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Research Article

Expression of SSEA4 and TRA1-60 as Marker of Induced Pluripotent Stem Cells by Small Molecule Compound VC6TFZ on Peripheral Blood Mononuclear Cell

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Abstract

Introduction: It is possible to induce pluripotent stem cells from somatic cells, offering an infinite cell resource with the potential for disease research and use in regenerative medicine. Due to ease of accessibility, minimum invasive treatment, and can be kept frozen, peripheral blood mononuclear cells (PBMC) were an attractive source cell. VC6TFZ, a small molecule compound, has been successfully reprogrammed from mouse fibroblast induced pluripotent stem cells (iPSCs). However, it has not been confirmed in humans.

Methods: Mononuclear cells were separated from peripheral venous blood using the centrifugation gradient density process. Mononuclear cells were cultured for six days in the expansion medium. The cells were divided into four groups (P1-P4). The induction of pluripotency using small-molecule compound VC6TFZ was completed within 14 days. Then for seven days, the medium shifted to 2i medium. iPSCs identification is based on colony morphology and pluripotent gene expression, SSEA4 and TRA1-60 marker using immunocytochemistry.

Results: Colonies appeared on the reprogramming process on day seventh. These colonies had round, large, and cobblestone morphology like ESC. Gene expression of SSEA4 and TRA1-60 increased statistically significant than control group (SSEA4 were P2 $p = 0.007$; P3 $p = 0.001$; P4 $p = 0.009$ and TRA1-60 were P2 $p = 0.002$; P3 $p = 0.001$; P4 $p = 0.001$).

Conclusion: Small molecule compound VC6TFZ could induce pluripotency of human PBMC to generate iPSCs. Pluripotency marker gene expression, SSEA 4 and TRA 1-60, in the experimental group, was statistically significantly higher than in the control group.

Keywords: *VC6TFZ; Pluripotent Stem Cells; Peripheral Blood Mononuclear Cells; Reprogramming; SSEA4; TRA1-60*

Introduction

Pluripotent stem cells are expected to become future regenerative therapies that can differentiate into all cell types derived into three germinal layers. Embryonic stem cells (ESCs) that are produced at the blastocyst stage from the internal cell mass of the embryo are the

common source of pluripotent stem cells. Because of ethical issues regarding embryo mortality, the risk of immunological rejection, and a limited supply owing to embryo origin, the use of ESCs has certain limitations. Because they are made from somatic cells, induced pluripotent stem cells (iPSCs) offer an alternate source of pluripotent stem cells that overcomes the ethical problem. Potential use of iPSCs such as cells for cell transplantation therapy, diseases modelling, and drug screening [1].

Induced pluripotent stem cells were produced by reprogramming process using different reprogramming factors from somatic cells. The first source cells were skin fibroblast cells, now the most commonly used [2,3]. Skin biopsy procedures, however, have been unconvertable, leave scar tissue, and take a long time for the expansion of fibroblast cells, restricting the use of fibroblasts as an iPSC source. Peripheral blood cells have become an attractive source of iPSCs because they are easy to extract, minimally invasive, and preserved in a frozen state [4].

Exogenous transcription factors OCT4, SOX2, Klf4, and c-Myc (OSKM) were transduced into the nucleus of somatic cells transplanted by retroviruses, resulting in iPSCs [2,3]. Exogenous transcription factors and integrative systems are used in a reprogramming process linked with low effectiveness, mutagenesis, and teratogenesis, limiting its utility in clinical applications. Small compounds that acted on signalling pathways, epigenetic alterations, and metabolic processes were extensively employed to improve reprogramming efficiency utilizing exogenous transcription factors. Despite the lack of foreign transcription factors, a mixture of small molecules may be enough to generate pluripotency [5].

VC6TFZ was a combination of small molecules consisting of deazaneplanocin (DZnep), CHIR990210 (CHIR), valproic acid (VPA), 616452 (Repsox), tranylcypromine, and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenylbenzoic acid (TTNPB). Combining these small compounds might generate pluripotency in mouse embryonic fibroblasts (MEFs) more effectively than the Yamanaka technique, which included exogenous transcription factors (0.02 vs 0.001 - 0.001) [6].

Induced pluripotent stem cells have several heterogeneous characteristics and morphology so that several markers can be used to identify the expression of iPSCs. Alkaline phosphatase staining is a marker for surface cell antigen on iPSCs. SSEA4 and TRA 1-60 are antibodies that express cell surface antigens, particularly against iPSCs. Surface cell antibodies are the most widely used method to identify stem cells in heterogeneous cultures, specifically SSEA4 and TRA 1-60 antibodies present as a benchmark for identifying iPSCs by immunostaining methods.

Aim of the Study

This research aimed to identify the expression of SSEA4 and TRA1-60 as a marker of iPSCs by small-molecule compound VC6TFZ in human peripheral blood mononuclear cells (PBMC).

Methods

This study is an experimental laboratory study (*in vitro* study) randomized study, with a "post-test only control group design" approach (Figure 1). We administrated small molecule compound VC6TFZ to induce pluripotency of PBMC and generate iPSCs. The blood sample for this study was derived from a 29-year-old male volunteer. Figure 2 shows the study methodology, which included PBMC isolation, culture, pluripotency induction using small molecules, and identification of iPSCs. This research was conducted at the Laboratory of the Center for Research and Development of Stem Cell, Institute of Tropical Disease (ITD), Airlangga University, Surabaya, Indonesia.

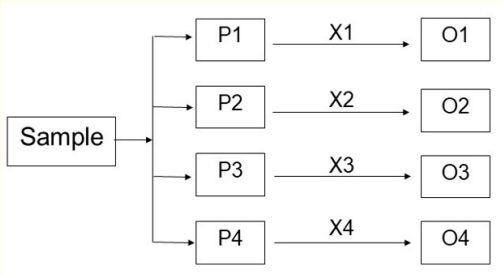

Figure 1: *Study design "post-test only control group design".*

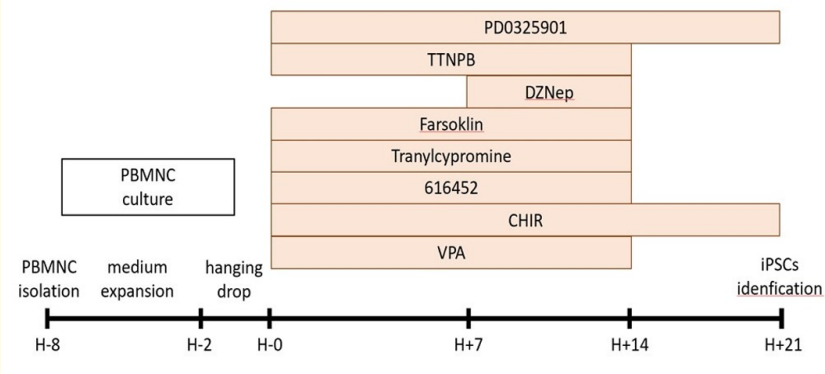

Figure 2: *Study protocol.*

PBMCs isolation

Heparin-coated tubes were used to collect blood, then diluted with 5 cc phosphate-buffered saline (PBS), and centrifuged for 30 minutes at 1600×g via a Ficoll gradient. PBMCs were collected, transferred to a fresh tube, and 10 cc PBS was added before centrifuging for 5 minutes at 2000-2500×g. Aspirate the supernatant and resuspend it in 10 cc PBS.

Mononuclear cell reprogramming using small molecule compound VC6TFZ

Three drops of mononuclear cell solution were added to an M-24 well plate that had been pre-coated with vitronectin overnight and feeder cells. Feeder cells were created from mitosis-inactivated rabbit adipose mesenchymal cells for 30 minutes using mitomycin-C 2 g/mL. We added ReproTesR medium, which contained small molecule VC6TF. DZnep was added on day 7, and for 14 days, cells were exposed to small molecules. Every four days, the medium was changed. VC6TFZ was changed to 2i medium (CHIR and PD0325901) on day 14.

Mononuclear cells were divided into four groups consisting of the control group (P1), and the experimental group (P2, P3, and P4) who received the small molecule compound VC6TFZ with different dosage variations; such as:

- Group 2 (P2) was the dosage VPA 0.5 mM, CHIR 5 µM, 616452 5 µM, tranylcypromine 2.5 µM, FSK 20 µM, DZnep 20 µM, and TTNPB 5 µM.
- Group 3 (P3) was the experimental group which exposed to small-molecule dosage VPA 0.75 mM, CHIR 10 µM, 616452 7.5 µM, tranylcypromine 5 µM, FSK 40 µM, DZnep 50 nM, and TTNPB 5 µM.
- Group 4 (P4) was the experimental group which exposed to small-molecule VPA 1 mM, CHIR 20 µM, 616452 10 µM, tranylcypromine 10 µM, FSK 60 µM, DZnep 100 µM, and TTNPB 5 µM.

PBMCs identification

iPSCs were identified using morphology and the expression of pluripotency markers (SSEA4 and TRA 1-60). Induced pluripotent stem cells colonies had large characteristics, tight and clear borders, and a cobblestone-like appearance. Cells in iPSC colonies were tiny and had a high ratio of cytoplasmic nuclei.

Immunocytochemical staining

Cells were fixed in methanol for 10 minutes before being washed twice with cold PBS. The cells were then stabilized for 10 minutes at room temperature with 0.25% Triton X-100 in PBS. PBS was used to wash the cells three times for five minutes. For 30 minutes, cells were treated with 1% bovine serum albumin (BSA) in phosphate-buffered saline with Tween (PBST). Cells were then treated in a humidified chamber for 1 hour at room temperature or overnight at 4°C in antibody solution (SSEA4 and TRA1-60) in 1% BSA in PBST. The cells were then washed for 5 minutes in PBS, which was done three times. The cell was then treated with the Alexa Fluor 488 goat anti-mouse IgG secondary antibody. A microscope is then used to examine the fluorescent signal.

Statistical analysis

The data were coded, tabulated, and statistically analyzed using SPSS version 24. SSEA4 and TRA1-60 expressions will be presented mean ± SD. Normality data test was done using Kolmogorov-Smirnov. Differences in SSEA4 and TRA1-60 expression across the four groups will be examined using a one-way ANOVA test if the data is normally distributed and a Kruskal-Wallis test if the data is not normally distributed. Significance cut off value is $\alpha = 0.05$.

Results

Isolation and culture of peripheral blood mononuclear cells

The isolation process separates mononuclear cells from other cells in the peripheral blood, such as erythrocytes and platelets. This isolation process is carried out by the centrifugation gradient density method, where the mononuclear cells are in the buffy coat layer (Figure 3A). Mononuclear cell culture was carried out for 6 days by culturing it on basal medium (RPMI) enriched with L-Glutamine 1 mL/100 mL, ITS 1 mL/100 mL, FGF 5 ng/uL, ascorbic acid 5 mg/100 mL, GMCSF 50 uL/100 mL and dexamethasone 100 uL/100 mL.

Mononuclear cells cultured on a medium enriched with cytokine cocktails for six days were then cultured using the hanging drop method for 48 hours (Figure 3B). Hanging drop is done by dripping mononuclear cells vertically one drop 25 µL of mononuclear cell suspension on a sterile petri-dish cover with a diameter of 5 cm and then turning it over (Figure 4A).

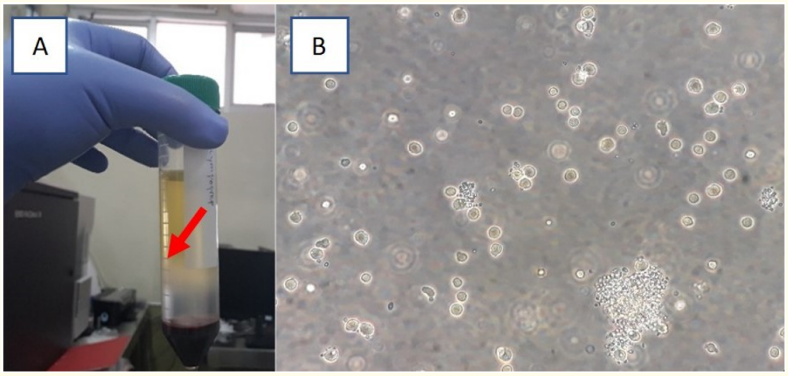

Figure 3: *(A) Buffy coat layer between plasma and Ficoll, (B) Peripheral blood mononuclear cells (200x magnification).*

Induction of peripheral blood mononuclear cell pluripotency

Five drops of mononuclear cell suspension that had been cultured by the hanging drop method were plated on M-6 well plates that had been previously coated with vitronectin and feeder cells (Figure 4B). In this study, adipose mesenchymal stem cells (AMSC) were used in rabbits that had been pre-treated with 2 ug/mL of Mitomycin C (MMC).

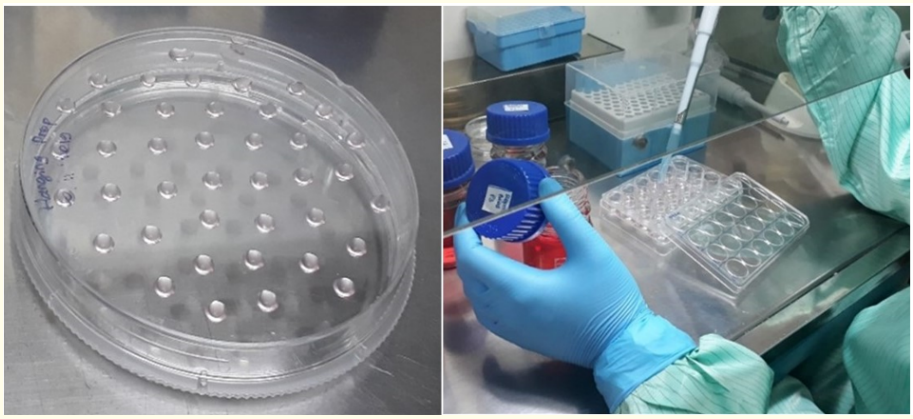

Figure 4: *(A) Hanging drop method, (B) M6 well plate which was previously coated with vitronectin and feeder cell.*

The induction of mononuclear cell pluripotency was carried out for 21 days. During the first seven days, the cells received small molecule VPA, CHIR, 616452, Tranylcypromine, Farsocline, and TTNPB. Deazaneplanocin (DZnep) was added on day 7 to day 14. On day 14, the small molecule compound VC6TFZ was replaced with a 2i medium consisting of PD0325901 and CHIR. ReproTesr medium is changed every four days.

Identification of induced pluripotent stem cells

The identification of iPSCs in this study was carried out by identifying pluripotent stem cell colonies and the expression of markers SSEA4 and TRA1-60. The induced pluripotent stem cells tend to form colonies with large sizes and clear edges. With the hanging drop method, it is possible to form colonies from the first day of plating. Colonies with large and round morphology, cobblestone-like appearances, and clear boundaries have been seen on the seventh day of the reprogramming process (Figure 5).

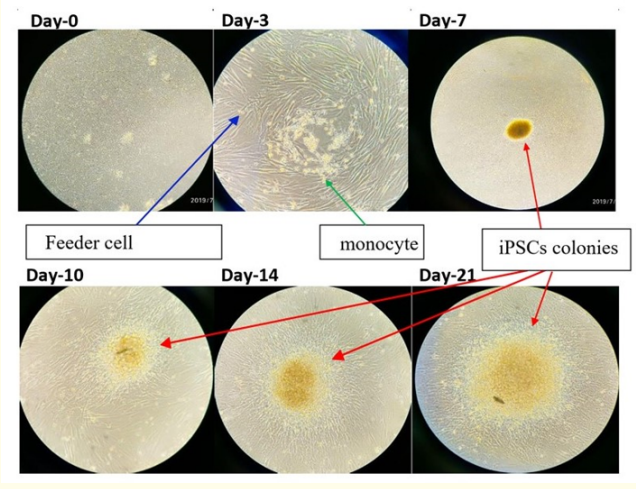

Figure 5: *Changed of colonies morphology at day 0 until day 21.*

Pluripotency markers were expressed in all colonies. S SSEA4 expression was identified in all groups, with the P3 group having the strongest levels. Cells that glow bright green had strong expressions of SSEA4 (Figure 6). ImageJ software was used to measure the luminance degree quantitatively. From Kolmogorov-Smirnov, the quantitative data of SSEA 4 marker expression at each dose was normally distributed. Significant differences were found in all experimental groups (P2: p = 0.007, P3: p = 0.001, P4: p = 0.009), as shown in table 1.

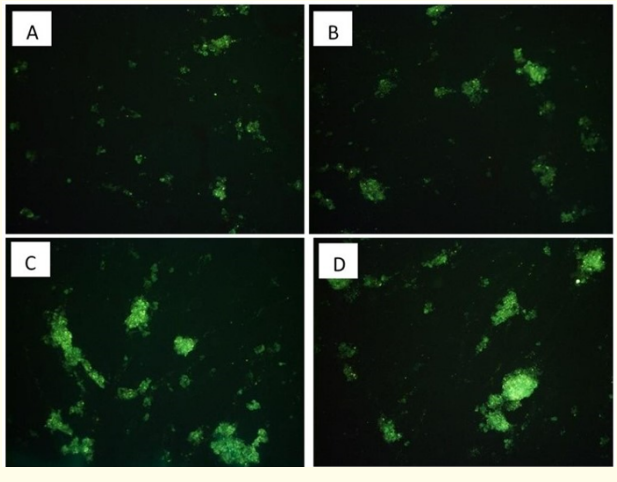

Figure 6: *SSEA4 expression in each group (A-D) P1-P4 respectively.*

TRA1-60 expression was found in all treatment groups, with the strongest expression in P3 and P4 groups (Figure 7). The quantitative data of TRA1-60 marker expression at each dose was normally distributed. There were significant differences were found between all experimental groups (P2: p = 0.002, P3: p = 0.001, P4: p = 0.001), as shown in table 1.

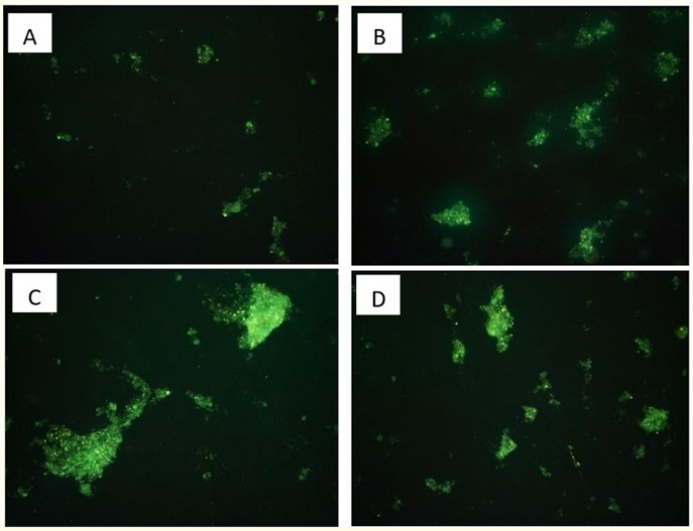

Figure 7: *TRA1-60 expression in each group (A-D) P1-P4 respectively.*

Marker group	VC6TFZ group (mean ± SD)	p
SSEA4	P1 (14.20 ± 0.389)	
	P2 (16.66 ± 0.112)	0.007
	P3 (17.37 ± 0.710)	0.001
	P4 (16.60 ± 0.212)	0.009
TRA1-60	P1 (12.09 ± 0.605	
	P2 (14.07 ± 0.722)	0.002
	P3 (14.33 ± 0.736)	0.001
	P4 (14.32 ± 0.113)	0.001

Table 1: *Comparison of expression between groups SSEA4 and TRA1-60.*

Discussion

Small-molecule compound VC6TFZ could induce pluripotency in human PBMC from this study. It was demonstrated by the production of colonies with the morphology of iPSCs and the expression of pluripotency markers SSEA4 and TRA1-60. A colony that began to develop on the seventh day of induction grew in size by the tenth day. The colonies had cobblestone-like, clear edges and round morphology. This colony's morphology is similar to iPSCs, as described in the guidelines and methodologies for generating iPSCs [7].

The colonies in this study express pluripotency markers, SSEA4 and TRA1-60. Higher expressions in all treatment groups compared to the control group was obtained. Statistically, there were significant differences between P3, P4, and control groups in SSEA4 expression ($p = 0,001$; $p = 0.009$) and TRA1-60 ($p = 0.001$; $p = 0.001$). This implies that SSEA4 and TRA1-60 are best expressed at small-molecule P3 and P4. Thus, a small molecule on the success of reprogramming was dose-dependent [6,8]. The concentration and combination of small molecules were crucial for effective reprogramming with small molecules. Because small compounds might be hazardous at certain concentrations, reprogramming effectiveness was often significantly lower at larger dosages [8].

The induction procedure we adopted in this investigation took 21 days, quicker than the Hou., *et al*. protocol. GFP-positive colonies developed around day 10 - 12 in Hou's work, while they appeared sooner in this study around day 6. The PBMC culture optimization procedure is critical to the reprogramming process. We cultured PBMC in an expansion medium for six days in this investigation. The longer the culture period, the more dead cells there are, but the number of surviving cells remains constant. Therefore, the ideal length of culture time was required. These six days were chosen based on studies by Gu., *et al*. who found that the 6-day culture time resulted in the highest number of colonies expressing TRA 1-60 and AP when compared to days 4, 8, or 10 [9].

Colonies may be formed using the hanging drop culture method, which uses gravity to cause cell aggregation [10]. The creation of spheroidal colonies is enabled by the concentration of cells in the drop. Spheroid colonies can produce extracellular matrices and habitats that imitate live tissue. Inside the drop cells, the resultant matrix holds the drop cells together. This technique improved intercellular connections as well as cell-extracellular matrix interactions [10]. This approach also enables more effective growth factor diffusion and metabolic waste elimination [11].

One of the difficulties in reprogramming PBMC was their non-adherent character [12]. To solve this difficulty, we used vitronectin and feeder cells to coat the well walls, allowing the PBMCs to adhere to the well. This connection was necessary to prevent cell loss during medium change. This feeder cell generated adhesion molecules and extracellular matrix, which help iPSCs stick together, allowing them to develop and survive [12,13].

The quantitative data analysis in this study was based on the immunocytochemistry (ICC) test results, which is a common laboratory examination technique used to anatomically visualize the localization of proteins or specific antigens in cells using specific primary antibodies that bind to these proteins. In this study, the method used for checking ICC is the indirect method. This method has a disadvantage, namely that secondary antibodies can cross-react with proteins other than the target so that it can provide a luminescence image that is not specific to the target cells. However, because the same conjugated secondary antibody may be used to detect multiple primary antibodies, this approach has the benefit of being less expensive [14].

Conclusion

The small chemical compound VC6TFZ has been shown to promote pluripotency in human PBMCs, allowing them to create iPSCs. SSEA 4 and TRA 1-60 gene expression in pluripotency markers was statistically more significant in the experimental group than in the control group. The chemical profile, differentiation, and self-renewal capabilities of these cells are required to be investigated further. The safety profile associated with the risk of teratogenesis and genetic instability has to be investigated further for clinical use.

Conflict of Interest

The authors declare that there is no conflict of interest.

Bibliography

1. Singh VK., *et al.* "Induced pluripotent stem cells: Applications in regenerative medicine, disease modeling, and drug discovery". *Frontiers in Cell and Developmental Biology* 3 (2015): 1-18.
2. Takahashi K and Yamanaka S. "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors". *Cell* 126.4 (2006): 663-676.
3. Takahashi K., *et al*. "Induction of pluripotent stem cells from adult human fibroblasts by defined factors". *Cell* 131.5 (2007): 861-872.
4. Raab S., *et al.* "A comparative view on human somatic cell sources for iPSC generation". *Stem Cells International* (2014): 768391.
5. Baranek M., *et al.* "Effect of small molecules on cell reprogramming". *Molecular Omics* 13.2 (2017): 277-313.
6. Hou P., *et al.* "Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds". *Science* 341.6146 (2013): 651-654.
7. Maherali N and Hochedlinger K. "Guidelines and techniques for the generation of induced pluripotent stem cells". *Cell Stem Cell* 3.6 (2008): 595-605.
8. Zhou J and Sun J. "A revolution in reprogramming: Small molecules". *Current Molecular Medicine* 19.2 (2019): 77-90.
9. Gu H., *et al*. "Optimizing the method for generation of integration-free induced pluripotent stem cells from human peripheral blood". *Stem Cell Research and Therapy* 9.1 (2018): 1-10.
10. Foty R. "A simple hanging drop cell culture protocol for generation of 3D spheroids". *The Journal of Visualized Experiments* 51 (2011): 2-6.
11. Jitcy SJ., *et al.* "2D and 3D Cell Culture in Drug Discovery". London: Intech (2016).
12. Yu G., *et al*. "Feeder cell sources and feeder-free methods for human iPS cell culture". In: Interface Oral Health Science. Berlin: Springer (2015): 351.
13. Li P., *et al*. "Effcient feeder cells preparation system for large-scale preparation and application of induced pluripotent stem cells". *Scientific Reports* 7.1 (2017): 1-15.
14. Richard WB. "Immunocytochemistry". Springer, New York, NY (2010): 7-16.

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