEA-4, mouse monoclonal IgG antibody (Cat. No. 09-0006) (1:100).
   Anti-SSEA-3, rat monoclonal IgM antibody (Cat. No. 09-0014) (1:100).
   Anti-SSEA-1, mouse monoclonal IgM antibody (Cat. No. 09-0005) (1:100).
   Anti-Oct3/4, mouse monoclonal IgG2b antibody (1:100).
   Anti-Sox2, mouse monoclonal IgG2a antibody (1:100).
   Anti-Nanog, rabbit polyclonal antibody (1:100).
   Anti-Lin28, goat IgG polyclonal antibody (1:100).

   Secondary antibodies:
   Goat anti-Mouse IgM, Cy3 conjugate (1:200).
   Goat anti-Mouse IgG, Cy3 conjugate (1:200).
   Goat anti-Rat IgM, Cy3 conjugate (1:200).
   Goat anti-Rabbit IgG, Cy3 conjugate (1:200).

2. **Alkaline Phosphatase (AP) Staining:** Alkaline phosphatase (AP) staining was performed using the Stemgent™ AP Staining Kit (Cat. No. 00-0009) following the recommended protocol provided in the kit.

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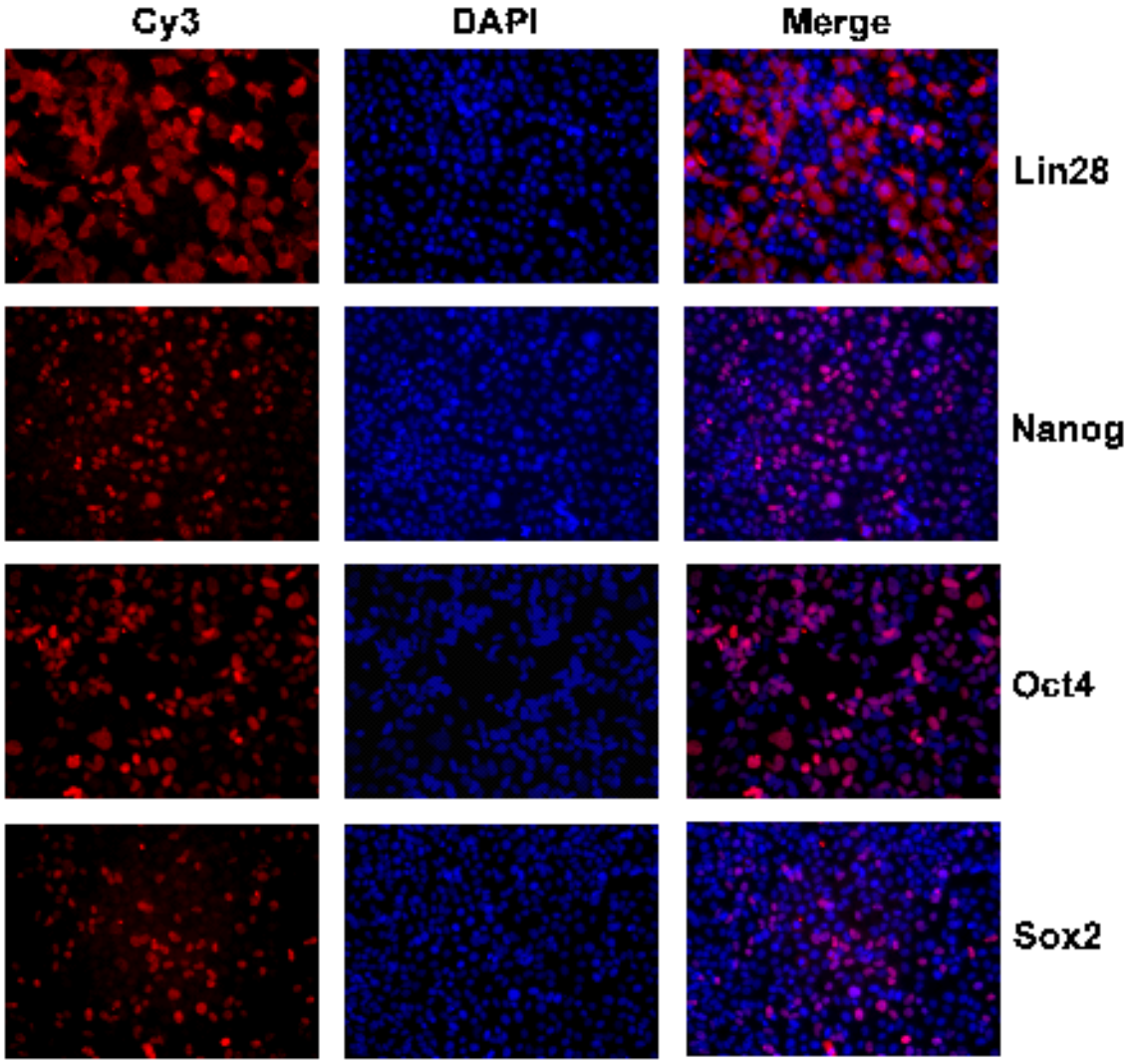

FIGURE 1:

Immunocytochemistry (ICC) detection of proteins ectopically expressed by human TF lentiviruses. HEK293-AD cells were transduced with the indicated lentiviruses (Sox2, Oct4, Nanog, and Lin28) at M.O.I. = 10. The cells were fixed 72 hours post-transduction, stained with corresponding antibodies, and visualized by Cy3-conjugated secondary antibodies. Cell nuclei were counter-stained with DAPI.

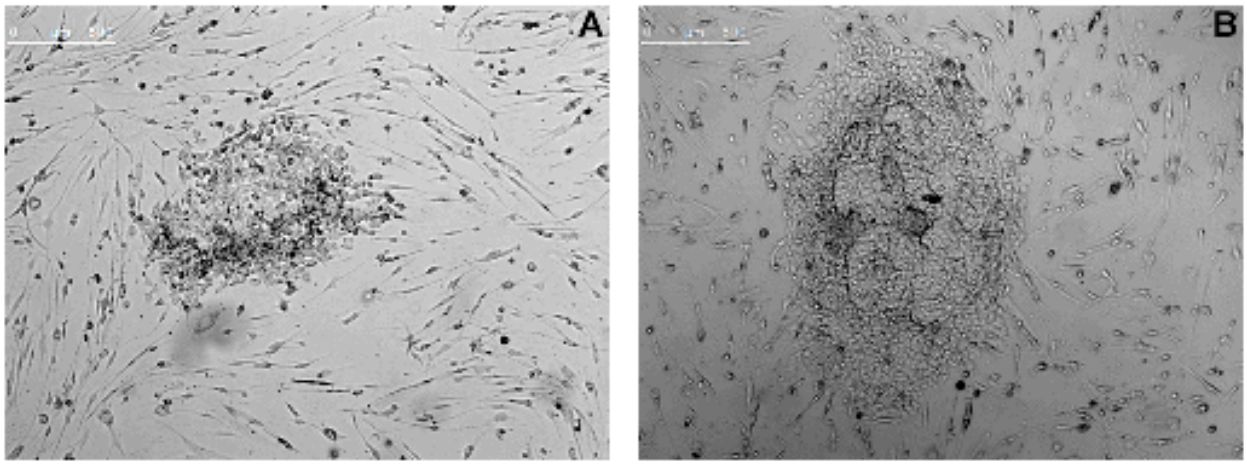

FIGURE 2:

iPS cell colony formation post viral transduction.

Bright field images of a typical iPS cell colony formed at (A) 4 days and (B) 17 days post-transduction.

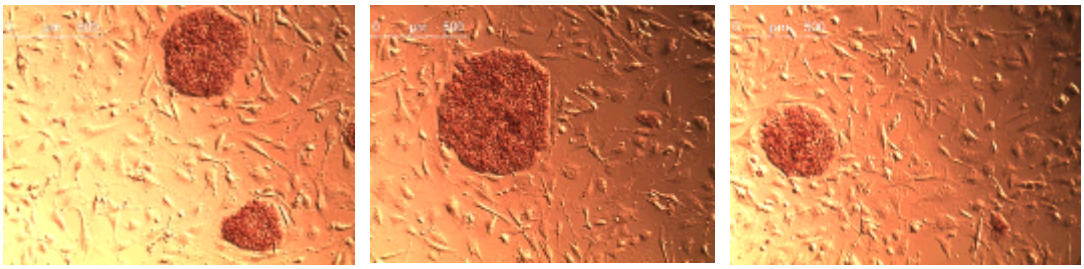

FIGURE 3:

Human iPS cell colonies are positive for alkaline phosphatase staining.

The images show three different colonies stained with the Stemgent™ Alkaline Phosphatase Staining Kit (Cat. No. 00-0009) that were passaged three times each.

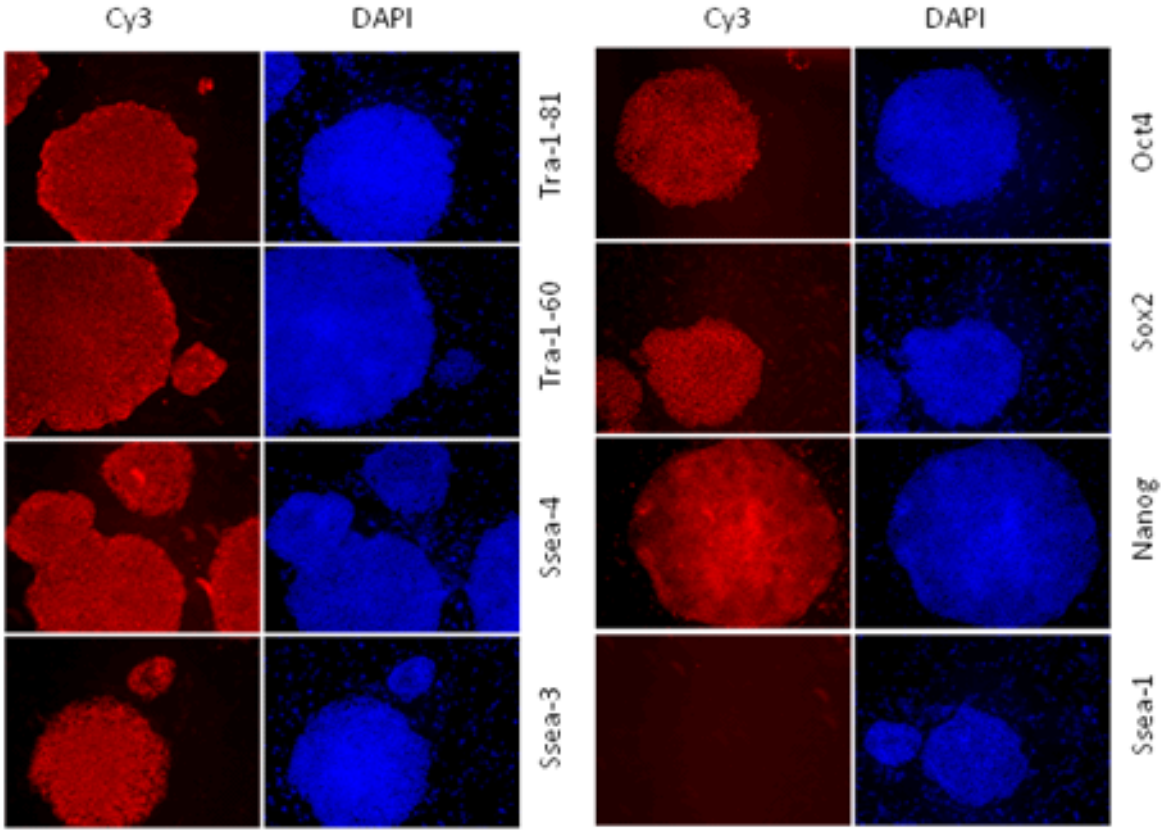

FIGURE 4:

Pluripotency marker detection for human iPS cells.

Human iPS cells express high level of the following ES cell specific surface markers: Tra-1-81, Tra-1-60, SSEA-4, and SSEA-3, and the following nuclear markers: Nanog, Sox2, and Oct4. As expected, human iPS cells were negative for SSEA-1.