Published in final edited form as:

Curr Mol Med. 2012 February ; 12(2): 126–137.

Direct conversion of dermal fibroblasts into neural progenitor cells by a novel cocktail of defined factors

Changhai Tian^{1,2,*}, Randall J Ambroz^{1,2}, Lijun Sun^{1,2}, Yongxiang Wang^{1,2}, Kangmu Ma^{1,2}, Qiang Chen^{1,2}, Bing Zhu^{1,2,4}, and Jialin C. Zheng^{1,2,3,*}

¹Laboratory of Neuroimmunology and Regenerative Therapy, University of Nebraska Medical Center, Omaha, NE, 68198-5930

²Department of Pharmacology & Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE, 68198-5800

³Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE, 68198-5900

⁴Department of Neurobiology, Zhejiang University School of Medicine, 388 Yu Hang-tang Road, Hangzhou, Zhejiang 310058, China

Abstract

The generation of functional neural progenitor cells (NPCs) independent of donor brain tissue and embryonic tissues is of great therapeutic interest with regard to regenerative medicine and the possible treatment of neurodegenerative disorders. Traditionally, NPCs are derived through the differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). However, the induction of NPCs from ESCs and iPSCs is a complicated process that increases the risk of neoplasia and undesired cell types. This process can be circumvented through the direct conversion of somatic cells from one cell type to another by ectopic expression of specifically defined transcription factors. Using gene expression profiling and parental cells from E/Nestin:EGFP transgenic mice as a monitoring system, we tested nine factors with the potential to directly convert fibroblasts into NPCs. We found that five of these factors can directly convert adult dermal fibroblasts into NPC-like cells (iNPCs), and the resulting iNPCs possessed similar properties as primary NPCs including proliferation, self-renewal and differentiation. Significantly, iNPCs also exhibit chemotactic properties similar to those of primary NPCs. These provide an important alternative strategy to generate iNPCs for cell replacement therapy of neurodegenerative diseases.

Keywords

Neural progenitor cells; direct conversion; iNPCs; neurodegenerative disease

Introduction

Regenerative medicine aims to repair diseased or damaged tissues by replacing affected cells with healthy, functional cells of the same type. Recent breakthroughs in the field of regenerative medicine have established the feasibility of reprogramming human and murine

To whom correspondence should be addressed to: Jialin C. Zheng or Changhai Tian, jzheng@unmc.edu or ctian@unmc.edu, 402-559-5656 or 402-559-5910 (office), 402-559-7480 (fax).

The authors declare no conflict of interest.

fibroblasts, especially disease-specific cell types into induced pluripotent stem cells (iPSCs) by ectopic expression of four transcription factors (Oct4, Sox2, either c-Myc and Klf4 or Nanog and LIN28) [1–5]. Reprogramming a committed cell back to a pluripotent cell and then instructing it toward a particular specialized cell type is demanding. A major challenge includes converting iPSCs from different origins into homogeneous cell populations without contamination of undesired cell types while also eliminating their tumorigenic potential.

Different strategies have recently been developed to avoid transition through an embryonic stem cell-like status by an induced transdifferentiation process based on the ectopic expression of some defined transcription factors. In addition to sustaining self-renewal of a progenitor cell for specific organ therapy by one regulatable gene [6], the inter-conversion from one differentiated cell type to another by the ectopic expression of defined transcription factors, and avoiding transition through an embryonic stem cell-like status, also provides an alternative strategy for repairing damaged tissue [7]. So far, functional neurons have been successfully converted from mouse and human fibroblasts [8–12], as well as from terminally differentiated hepatocytes [13]. In addition, the successful reprogramming of functional hepatocyte-like cells from fibroblasts [14, 15], neural progenitor cells from fibroblasts [16], and neural crest stem-like cells from melanocytes [17], as well as the development of multilineage blood progenitors [18] and cardiomyocytes [19, 20] from fibroblasts has been described.

Multipotent NPCs exist in the developing mammalian, as well as adult nervous systems, capable of migrating toward pathological sites and giving rise to the main phenotypes of the nervous system. Therefore, the transplantation of NPCs has become a promising source of therapies for various neurodegenerative diseases and brain injuries [21–23]. Recently, increasing evidence has suggested that the coordination of activities of cell-intrinsic factors and extracellular signaling molecules not only regulates neural stem cell maintenance, self-renewal and cell fate determination, but also controls the balance between NPC self-renewal and differentiation [24]. TLX is a transcription factor that is essential for NPC proliferation and self-renewal [25]. During development, TLX is expressed specifically in periventricular neural stem cells, regulating neural stem cell proliferation, self-renewal and differentiation through one of its downstream target genes, such as p21, Pten, or GFAP [26]. Importantly, by forming a negative regulatory loop with miR-9, TLX is also involved in neural stem cell fate determination [27].

Sox2 is one of earliest noted transcription factors and is highly expressed in neural progenitor cells. The combination of Sox and its partner factor Pou3f2 (Brn2), also provides a code of neural progenitor cell specification [28–30]. The basic helix-loop-helix factors Hes1 and Hes5 can inhibit neuronal differentiation and maintain neural progenitor cells by repressing expression of proneural genes and Notch ligand genes [31–33]. Moreover, the Myc family of transcription factors is well-established regulators of the cell cycle, which confers self-renewal and represses differentiation genes. Although myc also contributes to tumorigenesis, it might be the only gene required to be involved *in vitro* to enhance self-renewal of a neural progenitor, preserving its multipotency without inducing pluripotency [6].

Recently, Bim1 as a proto-oncogene was deemed to regulate the neural progenitor-like cell generation from astrocytes and could also be a replacer of Sox2, Klf4 and c-Myc in combination with Oct4 to reprogram fibroblasts into iPSCs [34, 35]. In view of these findings, we hypothesize that cell fate determinants will coordinate with genes contributing to self-renewal, proliferation and multipotency, to directly reprogram a terminally committed cell type back to NPCs. We tested this hypothesis by combining neural progenitor cell fate determinants with regulators of self-renewal, proliferation and

multipotency, and ectopically expressed these factors in adult dermal fibroblasts from Nestin-EGFP transgenic mice (E/Nestin:EGFP). We found that these factors can directly convert adult dermal fibroblasts into NPC-like cells (iNPCs). iNPCs possess the similar properties as primary NPCs including proliferation, self-renewal and differentiation. Importantly, iNPCs exhibit similar chemotactic properties with primary NPCs. These findings provide an alternative strategy to generate NPCs for cell replacement therapy of neurodegenerative diseases.

Methods and Materials

Tissue source and cell preparation

Dermal fibroblasts were isolated from adult Nestin-EGFP transgenic mice (provided by Richard J Miller from Northwestern University, Chicago, IL) aged 5.5–7.0 weeks, with approval of the University of Nebraska Medical Center Institutional Animal Care and Use Committee and utilizing National Institutes of Health (NIH) ethical guidelines. Briefly, skin was carefully dissected from the right rear flank of the transgenic mouse and put into a petri dish containing 20 ml of phosphate-buffered saline (PBS) with 100 U/ml penicillin and 100 µg/ml streptomycin. The skin was then cut into small pieces of approximately 0.5–1 mm³ using a scalpel under sterile conditions. The tissue pieces were seeded in 100 cm² gelatin-coated plates and cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 125 ng/ml amphotericin B at 37 °C in a 5% CO₂ humidified atmosphere. During the next 3–5 days, the cells grew out from the tissue pieces and gradually formed a confluent layer. After reaching confluence, the cells were subcultured by trypsinization using a 0.25% trypsin solution containing 0.01% EDTA in PBS. The experiments were performed using cell populations between passages 2 and 10. For MEFs isolation, we used E14.5 embryos (E/Nestin:EGFP). After the removal of the head, visceral tissues, gonads and spinal cord, the remaining tissues were washed and dissociated with trypsin-EDTA solution. Ten-million cells were plated on each gelatin-coated 100-mm dish and cultured in DMEM/F-12 (1:1) medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and non-essential amino acid at 37 °C in a 5% CO₂ humidified atmosphere. The next day, floating cells were removed by washing with PBS. In this study, MEFs were used within passage 2–5 to avoid replicative senescence.

Viral vectors, direct reprogramming and differentiation

pMXs-based retroviral vectors encoding Sox2 and c-Myc were purchased from Addgene (Cambridge, MA). Mouse Nr2e1 ORF (EcoRI + Not I), Pou3f2 ORF (BamHI + Xho I), Bmi1 ORF (BamHI + Xho I), Hes1 ORF (BamHI + Xho I) and Hes5 ORF (BamHI + Xho I) were amplified from SuperScript® Mouse 13.5 Day Embryos cDNA Library (Invitrogen, Carlsbad, CA) and then cloned into pMXs-retroviral vectors. Retroviruses (pMXs) were generated with Plat-E packaging cells as previously described [36]. In brief, Plat-E cells were seeded at 3.6×10^6 cells per 100-mm dish. 24 hours after seeding, 18 µg DNA of pMXs-based retroviral vectors encoding Sox2, c-Myc, Nr2e1, Pou3f2, Bmi1, Hes1 and Hes5 were independently introduced into Plat-E cells using 18 µl of Lipofectamine™ LTX transfection reagent (Invitrogen). The medium was replaced with 5 ml of DMEM containing 5% FBS 24h after transfection. Dermal fibroblasts or MEFs from Nestin-EGFP transgenic mice were seeded at 2×10^5 cells per 35-mm dish. At 48h and 72h post-transfection, virus-containing supernatants from these Plat-E cultures were recovered and filtered through a 0.45-µm cellulose acetate filter. Equal volumes of the supernatants were mixed and supplemented with polybrene at the final concentration of 10 µg/ml. Cells were incubated in virus/polybrene-containing supernatants overnight. The medium was changed 3 days after infection to NeuroCult® NSC Basal (Stem Cell Technologies, Inc., Vancouver, BC V5Z

1B3, Canada) Medium supplemented with NeuroCult® NSC Proliferation Supplements (Stem Cell Technologies, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF, BioWalkersville), and 20 ng/ml epidermal growth factor (EGF, BioWalkersville)). After 9–14 days, the predicted iNPC colonies were monitored by regular fluorescence microscope.

Astrocyte differentiation and culture medium was DMEM/F12 (1:1) with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS. Neuron differentiation was performed as previously described [37]. Briefly, iNPCs were plated at 5×10^4 cells/cm² on collagen IV-coated glass coverslips in freshly made pre-warmed medium A composed of a 1:3 mix of DMEM/F12 and Neurobasal media (Invitrogen) containing 0.5% N2 and 1% B27 supplements and containing bFGF-2 (10 ng/ml) and BDNF (20 ng/mL) for 3 days. Cells were then shifted to medium B composed of a 1:3 mix of DMEM/F12 and Neurobasal media containing 0.5% N2 and 1% B27 supplements and containing FGF-2 (6.7 ng/mL), and BDNF (30 ng/mL). After three days, medium B was replaced with fresh medium B with a reduced amount of FGF-2 (5ng/ml). The cells were maintained under these conditions for an additional 10–15 days; the medium was partially changed every 3 days.

Dopaminergic neuron differentiation

iNPCs were dissociated by 0.05% trypsin/0.04% EDTA, and plated on collagen IV-coated glass coverslips at a concentration of 5.0×10^5 cells/well in a 24-well plate in DMEM/F12(W/O HEPES, GIBCO) medium supplemented with 10 ng/ml of bFGF (R&D Systems, Minneapolis, MN) in the presence of murine N-terminal fragment of SHH (500 ng/ml) and murine FGF8 isoform b (100 ng/ml; both from R&D Systems). iNPCs were expanded for six days and the medium was changed every two days. Differentiation was induced by removal of bFGF and the differentiation medium consisted of DMEM/F12 medium supplemented with cAMP (1 mM), AA (200 mM, both from Sigma, St. Louis, MO). The cells were incubated under differentiation conditions for 6–15 days.

Immunocytochemistry

Cells grown on collagen IV-coated cover-slips were fixed in 4% paraformaldehyde (Sigma-Aldrich), and then permeabilized with 0.2% Triton X-100 in PBS at room temperature for 20 min and incubated for 1–2 hours in blocking solution (2% BSA in PBS). The primary antibodies, including βIII-tubulin (rabbit polyclonal Ab, 1:1000, Sigma), βIII-tubulin (monoclonal Ab, 1:1000, Sigma), Na v1.6 (Rabbit polyclonal Ab, 1:200, abcam®), GFAP (mouse monoclonal Ab, 1:1000, Sigma), Oct3/4 (mouse monoclonal Ab, 1:1000, Santa Cruz) and tyrosine hydroxylase1 (TH1, mouse monoclonal Ab, 1:1000, Covance), CXCR4 (mouse monoclonal Ab, 1:500, R&D Systems) were diluted in 1% BSA in PBS with 0.1% Triton X-100 and applied overnight at 4 °C. Fluorophore-conjugated secondary antibodies (molecular Probes) were diluted in PBS, and then incubated for two hours. After washing, cells were mounted with SlowFade light antifade reagent (Molecular Probes) and analyzed by Zeiss Axiovert microscopy and Zeiss Meta 510 confocal microscopy.

RNA extraction and TaqMan real-time RT-PCR

Total mRNA was isolated with TRIzol Reagent (Invitrogen) and RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). Assays-on-Demand primers were purchased from Applied Biosystems Inc. (Foster City, CA). Real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using the one-step quantitative TaqMan Real-time RT-PCR system (Applied Biosystems Inc. Foster City, CA). Gene mRNA levels were determined and standardized with GAPDH as internal control.

Chemotaxis assay

Cell migration assays were performed with Costar Transwell inserts (8- μ m pore filter, 24-well cell clusters) as previously described with some modifications [38]. Briefly, cells were plated in a density of 10^5 per cell insert in fibronectin (5ng/mL) pre-coated wells. After an attachment period of 12 hours, cells were starved for 2 hours with basal media before adding SDF-1 α in the lower wells. After incubation for 12 hours, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed in 4% paraformaldehyde for 20 minutes at room temperature and then stained with Hoechst 33342 solution (Sigma-Aldrich) for 30 minutes. After being washed with PBS three times, the cells were examined under a Zeiss Axiophoto microscopy (Carl Zeiss, Oberkochen, Germany). Pictures (11) were taken randomly from each insert, and the number of the Hoechst 33342-positive cells was counted manually on three independent experiments.

Results

Defined factors convert adult dermal fibroblasts into iNPCs

We hypothesized that defined factors known to instruct NPCs during brain development might also facilitate the direct conversion of a committed cell type into iNPCs. To test this idea, we selected nine candidates including Pou3f2 (Brn2), Nr2e1 (TLX), Sox2, Bmi1, Hes1, Hes5, Gli2, Klf4 and c-Myc that participate in neural progenitor cell fate determination, proliferation and self-renewal. A comparison of gene expression profiles between primary NPCs and mouse embryonic fibroblasts (MEFs) revealed that seven of nine factors are highly expressed in neural progenitor cells rather than in MEFs, including Pou3f2 (Brn2), Nr2e1 (TLX), Sox2, Bmi1, Hes1, Hes5 and c-Myc (Fig. S1). Next, we used adult dermal fibroblasts (SF) derived from Nestin-EGFP transgenic mice (E/Nestin:EGFP) to monitor the conversion process from fibroblasts to iNPCs (Fig. 1A). We also used MEFs as parental cells to confirm this process (Fig. S2A). Cultures of dermal fibroblasts and MEFs were carefully screened for the presence of any contaminating EGFP⁺ cells (Fig. 1B and Fig. S2B).

The ability of each of the seven factors to facilitate the direct conversion of fibroblasts into iNPCs, was tested by constructing pMXs-based retrovirus vectors encoding Pou3f2 (Brn2), Nr2e1 (TLX), Sox2, Bmi1, Hes1, Hes5 and c-Myc, and transduced fibroblasts with the combinations of different factors (TF7: Pou3f2, Nr2e1, Sox2, c-Myc, Bmi1, Hes1 and Hes5; TF5: Pou3f2, Nr2e1, Sox2, c-Myc and Bmi1; TF4: Pou3f2, Nr2e1, Sox2 and Bmi1; TF3: Pou3f2, Nr2e1 and Sox2) according to a schematic procedure and timeline designed to monitor the occurrence of EGFP⁺ cells (Fig. 1C and Fig. 1D). Interestingly, sporadic GFP⁺ cells were observed in the TF3, TF4, TF5 and TF7 groups, but not in the retrovirus-infected and control groups after 3 days infection (Fig. 2 and Fig. S3, left panel). After 5 days infection, we found that GFP⁺ colonies were formed only in the TF7 and TF5 groups (Fig. 2 and Fig. S3, right panel). These results suggest that the direct conversion of fibroblasts into NPCs can be achieved through utilizing five (TF5) of the seven candidates. However, TF3 and TF4 could initiate iNPC fate from fibroblasts, and they were unable to promote the formation of colonies, indicating an inability to self-renew and proliferate.

Bmi1 is essential for the direct conversion of fibroblasts into iNPCs

Bmi1 is a component of the Polycomb group (PcG) multiprotein PRC1 complex, which is required to maintain a transcriptionally repressive state of many genes, including Hox genes, throughout development [39], and it is also required for the self-renewal and proliferation of NPCs [40, 41]. Recently, Bim1 has been deemed to have dual effects on iPSCs generation from fibroblasts through the suppression of p16^{INK4} and p19^{Arf} expression and the

augmentation of Sox2 and N-Myc expression [35]. We next investigated whether Bmi1 was necessary for the direct conversion of fibroblasts into iNPCs. Adult dermal fibroblasts from Nestin-EGFP transgenic mice were infected with a combination of different factors (TF-v: Pou3f2, Nr2e1, Sox2, c-Myc and Hes5; TF-iv: Pou3f2, Nr2e1, Sox2, c-Myc; TF-iii: Pou3f2, Nr2e1, Sox2), and monitored for the occurrence of GFP⁺ colonies at day 3, day 9 and day 16 after infection. Unlike the combinations of factors containing Bmi1, no GFP⁺ colonies were observed in three groups without Bmi1, and only a few GFP⁺ cells were found even after infected cells were cultured in NPC medium (NPCM) for 16 days (Fig. 3) or 21 days (data not shown). These results suggested that Bmi1 is essential for the direct conversion process among selected candidates and requires a cooperative effort with other factors to complete cell proliferation and self-renewal.

iNPCs are capable of proliferation, self-renewal and differentiation

Multipotent neural stem cells have the ability to self-renew and are capable of giving rise to both neurons and glia. These cells line the cerebral ventricles of all adult animals, including humans [42]. The assessment of whether iNPCs have the characteristics of primary NPCs, we cultured mouse dermal fibroblasts infected with TF7, TF5, TF4, TF3 and retrovirus alone with NPCM for 12 days. We observed that GFP⁺ neurospheres were formed in the TF7 and TF5 groups only (Fig. 4A), suggesting that GFP⁺ cells in these groups possess the primary NPC morphology and can proliferate normally. We then passaged adult mouse skin fibroblast-derived iNPCs from TF7 and TF5 (named SF-TF7 and SF-TF5) and cultured these cells with NPCM for 6 days. These cells formed GFP⁺ neurospheres (Fig. 4B and Fig. S3 top panels), suggesting that iNPCs possess a normal capacity for self-renewal similar to primary NPCs. Next, we carefully examined the differentiation potential of iNPCs made with five factors (TF5) and seven factors (TF7, data not shown). The results demonstrated that iNPCs can be normally differentiated to not only neurons (β -tubulin III⁺) and astrocytes (GFAP⁺) (Fig. 4C and Fig.S3 low panels), but also region-specific neurons (TH1⁺/ β -tubulin III⁺) (Fig. 4D). Together, these data indicate that the iNPCs produced by direct conversion from adult mouse dermal fibroblasts possessed characteristics of primary NPCs.

iNPCs lose pluripotency and possess a NPC gene expression signature

The direct conversion of fibroblasts into iNPCs is necessary to avoid the transition through an embryonic stem cell-like status and reduce the risks of tumor formation. It is well-known that three transcription factors including Oct4, Nanog, and Sox2 are critical in the maintenance of ESC pluripotency. Moreover, Oct4 (Pou5f1) has a highly conserved role in maintaining pluripotent cell populations [43, 44], and its expression level dictates ESC fate [45]. Oct4 expression in iNPCs made with five factors was examined by comparing levels of Oct4 in fibroblast-derived iPSCs and iNPCs through immunostaining with Oct4 specific antibody. The results demonstrated that Oct4 was highly expressed in MEF-derived iPSCs (MEFiPSCs) rather than in iNPCs (Fig.5), which decreases the risk of teratoma formation. The determination of whether iNPCs truly adopted a new cellular identity through trans-differentiation was examined by isolating skin fibroblasts from adult pregnant female Nestin-EGFP transgenic mice and GFP⁺ NPCs from pups (E14.5d) of the same female, and generating iNPCs with seven factors from skin fibroblasts. When we quantified the mRNA levels of all five factors used for conversion, we found that all five specific genes and Hes5 were expressed at tendencies similar to those in primary NPCs in comparison to skin fibroblasts (Fig. 6). In addition, the level of Bmi1 expression was decreased in iNPCs in comparison with skin fibroblasts (Fig.6 left panel), indicating that Bmi1 may be essential only for the initiation of the trans-differentiation process. The expression level of Bmi1 in adult mouse dermal fibroblasts was different from that in MEFs.

iNPCs express functional CXCR4 and show chemotactic properties to stromal cell-derived factor 1 α (SDF-1 α) stimulation

It has been shown that NSCs have a pronounced migratory capacity towards areas of pathology in the central nervous system (CNS) [46, 47]. SDF-1 α , a CXC chemokine, is the only known ligand for the chemokine receptor CXCR4 [48]. Over the past years, the CXCL12/CXCR4 ligand/receptor system has been identified as one key regulator of NPC migration. A comparison of iNPCs migratory capacity with that of NPCs was checked by following the expression of CXCR4 in iNPCs using a monoclonal CXCR4 antibody. The results demonstrated that CXCR4 protein was highly expressed in the membrane of iNPCs (Fig. 7A), consistent with the expression pattern of CXCR4 in primary NPCs. We then tested whether SDF-1 α stimulation acts on iNPCs and regulates iNPC migration. As expected, SDF-1 α induced the migration of iNPCs in a dose-dependent manner, without a significant difference between 50 ng/ml and 100 ng/ml, which was similar to that of NPCs (Fig. 7B). Together, these data suggest that SDF-1 α can influence iNPC function through CXCR4 and that CXCR4 is functional in iNPC migration.

Discussion

Collectively, our data demonstrate that mouse somatic cells, such as embryonic fibroblasts and adult dermal fibroblasts, can be directly converted into functional neural progenitor cells by using a novel cocktail of defined factors. This process was different from the generation of iPSCs and bypassed the transition through an embryonic stem cell-like status. This suggests that adult dermal fibroblasts can be directly reprogrammed to multipotent iNPCs through the introduction of cell fate determinants and factors responsible for cell proliferation and self-renewal. The brains and spinal cords of Nestin-EGFP transgenic embryos were removed from the bodies to avoid the contamination of endogenous neural progenitor cells in our originating cultures. Fibroblasts were then carefully isolated and screened with fluorescence microscopy to exclude the existence of GFP⁺ cells before the cultures were utilized in this study. Although few GFP⁺ cells were observed in MEF cultures (Fig. S2 A-c; Fig. 1B right panel), they would disappear after several passages. This cell model provides a promising tool to monitor the conversion process and to identify potential candidates contributing to the conversion.

Functional neurons, hepatocyte-like cells and cardiomyocytes have been successfully converted from fibroblasts by expressing cell fate determinants collaborating in the direct conversion, with other factors [8, 14, 20, 49]. These findings support the possibility of reprogramming adult dermal fibroblasts into iNPCs through genetic-based approaches. It was reported that the nuclear orphan receptor TLX not only determined NPC fate through a negative regulatory loop with miR-9, but also contributed to proliferation and self-renewal of NPCs [25–27]. In addition, Nestin is a molecular marker for NPCs and *nestin* genes possess a central nervous system-specific enhancer within their second introns. It was well-known that Brn2 plays an important role in determining *nestin* gene expression by collaborating with Sox2 or Sox11 [28–30]. In this study, we used Brn2 and TLX as NPC fate determinants alone or with other factors as regulators of cell proliferation and self-renewal to see if they can work together and achieve the direct conversion of fibroblasts into iNPCs. Our results demonstrated that these two cell fate determinants with three other regulators were enough to reprogram terminally differentiated fibroblasts into iNPCs (Fig. 3, Fig. 4). We assume that this combination of five factors is not the only candidate group contributing to the direct generation of iNPCs. Other combinations of candidates or fewer candidates will be identified soon and may contribute to accomplishing this conversion process. Moreover, Bmi1 is a key regulator in our approach and recently has been deemed to replace Sox2, Klf4 and c-Myc, and promote iPSC generation in combination with Oct4 in addition to the trans-differentiation of mouse fibroblasts into NSC-like cells [35]. Although

our data demonstrated that Bmi1 was essential for the direct conversion of dermal fibroblasts into iNPCs, Bmi1 alone was not enough to accomplish the whole conversion even when combined with Pou3f2, Nr2e1 and Sox2 (Fig.2 and Fig.3). This suggests that Bmi1 plays a critical role in the initiation of the trans-differentiation of fibroblasts into iNPCs by transcriptional regulation, and its cooperation with c-Myc was also important for cell proliferation and self-renewal.

Our ultimate goal will be to use these resulting iNPCs for disease modeling and cell therapy of neurodegenerative diseases. In theory, the trans-differentiation of somatic cells should avoid reprogramming via a pluripotent state and reduce the risk of tumor formation that is associated with the use of ESCs and iPSCs. Our resulting iNPCs not only showed the abilities to proliferate, self-renew, differentiate and migrate, but also lost the expression of pluripotent markers (Fig.5). Thus, iNPCs may provide an alternative and safer cell source for transplantation in neurodegenerative disorders. Migration toward pathology is the first critical step in NPC engagement during regeneration. NPCs express CXC chemokine receptor CXCR4, the cognate receptor for SDF-1 α . The exposure of quiescent NPCs to SDF-1 α enhanced proliferation and promoted chain migration and transmigration, and activated intracellular molecular pathways mediating engagement [47]. In our study, iNPCs, compared to induced functional neurons derived from fibroblasts, not only possess the potential to generate the diverse neuronal subtypes, but also have migratory capacity after responding to a chemotactic agent (Fig. 4 and Fig.7). This will give iNPCs a broad application prospect in cell replacement therapy of neurodegenerative diseases. Before clinical application of iNPCs can be considered, it will be critical to intensively investigate the safety and effectiveness of iNPCs, optimize the combination of defined candidates and confirm the functionality of iNPCs in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We kindly thank Dr. Terry D. Hexum for comments on the manuscript and Drs. Yunlong Huang, Hui Peng, Qiang Liu, Jie Wu, Jie Chao, Lie Gao, Jinxiu liu, Yulong Li and Kristin M. Leland Wavrin, who provided technical support for this work. Julie Ditter, Lenal Bottoms, Johna Belling, and Robin Taylor provided outstanding administrative and secretarial support. This work was supported in part by research grants from the National Institutes of Health: R01 NS 41858-01, R01 NS 061642-01, 3R01NS61642-2S1, R21 MH 083525-01, P01 NS043985, and P20 RR15635-01 (JZ), the State of Nebraska, DHHS – LB606 Stem Cell 2009-10 (JZ), NEHHS-LB606 Stem Cell 2010-11 (SD and CT) and National Natural Science Foundation of China (NSFC) # 81028007 (JZ).

References

1. Blöchl R, Vénere M, Yen J, Ramalho-Santos M. Generation of induced pluripotent stem cells in the absence of drug selection. *Cell Stem Cell*. 2007; 1:245–247. [PubMed: 18371358]
2. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861–872. [PubMed: 18035408]
3. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006; 126:663–676. [PubMed: 16904174]
4. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–1920. [PubMed: 18029452]
5. Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. *Cell*. 2008; 134:877–886. [PubMed: 18691744]

6. Kim KS, Lee HJ, Jeong HS, et al. Self-renewal induced efficiently, safely, and effective therapeutically with one regulatable gene in a human somatic progenitor cell. *Proc Natl Acad Sci U S A*. 2011; 108:4876–4881. [PubMed: 21378266]
7. Blelloch R. Regenerative medicine: short cut to cell replacement. *Nature*. 2008; 455:604–605. [PubMed: 18833266]
8. Ambasudhan R, Talantova M, Coleman R, et al. Direct reprogramming of adult human fibroblasts to functional neurons under defined conditions. *Cell Stem Cell*. 2011; 9:113–118. [PubMed: 21802386]
9. Caiazzo M, Dell'Anno MT, Dvoretzskova E, et al. Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. *Nature*. 2011; 476:224–227. [PubMed: 21725324]
10. Pang ZP, Yang N, Vierbuchen T, et al. Induction of human neuronal cells by defined transcription factors. *Nature*. 2011; 476:220–223. [PubMed: 21617644]
11. Pfisterer U, Kirkeby A, Torper O, et al. Direct conversion of human fibroblasts to dopaminergic neurons. *Proc Natl Acad Sci U S A*. 2011; 108:10343–10348. [PubMed: 21646515]
12. Son EY, Ichida JK, Wainger BJ, et al. Conversion of mouse and human fibroblasts into functional spinal motor neurons. *Cell Stem Cell*. 2011; 9:205–218. [PubMed: 21852222]
13. Marro S, Pang ZP, Yang N, et al. Direct lineage conversion of terminally differentiated hepatocytes to functional neurons. *Cell Stem Cell*. 2011; 9:374–382. [PubMed: 21962918]
14. Huang P, He Z, Ji S, et al. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature*. 2011; 475:386–389. [PubMed: 21562492]
15. Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature*. 2011; 475:390–393. [PubMed: 21716291]
16. Kim J, Efe JA, Zhu S, et al. Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci U S A*. 2011; 108:7838–7843. [PubMed: 21521790]
17. Zabierowski SE, Baubet V, Himes B, et al. Direct Reprogramming of Melanocytes to Neural Crest Stem-Like Cells by One Defined Factor. *Stem Cells*. 2011
18. Szabo E, Rampalli S, Risueno RM, et al. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*. 2010; 468:521–526. [PubMed: 21057492]
19. Efe JA, Hilcove S, Kim J, et al. Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nat Cell Biol*. 2011; 13:215–222. [PubMed: 21278734]
20. Ieda M, Fu JD, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*. 2010; 142:375–386. [PubMed: 20691899]
21. Bjorklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. *Nat Neurosci*. 2000; 3:537–544. [PubMed: 10816308]
22. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature*. 2006; 441:1094–1096. [PubMed: 16810245]
23. Ryu JK, Cho T, Wang YT, McLarnon JG. Neural progenitor cells attenuate inflammatory reactivity and neuronal loss in an animal model of inflamed AD brain. *J Neuroinflammation*. 2009; 6:39. [PubMed: 20030829]
24. Qu Q, Shi Y. Neural stem cells in the developing and adult brains. *J Cell Physiol*. 2009; 221:5–9. [PubMed: 19562676]
25. Qu Q, Sun G, Li W, et al. Orphan nuclear receptor TLX activates Wnt/beta-catenin signalling to stimulate neural stem cell proliferation and self-renewal. *Nat Cell Biol*. 2010; 12:31–40. sup pp 1-9. [PubMed: 20010817]
26. Sun G, Yu RT, Evans RM, Shi Y. Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. *Proc Natl Acad Sci U S A*. 2007; 104:15282–15287. [PubMed: 17873065]
27. Zhao C, Sun G, Li S, Shi Y. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat Struct Mol Biol*. 2009; 16:365–371. [PubMed: 19330006]
28. Miyagi S, Saito T, Mizutani K, et al. The Sox-2 regulatory regions display their activities in two distinct types of multipotent stem cells. *Mol Cell Biol*. 2004; 24:4207–4220. [PubMed: 15121842]

29. Tanaka S, Kamachi Y, Tanouchi A, et al. Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol Cell Biol*. 2004; 24:8834–8846. [PubMed: 15456859]
30. Miyagi S, Nishimoto M, Saito T, et al. The Sox2 regulatory region 2 functions as a neural stem cell-specific enhancer in the telencephalon. *J Biol Chem*. 2006; 281:13374–13381. [PubMed: 16547000]
31. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999; 284:770–776. [PubMed: 10221902]
32. Gaiano N, Fishell G. The role of notch in promoting glial and neural stem cell fates. *Annu Rev Neurosci*. 2002; 25:471–490. [PubMed: 12052917]
33. Selkoe D, Kopan R. Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu Rev Neurosci*. 2003; 26:565–597. [PubMed: 12730322]
34. Moon JH, Yoon BS, Kim B, et al. Induction of neural stem cell-like cells (NSCLCs) from mouse astrocytes by Bmi1. *Biochem Biophys Res Commun*. 2008; 371:267–272. [PubMed: 18439910]
35. Moon JH, Heo JS, Kim JS, et al. Reprogramming fibroblasts into induced pluripotent stem cells with Bmi1. *Cell Res*. 2011; 21:1305–1315. [PubMed: 21709693]
36. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther*. 2000; 7:1063–1066. [PubMed: 10871756]
37. Spiliotopoulos D, Goffredo D, Conti L, et al. An optimized experimental strategy for efficient conversion of embryonic stem (ES)-derived mouse neural stem (NS) cells into a nearly homogeneous mature neuronal population. *Neurobiol Dis*. 2009; 34:320–331. [PubMed: 19236914]
38. Xu Q, Wang S, Jiang X, et al. Hypoxia-induced astrocytes promote the migration of neural progenitor cells via vascular endothelial factor, stem cell factor, stromal-derived factor-1alpha and monocyte chemoattractant protein-1 upregulation in vitro. *Clin Exp Pharmacol Physiol*. 2007; 34:624–631. [PubMed: 17581219]
39. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev*. 2002; 16:2893–2905. [PubMed: 12435631]
40. Molofsky AV, Pardal R, Iwashita T, et al. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature*. 2003; 425:962–967. [PubMed: 14574365]
41. Park IK, Qian D, Kiel M, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature*. 2003; 423:302–305. [PubMed: 12714971]
42. Goldman S. Stem and progenitor cell-based therapy of the human central nervous system. *Nat Biotechnol*. 2005; 23:862–871. [PubMed: 16003375]
43. Nichols J, Zevnik B, Anastasiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998; 95:379–391. [PubMed: 9814708]
44. Morrison GM, Brickman JM. Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development. *Development*. 2006; 133:2011–2022. [PubMed: 16651543]
45. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*. 2000; 24:372–376. [PubMed: 10742100]
46. Aboody KS, Brown A, Rainov NG, et al. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci U S A*. 2000; 97:12846–12851. [PubMed: 11070094]
47. Imitola J, Raddassi K, Park KI, et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci U S A*. 2004; 101:18117–18122. [PubMed: 15608062]
48. Tran PB, Miller RJ. Chemokine receptors: signposts to brain development and disease. *Nat Rev Neurosci*. 2003; 4:444–455. [PubMed: 12778117]
49. Vierbuchen T, Ostermeier A, Pang ZP, et al. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*. 2010; 463:1035–1041. [PubMed: 20107439]

Figure 1. The isolation and characterization of adult skin fibroblasts from Nestin-EGFP transgenic mice

(A) Adult Nestin-EGFP transgenic mice (E/Nestin:EGFP) were characterized by the IVIS optical imaging system. (B) Adult skin was dissected from the transgenic mouse. After 3–5 days in culture, the skin fibroblasts developed from the tissue and gradually formed a confluent layer (left panel, 20×), which was subjected to fluorescence microscopy (right panel, 20×). (C) General scheme of experimental design and timeline of iNPC induction.

Figure 2. Defined factors induce NPC-like cell (iNPC) generation from adult dermal fibroblasts

Dermal fibroblasts were isolated from adult Nestin-EGFP transgenic mice aged 5.5–7.0 weeks and infected with different combinations of pMXs-based retroviral vectors encoding Pou3f2, Nr2e1, Sox2, c-Myc, Bmi1, Hes1 and Hes5, respectively (TF7: Pou3f2, Nr2e1, Sox2, c-Myc, Bmi1, Hes1 and Hes5; TF5: Pou3f2, Nr2e1, Sox2, c-Myc and Bmi1; TF4: Pou3f2, Nr2e1, Sox2 and c-Myc; TF3: Pou3f2, Nr2e1 and Sox2). After 3–5 days in culture with NPC culture medium, GFP⁺ colonies were observed in cultures with TF7 or TF5 infection at day 5 rather than at day 3, and no colonies were observed in TF4 and TF3 cultures at day 3 or day 5.

Figure 3. Bmi1 is essential for the direct conversion of iNPCs from dermal fibroblasts

Dermal fibroblasts were infected with different combinations of pMXs-based retroviral vectors encoding Pou3f2, Nr2e1, Sox2, c-Myc and Hes5 except for Bmi1 (TF5: Pou3f2, Nr2e1, Sox2, c-Myc and Hes5; TF4: Pou3f2, Nr2e1, Sox2 and c-Myc; TF3: Pou3f2, Nr2e1 and Sox2). After 2, 9 and 16 days culture with NPC culture medium, all the conditions were subjected to regular fluorescence microscopy and all photographs were taken at 200× magnification.

Figure 4. Characterization of iNPCs in vitro

Dermal fibroblasts were infected with different combinations of defined factors as showed in (A). After a 12 day infection period, neurosphere formation was observed by fluorescence microscopy. Colonies from both TF7 and TF5 groups were trypsinized to single cells and cultured in NeuroCult® NSC Basal Medium supplemented with NeuroCult® NSC Proliferation Supplements, 20 ng/ml bFGF and 20 ng/ml bEGF. After 7 days, the self-renewal capacity was evaluated (B). iNPCs (from TF5) cultured on collagen IV-coated coverslips were incubated under neuronal or astrocyte differentiation conditions for 7 days, and then subjected to immunostaining with poyclonal β -tubulin III (C; upper panel) and monoclonal GFAP (C; lower panel) antibodies and nuclear staining with DAPI (blue). iNPCs were cultured on collagen IV-coated coverslips in NPC expansion medium

containing N2, SHH and fibroblast growth factor 8 (FGF8) at day 0 and in DA neuron differentiation medium containing N2 and ascorbic acid (AA) at day 4. The cells were cultured for another 3 days and fixed with 4% paraformaldehyde at day 7 and then subjected to double immunostaining with monoclonal tyrosine hydroxylase (TH1) (green) and polyclonal β -tubulin III (red) antibodies and nuclear staining with DAPI (blue) (**D**). All photographs were taken at 200 $\times$ magnification.

Figure 5. Lose of pluripotency in iNPCs

Mouse MEFs-derived iPSCs and iNPCs were cultured in 24-well plate containing collagen IV-coated coverslips. The cells were cultured for 3 days and fixed with 4% paraformaldehyde at room temperature for 30 minutes, then subjected to immunostaining with monoclonal Oct3/4 antibody (green) and nuclear staining with DAPI (blue). All photographs were taken at 200× magnification by fluorescence microscopy.

Figure 6. Candidate genes were expressed in iNPCs similar to those in primary NPCs
 iNPCs and primary NPC from the cortex of Nestin-EGFP transgenic mice were cultured in mouse NPC medium. Adult mouse fibroblasts from the skin of transgenic mice were cultured in DMEM with 10% FBS. Cells were collected and then subjected to total mRNA extraction and real-time PCR analysis with mouse Bmi1, Pou3f2, c-Myc, Sox2, Nr2e1, Hes1 and Hes5 specific primers. The comparisons were done between iNPCs (right panel), NPCs (left panel) and skin fibroblasts. Results are expressed as average $\pm$ SD of triplicate samples presented as fold change (compared to GAPDH) and are representative of 3 independent experiments. # denotes $p < 0.05$ in comparison with gene in skin fibroblasts; *denotes $p < 0.05$ in comparison to gene in NPCs or iNPCs.

Figure 7. iNPCs express functional CXCR4 and show chemotactic properties to SDF-1 α stimulation

iNPCs on collagen IV-coated coverslips were cultured in NPC medium, and fixed with 4% paraformaldehyde at room temperature for 30 minutes, and then subjected to immunostaining with monoclonal CXCR4 antibody (green) and nuclear staining with DAPI (blue). All photographs were taken with a Zeiss meta 510 confocal microscope (**A**, 60 $\times$ oil lens). iNPCs (10^5), re-suspended in 200 μ l of NeuroCult $^{\circ}$ NSC Basal Medium, were seeded in the upper chamber of a transwell. The lower chamber (a 24-well plate well) was filled with NeuroCult $^{\circ}$ NSC Basal Medium with mouse recombinant SDF-1 α . The 24-well plates containing cell culture inserts were incubated at 37 $^{\circ}$ C in a 5% CO $_2$ incubator. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed in 4% paraformaldehyde and then stained with Hoechst 33342 solution. The number of the Hoechst 33342-positive cells was counted in five randomly chosen fields of three independent experiments at 200 $\times$ magnification with a Zeiss Axiophoto microscope (**B**). # denotes $p < 0.05$ compared to control group.