

Research Paper



Generation of Human-induced Pluripotent Stem Cells Derived From Dermal Fibroblast of Schizophrenic Patients

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ABSTRACT

Introduction: Schizophrenia (SCZ) is a psychiatric disorder caused by environmental, social, and genetic factors. This phenomenon is a severe neuropsychiatric disorder with a 1% worldwide prevalence. As SCZ is an exclusively human disorder, animal models cannot mimic SCZ pathophysiology. Thus, it is crucial to develop a novel human-based specific model of SCZ to elucidate mechanisms of the occurrence of the disease. In this regard, the aim of this study was reprogramming somatic cells to human-induced pluripotent stem cells (hiPSCs), with possible potency to transformed to specific neural stem cells.

Methods: In the present study, we directly reprogrammed the isolated human ear dermal fibroblasts (HDFs) from schizophrenic patients into hiPSCs using some episomal agents in Matrigel-coated plates. The existence of pluripotency markers was confirmed by the immunocytochemistry (ICC) test and alkaline phosphatase protocol. We performed karyotype analysis to ensure the maintenance of the normal chromosomes.

Results: Analysis of colonies exhibited intense alkaline phosphatase engagement and Oct4, SSEA4, Nanog, and Tra-1-60. HiPSCs showed normal karyotypes and were potent to differentiate into ectoderm, endoderm, and mesoderm.

Conclusion: This study showed human dermal mesenchymal fibroblasts taken from schizophrenic patients can be reprogrammed to hiPSCs, with potential to transformation to three germ layers with sufficient expression of relate molecular markers. This is the first steps to produce SCZ specific neural stem cells, which can be used in the assessment of cellular changes in schizophrenia and possible effects of antipsychotic agents. .

Keywords:

Schizophrenia, Human-induced pluripotent stem cells hiPSCs, Fibroblast, Episomal vectors

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Highlights

- Human dermal mesenchymal fibroblasts taken from schizophrenic patients can be reprogrammed to human-induced pluripotent stem cells (hiPSCs).
- hiPSCs have the potential to transformation to three germ layers.
- This is the first steps to produce schizophrenia specific neural stem cells, as a cellular model of this disorder.

Plain Language Summary

Schizophrenia is a mental disorder associated with abnormal communication between and within brain regions. Since it is an exclusively human disorder, animal models cannot mimic all schizophrenia-like symptoms. Thus, it is crucial to develop a human-based model of schizophrenia to elucidate mechanisms of the occurrence of the disease and evaluate the treatment efficacy of this method. In this regard, we aimed to produce human-induced pluripotent stem cells from dermal fibroblast of schizophrenic patients. The generated cells could express pluripotent markers at the protein level, including SSEA-4, Oct-4, Nanog, and Tra-1-60. It seems that the use of human-induced pluripotent stem cells derived from schizophrenic patients can be a promising approach to treat Schizophrenia.

1. Introduction

Schizophrenia (SCZ) is a severe neuropsychiatric disorder with a global prevalence of 1% and 80-85% heritability (Sullivan et al., 2003). Multiple reasons, including genetic and environmental factors, have roles in the pathogenesis of SCZ (Tsuang et al., 2001; Palha & Goodman, 2006), and perturbation of these agents could cause the disturbance of information of the neural network and brain function (Christian et al., 2010). SCZ onset often happens in early adulthood (Sawa & Snyder, 2002), but in some patients, the abnormal neurodevelopmental process may start in childhood (Weinberger, 1987; Marin, 2012). Furthermore, most patients show different responses to treatment (Schennach et al., 2012), and only a few percent effectively respond to the treatment (Emsley et al., 2011). Studies have shown that SCZ is accompanied by abnormal neuronal communication with a significant role for oligodendrocyte and progenitor cells in the pathogenesis (Rubinov, 2013; Federspiel et al., 2006; Begre & Konig, 2008). As SCZ is an exclusively human disorder, animal models cannot mimic all SCZ pathophysiology (Stachowiak et al., 2013; Jones et al., 2011). Some curative animal model studies have failed in human clinical trials (Thomsen et al., 2010; Franco & Cedazo-Minguez, 2014). Thus, it is crucial to develop a novel human-based specific model of SCZ to elucidate mechanisms of the occurrence of the disease to find a neoteric therapy. First time in 2007,

Yamanaka et al. reprogrammed human fibroblasts into induced pluripotent stem (iPS) (Takahashi et al., 2007). These cells could be cultured on mouse embryonic fibroblast as feeders (Ellerstrom et al., 2006; Takahashi et al., 2007; Crook et al., 2007). Sun et al. used Matrigel as a feeder-free culture condition to generate individual-specific hiPSCs from the autologous source of cells (Sun et al., 2009). Both human and mouse iPS cells could transform into three germ layers (Maherali et al., 2007), (Meissner et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). HiPSCs develop a source of cells genetically similar to the original cell and are useful for tissue regeneration and drug screening. However, applying viruses to produce hiPSCs has shown a high rate of conveyance utility (Takahashi et al., 2007; Yu et al., 2007). Viruses can also induce a viral reservoir into the host chromosome. Scientists use non-integrating episomal vectors to produce hiPSCs to keep away from accidental genomic pools (Yu et al., 2009).

Considering the importance of developing new approaches to treat SCZ, we aimed to produce hiPSCs in SCZ patients using episomal vectors to evaluate the treatment efficacy of this method in our future studies.

2. Materials and Methods

Preparation of human dermal fibroblasts (HDFs)

Human skin biopsies were obtained from SCZ patients through dermal punch biopsies after receiving approval from the ethical committee of [Iran University of Medical Sciences](#), Tehran, Iran. The diagnosis of SCZ was confirmed by psychiatrists' assessment according to the SCID-I criteria. All participants were patients at the [Psychiatry Center of Iran](#). The earlobe tissues (4 mm) were collected, preserved in phosphate-buffered saline (PBS; Medicago, Canada) with 2% pen/strep (Gibco, USA), and transported to the cell culture laboratory at [Iran University of Medical Sciences](#). Tissues were minced and then centrifuged for five minutes (1200 rpm). The supernatant was discarded, and the tissues were digested overnight with Dispase II (sigma; USA). Subsequently, enzyme activity was neutralized with DMEM (Gibco, USA) containing 10% FBS (Gibco, USA), and the mixture was centrifuged for five minutes at 1200 rpm. After discarding the supernatant, the tissues were incubated for 30 minutes with collagenase I (Sigma, USA). The collagenase activity was then neutralized as described above. The suspension was filtered through a 70 μm filter (SPL, China) and centrifuged for five minutes at 1200 rpm. The supernatant was removed, and the cell pellets were cultured in 6-well plates containing DMEM with 10% FBS and 1% pen/strep antibiotics (PAN Biotech, Germany), and incubated at 37°C in an atmosphere of 5% CO₂. Three dermal biopsies were used in this study. Generation of dermal fibroblast-derived hiPSCs from SCZ patients.

In this study, hiPSCs were generated from dermal fibroblasts using episomal vectors (pEP4 E02S CK2M EN2L, Addgene) containing six factors (OCT-4, SOX-2, KLF-4, c-MYC, Nanog, LIN-28). Approximately 5000 human dermal fibroblasts (HDFs) per cm² were seeded in each well of a 12-well plate and transfected once using Lipofectamine (Invitrogen, L3000-001). On day 0 or 1, when fibroblasts reached 70-90% confluency, they were transfected according to the kit instructions. Initially, 2.5 $\mu\text{g}/\mu\text{L}$ of episomal vectors and 5 μL of Lipofectamine were mixed for 30 minutes in a microtube, then the mixture was added to the cell culture. After 12 hours, the media was replaced.

Six days later, colonies were transferred onto plates coated with Matrigel (1:30; Sigma, USA) containing DMEM/F12, 20% Knockout Serum Replacement, 100 μM non-essential amino acids, 1% penicillin/streptomycin, 2 mM L-glutamine (all from Gibco, USA), 100

μM β -mercaptoethanol (Sigma, USA), and 10 ng/ml human basic fibroblast growth factor (b-FGF; PeproTech, USA). The plates were incubated at 5% CO₂ with 95% of humidity. To monitor the transfection efficiency, only 6 columns were used for hiPSCs reprogramming, with the remaining serving as controls. After 4 to 5 weeks, colonies were visible. For passaging, colonies were rinsed with PBS and then incubated with DMEM/F12 containing collagenase I (Sigma, USA) at 37°C for 30 minutes. Then, the enzyme was removed, and the plates were rinsed with PBS. Colonies were gently dislodged mechanically and then transferred to new 24-well plates coated with Matrigel. These samples underwent immunocytochemical analysis to assess the expression of Tra1-60, Nanog, Oct3/4, SSEA4, and alkaline phosphatase staining, with at least three clones tested per patient. The schematic diagram of research is shown in [Figure 1](#).

Characterization of the established hiPSCs

Immunocytochemistry (ICC)

To assess the expression of pluripotency markers, colonies were fixed for twenty minutes using 4% paraformaldehyde, then permeabilized with 0.1–0.2% Triton X-100 for 30 minutes. Blocking was performed using 10% mouse serum in PBS for 1 hour at 37°C. For SSEA-4 and TRA-1-60 expression analysis, cells were incubated with Anti-SSEA-4 PE (1:250; Cat. No. CS204438) and Anti-TRA-1-60 FITC (1:200; Cat. No. CS204460) antibodies for 30 minutes at 37°C in a 5% CO₂ chamber.

For Oct-3/4 and Nanog expression analysis, the Human Pluripotent Stem Cell 3-Color Immunocytochemistry Kit (Cat No. SC021; bio-technie, USA) was used. Briefly, blocking was done with 10% normal donkey serum in 0.3% Triton® X-100. After removing the blocking buffer, cells were stained with NL637-conjugated Goat Anti-Human Oct-3/4 and NL493-conjugated Goat Anti-Human Nanog antibodies.

Derived HiPSCs were differentiated into three germ layers. Differentiation was verified using the Human Pluripotent Stem Cell Functional Identification Kit (Cat. No. SC027B), following the manufacturer's instructions. We used goat anti-human Otx2, goat anti-human Brachyury, and goat anti-human Sox17 as primary antibodies to confirm differentiation into ectoderm, mesoderm, and endoderm, respectively. NL557-conjugated donkey anti-goat (R&D systems, Cat.No.NL001) served as the secondary antibody (red). All nuclei were counterstained with DAPI (blue).

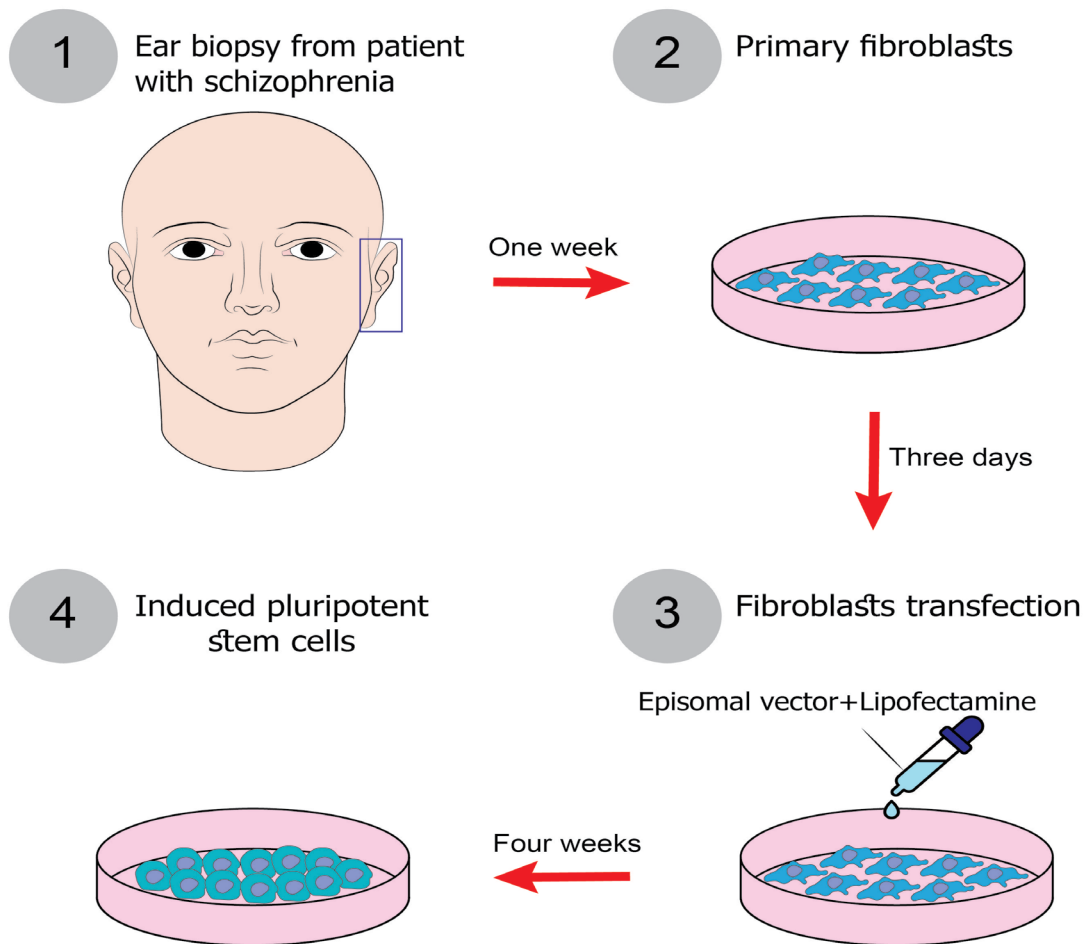


Figure 1. The schematic diagram of research showing all steps

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Alkaline phosphatase test

The alkaline phosphatase test was conducted using the Alkaline Phosphatase Staining Kit II (Reprocell, USA) to evaluate AP expression in hiPSC colonies. Briefly, cells were washed with PBST (PBS+triton, final concentration: 0.05%). Then, they were fixed in fixative solution for 2-5 minutes and then incubated in freshly prepared AP solution for 15 minutes at room temperature. The reaction was halted when a bright color developed.

Karyotype analysis

Cells underwent karyotype analysis by being cultured with thymidine (Sigma, USA) for 16 hours at 37°C in 5% CO₂. Three hours after removal, cells were exposed to colcemid (Gibco, 0.15 µg/ml, 30 minutes), followed by treatment with 0.075 M KCl at 37°C for 16 minutes. Cells

were then fixed three times in ice-cold 3:1 methanol: Glacial acetic acid and dropped onto pre-cleaned, chilled slides. At least 20 metaphase spreads were screened, and 10 were evaluated for chromosomal rearrangements.

Differentiation of hiPSCs into three germ layers

To confirm the pluripotency of hiPSC clones, three lineage differentiation assays were performed using the Human Pluripotent Stem Cell Functional Identification Kit (Cat. No. SC027B). Cells were harvested and prepared for analysis of lineage-specific markers on day 5 (for mesoderm and endoderm lineages) and day 7 (for the ectoderm lineage), as per the manufacturer's instructions. Immunofluorescence assay was carried out with antibodies to lineage-specific markers for endoderm, ectoderm, and mesoderm. Goat anti-human Otx2, goat anti-human Brachyury, and goat anti-human Sox17 were used as antibodies to verify ectoderm, mesoderm, and endoderm differentiation. Also, we used NL557-conju-

Table 1. The sequences of the primers used in this investigation

Gene	Sequence	Accession Number
<i>GAPDH</i>	CTCATTTCCTGGTATGACAACGA CTTCCTCTGTGCTCTTGCT	NM_002046.3
<i>Sox17</i>	CGGTATATTACTGCAACTAT GGATTTCCTTAGCTCCTCCA	NM-022454.3
<i>Brachyury</i>	AATCCTCATCCTCAGTTTGG GTCAGAATAGGTTGGAGAATTG	NM_003181.2
<i>Ppar</i>	CTAAAGAGCCTGCGAAAG TGTCTGTCTCCGTCTTCTTG	NM_005037
<i>Islet-1</i>	ATATCAGTTGTACGGGATCAAATG CACGCATCACGAAGTCGTTCT	NM_002202.2
<i>Pax-6</i>	CGGTTTCCTCCTTACAT ATCATAACTCCGCCATT	NM_000280.4

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gated donkey anti-goat (R&D systems, Cat.No.NL001) as the secondary antibody (red). All nuclei were counterstained with DAPI (blue). RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol and treated with DNase I to remove genomic DNA contamination. Per the manufacturer's instructions, 2 µg of total RNA was used for the reverse transcription reaction with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) using an oligo (dt) primer. Quantitative PCR reactions were conducted using Power SYBR Green Master Mix (Applied Biosystems), and results were analyzed on a 7500 real-time PCR system (Applied Biosystems). Gene expression levels were normalized to *GAPDH*, serving as an internal control, and compared with the same target gene in human dermal fibroblasts (HDF). The primer sequences are listed in [Table 1](#).

Statistical analysis

Data were analyzed by non-parametric Mann-Whitney test using SPSS software, version 16 and $P < 0.05$ were considered significant. Data were presented as Mean±SD.

3. Results

Generation of hiPSCs from dermal fibroblasts of patients with schizophrenia

Isolation and proliferation of human dermal fibroblasts

Human skin biopsies were cultured in 6-well plates ([Figure 2a](#)). Spindle-shaped cells migrated from the biopsies within 4-5 weeks of culture. Then, biopsies were

removed, and cells were allowed to reach approximately 75% confluence over 14 days, with medium changes every other day ([Figure 2b](#)).

Characterization of established hiPSCs

Immunocytochemistry, ALP test, and karyotype analysis

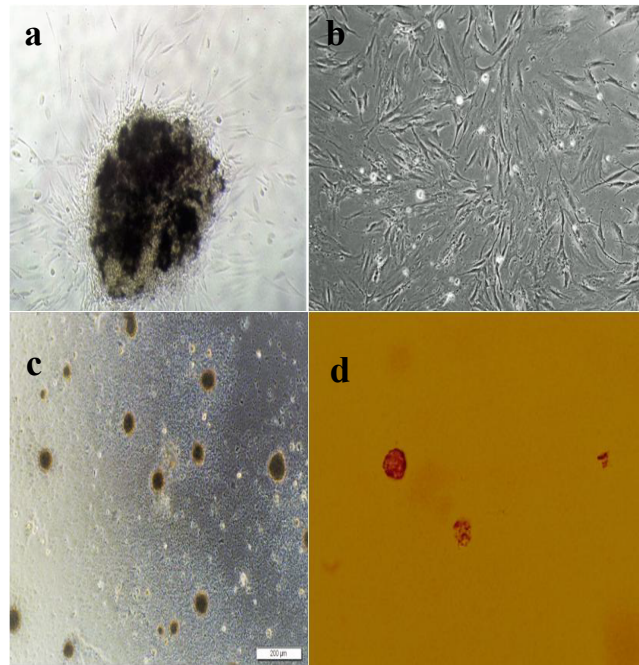
After introducing reprogramming factors, cells began to form colonies with hESC-like morphology ([Figure 2c](#)), which exhibited intense ALP activity ([Figure 2d](#)). These colonies were analyzed via immunocytochemistry and gene expression assays, expressing pluripotent markers including Tra-1-60, Oct4, SSEA4, and Nanog at the protein level ([Figure 3](#)). The putative hiPSCs maintained a normal karyotype ([Figure 4](#)).

Differentiation into three germ layers

The generated cells were continuously cultured with weekly passaging at a split ratio of 1:3. To confirm their multilineage differentiation potential, cells were induced to differentiate into ectoderm, mesoderm, and endoderm. Immunocytochemical staining demonstrated expression of Otx2, Brachyury, and Sox-17, markers of ectoderm, mesoderm, and endoderm, respectively ([Figure 5](#)). Gene expression was assessed for ectoderm differentiation markers Nestin and Pax-6; mesoderm markers Brachyury and PPAR; and endoderm markers Islet-1 and Sox-17. The differentiated hiPSCs showed upregulated gene expression compared to fibroblasts ([Figure 6](#)).

4. Discussion

SCZ is defined as a neurodevelopmental disorder that alters the processes of thought, perception, and emotion,



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Figure 2. Generation of human iPSC-like colonies from SCZ patients

A) Human dermal skin biopsy on day 14, B) SCZ patient-derived dermal fibroblast at passage three, C) Human iPSC colonies after induction with episomal vectors, D) ALP activity test on human iPSC colonies derived from SCZ patients

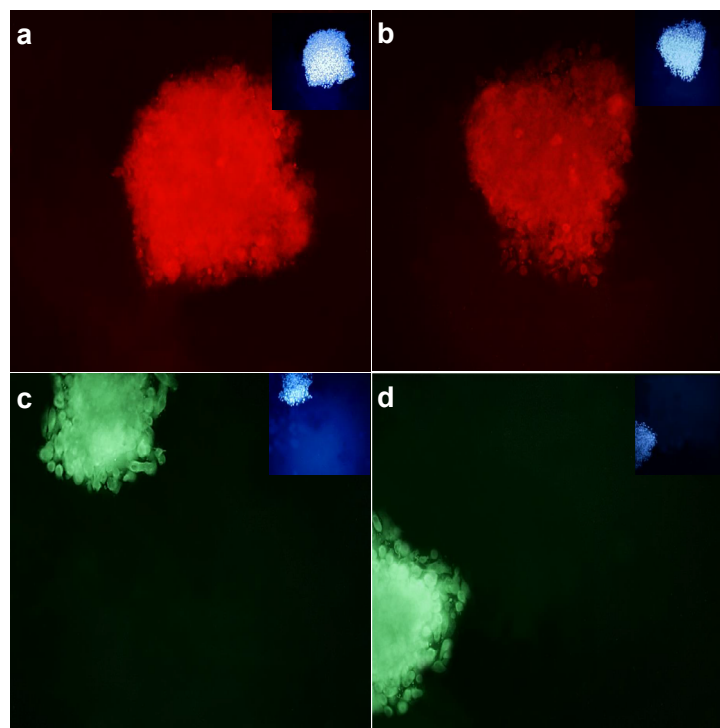
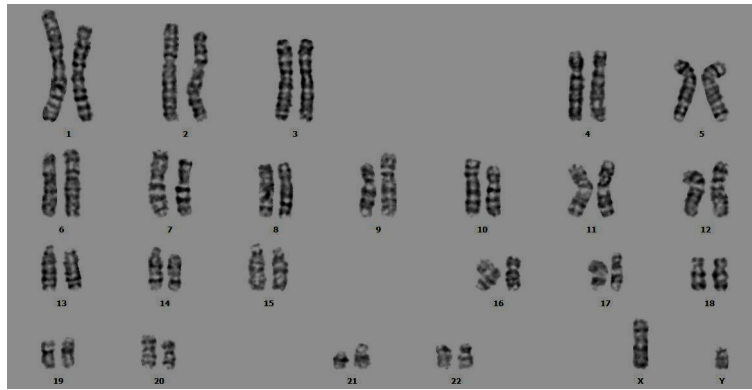


Figure 3. Characterization of human iPSC-like colonies using immunochemical staining

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Notes: The human iPSCs colonies were characterized based on the expression of pluripotency markers, including Oct-4 (a), SSEA-4 (b), Tra-1-60 (c), and Nanog (d).



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Figure 4. Karyotype analysis

Notes: Generated human iPSCs also maintained a normal karyotype, and cells maintained their normal chromosome morphology.

leading to mental deterioration. In the current study, we generated human induced pluripotent stem cells (hiPSCs) from dermal fibroblasts of patients with SCZD using episomal vectors on Matrigel-coated plates. Our results demonstrated that the derived colonies could express pluripotent markers at the protein level, including SSEA-4, Oct-4, Nanog, and Tra-1-60. The generated hiPSCs maintained their normal karyotype, and we could also detect strong ALP expression in the colonies. After induction, the cells could express ectodermal (Nestin), endodermal (Islet-1, Sox-17), and mesodermal (Brachyury and PPAR) markers at the level of mRNA when the results were compared with fibroblasts con-

firming the pluripotent properties of the generated cells. Previous studies have shown that iPSCs can be cultured on mouse embryonic fibroblast feeders (Ellerstrom et al. 2006; Takahashi et al. 2007; Crook et al. 2007). Alternatively, feeders can be replaced by Matrigel, as we have done in our project (Kleinman & Martin 2005). In an investigation, Sun et al. used Matrigel as a feeder-free culture condition to generate individual-specific hiPSCs from an autologous source of cells (Sun et al., 2009). Moreover, using conditioned media and Matrigel supported with essential growth factors could reduce the contamination of stem cells (Ghasemi-Dehkordi et al., 2015). The breaking points for hiPSCs for investigating

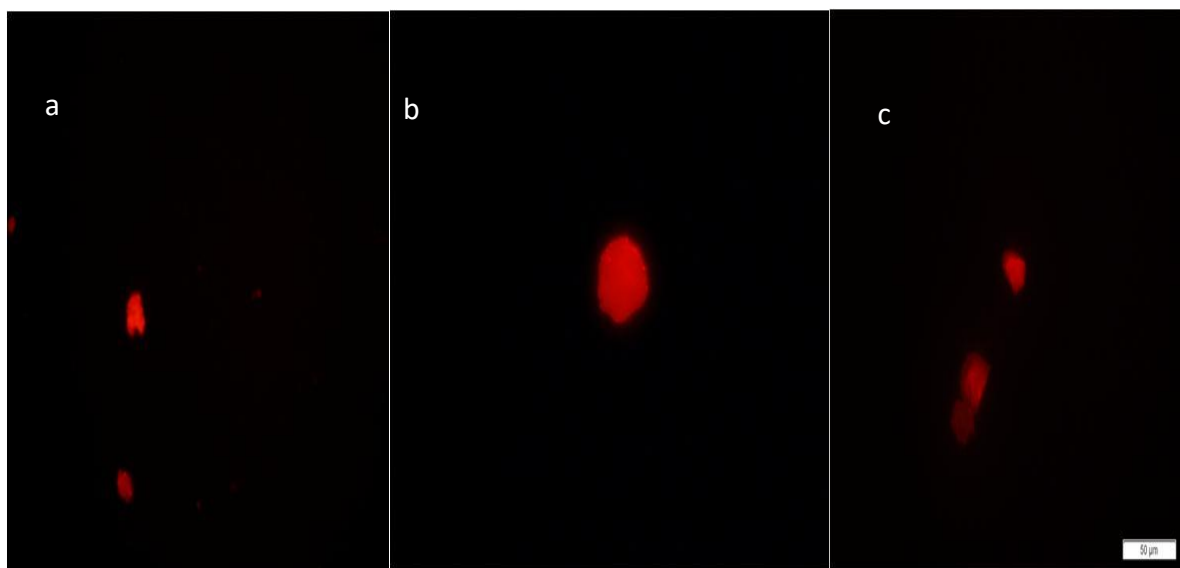
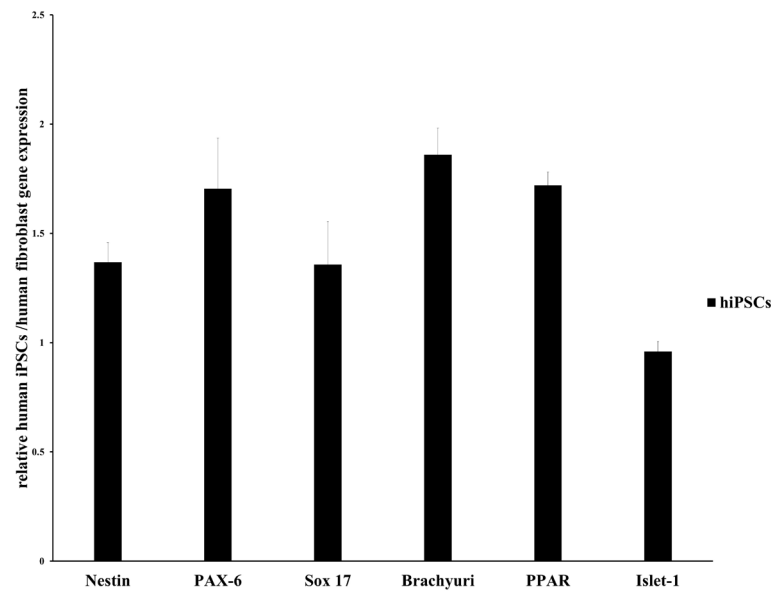


Figure 5. Differentiation of human iPSC-like colonies

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Notes: Differentiating human iPSC colony into mesoderm, ectoderm, and endoderm. The cells could express Otx2 (a), Brachyury (b), and Sox-17(c) at the level of protein.



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Figure 6. Gene expression

Notes: Relative gene expression analysis showed the expression of the ectodermal (Nestin and Pax-6), mesodermal (Brachyuri and PPAR), and endodermal (Sox-17 and Islet-1) markers in human iPSCs derived from SZD patients after induction with the corresponding differentiation media. The expression of the same markers in human fibroblast was assumed as the control.

brain disturbance are neuron and patient variability. This variation may be due to differences in the type of viruses used, epigenetic factors, spontaneous mutation, differences in techniques, and cell type of origin. Some scientists have investigated the advantages and difficulties of applying viral integration methods versus episomal or other methods. Viral methods induce genomic deviation (Hussein et al., 2011). They are more efficient than other methods consisting of recombinant protein (Zhou et al., 2009), miRNA (Miyoshi et al., 2011), and mRNA (Warren et al., 2010). Most SCZ patients show different responses to different treatments (Schennach et al., 2012), and only a few percent effectively respond to the therapy (Emsley et al., 2011). The application of new methods for treating this disease is critical.

Conclusion

The use of hiPSCs derived from patients with schizophrenia could be a promising approach for treating the disease and screening drugs. Further studies are suggested to investigate the behavior of these cells during neurogenesis.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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