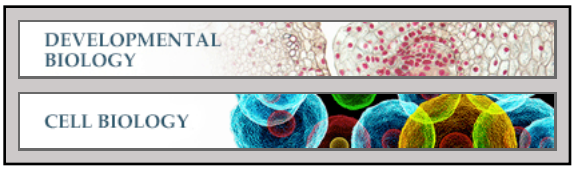
Developmental Biology:

Direct Conversion of Human Fibroblasts into Neuronal Restricted Progenitors



Qingjian Zou, Quanmei Yan, Juan Zhong, Kepin Wang, Haitao Sun, Xiaoling Yi and Liangxue Lai

J. Biol. Chem. *published online January 2, 2014*

Access the most updated version of this article at doi: 10.1074/jbc.M113.516112

JBC Papers in Press. Published on January 2, 2014 as Manuscript M113.516112
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M113.516112

Direct Conversion of Human Fibroblasts into Neuronal Restricted Progenitors*

Qingjian Zou[†,‡], Quanmei Yan[†], Juan Zhong[†], Kepin Wang[†], Haitao Sun[§], Xiaoling Yi[†], and Liangxue Lai[†,1]

[†] Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China;

[‡] School of Life Sciences, University of Science and Technology of China, Hefei 230027, China;

[§] Department of Neurosurgery, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, China

*Running title: *Generation of hiNRPs*

[1]To whom correspondence should be addressed: Liangxue Lai, Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China. TEL: +86-20-32015346; FAX: +86-20-32015346; Email: Lai_liangxue@gibh.ac.cn.

Key Words: induced neuronal restricted progenitors; trans-differentiation; Sox2; c-Myc; Brn4; Brn2

Background: Neuronal Restricted Progenitors have not been generated from fibroblasts by transdifferentiation.

Results: Human induced neuronal restricted progenitors (hiNRPs) were efficiently generated from fibroblasts by transfection of three defined factors: Sox2, c-Myc, and either Brn2 or Brn4.

Conclusion: Unipotent neuronal restricted progenitors can be rapidly and efficiently produced from fibroblasts.

Significance: This novel method will provide a new source of neurons for cellular replacement therapy of human neurodegenerative diseases.

ABSTRACT

Neuronal restricted progenitors (NRPs) represent a type of transitional intermediate cells that lie between multipotent neural progenitors (NPs) and terminal differentiated neurons during neurogenesis. These NRPs have the ability to self-renew and differentiate into neurons, but not into glial cells, which is considered as an advantage for cellular therapy of human neurodegenerative diseases. However, difficulty in the extraction of highly purified NPRs from normal nervous tissue prevents further studies and applications. In this study, we reported conversion of human fetal fibroblasts into human induced neuronal restricted progenitors (hiNRPs) in eleven days by using just three defined factors: Sox2, c-Myc, and either Brn2 or Brn4. The hiNRPs exhibited distinct neuronal characteristics, including cell morphology, multiple neuronal markers expression, self-renewal capacity, and genome-wide transcriptional profile. Moreover, hiNRPs were able to differentiate into various terminal neurons with functional membrane properties, but not glial cells. Direct generation of hiNRPs from somatic cells will provide a new source of cells for cellular replacement therapy of human neurodegenerative diseases.

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are expected to be promising sources of cells for cell therapy of human diseases due to their abilities to differentiate into various cell types. However, the tumorigenic potential and impurity of the differentiated cell types increase the risk for clinical application (1).

Mouse and human fibroblasts have been directly induced into terminal-differentiated neurons with different combinations of transcription factors (2-8). When the induced neurons (iNs) were transplanted into host, only few cells could survive and play functions due to their limited ability to proliferate. As a result, the treatment effectiveness of iN transplantation is not ideal. Many studies have focused on the generation of multi-lineage neural stem cells (NSCs) or neural progenitors (NPs) from fibroblasts (9-14). NSCs or NPs can differentiate into neurons and glial cells, the two major types of cells in nerve system (15). However, studies show that NSCs are more likely to differentiate into glial cells rather than functional neurons after transplantation (16,17), which is a disadvantage for neuron-replacement therapy of neurodegenerative diseases.

In neurogenesis, another major type of cell called neuronal restricted progenitor (NRP, also known as neuroblast), which has the abilities of proliferation and migration through rostral migratory stream in the nervous system (18,19), can develop into neurons rather than glial cells or other cell types *in vivo* and *in vitro* (20,21). When injected into the subventricular zone (SVZ), NRPs can migrate extensively and incorporated with the different regions of the brain to differentiate into various subtypes of neurons, contributing to brain plasticity and repair (19). However, the traditional acquisition of well-purified NRPs through isolation from normal nervous tissue is difficult and cumbersome (18,22), which makes it impossible to achieve sufficient cells for clinical and commercial application.

In this study, we attempt to establish an approach to directly convert human fetal fibroblasts (HFFs) into human induced neuronal restricted progenitors (hiNRPs). To change the fate of fibroblasts into hiNRPs, three processes must be considered. The first one is to use factors to convert the fibroblast into stem cells with proliferative features. Previous report showed that Sox2, Klf4 and c-Myc were critical for proliferation and NSC induction (10,12,13). The second one is to choose the factors to promote fibroblasts to acquire the characters of NPs. Bmi1, TLX and FoxG1 had been proven to be key factors NP cell induction (11,23,24). The third one is to make the induced cells achieve the capacity to become neurons. The POU III family Brn2 and Brn4 conferred the cells the tendency to become neurons (5,25). Therefore we chose these 8 factors for initial transdifferentiation trials and achieved the hiNRPs successfully. After a serial of further experiments, we found that by using just three defined factors: Sox2, c-Myc, and either Brn2 or Brn4, HFFs were able to be converted into hiNRPs. The successful generation of hiNRPs from somatic cells may provide a new source of neurons for replacement therapy of human neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and Huntington's chorea.

EXPERIMENTAL PROCEDURES

Construction of viral vectors--We selected eight factors, including Sox2 (NM_011443), c-Myc (NM_010849), Klf4 (NM_010637), TLX (NM_003269), Bmi1 (NM_005180), Brn2 (NM_005604), Brn4 (NM_000307) and FoxG1 (NM_005249). The factors were amplified and cloned into the lentiviral vector FUGW (Addgene) replacing the EGFP gene. Lentiviruses were

produced as previously reported (26). The 293T cells were seeded at 4×10^6 cells per 100 mm dish. After 1 day, the lentiviral vectors were packaged by co-transfecting them with auxiliary packaging vectors psPAX2 and pMD2-G. Lentiviruses were harvested after 48 h and centrifuged at 80,000 g for 2 h at 4℃ in an SW28 swinging bucket rotor (Beckmann, USA). After centrifugation, the supernatant was carefully aspirated and the pellet was suspended in 200μl Opti-MEM® Reduced Serum Medium (GBICO).

Generation of hiNRPs--H1 ES-derived human neural progenitor (hNP) cell lines, which are gifts of Dr. Guangjin Pan from GIBH, were used as control for characterization of hiNRPs. Human fetal fibroblasts were derived from an eight-week fetus retrieved from elective termination of pregnancy following local ethical approval. The previously described culture condition and procedures of fibroblasts were used (27). The timeline of hiNRP induction is shown in Fig. 1A. HFFs were seeded at 1×10^4 cells per well in 12-well plates 1 day before infection. Viruses were added to each well. The MOI (MOI= viral titer/cell number) is about 20~30 for each lentivirus. The next day, fresh fibroblast medium were added to replace previous medium. Two days later, induction medium were changed to complete hiNRP medium containing KnockOut™ DMEM/F-12 supplemented with StemPro® NSC SFM Supplement, Glutamax (2 mM), human bFGF (10 ng/ml) and EGF (10 ng/ml).

Reverse transcriptional PCR and quantitative PCR (qPCR)--Total RNA was extracted using Total RNA Kit II (Omega). Then first-strand cDNA was synthesized using a PrimeScript® RT Reagent Kit (TAKARA). Synthesized cDNA was subjected to RT-PCR using Premix Ex Taq™ Version 2.0 (TAKARA) with specific primers. And the qPCR was performed with SYBR® Premix Ex Taq™ (TAKARA) on CFX96 (Bio-Rad) as the manufacturer's protocol. Primers for RT-PCR and qPCR are listed in Table S1.

Methylation analysis--To determine the methylation level of the nestin enhancer in different cell types, about 500 ng genome DNA was treated with EZ DNA Methylation™ Kit (ZYMO RESEARCH) according to the manufacturer's protocol, followed by PCR. The primer sets are described in Table S1. Amplified products were purified and cloned into pMD-18T vectors (TAKARA). Vectors were transformed into *E. coli*. Nine to thirteen clones for each sample were randomly picked and sequenced.

Karyotype analysis--The hiNRPs were cultured in 100 mm dishes for 2 days, and incubated with 50 μg/ml demecolcine (Dahui Biotech) for 1 h. Cells were carefully treated with accutase (Sigma), and collected by centrifugation at 200g for 5 min. Cell precipitation was resuspended in 8 ml of 0.075 M KCl and incubated for 20 min at 37℃, followed by lysis with hypotonic buffer. Cells were then fixed in acetic acid/methanol (vol/vol=1:3). Metaphase chromosomes were stained with 5% Giemsa (Invitrogen) for 15 min. Cells were dropped on a cold slide and incubated at 75℃ for 3 h. Finally, metaphase state chromosomes were photographed under an Olympus BX51 microscope and analyzed using Karyo 3.0 software.

Microarray assay--Fibroblasts, hNPs and hiNRPs were collected in TRIzol (Invitrogen), followed by total RNA extraction. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Double-stranded cDNA (ds-cDNA) was synthesized from total RNA and labeled with Cy3 using a NimbleGen One-Color DNA Labeling Kit as the manufacturer's protocol. Array hybridization was performed with the NimbleGen Hybridization System. The arrays were scanned with an Agilent Scanner G2505C microarray scanner. Data were further analyzed using Agilent

GeneSpring GX software (Version 12.1).

Differentiation of hiNRPs in vitro--The differentiation of hiNRPs was performed as previously reported methods (12) with slight modifications. For generation of astrocytes, cells were cultured on matrigel-coated culture dishes in complete hiNRP medium with growth factors bFGF and EGF. The next day, medium was replaced by astrocyte differentiation medium (DMEM supplement with 1% N2, 2 mM GlutaMAX™-I, and 1% FBS) and cultured for 9 days. Medium shifting was conducted every other day. For differentiation of oligodendrocytes, hiNRPs were cultured on Poly-L-Ornithine/laminin (Sigma)-coated dishes for 1 day. Then, the medium was replaced by an oligodendrocyte medium containing Neurobasal® medium supplement with 2% B27, 2 mM GlutaMAX™-I, and 30 ng/ml T3 (Sigma), and cultured for 9 to 12 days. For the production of terminal neurons from hiNRPs, the cells were plated on poly-L-ornithine/laminin -coated plates in complete hiNRP medium for 1 day, followed by a medium change to Neurobasal medium supplemented with 2% B27 and 2 mM GlutaMAX™-I. To improve neuron formation, 100 μM dbcAMP (Sigma), 1 μM retinoic acid (Sigma), 10 ng/ml NT-3 (PeproTech) or GDNF (R&D) were added into the differentiation medium. The medium was changed every other day for at least two weeks.

Immunocytochemistry--For immunocytochemistry staining, cells were fixed using 4% Paraformaldehyde (PFA), washed three times with Phosphate Buffer Solution (PBS) and incubated for 45 minutes in blocking buffer (5% goat serum, 1% BSA, and 0.2% Triton-X 100 in PBS) at room temperature (RT). After removing the blocking buffer, cells were incubated overnight with primary antibody at 4℃. The following primary antibodies were used: Mouse anti-Oct4 (Santa Cruz, 1:100), rabbit anti-S100 (Sigma, 1:400), mouse anti-GFAP (Millipore, 1:200), mouse anti-O4 (Millipore, 1:100), mouse anti-nestin (Millipore, 1:200), mouse anti-Sox2 (Millipore, 1:200), rabbit anti-Pax6 (BD, 1:50), mouse anti-N-CAM (Thermo, 1:50), mouse anti-Tuj1 (sigma, 1:1000), rabbit anti-Tuj1 (Convance, 1:1000), mouse anti-TH (Sigma, 1:800), rabbit anti-Musashi1 (Millipore, 1:500), rabbit anti-Ki-67 (GenScript, 1:100), rabbit anti-DCX (GenScript, 1:100), rabbit anti-MAP2 (Sigma, 1:200), rabbit anti-NF (Millipore, 1:300), rabbit anti-NSE (ABCAM, 1:200), rabbit anti-CHAT (Millipore, 1:50), rabbit anti-5-HT (Immunstar, 1:800), rabbit anti-Glutamate (Sigma, 1:4000), rabbit anti-GABA (Sigma, 1:2000), rabbit anti-Synapsin I (Calbiochem, 1:1000). The next day, the slides were washed with PBS and added with fluorescence-labeled secondary antibody in the dark at RT for 1 h. The cells were then washed with PBS and stained with DAPI solution (1 μg/ml).

Electrophysiology--The whole cell patch clamp was performed on hiNRP-derived neurons using an Axopatch 200B amplifier and Digidata 1440A interface. Data were analyzed by pClamp 10.2 software. Neurons derived from hiNRPs were attached on coverslips, which were submerged in a recording chamber continually perfused with artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2. Neurons with big soma and smooth surfaces were recorded. Patch electrodes exhibited resistances of 4 MΩ to 9 MΩ when filled with a solution containing the following (in mM): 140.0 potassium methanesulfonate, 10.0 HEPES, 5.0 NaCl, 1.0 $CaCl_2$, 0.2 EGTA, 3.0 ATP-Na_2, 0.4 GTP-Na_2; at pH 7.3 (adjusted with KOH). The voltage was clamped at -80 mV.

For the voltage-gated currents (Na^+ and K^+ current) recording, the cells were delivered in 500

ms stepped from -80 mV to +80 mV. For the action potential recording, a series of 300 ms hyperpolarizing and depolarizing step currents from -20 pA to +50 pA was injected to elicit APs. Spontaneous postsynaptic currents were recorded without current injection.

Transplantation and immunohistochemistry--The hiNRPs were labeled with green fluorescence by transfecting with lentiviral vector FUGW. GFP-hiNRPs were collected at a density of 1×10^5 cells/μl for transplantation. The wild-type C57BL/6 mice (8 weeks, 25g to 30g) were anesthetized and fixed on the Kopf stereotaxic frame. Up to 2.5μl of cell suspension was microinjected into the lateral ventricle (anteroposterior: +1.5 mm; mediolateral: ±0.9 mm; dorsoventral: 3 mm below skull) for approximately 5 min by using a Hamilton 7005KH 10-μl syringe. After microinjection, the mice were placed under a lamp and warmed for 4 h. Then they were immunosuppressed with cyclosporine A (10 mg/ kg) each day.

Three to four weeks after transplantation, the mice were anesthetized and perfused with PBS (PH 7.4) followed by 4% PFA. The brains were collected and post-fixed for 24 hours at 4℃. After embedded in low melting-point agarose, the brains were cut into 40-μm consecutive sagittal sections using a Leica VT 1000S Vibratome. Free floating sections were immersed into 80℃ Antigen Retrieval Solution (Beyotime) for 25 min and washed three times in PBS. Then, the sections were permeabilized and blocked using TBS (0.05 M Tris, 150 mM NaCl and 0.5% Triton-X 100) supplemented with 0.1% Na-azide, 1.0% BSA and 5.0% normal goat serum for 1 h. Sections were incubated with primary antibodies for 48 h on a shaker at 4℃, then washed in TBS for 3 times and incubated with fluorescence-labeled secondary antibody at RT for 2 h. Finally, the sections were washed with PBS and stained with DAPI solution (2 μg/ml). At last, sections were observed under Zeiss 710 NLO confocal microscope and Olympus IX 51 fluorescence microscope.

RESULTS

Conversion of human fibroblasts into hiNRPs with eight transcription factors--The protocol for generation of hiNPRs from human fibroblasts is shown in Figure 1A. We chose eight key transcription factors, including Sox2, c-Myc, Klf4, TLX, Bmi1, Brn2, Brn4, and FoxG1 as the transdifferentiation inducer for pilot studies. After 5 to 7 days upon the transfection of these factors into HFFs, colonies with small and round cells occurred. The cells in the colonies proliferated more rapidly than the original fibroblasts. After culture for five days more, the large colonies with compacted cells were picked and seeded to a new plate. The cells were able to maintain strong self-renewal ability after we passaged more than 15 times and cultured for more than 2 months on matrigel-coated plates in the presence of bFGF and EGF. On passage 15, nestin antibody was used to conduct the immunostaining, we found that nestin was homogeneously expressed in the induced cells, but not in fibroblasts (Fig. 1B). We chose three colonies the nestin-positive cells to test their differentiation capacities. After cells were allowed to differentiate in the neuron-generating medium for 5 to 6 days, the cells with neuronal morphology occurred. After cultured for 8 to 10 days more, the resulted cells were Tuj1 positive, displaying their neuronal identity. However, when the nestin-positive cells were cultured in astrocyte differentiating condition for two weeks, they were not able to become GFAP positive glial cells, while as a control test, ES-derived NPs could differentiate into both neurons in neuron-differentiating medium and glial cells in glial cell-differentiating medium (Fig. 1C). These results indicated that the

nestin-positive cells were not multipotent neural progenitors, but neuronal restricted progenitors.

Narrowing down the candidate factors--To determine whether all the eight factors are necessary for inducing the fibroblasts into NRPs, we limited the number of factors by omitting the factors one by one in the next serial of induction trials. After 11 days of induction, formation of the NRP-like colonies was not affected when TLX, FoxG1, Bmi1, or Klf4 was absent. NRP-like colonies were not able to form in absence of either Sox2 or c-Myc. Therefore, both Sox2 and c-Myc were necessary factors for the trans-differentiation. We found when only Sox2 and c-Myc were used to conduct the induction, no NRP-like colonies were observed. But when either Brn2 or Brn4 was transferred into fibroblasts together with Sox2 and c-Myc, the NRP colonies occurred, meaning that Brn2 and Brn4 can replaced each other and are indispensable for the trans-differentiation from fibroblasts to hiNRPs (Fig. 1D). These results confirmed that the direct conversion of HFFs into hiNRPs can be achieved by transfection with only three factors c-Myc, Sox2, and Brn2 or Brn4 (MSB2 or MSB4 for short).

Characterization of 3F-hiNRPs--After trans-differentiation with the three transcription factors, many NRP-like colonies emerged. The cells in 3F-hiNRP colonies were round, small, and could be easily distinguished from the original fibroblasts (Fig. 2A and B). The colonies were sensitive to the surrounding environment at the first five passages. The hiNRPs were able to grow and proliferate when cultured as monolayers in the matrigel-coated plates (Fig. 2C). But if grew in suspension, they were more likely to die and could hardly expanded for further analysis (Fig. 2D). Most initial NRP-like colonies were hard to maintain over five passages. Some of them turned back to fibroblast-like morphology, and some of them differentiated into typical neuronal morphologies (Fig. 2E and 2F). We named these cells as intermediate differentiated hiNRPs (Diff-hiNRPs). However, about 18% (11/59) of the colonies with homogeneous population could maintain their NRP characteristics for more than 20 passages (Fig. 2G and 2H).

We collected ten hiNRP colonies (four MSB2 colonies and six MSB4 colonies) to differentiate into neurons and glia, respectively. After 10 days, immunostaining showed that 59% to 97% cells expressed Tuj1 after neuronal differentiation and less than 3% cells expressed GFAP after glial differentiation. Particularly, four hiNRP colonies (MSB2-10, MSB4-3, MSB4-5 and MSB4-6) did not form GFAP positive cells. Conversely, hNPs can differentiated into both $Tuj1^+$ neurons and $GFAP^+$ glia (over 60%), and HFFs were negative for Tuj1 and GFAP (Fig. 2I).

We then tested the expression status of introduced foreign genes, and found that the exogenous Sox2, c-Myc, Brn2, and Brn4 were expressed in most hiNRP cell lines. RT-PCR and qPCR analysis showed that hiNRPs expressed many neural and neuronal markers, such as Sox2, nestin, Msi1, CD133, N-CAM, DCX, Tuj1, and MAP2, but were negative for neural stem cell-specific marker, Pax6 (Fig. 3A and 3B). These results were double confirmed by immunofluorescence analysis, which displayed that the hiNRPs could express neuronal specific markers such as nestin, Sox2, DCX, Tuj1, MAP2 and Msi1 (Fig. 3C), while silenced with Pax6 (Fig. 3D), which did express in NPs (data not shown). In addition, the hiNRPs did not express Oct4, NeuN and GFAP either, the markers for ESC, mature neuron and glia, respectively (Fig. 3D). Chemokine receptor CXCR4, a key regulator of NPC migration, was also expressed in hiNRPs (Fig. 3C), suggesting that hiNRPs have migratory capacity. Most of the cells expressed the proliferation marker Ki-67 even after 25 passages

(Fig. 3C), indicating that hiNRPs could maintain self-renewal ability for a long time. All hiNRPs showed a normal diploid and male chromosomal karyotype at the 25th passage (Fig. 3E). These hiNRPs were hypodermically injected into four nude mice, no teratoma was formed in any of them even as long as ten months (data not shown).

The conserved region of the second nestin intron (located from +3314 to +3918), which is part of nestin enhancer, was largely methylated in hiNRPs, consistent with hNPs, but in a highly methylated status in human fibroblasts (Fig. 3F). The demethylation of nestin enhancer reactivated nestin promoter, resulting in nestin expression in hiNRPs.

Genome-wide transcriptional profiling of 3F-hiNRPs--We compared the global gene expression pattern of 3F-hiNRPs with the original fibroblasts and hES-derived hNPs to characterize the entire transcriptome. Both hierarchical clustering and pairwise scatterplots revealed that the global gene expression profiles of MSB2-hiNRPs and MSB4-hiNRPs were almost the same, but different from those of parental fibroblasts, Diff-hiNRPs and hNPs. The global gene expression profiles from both types of hiNRPs were closer to hNPs than to the parental fibroblasts. The profiles of Diff-hiNRPs were in the intermediate state between hiNRPs and the parental fibroblasts (Fig. 4A). The hiNRPs and control NPs showed high expression of neuronal-specific genes, such as nestin, DCX and Sox11, which were less expressed in fibroblasts. Fibroblast-specific genes such as Col1a1, Dkk3 and Sphk1 were significantly down-regulated in hiNRPs.

Differentiation potential of 3F-hiNRPs in vitro--To test the differentiating potential of 3F-hiNRPs, we seeded hiNRPs at passage 15 on to polyornithine/laminin- or matrigel-coated plates in neuronal medium without growth factors, in the presence of cAMP, NT-3, GDNF, or RA. The cells with the morphology of typical mature neuron occurred after cultured for 2 to 4 weeks. Neuron-like cells expressed the neuronal markers Tuj1, MAP2, neuron-specific enolase (NSE), and neurofilament light subunit (NF-L). Some of them expressed the synaptic proteins Synapsin1 (Fig. 5A). Furthermore, Neuron-like-cells positive for Glutamine (Glu), γ-aminobutyric acid (GABA), tyrosine hydroxylase (TH), 5-hydroxytryptamine (5-HT), or choline acetyltransferase (ChAT), could be found among the hiNRP derived cells (Fig. 5B), suggesting that hiNRPs could differentiate into neurons with various neurotransmitter phenotypes *in vitro*. Cells positive for the astrocyte markers S100 and GFAP were not found even after cultured for 2 to 4 weeks within the astrocyte-differentiation medium. Similarly, cells expressing oligodendrocyte marker O4 were not found either after cultured for the same time under oligodendrocyte-differentiation medium. Nevertheless, many differentiated cells expressed neuronal marker Tuj1, under either serum or T3 medium condition (Fig. 5C). It indicated that hiNRPs are confined to neuronal lineage no matter under what kinds of inducing medium were used.

To test whether hiNRP-derived neurons exhibit functional membrane properties, whole-cell patch-clamp recordings were performed after 4 to 6 weeks of hiNRP differentiation (Fig. 5D). The voltage-clamp mode records showed rapidly inactivating inward currents (Na^+) and persistent outward currents (K^+) in response to depolarizing voltage steps (Fig. 5E). The neurons also exhibited strong spontaneous postsynaptic currents (PSCs) (Fig. 5F). The neurons generated repetitive traces of action potentials through current-clamp records (Fig. 5G). Therefore, the hiNRP-derived neurons exhibited functional membrane properties of mature neurons *in vitro*.

Differentiation potential after Transplantation of 3F-hiNRPs into mouse brain--HiNRP cell lines expressing GFP were established by transfecting with EGFP gene. These GFP-expressing cells were then transplanted into the left lateral ventricle of two-month-old mice to test their survival ability and differentiation potential *in vivo*. After 4 weeks of transplantation, the grafts survived and integrated in the host brain around lateral ventricle (Fig. 6A). Furthermore, the integrated grafts migrated inside into the brain (Fig. 6B, above) and formed many long neuritis (Fig. 6B, below). Progenitor marker nestin was expressed in a few grafts, and neuronal markers (MAP2, NF-L, and NeuN) were observed in most grafts (Fig. 6C), indicating that hiNRPs could sustain self-renewal and differentiate into terminal neurons in the brain. Nevertheless, GFP-positive cells showed negative results for GFAP expression (Fig. 6C), indicating that the grafts had been induced to the neuronal lineage, but could not form glial cells *in vivo*.

DISCUSSION

Our study shows that hiNRPs can be generated directly and efficiently from fibroblasts. In the previous efforts, NRP-like cells have been induced from human ESCs (28), the small amount of residual pluripotent stem cells among the induced cells can be a safety concern due to their risk of tumorigenic tendency (29,30). NRPs could be sorted from NSC progeny as well (31), but the resource is limited and the procedures are cumbersome. We provided a simple method to obtain high purity NRPs, which can specifically differentiate into neurons rather than glial cells *in vitro* and *in vivo*.

We initially induced hiNRPs using 8 transcription factors (Bmi1, TLX, FoxG1, Klf4, Sox2, c-Myc, Brn2 and Brn4), all of which were proven to be key factors for NSCs/NPs or neuron induction. We successfully reached the goal of achieving NRPs by using these eight transcription factors in the initial trials. In the next step, we narrowed down the candidate factors to figure out which factors were essential in the generation of hiNRPs. We found three factors including Sox2, c-Myc, and either Brn2 or Brn4 were sufficient to induce the NRPs from the fibroblasts.

The factors we used are similar to previous report (24), in which five factors (Sox2, c-Myc, Bmi1, TLX and Brn2) were used to induce NPCs. The different fate of cells might be because we used the human fibroblasts, instead of mouse fibroblasts. The other report from Lujan et al. (11) also use the mouse fibroblasts as the starting cells. They found that out of nine Foxg1/Sox2 colonies, eight could differentiated into $Tuj1^+$ and $MAP2^+$ neurons, not $GFAP^+$ astrocytes, while only one could differentiated into both neurons and $GFAP^+$ astrocytes. Besides, they also found that only one out of four Foxg1/Brn2 NPC colonies showed tripotent character. The tendency prone to NRP appeared to exist in their induced cells. As for human cells, Giorgetti et al. (32) induced blood cord cells into neuronal cells, not NPCs, by only Sox2 and c-Myc. The cells exhibited a neuron-restricted phenotype, which indirectly supports our findings that human neuronal restricted progenitors can also be generated by related factors we used.

We first identified that NRPs could be generated from human fibroblasts by Sox2, c-Myc, and either Brn2 or Brn4. Based their functions reported previously, we speculate that Sox2 and c-Myc induce fibroblasts into progenitors with capacity of proliferation. Then, Brn2 or Brn4 plays the rule of driving progenitors toward neuronal lineage. Sox2 plays essential role in the maintenance of both ESCs and NSCs and prevent cell differentiation. As one member of Myc family, c-Myc can significantly improve proliferation and

promote reprogramming. Myc family is also a pivotal target of Wnt-β-catenin signaling, which regulates neuronal differentiation of NPCs and expansion of basal progenitors (33). Notably, Brn2 and Brn4 are both expressed in early-born neurons and play key role in the initiation of neuronal differentiation (34,35) and they share high homology in their primary structure with each other and have complementary functions (36,37). Brn-2 expression is restricted to late neural precursor cells, and a wide range of the postmitotic neurons (38). Brn2 promotes neurogenesis by upregulating other proneural genes (eg. Tbr2), as well as by diminishing notch-directed transcription of Hes5 (39). The level of Brn4 is significantly up-regulated in newborn neurons originating from embryonic striatal NSCs stimulated by IGF-1 and BDNF (35). Overexpression of Brn4 elevates neuronal differentiation and maturation from NSCs, while suppression expression of Brn4 using RNAi markedly decreases the number of newborn neurons (25,40,41). Brn4 co-expresses with MAP2 or beta-tubulin-III in neurons, while almost all of the GFAP positive astrocytes have weak immunoreactive intensity of Brn4 (25). Taken together, Brn2 and Brn4 are required for neuronal differentiation and development.

The specific gene expression pattern ($Nestin^+/PAX6^-/DCX^+$) in our hiNRPs is similar to that in NRPs. Nestin is a broad-spectrum marker for NSCs/NPs and NRPs, while Pax6 is specifically expressed in NSCs/NPs, and substantially downregulated along with the initiation of differentiation into intermediate progenitor cells (42,43). In our nestin-positive hiNRPs, absence of Pax6 indicated that multipotent NSCs/NPs did not exist in the hiNRP colonies. DCX has a binding site of transcription factors Brn2 and Brn3 in its promoter region (44) and is transiently and specifically expressed in proliferating neuronal precursors during neurogenesis (45). The expression of DCX in our hiNRPs further confirmed their neuronal precursor identity.

The functions of hiNRPs were further validated by a serial of in vitro and in vivo experiments. When cultured in vitro in a neuron –differentiation condition, hiNRPs were able to differentiate into a variety of sub-types of mature neurons including cholinergic, serotonergic, dopaminergic and GABAergic neurons with functional membrane properties. When transplanted to lateral ventricle, similarly, hiNRPs were able to differentiate into NeuN positive terminal neurons by just responding to induction of surrounding environment. These differentiated terminal neurons were found in a variety of locations, displaying their migration ability in the brain.

Transdifferentiation of hiNRPs from human fibroblasts with a high efficiency provided a new approach for creation of a large number of neurons, which can be used for neuronal developmental studies as well as therapeutic strategies addressing neurodegenerative diseases.

REFERENCES

1. Miura, K., Okada, Y., Aoi, T., Okada, A., Takahashi, K., Okita, K., Nakagawa, M., Koyanagi, M., Tanabe, K., Ohnuki, M., Ogawa, D., Ikeda, E., Okano, H., and Yamanaka, S. (2009) Variation in the safety of induced pluripotent stem cell lines. *Nat Biotechnol* **27**, 743-745
2. Caiazzo, M., Dell'Anno, M. T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T. D., Menegon, A., Roncaglia, P., Colciago, G., Russo, G., Carninci, P., Pezzoli, G., Gainetdinov, R. R., Gustincich, S., Dityatev, A., and Broccoli, V. (2011) Direct generation of functional dopaminergic neurons

from mouse and human fibroblasts. *Nature* **476**, 224-227

3. Kim, J., Su, S. C., Wang, H., Cheng, A. W., Cassady, J. P., Lodato, M. A., Lengner, C. J., Chung, C. Y., Dawlaty, M. M., Tsai, L. H., and Jaenisch, R. (2011) Functional Integration of Dopaminergic Neurons Directly Converted from Mouse Fibroblasts. *Cell Stem Cell* **9**, 413-419
4. Pang, Z. P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D. R., Yang, T. Q., Citri, A., Sebastiano, V., Marro, S., Sudhof, T. C., and Wernig, M. (2011) Induction of human neuronal cells by defined transcription factors. *Nature* **476**, 220-223
5. Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Sudhof, T. C., and Wernig, M. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* **463**, 1035-1041
6. Yoo, A. S., Sun, A. X., Li, L., Shcheglovitov, A., Portmann, T., Li, Y., Lee-Messer, C., Dolmetsch, R. E., Tsien, R. W., and Crabtree, G. R. (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature* **476**, 228-231
7. Marro, S., Pang, Z. P., Yang, N., Tsai, M. C., Qu, K., Chang, H. Y., Sudhof, T. C., and Wernig, M. (2011) Direct lineage conversion of terminally differentiated hepatocytes to functional neurons. *Cell Stem Cell* **9**, 374-382
8. Qiang, L., Fujita, R., Yamashita, T., Angulo, S., Rhinn, H., Rhee, D., Doege, C., Chau, L., Aubry, L., Vanti, W. B., Moreno, H., and Abeliovich, A. (2011) Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. *Cell* **146**, 359-371
9. Wang, L., Wang, L., Huang, W., Su, H., Xue, Y., Su, Z., Liao, B., Wang, H., Bao, X., Qin, D., He, J., Wu, W., So, K. F., Pan, G., and Pei, D. (2012) Generation of integration-free neural progenitor cells from cells in human urine. *Nat Methods* **10**, 84-89
10. Ring, K. L., Tong, L. M., Balestra, M. E., Javier, R., Andrews-Zwilling, Y., Li, G., Walker, D., Zhang, W. R., Kreitzer, A. C., and Huang, Y. (2012) Direct reprogramming of mouse and human fibroblasts into multipotent neural stem cells with a single factor. *Cell Stem Cell* **11**, 100-109
11. Lujan, E., Chanda, S., Ahlenius, H., Sudhof, T. C., and Wernig, M. (2012) Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells. *Proc Natl Acad Sci U S A* **109**, 2527-2532
12. Han, D. W., Tapia, N., Hermann, A., Hemmer, K., Hoing, S., Arauzo-Bravo, M. J., Zaehres, H., Wu, G., Frank, S., Moritz, S., Greber, B., Yang, J. H., Lee, H. T., Schwamborn, J. C., Storch, A., and Scholer, H. R. (2012) Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* **10**, 465-472
13. Thier, M., Worsdorfer, P., Lakes, Y. B., Gorris, R., Herms, S., Opitz, T., Seiferling, D., Quandel, T., Hoffmann, P., Nothen, M. M., Brustle, O., and Edenhofer, F. (2012) Direct conversion of fibroblasts into stably expandable neural stem cells. *Cell Stem Cell* **10**, 473-479
14. Sheng, C., Zheng, Q., Wu, J., Xu, Z., Wang, L., Li, W., Zhang, H., Zhao, X. Y., Liu, L., Wang, Z., Guo, C., Wu, H. J., Liu, Z., Wang, L., He, S., Wang, X. J., Chen, Z., and Zhou, Q. (2012) Direct reprogramming of Sertoli cells into multipotent neural stem cells by defined factors. *Cell Res* **22**, 208-218
15. Reynolds, B. A., and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710
16. Holmin, S., Almqvist, P., Lendahl, U., and Mathiesen, T. (1997) Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. *Eur J Neurosci* **9**, 65-75
17. Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisen, J. (1999) Identification

of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**, 25-34

18. Mayer-Proschel, M., Kalyani, A. J., Mujtaba, T., and Rao, M. S. (1997) Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. *Neuron* **19**, 773-785
19. Yang, H., Mujtaba, T., Venkatraman, G., Wu, Y. Y., Rao, M. S., and Luskin, M. B. (2000) Region-specific differentiation of neural tube-derived neuronal restricted progenitor cells after heterotopic transplantation. *Proc Natl Acad Sci U S A* **97**, 13366-13371
20. Mo, Z., Moore, A. R., Filipovic, R., Ogawa, Y., Kazuhiro, I., Antic, S. D., and Zecevic, N. (2007) Human cortical neurons originate from radial glia and neuron-restricted progenitors. *J Neurosci* **27**, 4132-4145
21. Mujtaba, T., Piper, D. R., Kalyani, A., Groves, A. K., Lucero, M. T., and Rao, M. S. (1999) Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev Biol* **214**, 113-127
22. Kalyani, A. J., Piper, D., Mujtaba, T., Lucero, M. T., and Rao, M. S. (1998) Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture. *J Neurosci* **18**, 7856-7868
23. Moon, J. H., Heo, J. S., Kim, J. S., Jun, E. K., Lee, J. H., Kim, A., Kim, J., Whang, K. Y., Kang, Y. K., Yeo, S., Lim, H. J., Han, D. W., Kim, D. W., Oh, S., Yoon, B. S., Scholer, H. R., and You, S. (2011) Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1. *Cell Res* **21**, 1305-1315
24. Tian, C., Ambroz, R. J., Sun, L., Wang, Y., Ma, K., Chen, Q., Zhu, B., and Zheng, J. C. (2012) Direct conversion of dermal fibroblasts into neural progenitor cells by a novel cocktail of defined factors. *Current molecular medicine* **12**, 126-137
25. Shi, J., Jin, G., Zhu, H., Tian, M., Zhang, X., Qin, J., and Tan, X. (2010) The role of Brn-4 in the regulation of neural stem cell differentiation into neurons. *Neurosci Res* **67**, 8-17
26. Tiscornia, G., Singer, O., and Verma, I. M. (2006) Production and purification of lentiviral vectors. *Nature protocols* **1**, 241-245
27. Rolfe, K. J., Cambrey, A. D., Richardson, J., Irvine, L. M., Grobbelaar, A. O., and Linge, C. (2007) Dermal fibroblasts derived from fetal and postnatal humans exhibit distinct responses to insulin like growth factors. *BMC Dev Biol* **7**, 124
28. Nistor, G., Siegenthaler, M. M., Poirier, S. N., Rossi, S., Poole, A. J., Charlton, M. E., McNeish, J. D., Airriess, C. N., and Keirstead, H. S. (2011) Derivation of high purity neuronal progenitors from human embryonic stem cells. *PLoS One* **6**, e20692
29. Fong, C. Y., Gauthaman, K., and Bongso, A. (2010) Teratomas from pluripotent stem cells: A clinical hurdle. *J Cell Biochem* **111**, 769-781
30. Vescovi, A. L., Galli, R., and Reynolds, B. A. (2006) Brain tumour stem cells. *Nat Rev Cancer* **6**, 425-436
31. Azari, H., Osborne, G. W., Yasuda, T., Golmohammadi, M. G., Rahman, M., Deleyrolle, L. P., Esfandiari, E., Adams, D. J., Scheffler, B., Steindler, D. A., and Reynolds, B. A. (2011) Purification of immature neuronal cells from neural stem cell progeny. *PLoS One* **6**, e20941
32. Giorgetti, A., Marchetto, M. C., Li, M., Yu, D., Fazzina, R., Mu, Y., Adamo, A., Paramonov, I., Cardoso, J. C., Monasterio, M. B., Bardy, C., Cassiani-Ingoni, R., Liu, G. H., Gage, F. H., and Izpisua Belmonte, J. C. (2012) Cord blood-derived neuronal cells by ectopic expression of Sox2 and c-Myc. *Proc Natl Acad Sci U S A* **109**, 12556-12561
33. Kuwahara, A., Hirabayashi, Y., Knoepfler, P. S., Taketo, M. M., Sakai, J., Kodama, T., and Gotoh, Y. (2010) Wnt signaling and its downstream target N-myc regulate basal progenitors in the developing neocortex.

Development **137**, 1035-1044

34. Nakai, S., Kawano, H., Yudate, T., Nishi, M., Kuno, J., Nagata, A., Jishage, K., Hamada, H., Fujii, H., Kawamura, K., and et al. (1995) The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. *Genes Dev* **9**, 3109-3121
35. Shimazaki, T., Arsenijevic, Y., Ryan, A. K., Rosenfeld, M. G., and Weiss, S. (1999) A role for the POU-III transcription factor Brn-4 in the regulation of striatal neuron precursor differentiation. *EMBO J* **18**, 444-456
36. Rhee, J. M., Gruber, C. A., Brodie, T. B., Trieu, M., and Turner, E. E. (1998) Highly cooperative homodimerization is a conserved property of neural POU proteins. *J Biol Chem* **273**, 34196-34205
37. Andersen, B., and Rosenfeld, M. G. (2001) POU domain factors in the neuroendocrine system: lessons from developmental biology provide insights into human disease. *Endocrine reviews* **22**, 2-35
38. Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., Mori, K., Ogawa, M., and Noda, T. (2002) Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. *Genes Dev* **16**, 1760-1765
39. Dominguez, M. H., Ayoub, A. E., and Rakic, P. (2013) POU-III Transcription Factors (Brn1, Brn2, and Oct6) Influence Neurogenesis, Molecular Identity, and Migratory Destination of Upper-Layer Cells of the Cerebral Cortex. *Cereb Cortex* **23**, 2632-2643
40. Tan, X. F., Jin, G. H., Tian, M. L., Qin, J. B., Zhang, L., Zhu, H. X., and Li, H. M. (2011) The co-transduction of Nurr1 and Brn4 genes induces the differentiation of neural stem cells into dopaminergic neurons. *Cell Biol Int* **35**, 1217-1223
41. Tan, X. F., Qin, J. B., Jin, G. H., Tian, M. L., Li, H. M., Zhu, H. X., Zhang, X. H., Shi, J. H., and Huang, Z. (2010) Effects of Brn-4 on the neuronal differentiation of neural stem cells derived from rat midbrain. *Cell Biol Int* **34**, 877-882
42. Kowalczyk, T., Pontious, A., Englund, C., Daza, R. A., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttner, W. B., and Hevner, R. F. (2009) Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. *Cereb Cortex* **19**, 2439-2450
43. Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., Kowalczyk, T., and Hevner, R. F. (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci* **25**, 247-251
44. Karl, C., Couillard-Despres, S., Prang, P., Munding, M., Kilb, W., Brigadski, T., Plotz, S., Mages, W., Luhmann, H., Winkler, J., Bogdahn, U., and Aigner, L. (2005) Neuronal precursor-specific activity of a human doublecortin regulatory sequence. *J Neurochem* **92**, 264-282
45. Brown, J. P., Couillard-Despres, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L., and Kuhn, H. G. (2003) Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol* **467**, 1-10

FOOTNOTES

We thank the following for their significant contributions: Miguel Esterban for helpful suggestions, Wen Li for cell transplantation, Longmei Zhang for electrophysiology, and Keyu Lai for karyotype assay.

This work was supported by grant from the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA01020107), the Ministry of Science and Technology National Basic Research Program of China (973 program) (2011CB944203) and the National Natural Science Foundation of China

(31071293).

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE51580.

Supplemental information includes a table of primers for RT-PCR and qPCR in our works.

FIGURE LEGENDS

FIGURE 1. Direct reprogramming of human fibroblasts into hiNRPs. (A) Timeline of hiNRP induction using eight factors. (B) Immunofluorescence assay showed that fibroblasts were negative for nestin, which was activated when hiNRPs were formed by induction with eight factors (20×). (C) Neural progenitors could differentiate into neuronal and glial cells ($Tuj1^+$, $GFAP^+$ and $O4^+$), while 8F-hiNRPs only differentiated into neuronal cells ($Tuj1^+$, $GFAP^-$ and $O4^-$). (D) Quantification of colonies from human fibroblasts infected with different combinations of the eight genes. (g: EGFP; S: Sox2; M: c-Myc; B: Bmi1; T: TLX; K: Klf4; F: FoxG1; B2: Brn2; B4: Brn4). Error bars, S.D., based on three replicates (n= 3).

FIGURE 2. Generation, expansion and differentiation of 3F-hiNRPs. (A to G) Process of generation, expansion of 3F-hiNRPs. After induction, cells proliferated to form colonies and decreased in size compared with the original fibroblasts (A and B). Colonies were picked and seeded onto matrigel-coated plates or low-attachment plates. Cells grew well in adherent culture (C) Many of cells died after being placed in a suspension culture (D). After several passages, most colonies were de-differentiated into fibroblast-like cells (E) or differentiated into neuronal like cells (F), while a few colonies still maintained homogenous status (G). Scale bar= 50 μm. (H) The number of hiNRP colonies can passage at most. (I) The potential of hNPs and hiNRP colonies differentiated into neurons and glia, respectively.

FIGURE 3. Characteristics of hiNRPs. (A) RT-PCR showed that markers of neuronal lineage, such as Sox2, Nestin, N-CAM, Musashi1, CD133, Tuj1, MAP2, and DCX, were expressed in both 4F and 3F hiNRPs, whereas DCX were not activated in Diff-hiNRPs. As for the transgenes, most exogenous genes were expressed in hiNRPs. (B) Analysis of qPCR also showed that hiNRPs expressed markers of neuronal lineage (Sox2, Nestin, N-CAM, Musashi1, CD133, MAP2, and DCX), but did not express Pax6. (n = 3). (C and D) The 3F-hiNRPs were immunopositive for neuronal markers such as Sox2, Nestin, N-CAM, Msi1, Tuj1, MAP2, and DCX, proliferation marker Ki-67, and chemokine receptor CXCR4, whereas the hiNRPs did not express Oct4, Pax6, GFAP, and NeuN, the markers of ESCs, NSCs, glial cells, and terminal neurons, respectively. Scale bar= 50 μm. (E) Karyotypes of 3F-hiNRPs (MSB2-hiNRPs P23, MSB4-hiNRPs P25) were normal. (F) Analysis of DNA methylation status of nestin in human fibroblasts, hNPs, and hiNRPs. Blank and filled circles represent unmethylated and methylated CpG islands, respectively. (hNP: human neural progenitors; hiNRP: human induced neuronal restricted progenitor; HFF: human fetus fibroblast; Diff: intermediate differentiated-like hiNRPs; 4F: c-Myc, Sox2, Brn2 and Brn4).

FIGURE 4. Genome-wide transcriptional profiling of hiNRPs. (A) Hierarchical cluster analysis of human fibroblasts, intermediate differentiated hiNRPs (Diff-hiNRPs), two different types of hiNRPs (MSB2-hiNRPs and MSB4-hiNRPs), and hES-derived hNPs. The color bar at the top indicates gene expression in log2 scale. Red and green colors represent higher and lower gene expression levels, respectively. (B) Scatterplot analysis of the global gene expression profiles of hiNRPs, fibroblasts, and hNPs. Black lines indicate boundaries of threefold change in gene expression levels. Gene expression levels are depicted in log2 scale. The number of differentially expressed genes is indicated under each scatterplot.

FIGURE 5. *In vitro* differentiation of 3F-hiNRPs. (A) After differentiation of hiNRPs for two to four weeks, neuronal like cells with synapse occurred. Immunostaining showed that differentiated hiNRPs

were positive for pan neuronal markers (Tuj1, MAP2) and mature neuronal markers (NSE, Syn1 and NF-L). (B): Differentiated hiNRPs were also positive for subtype-specific neuronal markers (TH, 5-HT, ChAT, Glutamate and GABA). (C) Most of the differentiated hiNRPs formed neuronal cells rather than glial cells even under standard glial differentiation procedure (positive for Tuj1, negative for S100, GFAP and O4). (A–C: Scale bar=50 μm). (D) A patched neuron differentiated from hiNRPs *in vitro*. (E) Representative traces of action potentials of an hiNRP-derived neuron in response to step current injections (−20 pA to 50 pA). Membrane potential was maintained at approximately -80 mV. The lower panel showed a single current trace at 40 pA injected current. (F) Representative traces of spontaneous postsynaptic currents (PSCs) were recorded. (G) Representative recordings of voltage-gated ion channels (K^+, left and Na^+, right) were recorded.

Figure 6. *In vivo* transplantation of 3F-hiNRPs. (A) The hiNRPs labeled with EGFP were transplanted into the lateral ventricle of adult mice. The GFP-positive grafts survived in the brains after three weeks. (B) Grafts integrated and migrated inside the brain from the lateral wall of the lateral ventricle. Grafts differentiated into neurons and form many neuritis which extend inside of the brain. (C) The GFP-expressing cells were found in a variety of locations of the host brain. The transplanted grafts remained positive for nestin in hippocampus, and the grafts showed differentiation into MAP2, NF-L, and NeuN positive neurons in thalamus and cerebral cortex. Nevertheless, the GFP-positive integrated grafts were negative for GFAP. Scale bar= 50 μm.

FIGURE 1

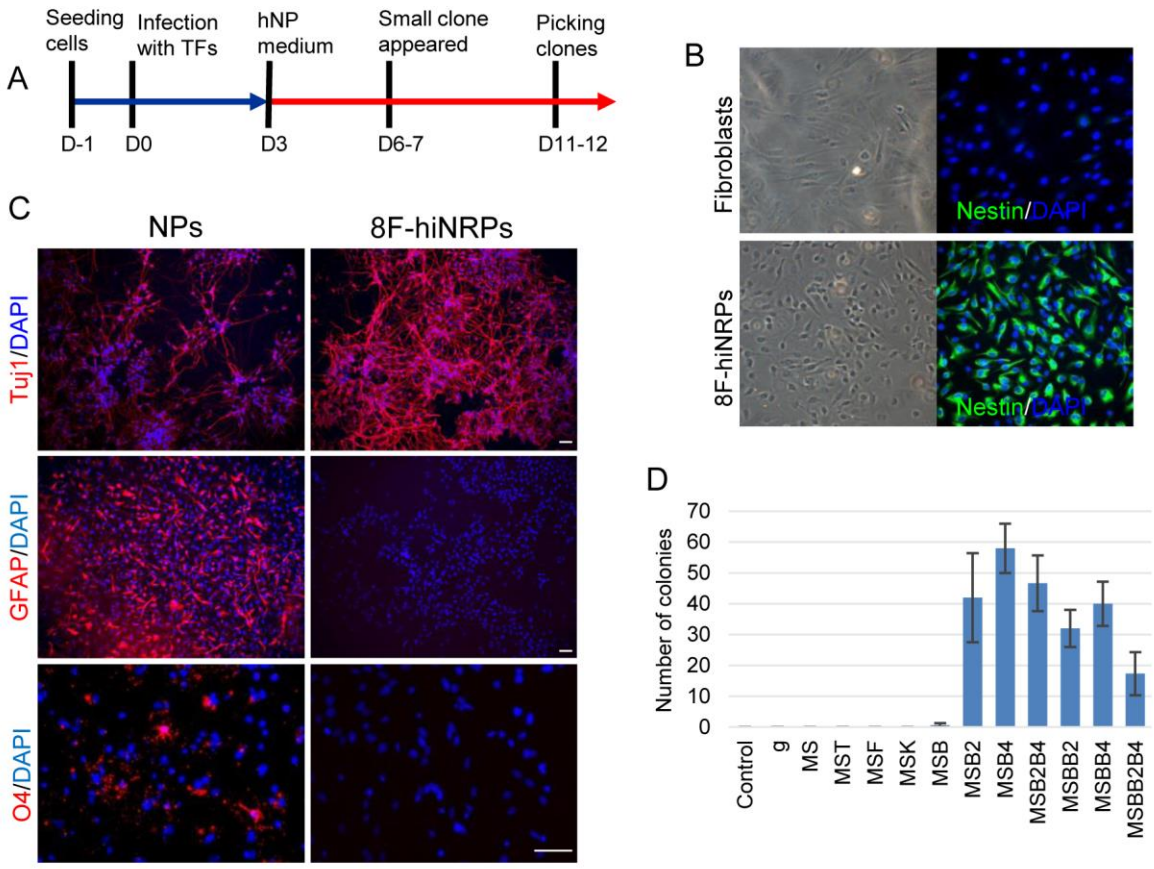


FIGURE 2

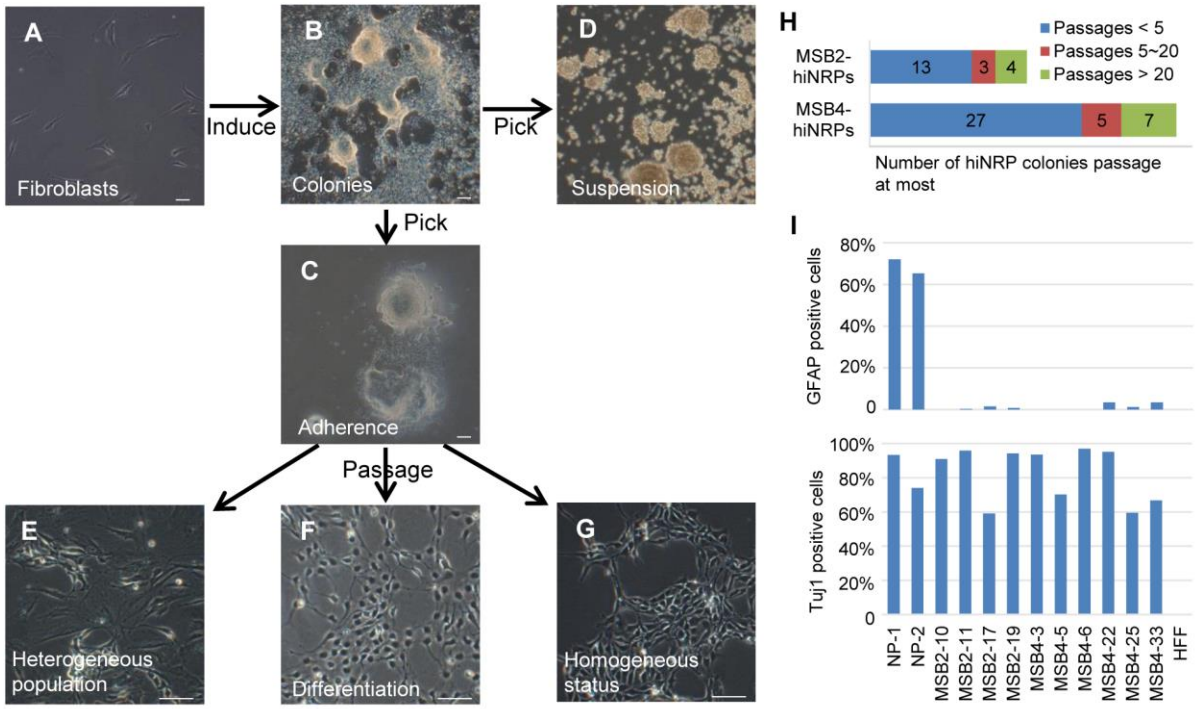


FIGURE 3

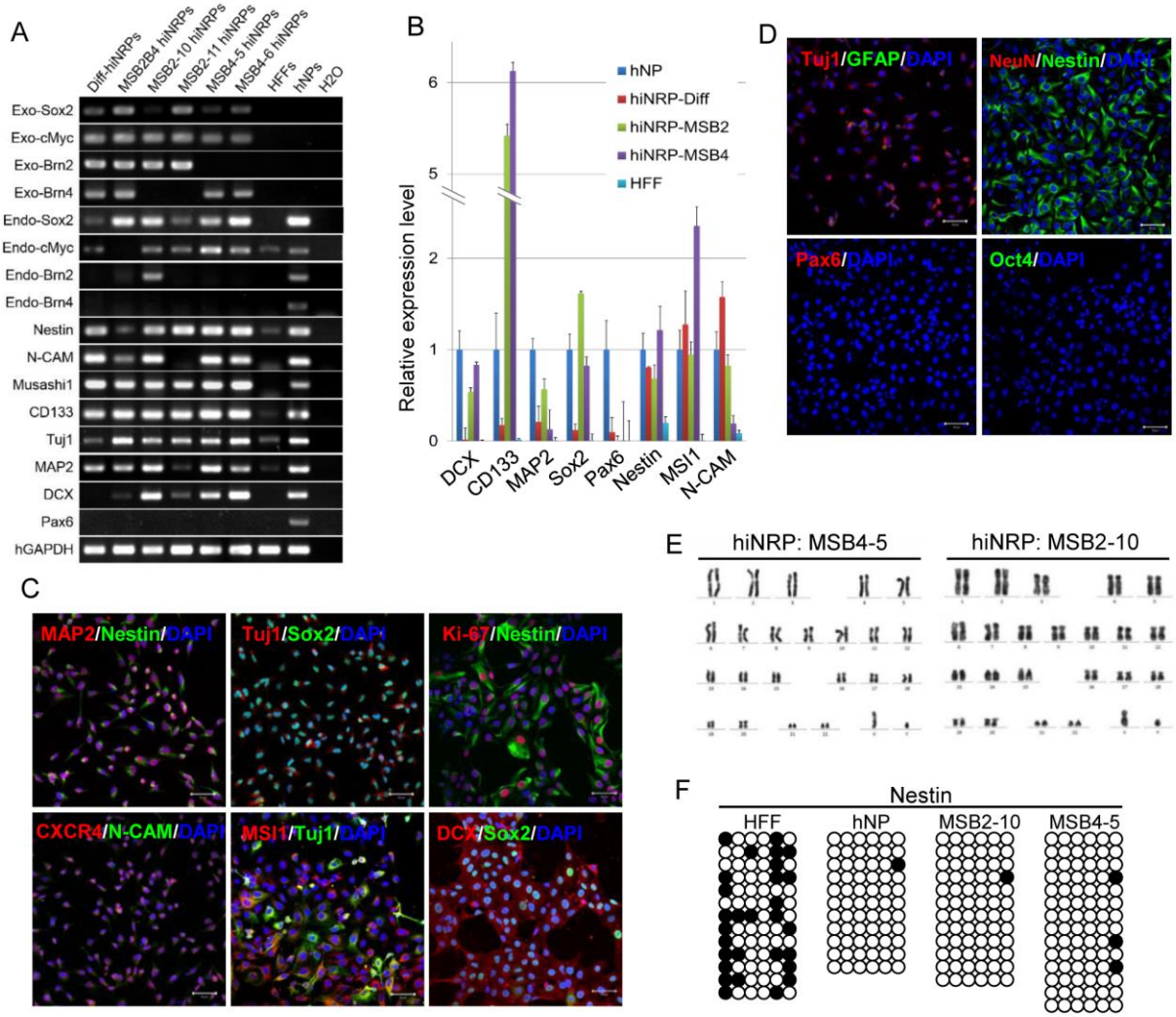


FIGURE 4

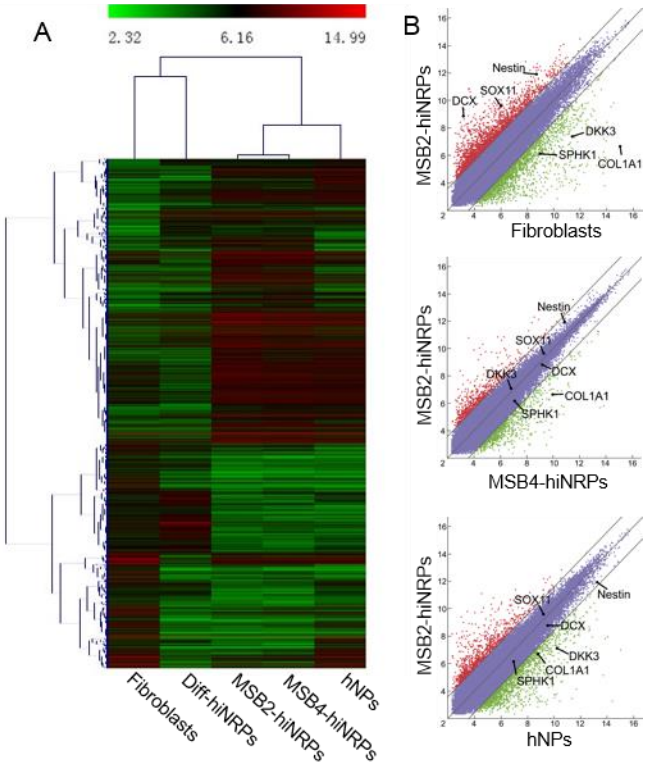


FIGURE 5

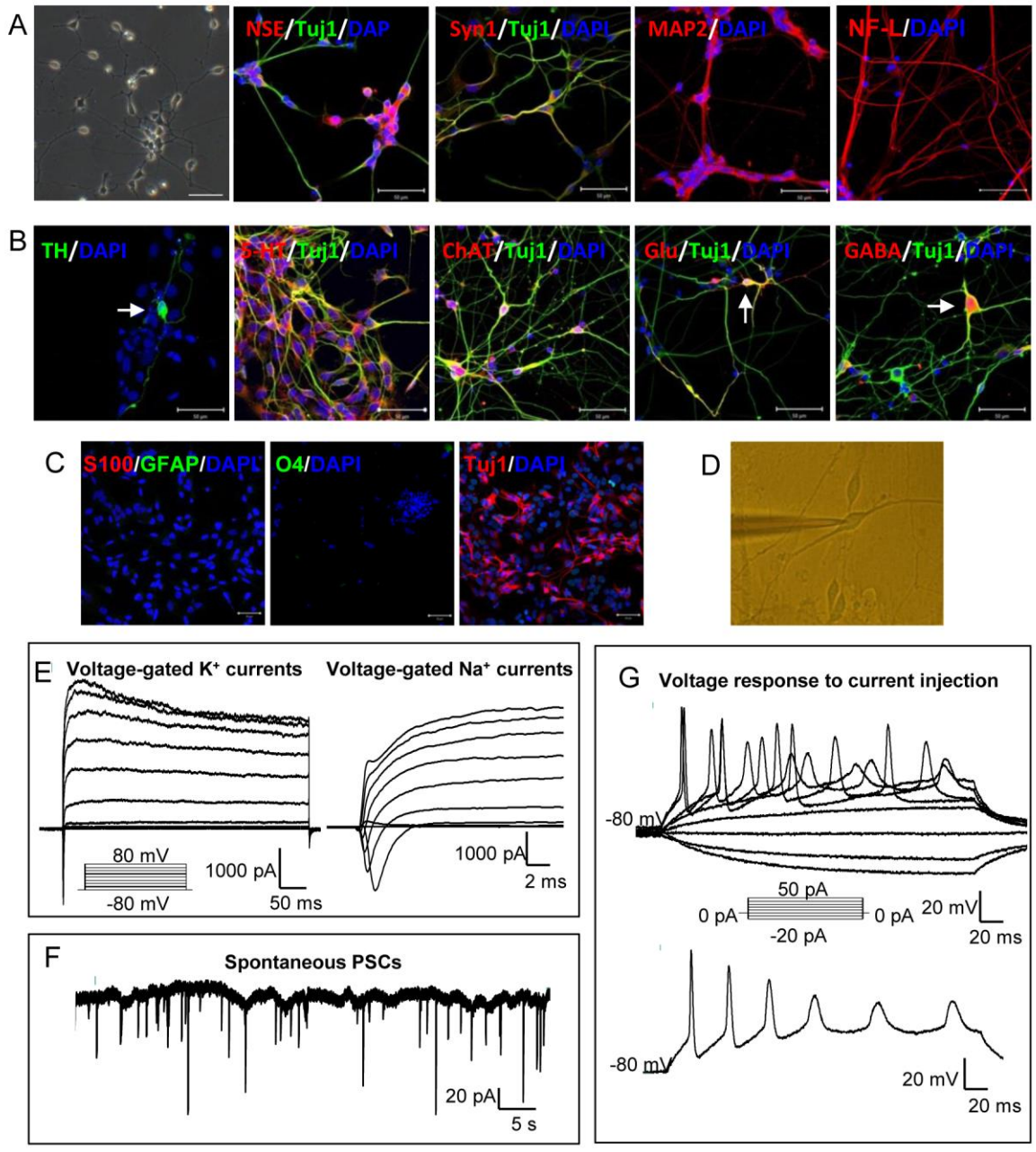


FIGURE 6