



Comparison of Reprogramming Methods for Generation of Induced-Oligodendrocyte Precursor Cells

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Abstract

Direct conversion by trans-differentiation is of growing interest in cell therapy for incurable diseases. The efficiency of cell reprogramming and functionality of converted cells are important considerations in cell transplantation therapy. Here, we compared two representative protocols for the generation of induced-oligodendrocyte progenitor cells (iOPCs) from mouse and rat fibroblasts. Then, we showed that induction of Nkx6.2, Olig2, and Sox10 (NOS) was more effective in mouse fibroblasts and that induction of Olig2, Sox10, and Zfp536 (OSZ) was more effective at reprogramming iOPCs from rat fibroblasts. However, OSZ-iOPCs did not show greater proliferation than NOS-induced cells. Because the efficiency of iOPCs generation appears to differ between cell species depending on transcription factors and culture conditions, it is important to select appropriate methods for efficient reprogramming.

Key Words: Direct conversion, Oligodendrocyte, iOPC, Efficiency

INTRODUCTION

Oligodendrocytes (OLs) differentiate into myelin-forming oligodendroglia in the brain (Nave, 2010). Demyelinating disorders causing OL dysfunction include multiple sclerosis and various leukodystrophies, which result in the destruction or failed development of central white matter (Franklin and Ffrench-Constant, 2008). Examples of leukodystrophies include adrenoleukodystrophy (ALD), metachromatic leukodystrophy, Krabbe disease, Alexander disease, and Pelizaeus-Merzbacher disease. These typically include symptoms such as convulsions, muscle weakness, motor disturbance, ataxia, and visual and auditory defects (Barkovich, 2000; Maria *et al.*, 2003; Lossos *et al.*, 2015). Moreover, defects of myelin can bring about mental disorders such as schizophrenia (Karoutzou *et al.*, 2008; Takahashi *et al.*, 2011).

Direct conversion of somatic cells into other lineage cells has recently been noted for cell therapy. This approach includes trans-differentiation to a desired cell type from autologous cells (Graf and Enver, 2009; Lee *et al.*, 2015). For example, many papers achieved trans-differentiation of fibroblasts to functional and specific cell types such as neurons, neural precursor cells (NPC), astrocytes, oligodendrocytes, cardio-

myocytes, and hepatocytes (Ieda *et al.*, 2010; Vierbuchen *et al.*, 2010; Sekiya and Suzuki, 2011; Caiazzo *et al.*, 2015; Lim *et al.*, 2015a, 2015b; Prasad *et al.*, 2017). Important factors in trans-differentiation include the efficiency of reprogramming and suitability for experimental implementation. Thus, we would like to compare two methods of direct conversion to iOPC.

Successful iOPC generation was first reported in 2013 by two groups. One group used the transcription factors (TFs) Nkx6.2, Olig2, and Sox10 while another group also used Olig2 and Sox10, but chose Zfp536 instead of Nkx6.2 (Najm *et al.*, 2013; Yang *et al.*, 2013). Both papers generated iOPCs and iOLs successfully. We repeated these in mouse and rat fibroblasts and examined their effectiveness.

In this paper, we demonstrate that the NOS TFs are suitable for iOPC production from MEFs, and NOS-iOPCs have the potential to proliferate and differentiate. In REFs, the OSZ TFs are better than NOS, but OSZ-iOPCs cannot proliferate properly. Taken together, our study suggests that the efficiency of iOPC generation differs based on species and transcription factors, so suitable methods for each circumstance are needed for remyelinating cell-based therapy.

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MATERIALS AND METHODS

Isolation of skin fibroblasts and cell culture

Animals were housed and treated according to the Institutional Animal Care and Use Committee (IACUC, 2015-0083A) guidelines of Hanyang University (Seoul, Korea).

We took separate embryos from female Sprague Dawley (SD) rats at embryonic day 14 (E14), removed the head, limbs, spinal cord, and all internal organs, and dissected skin tissues and isolated dermal fibroblasts (REFs). CF1 mice (Charles River Laboratories, Wilmington, MA, USA) were isolated from E13.5 embryos (MEFs), and the dissection method was the same. Cells were plated in a T75-flask and cultivated in fibroblast medium composed of high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Invitrogen), 0.1 mM (1x) nonessential amino acids (Invitrogen), 0.1 mM β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-Glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). We used fibroblasts for experiments at least at passage 3.

Retroviral vector construction, production and transduction

Retroviral constructs for *Nkx6.2*, *Olig2*, and *Sox10* and *Olig2*, *Sox10*, and *Zfp536* were constructed by engineering the appropriate DNA fragments into the pCL retroviral vector with a P2A sequence. Retroviruses were produced from 293GPG packaging cells using the transfection reagent Lipofectamine 2000 (Invitrogen). Supernatants containing viral particles were harvested 72 h after transfection. Viral supernatant was added to fibroblasts in the presence of polybrene (8 μ g/ μ l, Sigma-Aldrich) for 16-20 hours and then the medium was changed to fresh medium.

Generation of induced-oligodendrocyte precursors

According to the NOS protocol, fibroblasts were seeded at 1×10^5 cells on 6-well plates (Corning). The next day, attached cells were transduced with *Nkx6.2*, *Olig2*, and *Sox10*-expressing retroviruses overnight. At day 3, transduced cells were passed on 15 μ g/mL poly-L-ornithine (PLO; Sigma-Aldrich) and 10 μ g/mL Laminin (Invitrogen) coated dishes and cultured in OPC medium composed of N2 medium with 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), 200 ng/ml sonic hedgehog (SHH; R&D Systems), 20 ng/ml PDGF-AA (PeproTech, Rocky Hill, NJ, USA), 2mM L-Glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). In MEF reprogramming, we added $1 \times$ B-27 without vitamin A (Invitrogen) to the medium, which was changed every other day.

According to the OSZ protocol, fibroblasts were seeded at 3×10^3 on a 25 μ g/mL poly-L-lysine (PLL; Sigma-Aldrich)-coated 24-well plate. The next day, the attached cells were transduced with *Olig2*, *Sox10*, and *Zfp536*-expressing retroviruses overnight. At day 2, culture conditions were changed from fibroblast medium to OPC medium, which consisted of DMEM (Invitrogen) with 100 μ g/ml human transferrin (Sigma-Aldrich), 100 μ g/ml bovine serum albumin (Affymetrix, Cleveland, OH, USA), 16 μ g/ml putrescine (Sigma-Aldrich), 60 ng/ml progesterone (Sigma-Aldrich), 40 ng/ml sodium selenite (Sigma-Aldrich), 5 μ g/ml N-acetyl-L-cysteine (Sigma-Aldrich), 10 ng/ml D-biotin (Sigma-Aldrich), 4.2 μ g/ml forskolin (Sigma-Aldrich), 5 μ g/ml bovine insulin (Sigma-Aldrich), 1mM sodium pyruvate (Sigma-Aldrich), 1x Trace Elements B (Mediatech,

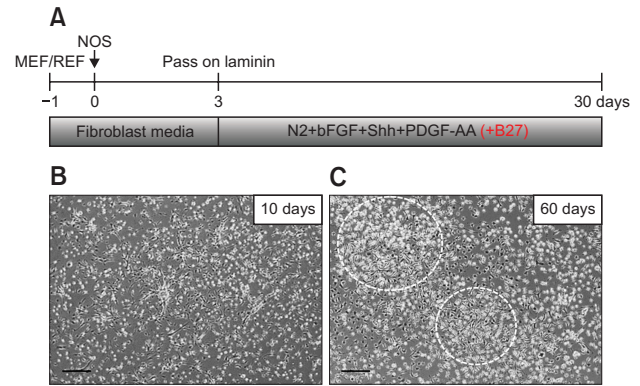


Fig. 1. Generation of iOPCs from fibroblasts through NOS induction. (A) Schematic diagram representing the experimental design and timeline of iOPCs generation using NOS TFs. (B-C) Phase contrast images of reprogrammed cells from MEFs by date. (B) is at day 10, and (C) is at day 60. White circles indicate proliferating cell clusters. Scale bar=100 μ m.

Herndon, VA, USA), and 10 ng/ml CNTF (PeproTech), 10 ng/ml PDGF-AA (PeproTech), 1 ng/ml NT-3 (PeproTech), 2 mM L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). Differentiation medium contained 40 ng/ml triiodothyronine (T3; Sigma-Aldrich) without PDGF-AA or NT-3. In MEF reprogramming, we added $1 \times$ B-27 (Invitrogen). Medium was changed every other day.

Immunostaining

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich). After 15-20 minutes, we washed cells with 0.1% BSA/PBS washing buffer 3 times and blocked for 1 hour by adding 0.3% triton X-100 (Sigma-Aldrich) and 10% normal goat serum (Invitrogen). After blocking, cells were incubated with primary antibodies at 4°C overnight. Anti-NG2 antibody (1: 200; Chemicon, Temecula, CA, USA), anti-Nestin antibody (1:500; BD Biosciences, NJ, USA), anti-Olig2 antibody (1:1000; Chemicon), anti-MBP antibody (1:1000; Abcam, Cambridge, UK), anti-A2B5 antibody (1:200; R&D systems), anti-PDGF-R antibody (1:200; R&D systems), and anti-O4 antibody (1:500; R&D systems) were used. Biotin was reacted for 30 minutes, and fluorescence-tagged antibody was reacted for one hour. Cells were mounted in VECTASHIELD with DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA).

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 with at least three independent experiments. Data are expressed as mean \pm SEM.

RESULTS

Nkx2.1, *Olig2*, and *Sox10* transcription factors are more suitable for trans-differentiation of mouse fibroblasts into iOPCs

To investigate whether NOS 3TFs could generate OPCs, we tried to convert rodent fibroblasts to iOPCs using NOS 3TFs according to Paul J Tesar's protocol (Najm *et al.*, 2013). E13.5

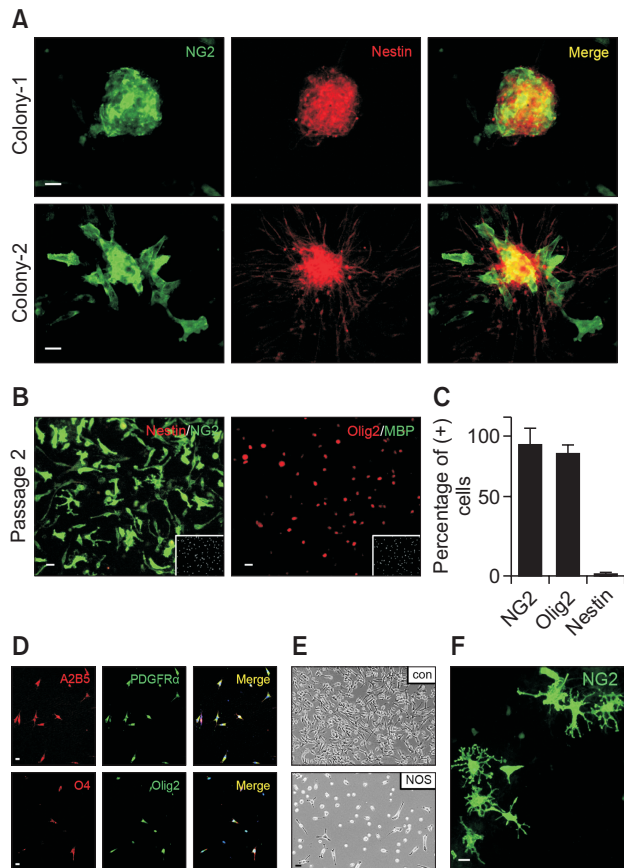


Fig. 2. Characterization of produced NOS-iOPCs. (A) Colonies generated from MEFs express an OPC marker, NG2, and an NSC marker, nestin. (B-C) After passage, dissociated single cells still express NG2 but not nestin. Another OPC marker, Olig2, is also expressed, while a mature OL marker, MBP, is not. (D) Reprogramming cells (day 30) from REFs show A2B5, PDGFR α , O4, and Olig2, which are all oligodendrocyte lineage markers. (E) In contrast with control REFs, only few iOPCs from REFs survived because of apoptosis. (F) At day 60, the morphology of iOPCs from REFs is similar to that of typical OLs. Scale bar=20 μ m.

MEFs and E14 REFs were used for reprogramming, and 3 TF retroviral constructs were connected by 2A sequences. After 3 days of 3 TF retrovirus transduction, cells were passed onto a new plate and changed to OPC medium, which included bFGF, Shh, and PDGF-AA for OPC-promoting culture conditions (Fig. 1A). Early in the conversion process, many MEF cells died and the surviving cells became smaller (Fig. 1B). Reprogrammed cells had a bipolar morphology that was similar to typical OPCs. When monitored continuously, smaller cells proliferated and formed several clusters (Fig. 1C).

On day 30, clusters were compacted like colonies, and they detached and floated on the plate. Colonies expressed the OPC marker NG2 and the neural stem cell marker nestin (Fig. 2A). In general, nestin is expressed at the early stage of neurogenesis more than in OPCs. Therefore, we assumed the generated colonies were still immature iOPCs that had the characteristics of neural stem cells. The colonies were passed and dissociated into single cells with reduced nestin expression. Furthermore, passaged cells expressed olig2, which

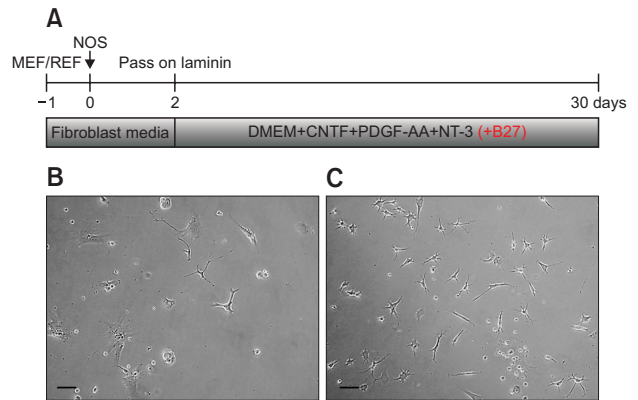


Fig. 3. Generation of iOPCs from fibroblasts through OSZ induction. (A) Schematic diagram representing the experimental design and timeline of iOPC generation using OSZ transcription factors (TFs). (B-C) Phase contrast images of reprogrammed cells from MEFs (B) and REFs (C) at 14 days. Scale bar=100 μ m.

is OPC marker, but not expressed the mature differentiated oligodendrocyte (OL) marker MBP (Fig. 2B, 2C, NG2; $82.2 \pm 10.6\%$, Olig2; $77 \pm 5.4\%$, Nestin; $1.1 \pm 1.3\%$). This result demonstrated that reprogrammed cells specifically converted into OPCs as time went on.

Overall, the induction of the NOS protocol was effective and specific to iOPC conversion from MEFs. In the case of REFs, induction of these transgenes still promoted reprogramming to iOPCs expressing OPC-associated proteins such as A2B5, PDGFR α , O4, and Olig2 (Fig. 2D). However, they had lower proliferation efficiency, as almost all of the cells were eliminated by apoptosis (Fig. 2E). Nevertheless, reprogrammed REFs were cultured for a long time, and the remaining cells formed elaborating branches like OLs (Fig. 2F). Taken together, these data suggest that the NOS TFs induction protocol is effective for iOPC conversion from MEFs.

Generation of induced-OPCs using the *Olig2*, *Sox10*, *Zfp536* transcription factors is more effective in REFs

Around the same time, Marius Wernig's group published a paper on iOPC generation using *Olig2*, *Sox10*, and *Zfp536* (OSZ) instead of *Nkx6.2* (Yang *et al.*, 2013). We examined the OSZ TFs induction protocol in E13.5 MEFs and E14 REFs. In this protocol, retrovirus-transduced cells were not passed, but were just changed to OPC medium at day 2. The reprogramming medium was composed of CNTF, PDGF-AA, and NT-3-like oligodendrocyte inducing factors (Fig. 3A). Both fibroblasts became smaller earlier (day 14) than they did with NOS induction (Fig. 3B, 3C); however, they did not proliferate well. On reprogramming day 14, almost all MEFs and REFs expressing NG2 had typical OPC morphologies, though a few of them expressed nestin (Fig. 4A). Importantly, after additional 2 weeks, approximately half of the rat cells were nestin+ (Fig. 4B, 4D), and they lost expression of nestin gradually and only had expression of NG2 until day 60 (Fig. 4C, 4D, NG2, 1 month; $95.9 \pm 3.7\%$, 2 months; $93.3 \pm 6.5\%$, nestin, 1 month; $59.3 \pm 7\%$, 2 months; $8.2 \pm 7.7\%$). Almost all mouse cells died during the reprogramming process. Taken together, these data suggest that the OSZ 3TF induction protocol is effective for iOPC conversion from REFs.

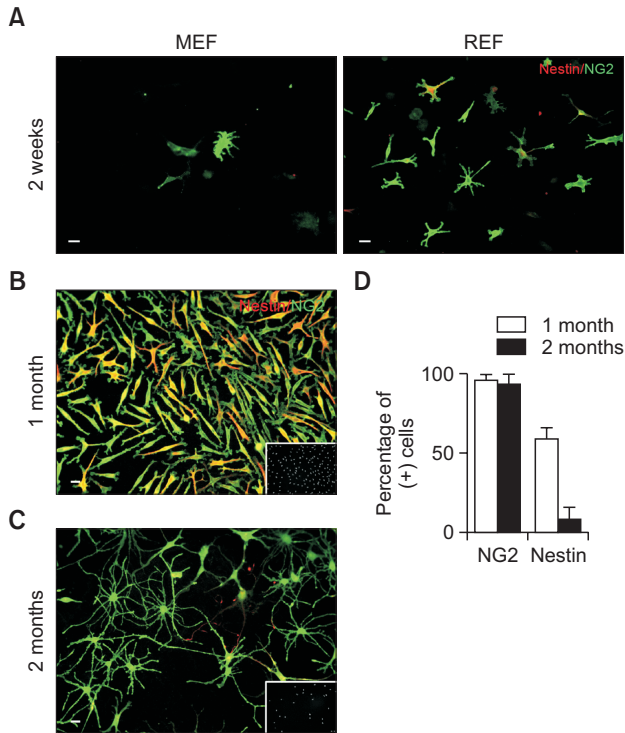


Fig. 4. Identification of OSZ-iOPCs for comparison with NOS-iOPCs. (A) Immunofluorescence microscopy images of iOPCs from MEFs and REFs. Approximately 2 weeks later, only a few MEFs survived and the remaining cells expressed NG2. (B-D) About half of the reprogrammed cells from REFs co-express NG2 and Nestin (B, D) under long-term culture (day 60), iOPCs form OLs, and the expression of nestin is decreased (C, D). Scale bar=20 μ m.

DISCUSSION

Many research groups have suggested that direct trans-differentiation is a valuable method for obtaining desired cell sources for cell-based therapy (Plath and Lowry, 2011; Xu *et al.*, 2015). However, this approach should be evaluated for efficiency and identification of reprogramming mechanisms (Vidal *et al.*, 2014; Prasad *et al.*, 2017).

Here, we have compared two representative protocols of iOPC generation from mouse and rat fibroblasts. In 2013, two groups published on the reprogramming of fibroblasts to OPCs around the same time. One of them used three transcription factors, *Nkx6.2*, *Olig2*, and *Sox10* (NOS), and another group used *Olig2*, *Sox10*, and *Zfp536* (OSZ) (Najm *et al.*, 2013; Yang *et al.*, 2013). The transcription factors were induced by retroviruses and we used a 2A sequence to produce virus construct for equal expression levels of the three genes.

Many transcription factor-induced fibroblasts died due to FBS depletion of OPC medium in both protocols. In the NOS protocol, however, the morphology of fibroblasts became smaller sooner and MEFs proliferated in clusters by 1 month. Interestingly, REFs became smaller too, but they did not proliferate well. The produced iOPCs had typical OPC morphology and characteristics, and they differentiated to OLs that formed elaborate branches.

Meanwhile, in the OSZ protocol, fibroblasts were smaller and faster when compared to the NOS protocol, and REFs

Table 1. The table represents conversion properties between the two protocols.

	MEF	REF
NOS-iOPC		
Efficiency	About 80%	About 70%
Reprogramming time	More than 1 month	More than 1 month
iOPC proliferation	++	Almost all cells died
OL differentiation	+	+
OSZ-iOPC		
Efficiency	No cells survived	About 90%
Reprogramming time	-	In 2 months
iOPC proliferation	-	+
OL differentiation	-	+

NOS: *Nkx6.2*, *Olig2*, *Sox10*, OSZ: *Olig2*, *Sox10*, *Zfp536*, iOPC: induced oligodendrocyte precursor cell, OL: oligodendrocyte.

converted to iOPCs better than MEFs because MEFs did not survive the reprogramming period. The remaining REFs also did not have the potential to proliferate. They expressed nestin, known as a neural stem cell marker, for quite some time. Approximately two months after OSZ induction, expression of nestin disappeared completely and only NG2 expression remained. We speculate that the OSZ protocol reprograms faster than the NOS protocol initially, but that it takes a long time to generate ideal iOPCs (Table 1).

In summary, MEF reprogramming occurs preferentially through the NOS protocol and REF reprogramming occurs preferentially through the OSZ protocol. This difference results from the induction of transcription factors and culture conditions in each species. On the basis of these data, the rate and efficiency of iOPC reprogramming vary under different circumstances; therefore, appropriate conversion methods need to be selected to better obtain converted cells.

CONFLICT OF INTEREST

There are no conflicts of interest.

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