Tech Science Press

Comparative characterization of human fetal neural stem cells and induced neural stem cells from peripheral blood mononuclear cells

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Key words: Human fetal neural stem cells, Human peripheral blood mononuclear cells, Induced neural stem cells

Abstract: Human-induced neural stem cells (iNSCs) transplantation is a potential treatment of neurodegeneration diseases. However, whether the reprogrammed cells have the same characterizations as human fetal neural stem cells needs further exploration. Here we isolated human fetal neural stem cells from aborted 12-week fetal brains and compared with iNSCs reprogrammed from human peripheral blood mononuclear cells in gene expression, proliferation ability, differentiation capacity, and the responses to tumor necrosis factor-α. We found that iNSCs and NSCs both expressed neural stem cell markers Nestin, SOX1, and SOX2. However, only iNSCs can be patterned into dopaminergic neurons and motor neurons. Furthermore, both iNSCs and NSCs can differentiate into oligodendrocyte progenitor cells. In addition, a low dose of tumor necrosis factor-α did not inhibit the proliferation and differentiation of iNSCs and NSCs. In conclusion, iNSCs have properties similar to, and even better than, fetal neural stem cells and may be suitable for disease modeling and transplantation.

Introduction

Neurodegenerative diseases are characterized by the loss of neurons in Alzheimer's disease (AD), loss of dopamine (DA) neurons in Parkinson's disease (PD), and loss of motor neurons in amyotrophic lateral sclerosis (ALS) (Muñoz, 2018). Transplanted DA neurons could restore striatal DA release and relieve symptoms in PD patients (Kordower *et al.*, 2008; Li *et al.*, 2008). The patient-specific DA neuroblasts derived from induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) could eliminate ethical concerns and avoid immune reactions (Elkabetz *et al.*, 2008; Frontini-López *et al.*, 2018). In addition, the induction of neural stem cells (iNSCs) from somatic cells reduces the risk of tumor development associated with stem cells (Thier *et al.*, 2012; Lu *et al.*, 2013). However, whether the reprogrammed NSCs have the same features as human fetal neural stem cells or not still needs further exploration.

In this study, we compared the iNSCs derived from human peripheral blood mononuclear cells (PB-MNCs) with NSCs derived from human fetal brain for gene expression, proliferation ability, differentiation capacity, and response to tumor necrosis factor-α (TNF-α). Our results showed that iNSCs derived from PB-MNCs may be suitable for disease modeling and transplantation for neurodegenerative disease therapy.

Materials and Methods

Isolation of fetal NSCs and PB-MNCs

Fetal brain NSCs were isolated from the women seeking abortion as described previously (Wang *et al.*, 2010). PBMNCs were isolated as described previously (Tang *et al.*, 2016). Briefly, 8 mL venous blood samples were collected from the donor who signed informed consent, and PBMNCs were isolated and cultured in the medium containing 100 ng/mL SCF, 40 ng/mL insulin-like growth factor 1 (IGF-1), 10 ng/mL interleukin 3 (IL-3), 2 U/mL erythropoietin (EPO) (all from Life Technologies, Carlsbad, USA), and 100 μg/mL transferrin and 1 μM dexamethasone (both from Sigma). For reprogramming of PB-MNCs, PBMNCs were transfected with episomal vectors as described previously (Yu *et al.*, 2009). Briefly, the cDNAs for human OCT4, SOX2, NANOG, LIN28, c-Myc, and KLF4 genes were subcloned into oriP/EBNA1-based pCEP4 episomal vector (Invitrogen). PBMNCs were transfected with the episomal vectors and then directly plated to irradiated mouse embryonic fibroblasts (MEFs) seeded dishes. The culture medium was exchanged every other day. On day 3 post-transfection, the culture medium was replaced with iNSCs medium. Colonies with morphology similar to iNSCs were visible on day 10 post-transfection.

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Immunocytochemical staining
Cells were fixed and blocked in 3% donkey serum (Jackson Immuno, West Grove, PA, USA) for 1 h, and then incubated with primary antibodies at 4°C overnight followed by incubation with secondary antibody at room temperature for 1 h. The nuclei were stained with DAPI (Sigma-Aldrich). The primary antibodies were: Goat anti-Sox2 (1:1,000, SC17320, Santa Cruz), Rabbit anti-GFAP (1:500, Z0334, Dako), Mouse anti-Nestin (1:500, 611658, BD bioscience), Goat anti-Sox1(1:400, SC17318, Santa Cruz), Rabbit anti-Ki-67 (1:500, AB9260, Millipore), Mouse anti-MAP2 (1:200, M9942, Sigma), Sheep anti-TH (1:300, AB1542, Millipore), Rabbit anti-Tuj-1 (1:600, ab6046, Abcam), Rabbit anti-Olig2 (1:200, AB9610, Millipore), Mouse anti-O1 (1:300, 14-6506, eBioscience), Mouse anti-HB9 (1:50, 81.5C10, DSHB), Mouse anti-Tuj-1 (1:400, MAB1637, Millipore), Mouse anti-NKX2.2 (1:400, SC-398951, Santa Cruz). The cells from 9 randomly selected fields from three coverslips in each group were observed under a Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany). The images were captured and analyzed using ImageJ software (NIH; http://imagej.nih.gov/ij/). The numbers of cells stained positively for different markers and of total cells stained with DAPI were determined, and the differentiation rate was calculated accordingly.

PCR
RNA was isolated from the cells using RNeasy plus Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with GoScript^{TM} Reverse Transcription System (Promega, Madison, USA). PCR was performed with Taq Master Mix (Promega) and primers listed below:
GAPDH, GTGGACCTGACCTGCCGTCT
and GGAGGAGTGGGTGTCGCTGT;
GBX2, CTCGCTGCTCGCCTTCTC
and GCCAGTCAGATTGTCATCCG;
OTX2, GGGCTGAGTCTGACCACTTC
and CTGGGTGGAAAGAGAAGCTG;
HOXB2, AATCTCCCCCTCCCAAAATCG
and GGCCGGCACAGGTACTTATT;
HOXB8 CGGCAATTTCTACGGCTACG
and GAGCTCACCTTGCGGGC; EN1
GACTCGGACAGGTGCTATCG and
AACGAAAACGAAACTGGGCG;
EMX1 GAGACGAGCTCAACCCTCG
and TCCAGCTTCTGCCGTTTGTA;
EMX2 AACCGCTGGGAGTTTTGCTA and
TGGCTTGATGATTGGTCGCT; FOXG1
GGCAAGGGCAACTACTGGAT and
CTGAGTCAACACGGAGCTGT.
Amplification parameters were as follows: initial denaturation for 2 min at 94°C, then 94°C for 30 s, 60°C for 30 s, 72°C for 50 s (30 cycles), and final extension for 5 min at 72°C.

Statistical analysis
Data were shown as mean ± SEM and analyzed by GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Comparison was made by two-way ANOVA with Dunnett's test. $p < 0.05$ was considered to be significant.

Results

Identification of fetal brain NSCs and iNSCs reprogrammed from PB-MNCs
When cultured in defined medium, fetal brain-derived and PB-MNCs-derived colonies showed either monolayers or spheres (Fig. 1(A)), and both expressed Sox1, Sox2, Nestin, which are the markers of NSCs (Fig. 1(B)). Both cells grew rapidly in a defined medium (Fig. 1(C)). In addition, iNSCs showed prominent expression of transcription factors typical of forebrain/midbrain/hindbrain identities, including OTX2, EN1, GBX2, HOXB2, and HOXB8, while fetal brain NSCs expressed anterior CNS markers EMX1, EMX2 and OTX2 (Fig. 1(D)).

*Only iNSCs generated dopaminergic neurons and motor neurons*
To assess for differentiation capacity of NSCs and iNSCs, they were cultured in a medium that promoted specific neural lineage differentiation. The majority of NSCs and iNSCs were differentiated into MAP2+ neurons, and only minor portion differentiated into GFAP+ astrocytes (Figs. 2(A) and 2(B)). For differentiation into dopaminergic neurons, 9.05% ± 1.89 % of iNSCs became TH^{+} dopaminergic neurons, but there were no TH^{+} positive cells in NSCs (Figs. 2(C) and 2(D)). For differentiation into motor neurons, 9.85% ± 1.95% of iNSCs expressed HB9, a marker of motor neurons, while no HB9 positive cells were found in NSCs (Figs. 2(E) and 2(F)). Therefore, iNSCs have a better differentiation capacity than NSCs.

*Both iNSCs and NSCs were patterned to oligodendrocyte progenitor cells*
Oligodendrocyte plays an important role in the generation of the myelin sheath around the axon. Thus, we explored whether NSCs and iNSCs can differentiate into oligodendrocytes. We cultured iNSCs and NSCs in special medium (Fig. 3(A)), and both iNSCs and NSCs appeared to be bipolar cells (Figs. 3(B) and 3(C)). Following further differentiation, both iNSCs and NSCs expressed OLIG2 NKX2.2 and O1, the markers of oligodendrocyte progenitors (Fig. 3(D)). The differentiation of iNSCs and NSCs into oligodendrocyte progenitors showed no significant difference (Fig. 3(E)).

Low dose of TNF-α did not inhibit iNSCs and NSCs proliferation and differentiation
Elevated TNF-α levels have been reported in many nervous diseases, and TNF-α had various effects on the proliferation and differentiation of NSCs (Walter *et al.*, 2011; Ye *et al.*, 2013; Ben-Hur *et al.*, 2003). We treated iNSCs and NSCs with TNF-α, and immunostaining of proliferation marker Ki-67 at 0, 24, 48, and 72 h time points showed that TNF-α did not affect the proliferation of iNSCs and NSCs at 1 ng/mL and 10 ng/mL (Figs. 4(A) and 4(B)). In addition, TNF-α did not affect the differentiation of iNSCs and NSCs into MAP2-positive neuronal cells at 10 ng/mL (Figs. 4(C) and 4(D)).
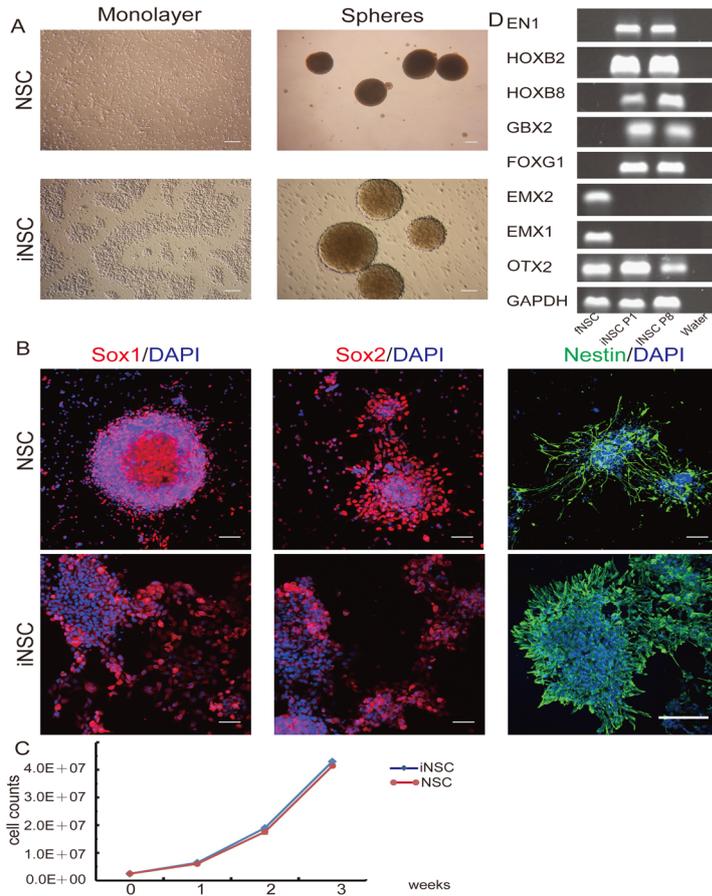
FIGURE 1. Identification of fetal brain NSCs and iNSCs. (A) The morphologies of NSCs and iNSCs. (B) NSCs and iNSCs were positive for Sox1 (left, red), Sox2 (middle, red) and Nestin (right, green). Nuclei were stained blue by DAPI. (C) Growth curve of iNSC and NSCs. (D) Region-specific gene expression of NSCs and iNSCs. Scale bars: 50 μm.
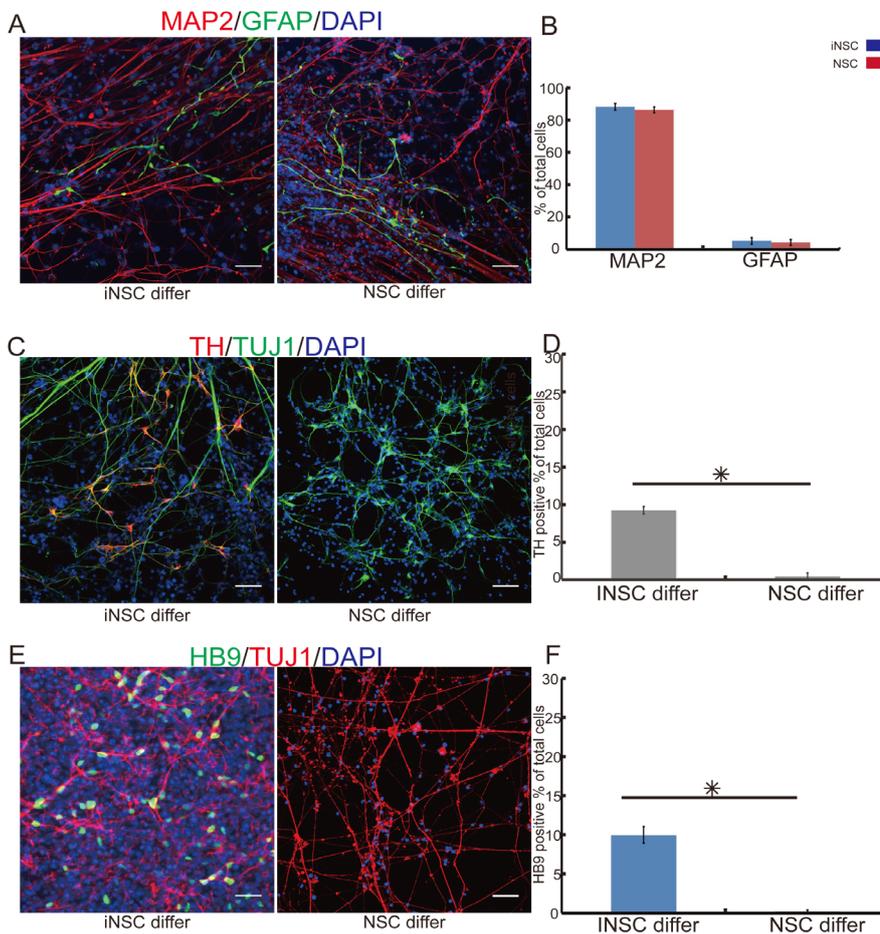
FIGURE 2. Differentiation ability of NSCs and iNSCs. (A) NSCs and iNSCs were positive for differentiation markers (MAP2, red; GFAP, green). Nuclei was stained blue by DAPI. (B) Percentage of neurons (MAP2-positive cells) and astrocytes (GFAP-positive cells) in differentiated NSCs and iNSCs. (C) Differentiated NSCs and iNSCs were positive for indicated markers (TH, red; TUJ1, green). Nuclei were stained blue by DAPI. (D) Percentage of TH-positive dopamine neurons in differentiated NSCs and iNSCs. (E) Differentiated NSCs and iNSCs were positive for indicated markers (HB9, green; TUJ1, red). Nuclei were stained blue by DAPI. (F) Percentage of HB9-positive motor neurons in differentiated NSCs and iNSCs. Scale bars: 50 μm. Data were presented as mean ± SD from 3 independent experiments. *$p < 0.05$.
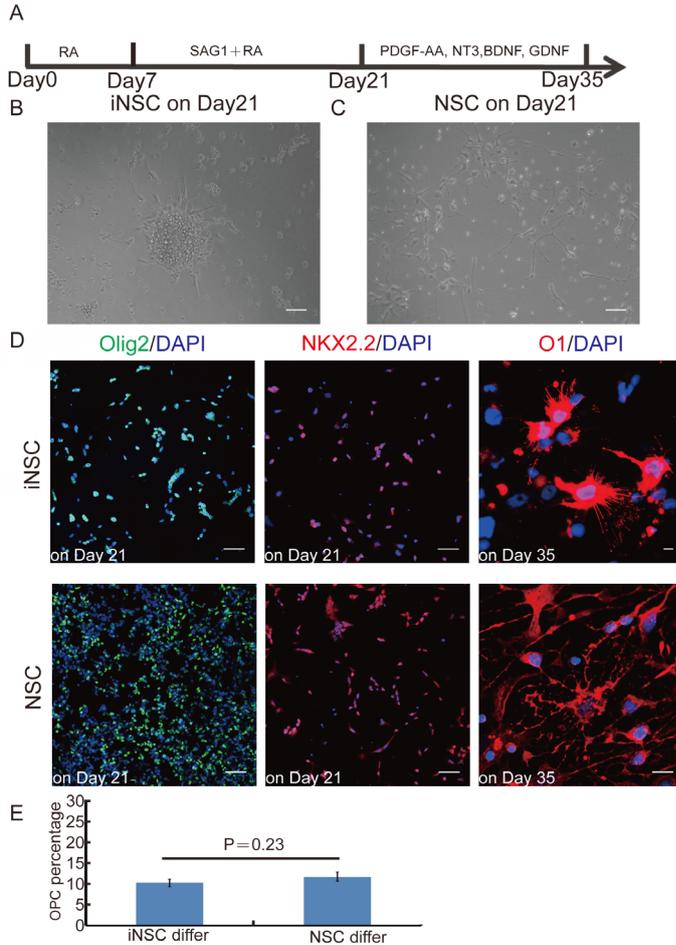
FIGURE 3. Differentiation of iNSCs and NSCs into oligodendrocyte progenitor cells. (A) Schematic presentation of differentiation protocol. (B-C) Morphology of differentiated NSCs and iNSCs onday 21. (D) Differentiated NSCs and iNSCs were positive for indicated markers (Olig2, green; NKX2.2, red; O1, red). Cell number was determined by DAPI staining. (E) Percentage of O1-positive oligodendrocyte progenitor cells in differentiated NSCs and iNSCs. Scale bars: 50 μm. Data were presented as mean ± SD from 3 independent experiments.
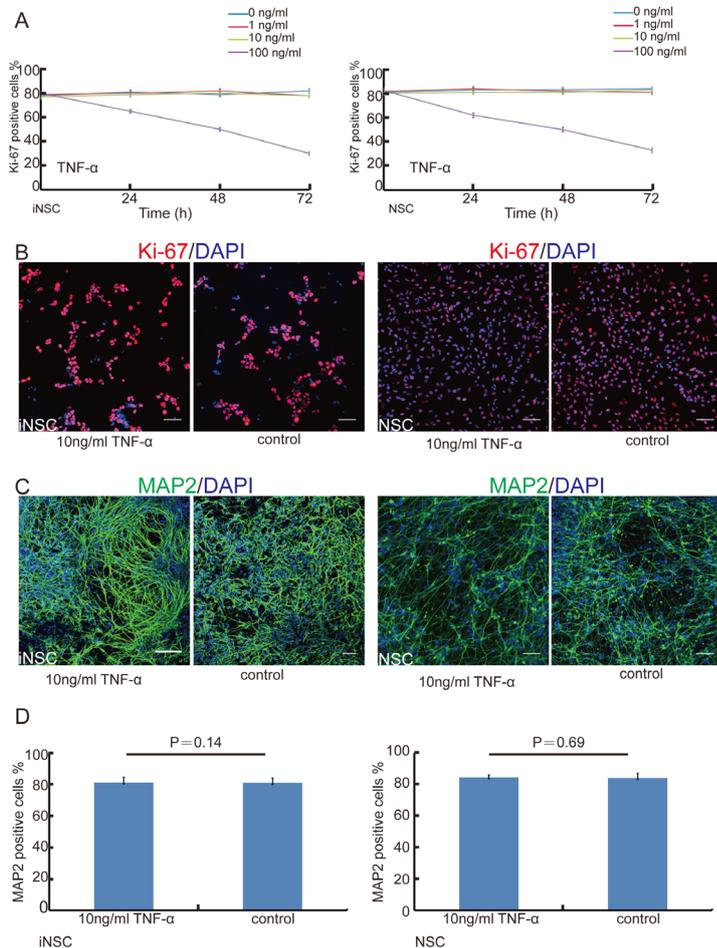
FIGURE 4. Response of iNSCs and NSCs to TNF-α. (A-B) Percentage of Ki-67 positive cells in differentiated iNSCs and NSCs after treatment with TNF-α for 72 h. Cell number was determined by DAPI staining. (C) Immunostaining of differentiated iNSCs and NSCs after treatment by 10 ng/mL TNF-α for 72 h with the indicated markers (MAP2, green). Nuclei were stained blue by DAPI. (D) Percentage of MAP2-positive neurons in differentiated NSCs and iNSCs. Cell number was determined by DAPI staining. Scale bars: 50 μm. Data were presented as mean ± SD from 3 independent experiments.

Discussion

Transplantation of patient-specific iPSCs has become a new approach in regenerative medicine (Peng *et al.*, 2018; Tang *et al.*, 2016; Thier *et al.*, 2012). Currently, a variety of methods have been developed to directly reprogram somatic cells to iNSCs, which greatly promote the application of iNSCs in the clinical (Shahbazi *et al.*, 2018). For example, a recent study reported that iNSCs reprogrammed from PBMNCs could be differentiated into specialized DA neurons, which can then be transplanted into a PD mouse model to evaluate the efficacy for PD treatment (Zheng and Chen, 2019).

In this study, we compared iNSCs derived from PBMNCs and human fetal NSCs. The results showed that iNSCs express higher levels of midbrain and hindbrain genes such as En1, Hoxb2, Hoxb8, while NSCs express higher levels of anterior brain genes. These findings suggest that iNSCs can be at an early stage of neural system development and have the potential of plasticity, consistent with previous reports (Koch *et al.*, 2009; Wang *et al.*, 2019). In contrast, human fetal NSCs could not respond to patterning cue to instruct spinal motor neuron or midbrain dopamine neuron fate. RA induced iNSCs to differentiate into oligodendrocytes (Okada *et al.*, 2008). The activation of the SHH pathway can promote iNSCs to a ventral and posterior progenitor cell fate. In our study, we used SAG1 (SHH agonist) and FGF8 to promote iNSCs to differentiate into TH positive midbrain neurons and used RA and SAG1 to pattern iNSCs to HB9-positive motor neurons, but NSCs could not be instructed into midbrain neurons or motor neurons. This may be due to the fact that human NSCs have regional specificity (Horiguchi *et al.*, 2004; Ono *et al.*, 2007). For example, TH positive neurons are mainly generated from the precursor cells from diencephalon and mesencephalon, while HB9 positive motor neurons are mainly from spinal progenitor cells (Li *et al.*, 2000; Yan *et al.*, 2007).

TNF-α is a key factor in the regulation of pathological processes such as inflammation, autoimmunity (Tchelingerian *et al.*, 1993). TNF-α has been reported to have diverse effects on the proliferation of human NSCs (Widera *et al.*, 2006; Liu *et al.*, 2005). TNF-α could promote the survival of NSCs by activating nuclear factor kappa B pathway (Shih *et al.*, 2015). In this study, we found that TNF-α at low dose had no significant effects on the proliferation and differentiation of NSCs and iNSCs. Further studies are needed to verify the *in vivo* effects of TNF-α on NSCs in order to facilitate the transplantation of NSCs into diseased condition to promote the functional recovery and evaluate the risk of cancer development following the transplantation. In addition, novel components of medicinal plants could be utilized to promote the proliferation and differentiation of iNSCs with an impact on regenerative medicine (Mahmoudi *et al.*, 2019; Montes *et al.*, 2019; Villa-Hernández *et al.*, 2018).

In conclusion, iNSCs have properties similar to and even better than fetal NSCs and may be suitable for disease modeling and transplantation.

Conflict of Interest

No conflict of interest.

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