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ORIGINAL ARTICLE

TERM

Efficient Induction of Neural Precursor Cells from Fibroblasts Using Stromal Cell-Derived Inducing Activity

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The direct lineage conversion of fibroblasts into neuronal or neural precursor cells (NPCs) has become a hot issue in recent years as an attractive approach in the field of stem cell regenerative medicine. In this study, we adopted the stromal feeder co-culture method during the early conversion period to enhance conversion efficiency. Stromal cells are often used in directed differentiation of dopaminergic (DA) neurons from pluripotent stem cells. We co-cultured rat embryonic fibroblasts (REFs) on γ-irradiated sonic hedgehog-overexpressing MS5 stromal (MS5-SHH) cells after transduction with *Brn2*, *Ascl1*, *Myt1L*, and *BclxL-GFP* (BAMX^{GFP}) transcription factors to REFs. One week after co-culture, transduced cells (GFP+ cells) that proliferated on MS5-SHH cells were separated from MS5-SHH cells through a 40 μm cell strainer. Subsequently, the converted cells (GFP+ cells) were expanded on fibronectin-coated culture plates in NPC expansion medium. The induced NPCs (iNPCs) expressed NPC potential (NESTIN+/SOX2+) earlier than seen with non-co-culture methods and were efficiently differentiated into DA neurons by overexpression of *Nurr1* and *Foxa2* genes, which are specific transcription factors for midbrain DA neuron development. These observations indicated that direct conversion to NPCs using an MS5 stromal cells co-culture method is a suitable technique for efficient generation of iNPC/DA neurons from fibroblasts. Tissue Eng Regen Med 2016;13(5):554-559

Key Words: Induced neural precursor cell; Direct conversion; Stromal cell; Co-culture; Dopaminergic neuron

INTRODUCTION

The generation of functionally differentiated cells is a long-standing goal for cell therapy. Directed differentiation of pluripotent stem cells has been extensively studied for this purpose and the techniques have progressed rapidly in recent years. However, there has been great interest in the development of alternative strategies for obtaining functionally differentiated cells [1-3]. Direct conversion, which is the direct induction of functional cell types from one lineage to another without passing through an intermediate pluripotent stage [4], could be an alternative way to produce desired cell types. In particular, the direct conversion of fibroblasts to neural precursor cells has become an attractive approach in neurodegenerative disease stem cell regenerative medicine. Various methods have been used for direct reprogramming using transcription factor gene or protein delivery [5-8] and chemical (small molecule) treatment [9-11]. Although several studies have reported the generation of induced neural precursor cells (iNPCs) from rodent and human fibroblasts, the conversion processes were not achieved in a neural-related environment. To overcome this limited capacity, we adopted the stromal cell-derived inducing activity (SDIA) approach. The SDIA method has been applied for efficient directed differentiation of human embryonic stem cells (hESCs) into dopaminergic (DA) neurons [12-15] because stromal cells produce several factors that support neural differentiation when these cells are used as feeders [16,17]. Furthermore, this method has the advantage of simplicity and rapidity compared with previous embryoid body methods [18,19]. The stromal cell lines PA6 and MS5 are derived from mouse skull bone marrow [20]. In this report, we introduce an efficient direct conversion method for *in vitro* NPC/DA neuron generation from rodent fibroblasts using a stromal cell co-culture di-

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rect conversion method.

MATERIALS AND METHODS

Fibroblast isolation

Animals were housed and treated according to the institutional animal care and use committee guidelines of Hanyang University (IACUC No. 2015-0082A). Fibroblasts were isolated from embryos of Sprague-Dawley rats (DaeHan BioLink, Korea) after removing the head, spinal cord, and all internal organs, including the gonads (REFs). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen, Grand Island, NY, USA) containing 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), 0.1 mM non-essential amino acids (Invitrogen, Grand Island, NY, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY, USA).

Retroviral production

Retroviral plasmids for *Brn2*, *Ascl1*, *Myt1L*, *BclxL*-IRES-*GFP*, *Nurr1*, and *Foxa2* were constructed by engineering the appropriate DNA fragments into the pCL retroviral vector [21]. Retroviral vectors were transfected into 293GPG packaging cells using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA). Supernatants containing viral particles were harvested 72 h after transfection.

iNPC generation using a co-culture method

The iNPC generation and isolation protocol was modified from the method reported in a previous study [22]. Briefly, fibroblasts were seeded on gelatin-coated culture dishes. The next day, the cells were transduced with *Brn2*, *Ascl1*, *Myt1L*, and *BclxL-GFP* (BAMX^{GFP}) retroviruses. After 16–20 h, the culture medium was replaced with fresh fibroblast medium containing 100 ng/mL human fibroblast growth factor 8 (FGF8; Peprotech, Rocky Hill, NJ, USA). After 2 days, transduced cells were placed in neural induction medium [NIM; N2 medium supplemented with 20 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), 100 ng/mL FGF8, 100 units/mL recombinant human leukemia inhibitory factor (Millipore, Billerica, MA, USA), and 2 μg/mL doxycycline (Sigma-Aldrich, St. Louis, MO, USA)]. After a further 2 days the transduced cells were transferred onto γ-irradiated sonic hedgehog-overexpressing MS5 stromal (MS5-SHH) cells [23] and co-cultured for 7 days. To collect transduced cells from co-cultures with MS5-SHH cells, whole cells were harvested, put through a 40 μm cell strainer (Falcon, NY, USA) two times, and re-seeded on poly(L-ornithine) (15 μg/mL, Sigma-Aldrich, St. Louis, MO, USA)/fibronectin (FN, 1 μg/mL, Sigma-Aldrich, St. Louis, MO, USA)-coated culture dishes in NIM medium for 1–2 weeks.

Dopaminergic neuron generation from iNPCs

iNPCs were transduced for 2 h with retroviruses carrying the dopaminergic neuron-specific factors, *Nurr1* and *Foxa2*, cultured overnight in NIM, and then differentiated in N2 medium containing 0.2 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL brain-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA), 20 ng/mL glial cell line-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA), and 250 μg/mL dibutyryl-cAMP (Sigma-Aldrich, St. Louis, MO, USA).

Immunostaining of cultured cells

Immunostaining of cultured cells was performed as described previously [22]. Information on primary antibodies is summarized in Table 1.

Table 1. Primary antibody information

Antibody	Dilution	Source	Location
Polyclonal antibody (rabbit)			
SOX2	1:500	Chemicon	Temecula, CA
Tyrosine hydroxylase (TH)	1:2,000	Pel-Freez	Rogers, AR
Synapsin I	1:1,000	Sigma-Aldrich	St. Louis, MO
Green fluorescent protein (GFP)	1:2,000	Life Technologies	Eugene, OR
Monoclonal antibody (mouse)			
Neuron-specific beta-III tubulin (TuJ1)	1:2,000	Covance	Richmond, CA
Microtubule-associated protein 2 (MAP2)	1:1,000	Sigma-Aldrich	St. Louis, MO
Nestin	1:500	BD Biosciences	Franklin Lakes, NJ
Other host (rat)			
Dopamine transporter (DAT), anti-rat	1:200	Abcam	Cambridge, UK

Cell counting and statistical analyses

Cell counting was performed with uniform random selection of 5–10 microscopic fields/well with 3–4 wells per experimental condition. All values were confirmed by at least three independent experiments. Data are expressed as means±SEM. When more than two groups were compared, a paired t-test was performed using SigmaPlot for Windows, version 10.0, (Systat Software GmbH, Erkrath, Germany).

RESULTS

Stromal cell co-culture method is suitable for direct conversion

Stromal cell co-culture protocols for DA generation of hESCs based on co-culture with PA6 or MS5 stromal cells have been reported [14-16]. To induce neural precursor cells from fibroblasts, we applied the co-culture method during the iNPC induction period. Direct conversion processes were followed by a BAMX-based conversion protocol [22] and the overall procedure is schematized in Figure 1. During co-culture on MS5-SHH cells, the number of transduced cells (GFP+ cells) increased and cell morphology changed to that of neural precursor-like cells, with transformation into small cells with fiber outgrowths (Fig. 2; GFP+ cells: 8.81±1.38% at 3 days, 14.15±0.79% at 7 days). These results indicate that stromal cell co-culture is a suitable technique for generating iNPCs.

iNPCs derived from the co-culture method are efficiently expandable and exhibit NPC properties

At the end of the MS5-SHH cell co-culture, transduced cells (GFP+) were mixed with stromal cells. To purify transduced iNPCs from stromal cells, the cells were passed through a 40-μm cell strainer. Before selection using the strainer, the cells were highly heterogeneous and cells of different sizes were observed (stromal cells were larger than transduced cells; Fig. 3A-A'). After selection, most of the iNPC cells were purified (1st selection; Fig. 3A-A"). To obtain a greater degree of purification of the transduced cells, we passed the purified transduced cells through the strainer one more time (2nd selection; Fig. 3A-A"'). Sorted cells were replated on FN-coated dishes and most of the transduced cells expressed GFP and proliferated during the expansion culture period (Fig. 3B). Furthermore, the sorted cells had a morphology highly similar to that of NPCs and expressed the NPC markers NESTIN and SOX2 (Fig. 3C-C'), whereas the mixture of cells that could not pass through the pores but remained on the surface of cell strainer

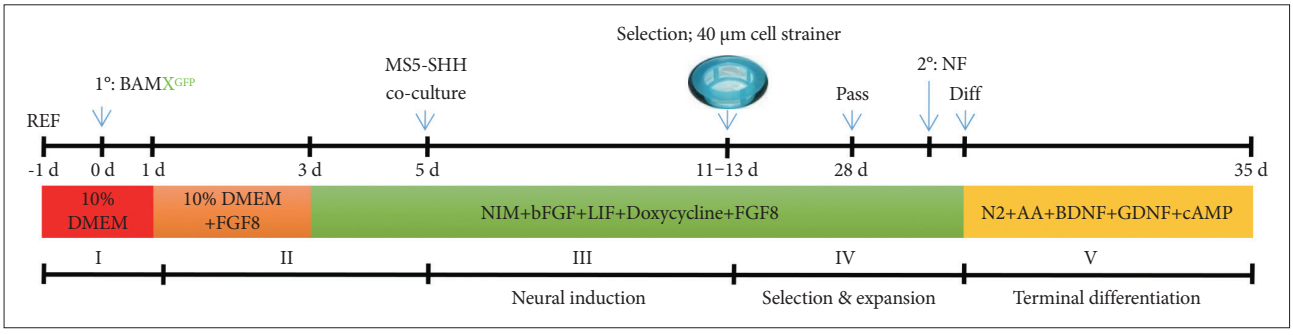

Figure 1. Strategies of iNPC/DA neuron differentiation from rat embryonic fibroblasts using a stromal cell co-culture system. Schematic diagram depicting the timeline of iNPC/DA neuron conversion processes. REF: rat embryonic fibroblast, BAMX^{GFP}: *Brn2*, *Ascl1*, *Myt1l*, *BclxL-GFP*, NF: *Nurr1*, *Foxa2*, Pass: cell passage, Diff: differentiation.

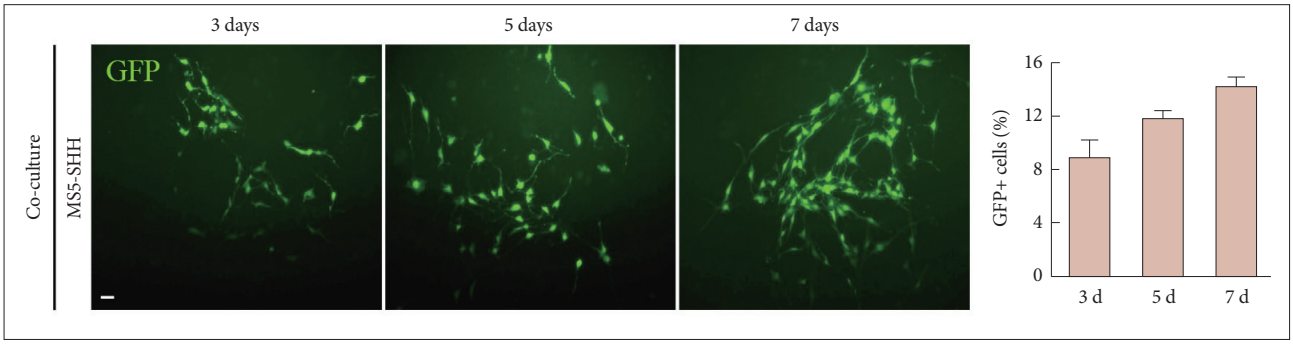

Figure 2. Transduced cells (GFP+ cells) acquire a neural precursor-like cell morphology and proliferate on stromal cells. The proportion of GFP+ cells increased during co-culture with MS5-SHH stromal cells. Error bars denote the standard error of the mean. Scale bar: 20 μm.

after the sorting process little expressed NESTIN and SOX2 and exhibited non-NPC morphology (Fig. 3C-C").

iNPCs derived from the co-culture method can be differentiated into DA neurons

One of the benefits of NPCs is that they are easy to manipulate genetically to produce desired cell types. We attempted to generate DA neurons from iNPCs by transducing the iNPCs with midbrain-specific factors *Nurr1* and *Foxa2*. After being transduced, the cells changed to a mature DA neuron morphology and, in addition, were differentiated toward TuJ1+ (34.72±3.65%), TH+ (22.40±2.26%) cells (Fig. 4A and 4A'; A' is a high-magnification image of the white dotted box in 4A). Microtubule-associated protein 2, dopamine transporter, and synapsin I proteins involved in DA homeostasis were also expressed and co-localized in TH+ neurons (Fig. 4B and C). Taken together, these findings indicate that iNPCs derived from the co-culture method could be used to generate functional DA neurons.

DISCUSSION

In the last several years, methods for fibroblast-to-NPC conversion using exogene expression [5-7] and/or small molecule treatment [9-11] have been reported. Consistent with these methods, we also generated iNPCs from rodent fibroblasts using a retroviral system [22]. Our previous study showed that iNPCs/DA neurons could be generated *in vitro* for cell transplantation therapies. In this study, we introduce a method for generating iNPCs/DA neurons from fibroblasts using SDIA and show that iNPCs/DA neurons can be efficiently induced using this approach. The SDIA method is not a novel method for directed differentiation of NPC/DA from pluripotent stem cells; however, this study is the first to describe direct conversion of iNPC/DA neurons using SDIA. Several studies have suggested

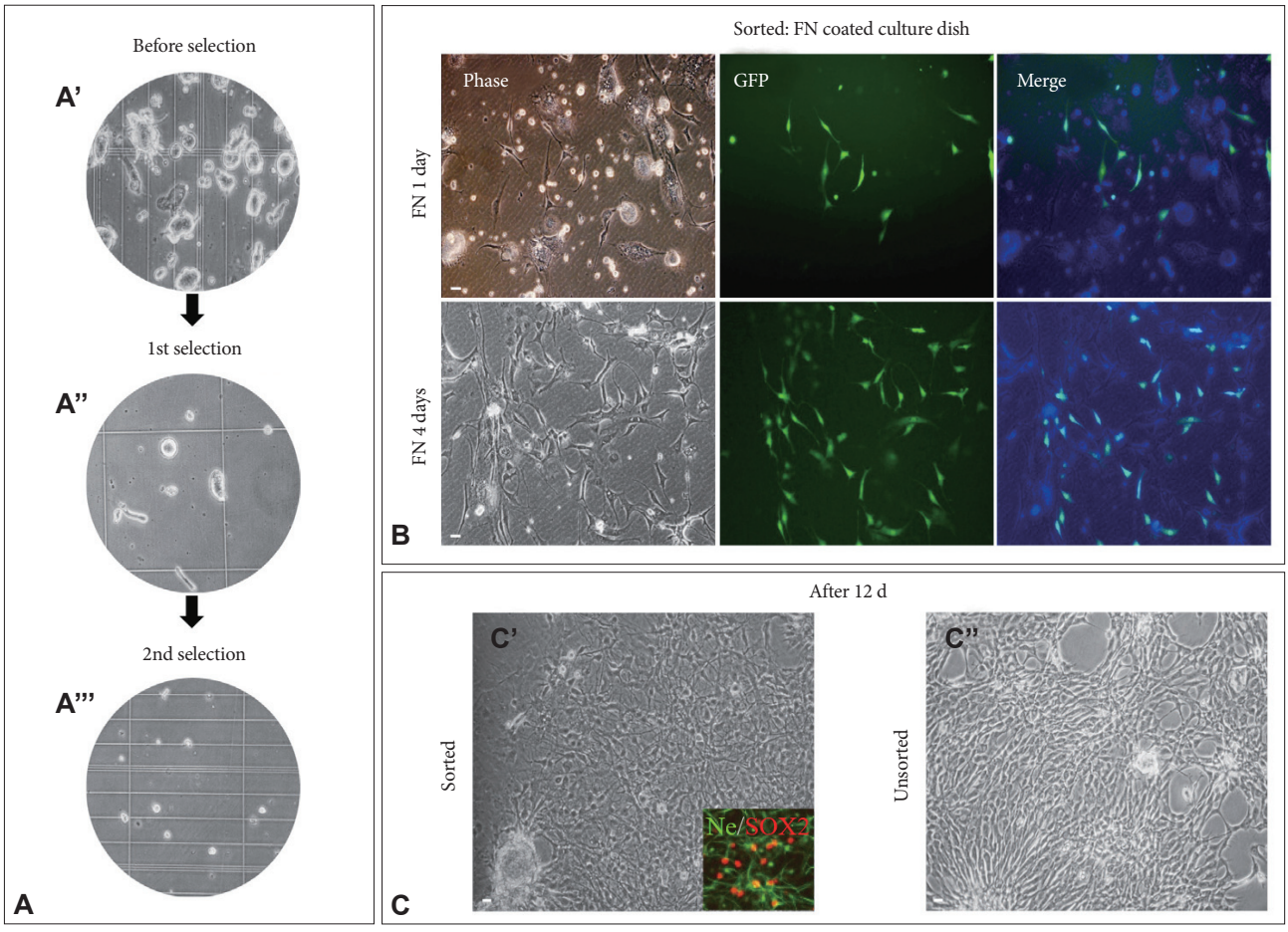

Figure 3. iNPCs derived from the co-culture method are expandable and exhibit NPC properties. (A) The purification process of GFP+ cells from stromal cells: (A') Before purification, (A") After 1st selection using cell strainer, (A"') After 2nd selection. (B) Purified GFP+ cells were proliferated on FN-coated dishes. (C) After 12 days the collected iNPCs (C') looked more like NPCs and expressed NESTIN (Ne-green) and SOX2 (red), whereas the uncollected cells little expressed NESTIN and SOX2 and exhibited a non-neuronal cell morphology (C"). Scale bar: 20 μm. FN: fibronectin, iNPCs: induced neural precursor cells.

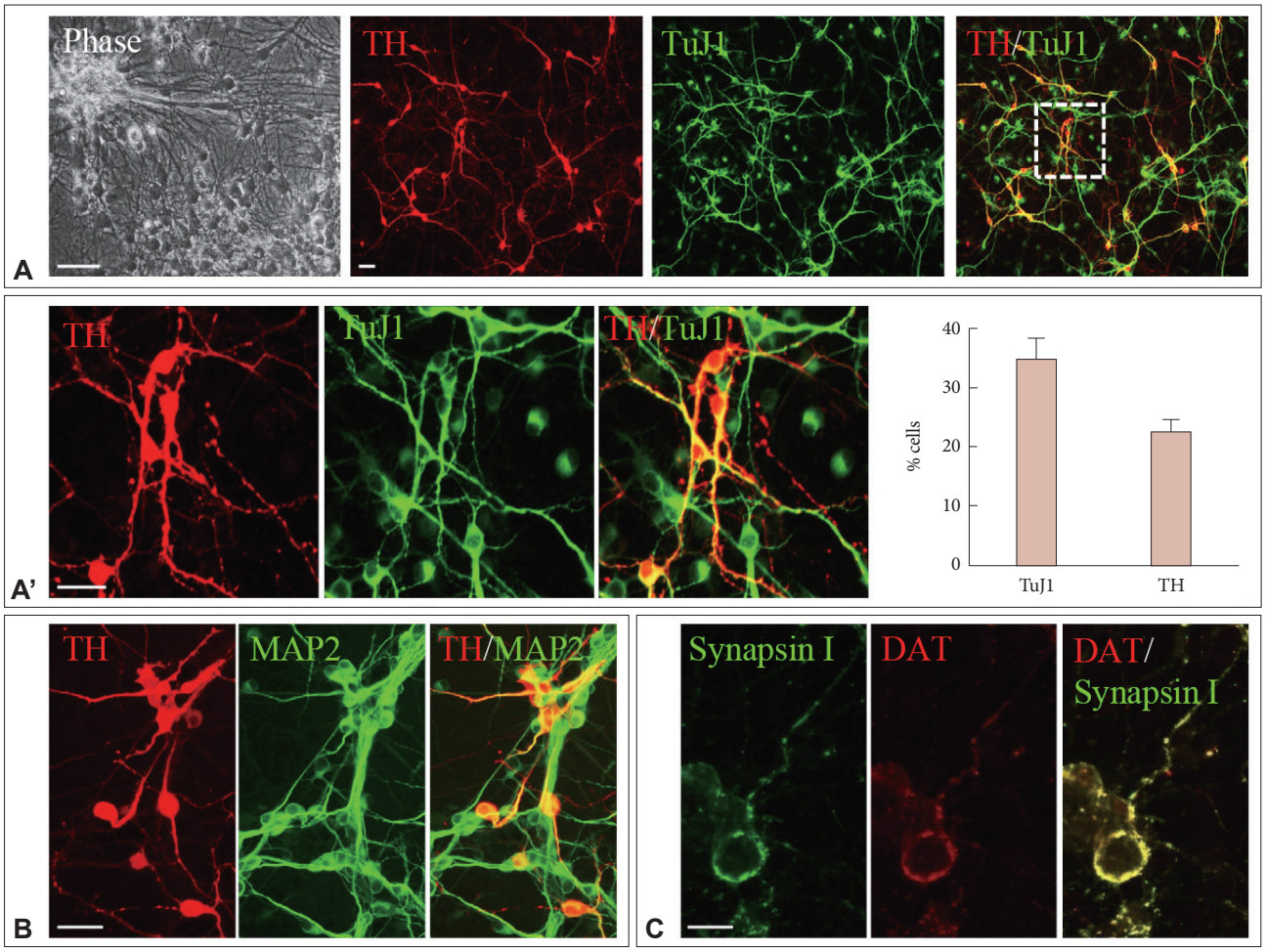

Figure 4. Functional DA neurons can be induced from iNPCs *in vitro*. (A) *Nurr1*- and *Foxa2*-transduced iNPCs differentiated over 10–14 days in culture. Cells showed mature DA neuron morphology and TH+ cells co-expressed TuJ1 (A) and MAP2 (B). (A') High magnification view of white dotted box in A. (C) Synapsin 1 and DAT were also expressed and were co-localized. Error bars denote the standard error of the mean (SEM). Scale bars: 30 μm (A, A', and B) and 10 μm (C). DA: dopaminergic, iNPCs: induced neural precursor cells, TuJ1: neuron-specific beta-III tubulin, MAP2: microtubule-associated protein 2, DAT: dopamine transporter.

that stromal cells produce a variety of factors that support neural differentiation without inducing mesodermal markers [24,25] and that the time course of neural marker induction by SDIA is similar to that observed in early neural development [16]. Although the SDIA method has beneficial advantages, it also has the unsolved problem that the stromal cells used are not from animal-free conditions. Although the current study involved rodent fibroblast conversion, when we advance to researching human fibroblast conversion using a co-culture system we will need to consider animal-free conditions. To overcome this problem, we suggest that human fibroblasts and/or human neural precursor cells might be alternative cell sources for use as co-culture feeder cells. Moreover, if a human feeder co-culture method shows positive effects in both *in vitro* and *in vivo* experiments, it could be applicable to cell replacement treatments.

Acknowledgements

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the institutional animal care and use committee of Hanyang University, Korea (IACUC No. 2015-0082A).

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