Direct reprogramming of fibroblasts into neural stem cells by single non-neural progenitor transcription factor Ptf1a

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Induced neural stem cells (iNSCs) reprogrammed from somatic cells have great potentials in cell replacement therapies and in vitro modeling of neural diseases. Direct conversion of fibroblasts into iNSCs has been shown to depend on a couple of key neural progenitor transcription factors (TFs), raising the question of whether such direct reprogramming can be achieved by non-neural progenitor TFs. Here we report that the non-neural progenitor TF Ptf1a alone is sufficient to directly reprogram mouse and human fibroblasts into self-renewable iNSCs capable of differentiating into functional neurons, astrocytes and oligodendrocytes, and improving cognitive dysfunction of Alzheimer's disease mouse models when transplanted. The reprogramming activity of Ptf1a depends on its Notch-independent interaction with Rbpj which leads to subsequent activation of expression of TF genes and Notch signaling required for NSC specification, self-renewal, and homeostasis. Together, our data identify a non-canonical and safer approach to establish iNSCs for research and therapeutic purposes.
Neurodegenerative diseases including Alzheimer’s disease (AD), Huntington’s, and glaucoma have become a global threat to human health. Traditional treatment attenuates disease progress but is overall ineffective since lost cells are not replenished in the lesion. Endogenous neurogenesis is insufficient for replenishment and results in only very limited self-repair in these diseases. Current focus of regenerative medicine emphasizes on how to generate a large number of neurons, glia or their progenitors that have the ability to integrate and function in the affected tissues, thereby providing a promising approach to lesion repair. At present, clinical application of human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) has been undermined by their tumorigenic risk1,2. By contrast, neural stem cells (NSCs) have proven to be a safe cell resource that is not prone to tumors3,4 and therefore provide a powerful strategy to patient-specific cell replacement therapies. They also provide a useful tool for drug discovery and in vitro disease modeling5.

Somatic cell reprogramming is a valuable tool for deriving patient-specific NSCs. Recent work has demonstrated that mouse and human somatic cells can be reprogrammed to transdifferentiate into induced NSCs (iNSCs)/neural progenitor cells by defined tissue-specific transcription factors (TFs)5-9 and/or chemicals10,11. In most cases of TF-induced iNSCs, reprogramming is achieved by Sox2 alone or Sox2 in combination with various other TFs12. More recently, a single zinc-finger TF, Zifp521, has been shown to directly reprogram human fibroblasts into iNSCs13. Thus, it appears that iNSC generation by TF-induced somatic cell reprogramming critically depends on Sox2 or Zifp521, which are normally expressed in proliferative neural progenitors and are key regulators of neurogenesis in vivo14-17. In fact, Sox2 has been postulated as a master regulator of direct iNSC reprogramming12. This then begs the question of whether neural progenitor TFs are the necessity for such direct reprogramming and whether it can be achieved by non-neural progenitor TFs.

Previously, we and others have identified numerous TFs, which are expressed in mitotic progenitors and/or postmitotic cells during retinal development, and have key roles in controlling retinal cell specification and differentiation18. We were interested in finding out whether any of these progenitor TFs and non-progenitor TFs was capable of transdifferentiating fibroblasts into iNSCs or functional neurons. Ptf1a (pancreas TF-1a) is a basic helix-loop-helix (bHLH) TF that has an indispensable role in the development of retina, cerebellum, spinal cord, and pan-creas19-23. Here we report that unlike other typical reprogramming TFs of iNSCs, Ptf1a is selectively expressed in postmitotic precursors in the central nervous system (CNS). Moreover, unlike a number of other retinal TFs that we tested, ectopic expression of Ptf1a directly converts mouse and human fibroblasts into self-renewable and tripotent iNSCs with high efficiency. This reprogramming activity requires Notch-independent interaction between Ptf1a and Rbpj, as well as subsequent activation of expression of TF genes and Notch signaling involved in NSC homeostasis. Further, transplantation of Ptf1a-reprogrammed iNSCs improves cognitive function of AD mouse models.

Reprogramming of MEFs by Ptf1a into self-renewable iNSCs.

Given the demonstrated reprogramming activities of some TFs, the expression of Ptf1a in postmitotic neurons suggests a possibility that it may be able to convert fibroblasts into mature and functional neurons. However, repeated attempts to use Ptf1a to reprogram MEFs into differentiated neurons by established procedures all failed. We then tested whether Ptf1a had the ability to directly reprogram MEFs into iNSCs, albeit somewhat counter-intuitively given the expression of Ptf1a almost exclusively in postmitotic precursors. However, when MEFs were infected with doxycycline (Dox)-inducible Ptf1a lentiviruses and cultured in the N3 medium containing epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and Dox, they started to change morphology and form clusters at day 6, from which numerous neurospheres emerged by day 9 (Fig. 1a, b, d). Control MEFs infected with Dox-inducible green fluorescent protein (GFP) lentiviruses barely formed any normally shaped neurospheres (Fig. 1b, d). When multiple Ptf1a-induced neurospheres were collected as a mixture, dissociated, and replated, they rapidly formed new secondary neurospheres starting from day 3 (Fig. 1c), indicating their self-renewal ability.

We picked primary neurospheres individually to clonally derive multiple reprogrammed mouse iNSC (miNSC) cell lines (Supplementary Fig. 3). We seeded them in separate plate wells in the presence of Dox and found that cells with NSC morphology grew gradually from adhered neurospheres. They were further expanded and passaged in the presence or absence of Dox for 30 generations. At about passage 10, all miNSC lines ceased to generate neurospheres, became homogeneous, and grew in a monolayer as typical NSCs would (Supplementary Fig. 3). Consistent with the self-renewal capability of each clonally derived miNSC line, we found that ~32.6% of miNSC10 cells were pulse-labeled by EdU, which was more than that (18.9%) of the wild-type mouse NSC line SCR029 (Fig. 1e, f). Thus, miNSCs reprogrammed from MEFs by Ptf1a were proliferative and self-renewable. By contrast, MEFs infected with GFP lentiviruses failed to generate neurospheres immunoreactive for typical NSC markers and the few spheroids likely formed spontaneously looked abnormal and lacked any ability to expand in culture (Supplementary Fig. 4).

To confirm that Ptf1a indeed directly induced miNSCs from MEFs, we performed immunofluorescent staining using antibodies against several NSC markers. This revealed strong expression of exogenous Ptf1a in neurospheres, which in turn induced marked expression of NSC marker proteins Sox2, Pax6, Olig2, and Nestin (Fig. 2a). In monocloned miNSCs, the expression of Sox2, Pax6, and Nestin remained high (Fig. 2a). Consistent with protein expression levels, quantitative reverse transcriptase PCR (qRT-PCR) assays demonstrated that the expression level of Sox2, Pax6, Olig2, and Nestin transcripts was
Quantil collected primary neurospheres formed secondary neurospheres in 3 days in culture and morphologically homogeneous larger neurospheres by day 7. Neurosphere-like structures gradually appeared in 9 neurospheres from MEFs by Ptf1a lentiviruses. MEFs were prepared from E12.5 C57BL6/J mouse embryos and infected with tet-on Ptf1a lentiviruses at day 0. Neurosphere-like structures gradually appeared in 9–14 days when cultured in N3 medium containing EGF, bFGF, and doxycycline. Morphological changes of MEFs infected with control GFP or Ptf1a lentiviruses. Numerous cell clusters could be seen in Ptf1a virus-infected MEFs by day 6 in culture, but rarely in GFP virus-infected MEFs. From day 9 onward, many typical neurosphere-like structures formed in Ptf1a-transduced MEFs, whereas in GFP-transduced MEFs there were only few cell aggregates, which were morphologically different from typical neurospheres. Dissociated cells from collected primary neurospheres formed secondary neurospheres in 3 days in culture and morphologically homogeneous larger neurospheres by day 7. Quantification of Ptf1a-induced neurospheres. MEFs (4 × 10^4) were seeded into each well of 12-well plates, infected with Ptf1a or GFP viruses, and neurosphere-like structures in each well were then counted at day 10 and 14 following virus infection. There were significantly more neurospheres in Ptf1a-induced samples at day 10 and 14. Data are presented as mean ± SD (n = 3). Asterisks indicate significance in unpaired two-tailed Student’s t-test: *P < 0.005, **P < 0.0001. EdU-labeling of Ptf1a-induced iNSCs (miNSC10) and control NSCs (SCR029). Cells were counterstained with nuclear DAPI. Quantification of EdU-labeled cells. Higher percentage of proliferative cells was found in miNSC10 than in control SCR029 NSCs. Data are presented as mean ± SD (n = 3). The asterisk indicates significance in unpaired two-tailed Student’s t-test: *P < 0.0001. Scale bars, 80 μm (b), (e) and 93.1 μm (c).

greatly elevated in miNSC5, 10, and 12 lines compared with MEFs, just like in the control mouse NSC line SCR029 (Fig. 2b). By contrast, there was only minimal or no expression of pluripotent factor genes Oct4, Nanog, and Klf4 in miNSC lines compared with mouse ESCs (Fig. 2c). Moreover, as a result of cell-fate change, the expression of MEF marker genes Snai1, Twist2, and Colla1 was drastically reduced in miNSC lines (Fig. 2d). In agreement with active transcription of the Nestin gene in miNSCs, its promoter was hypomethylated in miNSC and control SCR029 cells but hypermethylated in MEFs (Fig. 2e). However, the promoters of Oct4 and Nanog were hypermethylated in all three cell types (Fig. 2e), consistent with the absence of pluripotent factor gene expression in these cells. Six3 is a TF marker for ventral forebrain and retinal progenitor cells. We found by semi-qRT-PCR and qRT-PCR that there was a significant increase of Six3 expression in Ptf1a-reprogrammed miNSCs compared with MEFs (Supplementary Fig. 5a, b). To visualize Six3-positive progenitors in Ptf1a-induced neurospheres, we bred Six3-Cre transgenic driver mice with R26R-YFP reporter mice to obtain R26R-YFP; Six3-Cre embryos (Supplementary Fig. 5c). When MEFs prepared from these embryos were infected with Ptf1a lentiviruses, they formed neurospheres with many but not all cells positive for both yellow fluorescent protein (YFP) and Nestin (Supplementary Fig. 5d), suggesting a possibility that Ptf1a induced some stem cells characteristic of forebrain and retinal progenitors. To further confirm the NSC identity of the miNSCs, we profiled transcriptomes of miNSC10, SCR029, and MEF cells by RNA-seq analysis. Scatter plots of gene expression levels among miNSC10, SCR029, and MEF cells revealed that miNSC10 and SCR029 cells were similar to each other but were highly divergent from MEFs (Fig. 3a–c). In agreement with these results, hierarchical cluster analysis also showed a high degree of similarity between miNSC10 and SCR029 cells but great difference between them and MEFs (Fig. 3d). Numerous genes were downregulated or upregulated in expression levels in miNSCs compared with MEFs (Fig. 3e; Supplementary Data 1). We performed gene-set enrichment analysis (GSEA) of the upregulated genes followed by network visualization (Fig. 3f).

Two major groups of clustered networks emerged. One was enriched for neural development-relevant GO (Gene Ontology) terms such as neurogenesis, neuron differentiation, nervous
system development, regulation of neurogenesis, brain development, and axonogenesis. The other was enriched for cell cycle-relevant GO terms including cell division, mitotic cell cycle, nuclear division, sister chromatid segregation, and regulation of cell cycle. These data are consistent with miNSCs as NSCs and their capacity to proliferate and self-renew. In agreement with this, further analyses showed that miNSC10, SCR029, NS528 (mouse ES cell-derived NSCs), and ciNSC10 (NSCs chemically induced from MEFs) cells are, albeit somewhat distinct, more similar to each other than to MEFs (Supplementary Fig. 6).

Ptf1a is expressed in dividing pancreas primordia and required for the generation of multipotent pancreatic progenitor cells20,21. Moreover, pancreas stem cells and NSCs are both characterized by Nestin expression29. Thus, Ptf1a-reprogrammed miNSCs might also represent pancreas stem cells. This possibility was tested by semi-qRT-PCR assay for a series of markers for

![Graph showing relative expression levels to SCR029 for Nestin, Olig2, Pax6, and Sox2.](image)

![Graph showing relative expression levels to ES for Sox2, Klf4, Nanog, and Oct4.](image)

![Graph showing relative expression levels to MEF for Snai1, Twist2, Col1a1, Sox2, and Oct4.](image)
pancreatic progenitors and differentiated cells. None of the tested progenitor markers (Pdx1, Neurog3, Nkx2-2, Neurodi, Mafa, IslI, and Foxa2) showed any expression in miNSCs, and miNSCs were also negative for expression of the insulin genes (Ins1 and Ins2) and glucagon gene (Gcg) (Supplementary Fig. 7), effectively ruling out Ptf1a-reprogrammed miNSCs as pancreas stem cells.

Tripotency of Ptf1a-reprogrammed miNSCs. We asked whether miNSCs reprogrammed by the non-neural progenitor TF Ptf1a had the potential to differentiate into neurons, astrocytes, and oligodendrocytes as typical NSCs would (Fig. 4a). When cultured in neural differentiation medium, in a few days, miNSCs underwent robust morphological changes with decreased cell body size and pervasive neurite extension (Fig. 4b). At 2–3 weeks of culture, many cells differentiated into neurons immunoreactive for TuJ1, Map2, Dcx (doublecortin), NeuN, Tau, peripherin, or GABA (Fig. 4c). Quantification at 3 weeks showed that 83.3% of all cells were positive for TuJ1 (Fig. 4e). Furthermore, different miNSC lines (miNSC5, 10 and 12) displayed a similar capacity to differentiate into neurons immunoreactive for TuJ1 or Map2 (Supplementary Fig. 8). Under astrocyte differentiation condition, 87.2% of differentiated cells developed into glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes (Fig. 4c, e). In oligodendrocyte differentiation medium, miNSCs were able to differentiate into oligodendrocytes immunoreactive for O1, CNP, or MBP, and 26.6% of differentiated cells were O1-positive (Fig. 4c, e). Thus, Ptf1a-reprogrammed miNSCs are tripotent, being able to differentiate into neurons, astrocytes, and oligodendrocytes.

The neurons differentiated from miNSCs exhibited active membrane properties. Following 2 weeks of differentiation, most of the neurons (seven out of nine) generated potassium currents and small sodium currents but no action potentials, suggesting that they were functionally immature. At 3 weeks, whole-cell patch-clamp recording showed that some differentiated neurons (4 out of 11) had typical sodium and potassium currents, and exhibited multiple action potential responses (Fig. 4f-i). Moreover, some neurons (6 out of 26) showed spontaneous postsynaptic currents (Fig. 4j). Consistent with this observation, double-immunostaining revealed that numerous neurons were labeled by punctate synapsin staining (Fig. 4d), indicating the formation of synaptic connections between differentiated neurons. Therefore, Ptf1a-reprogrammed miNSCs are able to differentiate into functional neurons.

We assessed the potential of Ptf1a-reprogrammed miNSCs to differentiate into the three cell lineages in vivo. miNSCs were tagged by GFP-expressing lentiviruses and transplanted into the hippocampus of the APP/PS1 mouse models of AD30,31. One month after transplantation, a series of behaviors were tested to evaluate the cognitive function of the transplanted animals (Fig. 6a). No difference in nest quality was observed between saline- and miNSC-injected mice in the nest-building test (Fig. 6b). In the next open-field test, no difference in the total distance traveled or the center time was observed between saline- and miNSC-injected mice either (Fig. 6c).

To investigate the learning and memory behavior, novel object recognition test, Y-maze test, and Morris water maze test were carried out. The results showed that between saline- and miNSC-injected mice, there was no difference in the time spent on the novel object in the novel object recognition test and no difference in the total percentage of correct spontaneous alternations (SAP) in the Y-maze test (Fig. 6d, e). However, the Morris water maze test (Fig. 6f) showed that the average escape latency was significantly decreased for miNSC-transplanted animals compared with the control group at D6 (testing day) (Fig. 6f), and that miNSC-transplanted mice spent more time in the target quadrant (Fig. 6f). Target annulus crossovers revealed that miNSC-transplanted mice appeared to have a preference for the target platform location but this preference did not reach statistical significance (Fig. 6f). Together, these results suggest that transplanted miNSCs can significantly improve spatial learning and memory of the APP/PS1 AD mouse model.

**Fig. 2** Characterization of Ptf1a-reprogrammed iNSCs. a, Neurospheres induced from MEFs by Ptf1a at day 8 and 10 were immunoreactive for Ptf1a, Sox2, Pax6, Nestin, or Olig2. Monolayered miNSC10 cells were also immunoreactive for Ptf1a, Sox2, Pax6, or Nestin. Cells were counterstained with nuclear DAPI. Scale bars, 80 μm. b, qRT-PCR analysis showed that in comparison with MEFs, there was a great increase in expression of Nestin, Olig2, Pax6, and Sox 2 genes in Ptf1a-derived miNSCs, miNSC10, and miNSC12 cells, as well as in control SCR029 NSCs. Data are presented as mean ± SD (n = 4). Asterisks indicate significance in unpaired two-tailed Student’s t-test: *P < 0.0005. c, qRT-PCR assay showed that compared with mouse ESCs, there was only minimal or no expression of pluripotent factor genes Klf4, Nanog, and Oct4 in miNSC and control NSC cells. As a pluripotent factor gene and also NSC marker gene, Sox2 exhibited high levels of expression in both miNSC and ES cells. Data are presented as mean ± SD (n = 4). Asterisks indicate significance in unpaired two-tailed Student’s t-test: *P < 0.005, **P < 0.001, ***P < 0.0001. d, As revealed by qRT-PCR assay, expression of fibroblast cell marker genes Snai1, Twist2, and ColIα1 was dramatically downregulated in miNSC cells. Data are presented as mean ± SD (n = 4). Asterisks indicate significance in unpaired two-tailed Student’s t-test: *P < 0.001. e, DNA methylation status was examined in the promoter regions of Nestin, Oct4, and Nanog genes. Filled and empty circles represent methylated and unmethylated CpGs, respectively. Compared with MEFs, the promoter region of Nestin became hypomethylated in SCR029 and miNSC10 cells; however, the promoter regions of Oct4 and Nanog remain hypermethylated.
Fig. 3 Global gene expression profiles of iNSCs, control NSCs, and MEFs. a–c Pairwise scatter plot analysis of the global gene expression profiles of miNSC10, SCR029, and MEF cells. The transcriptome of each cell type was profiled by RNA-seq analysis. Gene expression levels (FPKM) are depicted in log_{10} scale. Pearson’s correlation coefficients (r) are indicated. d Heat map of the z-transformed gene expression values in miNSC10, SCR029, and MEF cells. miNSC10 and SCR029 cells are classified into the same hierarchical cluster. e Volcano plot (significance vs. fold change) of significantly altered genes (fold change ≥ 2 and FDR < 0.05) between miNSC10 and MEF cells. f Gene ontology (GO) enrichment analysis of the upregulated genes between miNSC10 and MEF cells. The upregulated genes were analyzed for GO term enrichment by gene-set enrichment analysis (GSEA). The result was visualized on a network of gene sets (nodes) connected by their similarity (edges). Node size represents the gene-set size and edge thickness represents the degree of overlap between two gene sets. Depicted are the two major groups of enriched gene sets.
We also evaluated the therapeutic potential of miNSCs in the Aβ1-40 (amyloid β peptide 1–40)-injured AD mouse model, which was created by injection of Aβ1-40 into the hippocampus32. Two weeks after Aβ1-40 injection, we performed a series of behavioral tests to assess the cognitive function of the Aβ1-40-injured mice (Supplementary Fig. 9). We found that in the novel object recognition test, the time spent on the novel object was significantly decreased for Aβ1-40-injected mice (Supplementary Fig. 9d). In Morris water maze test (Supplementary Fig. 9f), the average escape latency was significantly increased for Aβ1-40-injected mice compared with saline-treated controls at D6 (Supplementary Fig. 9f) and the injected mice spent markedly reduced time in target quadrant and failed to show a preference for the target platform location (Supplementary Fig. 9f). Therefore, Aβ1-40-injured mice display learning and memory impairment similar to that of AD.

To test whether transplanted miNSCs produce any therapeutic effect in the Aβ1-40-injured AD mouse model, GFP-tagged
Fig. 4 In vitro differentiation potential of Ptf1a-reprogrammed miNSCs. **a** Schematic showing that miNSCs reprogrammed directly from MEFs by Ptf1a are able to differentiate into neurons, astrocytes, and oligodendrocytes under proper culture conditions. **b** miNSC10 cells underwent drastic morphological changes to form neuron-like cells when the culture medium was switched from NSC medium to neural differentiation medium. **c** miNSC10 cells could be differentiated into neurons immunoreactive for TuJ1, Map2, Dcx, NeuN, Tau, Peripherin, or GABA. They were also capable of differentiating into astrocytes (immunoreactive for GFAP) and oligodendrocytes (immunoreactive for O1, CNP, or MBP). **d** In vitro differentiated neurons were immunoreactive for both TuJ1 and synapsin. Cells in **c** and **d** were counterstained with nuclear DAPI. **e** Quantification of Map2+ neurons, GFAP+ astrocytes, and O1+ oligodendrocytes differentiated from miNSC10 cells under different differentiation conditions. **f** A merged micrograph showing a typical GFP-tagged neuron differentiated from miNSCs that was chosen for patch-clamp recording. **g** Voltage-clamp recordings indicated fast activated and inactivated inward sodium currents as well as outward potassium currents on a differentiated neuron. **h** Current-clamp recordings revealed action potential responses of the differentiated neuron under current injection. **i** Multiple action potentials were induced after depolarization of the neuron. **j** Spontaneous postsynaptic currents recorded from an in vitro differentiated neuron. Scale bars, 80 μm (**b** and 40 μm (**c, d**)).

Fig. 5 In vivo differentiation potential of Ptf1a-reprogrammed miNSCs. **a** miNSC10 cells were infected with lentiviruses containing CMV promoter-driven GFP and neomycin. After screening with G418, most miNSC cells expressed GFP as shown. **b** GFP-tagged miNSC10 cells were injected into the hippocampus region of 2-month-old mice. One month after transplantation, GFP+ cells were found successfully integrated into the tissue. Some of them were immunoreactive for the oligodendrocyte cell marker Olig2. **c**–**f** Transplanted GFP+ cells co-expressing the astrocyte cell marker GFAP. **g** Immunolabeling for the presynaptic protein marker synapsin. **h** Single confocal plane images of the region outlined by the small rectangle in **h** show direct contact between the GFP+ dendrite and synapsin+ presynaptic terminals of the surrounding endogenous cells. **i** The 3D surface of confocal z-stacks of the region outlined by the large rectangle in **h** shows the formation of synaptic connections with endogenous cells. **k** The 3D surface of z-stacks of a dendrite shows the development of dendritic spines with typical head-neck structures (indicated by asterisks). Cells in **b**–**h** were counterstained with nuclear DAPI. Large arrows in **b**–**g** point to representative colocalized cells and/or processes. Small arrows in **i** and **j** indicate direct contact between neurites and multiple synapsin+ presynaptic terminals. **l** Quantification of GFP+ cells that are immunoreactive for NeuN, GABA, vGLUT3, GFAP, or Olig2. Scale bars, 80 μm (**a**) and 20 μm (**b–h**).
Fig. 6 miNSC transplantation in the hippocampus improved the cognitive dysfunction of APP/PS1 and Aβ₁-40-injured mice. a Time table of evaluating the behaviors of APP/PS1 mice after miNSC transplantation. b Nest-building test. The nest-building behavior of control and miNSC-transplanted mice was assayed by assessing the nest quality after 24 h exposure to a sheet of tissue cotton. Comparing the nest quality, no statistically significant difference was observed between groups. c Open-field test. An illustrative example of travel pathways for a saline-injected mouse and a miNSC-transplanted mouse in the open-field test. Distance traveled and center square duration in the open-field test show no difference between the groups. d Novel object recognition test. An illustrative example of travel pathways for a saline-injected mouse and a miNSC-transplanted mouse in the novel object recognition test. miNSC-transplanted mice show no difference in the preference for the novel. e Y-maze test. An illustrative example of travel pathways for a saline-injected mouse and a miNSC-transplanted mouse in the Y-maze test. Spontaneous alternation (SAP) in the Y-maze was unimproved for the miNSC-transplanted mice. f Morris water maze test. An illustrative example of travel pathways for a saline-injected mouse and a miNSC-transplanted mouse in the Morris water maze test. Learning curves for Morris water maze acquisition trials were obtained across a period of 6 days. Time spent in target quadrant in the Morris water maze shows that miNSC-transplanted mice spent more time in the target quadrant. Target annulus crossovers reveal that miNSC-transplanted mice show a preference for the target platform location without reaching statistical significance. g-l As in a-f, but tested after miNSC transplantation in Aβ₁-40-injured mice. Data are presented as mean ± SD (n = 6–11). Asterisks indicate significance in ANOVA test: *p < 0.05; **p < 0.01
miNSCs were transplanted into the same location 2 weeks after Aβ1-40 injection. Two months later, we performed the same set of behavioral tests described above (Fig. 6g). Notably, Morris water maze test (Fig. 6l) showed that the average escape latency was significantly decreased for miNSC-transplanted animals (Fig. 6l), and that miNSC-transplanted mice spent more time in the target quadrant and had a preference for the target platform location (Fig. 6l). There was no significant difference between groups in nest quality, total distance traveled and center time in open-field test, preference for novel object, and SAP in Y maze (Fig. 6h–k). Thus, transplanted miNSCs can significantly improve spatial learning and memory of both APP/PS1 and Aβ1-40-injured AD mouse models, producing a similar therapeutic effect as ESC-derived NSCs.

Dependency of Ptf1α reprogramming on Rbpj interaction. It has been shown previously that Ptf1α forms a trimeric DNA-binding complex among Ptf1a, Rbpj, and an E-protein, as well as the Ptf1αW298A C-terminal mutant that lacks the ability to interact with Rbpj. Unlike Ptf1α lentivirus-infected MEFs, MEFs transduced with Ptf1αW298A lentiviruses failed to undergo morphological changes by day 6 or form neurospheres by day 9. Quantification of neurospheres induced by Ptf1α and Ptf1αW298A. MEFs (4 × 10⁴) were seeded into each well of 12-well plates, infected with Ptf1α or Ptf1αW298A viruses, and neurospheres in each well were then counted at day 10 and 14 following virus infection. There was a dramatic decrease of neurospheres in Ptf1αW298A-induced samples at day 10 and 14. Data are presented as mean ± SD (n = 3). Asterisks indicate significance in unpaired two-tailed Student’s t-test: *P < 0.005, **P < 0.0001. d MEFs infected with Ptf1α lentiviruses generated neurospheres at day 8 that were immunoreactive for Sox2, Pax6, and Nestin, whereas MEFs infected with Ptf1αW298A viruses were negative for these NSC markers. Cells were counterstained with nuclear DAPI. e Neurosphere formation by Ptf1α in MEFs infected with lentiviruses expressing Rbpj shRNA or scrambled Rbpj shRNA. f Relative expression levels of Rbpj in MEFs infected with lentiviruses expressing Rbpj shRNA or scrambled Rbpj shRNA as determined by qRT-PCR assay. Data are presented as mean ± SD (n = 6). Asterisk indicates significance in unpaired two-tailed Student’s t-test: *P < 0.0001. g Quantification of neurospheres induced by Ptf1α in the presence of Rbpj shRNA or scrambled Rbpj shRNA. MEFs (4 × 10⁴) were seeded into each well of 12-well plates, infected with Ptf1α lentiviruses and viruses expressing Rbpj shRNA or scrambled Rbpj shRNA. Neurospheres in each well were counted at day 10 following virus infection. Data are presented as mean ± SD (n = 6). Asterisk indicates significance in unpaired two-tailed Student’s t-test: *P < 0.0005. Scale bars, 160 μm (e), 80 μm (b), and 40 μm (d).
binding complex with Rbpj and an E-protein independent of Notch signaling to specify pancreatic lineages and GABAergic neurons. We investigated whether Notch-independent interaction between Ptf1a and Rbpj is also required for the Ptf1a reprogramming activity. In MEFs, western blotting and co-immunoprecipitation assays showed that Rbpj protein was immunoprecipitated with Rbpj but not E-protein (Fig. 7a). We found that MEFs transduced with Dox-inducible Ptf1aW298A lentiviruses did not co-immunoprecipitate Rbpj (Supplementary Fig. 10). A mutant form of Ptf1a, Ptf1aW298A, which contains a substitution of a conserved amino acid residue tryptophan by alanine at the C-terminus, has previously been shown to disrupt Ptf1a interaction in a 300-bp window centered at the peak summit. We found that MEFs infected with Ptf1aW298A lentiviruses failed to express NSC protein markers Sox2, Pax6, or Nestin (Fig. 7d). Thus, interaction with Rbpj is a prerequisite to Ptf1a reprogramming activity. Consistent with this, knockdown of Rbpj expression in MEFs reduced the number of Ptf1a-induced neurospheres by more than twofold (Fig. 7e–g).

Regulation of iNSC homeostasis by TFs and Notch signaling. ATAC-seq profiles genome-wide accessible chromatin regions that can be bound by TFs. We performed ATAC-seq analysis of miNSC10 iNSCs and SCR029 NSCs, and found that peak-associated genes are enriched for those involved in neurogenesis, neuron development, brain development, gliogenesis, and glial cell development (Fig. 8a, b), consistent with iNSC features. De novo motif discovery in a 300 bp window centered at the peak summit revealed that both miNSC10 and SCR029 cells are enriched for similar sets of TF-binding motifs belonging to several TF families of TFs including Sox, bHLH (Ebox), Pou3f, homeobox, NFI, and RFX. Thus, interaction with Rbpj is a prerequisite to Ptf1a reprogramming activity. Consistent with this, knockdown of Rbpj expression in MEFs reduced the number of Ptf1a-induced neurospheres by more than twofold (Fig. 7e–g).
Nfi, and Rfx (Fig. 8c). Correspondingly, RNA-seq data show that multiple members of each of these TF gene families are upregulated or highly expressed in miNSC10 and SCR029 cells compared with MEFs (Supplementary Fig. 11a, c–f). Apart from Sox2, qRT-PCR validated that there is upregulation of Sox5, 6, 8, and 21 expression in miNSC10 and SCR029 cells (Supplementary Fig. 11b). In general, the chromatin accessibility profile of miNSC10 cells closely resembles that of SCR029 cells but is quite distinct from that of MEFs; and for upregulated TF genes, e.g., Olig2 (bHLH), Sox2, Pou3f2/Brn2, and Pax6 (homeobox), there are more peaks and/or differential peaks within these loci in miNSC10 and SCR029 cells compared with MEFs (Fig. 8d–g; Supplementary Fig. 12), indicating a more open chromatin configuration for elevated gene expression.

The six families of TFs corresponding to the most enriched motifs all contribute to maintaining NSC homeostasis such as NSC state specification, self-renewal, and prevention of quiescence and neuronal differentiation (Fig. 8h). Sox2 is required for the specification and maintenance of NSCs. It does so by binding to distal enhancers in cooperation with class three Pou domain TFs, especially Pou3f2 which is also involved in specifying the neural lineage from ESCs38,39. Olig2 promotes NSC self-renewal by activating pro-proliferation genes, as well as inhibits premature neuronal differentiation and stem-cell quiescence by repressing genes involved in these processes40. Pax6 is a well-known homeobox TF essential for the determination, self-renewal, multipotency, and neurogenesis of NSCs41,42. The Lhx2 homeobox TF has a role in the specification of NSCs by activating Pax6 expression, while attenuating bone morphogenetic protein (BMP) and Wnt signaling43. The Nfi and Rfx family members have been shown to co-occupy genomic sites with Sox2 and Pou3f2, and are essential for CNS development and quiescent state38,44,45.

Notch signaling is a classic cell contact-dependent signaling pathway required for NSC proliferation and self-renewal, as well as for prevention of untimely neuronal differentiation of NSCs46. Our RNA-seq data reveal a dramatic upregulation of Notch signaling component genes Notch1, Dll1, Hes1, and Hey1 in both miNSC10 and SCR029 cells compared with MEFs and these loci also display differential ATAC-seq profiles (Supplementary Fig. 13a–d), indicating that Dll1-Notch signaling is activated in both miNSCs and NSCs for their renewal and maintenance. Consistent with the upregulation of Notch1, Dll1, and Hes1 genes, their promoters are hypomethylated in miNSC10 and SCR029 cells compared with MEFs (Supplementary Fig. 13e). Notch signaling component genes Notch1, Dll1, and Hes1 expression in MEFs in a time-dependent manner but had no effect on Rbpj expression (Supplementary Fig. 14). To determine whether Notch signaling is involved in self-renewal and maintenance of neurospheres reprogrammed by Ptf1a from MEFs, we applied the Notch signaling inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyler ester (DAPT) in the reprogramming process. We found that DAPT reduced not only the number of neurospheres but also the expression of Notch signaling effector genes Hes1 and Hes5 in a dose-dependent manner (Supplementary Fig. 13f–j), suggesting that Ptf1a activates Notch signaling, which in turn is required for self-renewal and maintenance of Ptf1a-reprogrammed miNSCs.

Reprogramming of human fibroblasts by Ptf1a into iNSCs.

Using a protocol similar to MEF reprogramming, we generated neurospheres from human foreskin fibroblasts (HFFs) by transduction of Dox-inducible Ptf1a lentiviruses (Fig. 9a, b). Interestingly, completely monolayered hiNSCs (human iNSCs) were obtained only when we expanded and passaged neurospheres in the absence of Dox (Fig. 9c, d; Supplementary Fig. 15a). At passage 20 in the absence of Dox, hiNSCs became completely monolayered without forming any neurospheres, whereas in the presence of Dox there were still many neurospheres present in the culture up to passage 32 (Supplementary Fig. 15a), suggesting that Dox-induced exogenous Ptf1a had an inhibitory effect on the generation of typical monolayered iNSCs. qRT-PCR assays revealed that the expression levels of NSC markers Sox2, Pax6, Olig2, and NESTIN were several fold higher in hiNSCs cultured in the absence of Dox than in the presence of Dox (Supplementary Fig. 15b), providing an explanation why hiNSCs behaved more like typical NSCs in the absence of Dox. Notably, there was a higher level of endogenous Ptf1a expression in hiNSCs cultured in the absence of Dox than in the presence of it (Supplementary Fig. 15c).

Ptf1a-reprogrammed hiNSCs barely expressed pluripotent factor genes OCT4 and NANOG as determined by qRT-PCR (Supplementary Fig. 15d). By contrast, they exhibited high levels of NSC marker protein expression, including SOX2, PAX6, NESTIN, Olig2, and FABP7 (BLBP) (Fig. 9e–f). Under various differentiation conditions, hiNSCs were able to differentiate into neurons immunoreactive for TUJ1, MAP2, NEUN, TAU, or GABA (Fig. 9j–n), astrocytes immunoreactive for GFAP (Fig. 9o), or oligodendrocytes immunoreactive for O1 (Fig. 9p). Following differentiation in neuronal or glial cell differentiation media, 85.4%, 70.3%, and 33.7% of all cells developed into TUJ1-positive neurons, GFAP-positive astrocytes, and O1-positive oligodendrocytes, respectively (Supplementary Fig. 15e). Therefore, Ptf1a has a similar activity to reprogram human fibroblasts into tripotent NSCs.

We characterized the electrophysiological properties of hiNSC-derived neurons by whole-cell patch-clamp recording. Two weeks after in vitro differentiation, most of the neurons (11 out of 17) generated sodium and potassium currents, some of which looked typical of functional neurons (Fig. 9r). Some of the differentiated neurons showed action potentials (5 out of 17) and some of them (5 out of 17) exhibited spontaneous postsynaptic activities (Fig. 9s–u), suggesting that in vitro differentiated neurons can form synaptic connections. In agreement, many neurons displayed punctate immunolabeling for synapsin (Fig. 9q). Thus, similar to miNSCs, Ptf1a-induced hiNSCs are able to differentiate into mature functional neurons in vitro.

We further investigated the potential of Ptf1a-reprogrammed hiNSCs to differentiate into neuronal and glial cell lineages in vivo. GFP-tagged hiNSCs were transplanted into the hippocampus of adult mice and analyzed by immunostaining after 1.5 months. Similar to miNSCs, hiNSCs were able to differentiate into neurons immunoreactive for NeuN, GABA, and vGLUT3 in proportions of 5.7%, 2.7%, and 2.9%, respectively (Fig. 10a–c, f). They also differentiated into glial cells immunoreactive for GFAP and Olig2 in proportions of 82.2% and 7.6%, respectively (Fig. 10d–f). Therefore, Ptf1a-reprogrammed hiNSCs have the multipotency to differentiate into neuronal and glial lineages both in vitro and in vivo.

Discussion

To our knowledge, this study demonstrates for the first time that a single non-neural progenitor TF has the ability to directly convert somatic cells into iNSCs, casting doubt on the hypothesis of Sox2 as a master regulator of direct iNSC reprogramming12. We show that Ptf1a is absent from NSCs, exhibits very low abundance in the developing CNS, and is expressed only in a small population of postmitotic cells within a few selective CNS tissues. Thus, Ptf1a can hardly considered as a neural progenitor TF and is unlikely involved in NSC generation in vivo.
Yet, unexpectedly, it displays a strong activity in directly reprogramming somatic cells into tripotent iNSCs, suggesting that neural progenitor TFs are unnecessary in driving TF-mediated reprogramming of iNSCs, and that non-neural progenitor TFs may be able to do the same as long as they have a similar driving activity. Furthermore, Ptf1a appears to be very efficient in converting fibroblasts into neurospheres. The efficiency of Sox2 alone is < 0.1%4. In comparison, the efficiency of Ptf1a is > 0.5% at day 14 (Fig. 1d). In previous studies of TF-mediated iNSCs, reprogramming usually involved neural progenitor TFs Sox2 or Zfp521, either alone or in combination with other TFs12,13. However, both Sox2 and Zfp521 present tumorigenic risks. For example, Sox2 is associated with osteosarcoma47, skin squamous-cell carcinoma48, and various other cancers49. Zfp521 is linked with acute B-lineage leukemia50 and medulloblastoma51. By contrast, Ptf1a is inversely associated with pancreatic ductal adenocarcinoma52. Therefore, Ptf1a may offer a safer and more efficient solution than any other TF or combinations of TFs in

Fig. 9 Ptf1a reprograms human foreskin fibroblasts (HFF) into tripotent iNSCs. a, b Ectopic expression of Ptf1a in HFFs by lentiviruses induced the formation of neurospheres. c, d In the absence of doxycycline (Dox), Ptf1a-induced neurosphere cells were capable of forming neurospheres before passage 20 (c), but lost the capacity after passage 20 and became monolayered (d). e–i Ptf1a-induced human neural stem cells (hiNSCs) were highly immunoreactive for PTF1A, SOX2, PAX6, NESTIN, OLIG2, and FABP7. j–p Ptf1a-induced hiNSCs were capable of differentiating into neurons immunoreactive for TUJ1, MAP2, NEUN, TAU, or GABA, astrocytes labeled by GFAP, or oligodendrocytes marked by O1. q Neurons differentiated from hiNSCs were immunoreactive for both TuJ1 and synapsin. Cells in f–q were counterstained with nuclear DAPI. r Voltage-clamp recordings indicated fast activated and inactivated inward sodium currents as well as outward potassium currents on a differentiated neuron. s Current-clamp recordings revealed action potential responses of a differentiated neuron under current injection. t An action potential was induced after depolarization of the neuron. u Spontaneous postsynaptic currents recorded from a differentiated neuron. Scale bars, 80 μm (a–d) and 40 μm (e–q).
generating iNSCs from somatic cells for potential therapeutic purposes.

Our work has demonstrated the feasibility of using Ptf1a-reprogrammed iNSCs as a cell replacement therapy to treat neurodegenerative diseases. First, Ptf1a has the capacity to reprogram not only murine but also human fibroblasts into self-renewable iNSCs, providing a renewable human cell source. Second, Ptf1a-induced miNSCs exhibited proper survivability and tripotent differentiation potential (neuron, astrocyte, and oligodendrocyte) when transplanted into the hippocampus of normal mice. Third, we tested whether transplanted miNSCs had any therapeutic effect in treating murine AD models and found that transplantation of Ptf1a-reprogrammed miNSCs significantly improved cognitive dysfunction, in particular spatial learning and memory of the AD mice. The therapeutic effect achieved by Ptf1a-induced miNSCs is at least compatible with that of ESC-derived NSCs but without the potential tumorigenic risk.

Multiple mechanisms appear to be involved in Ptf1a-mediated reprogramming of iNSCs from somatic cells. First, the reprogramming activity of Ptf1a critically depends on its interaction with Rbpj, similar to its role in determining pancreas progenitor fates. When Rbpj was knocked down by RNA interference, there were many fewer neurospheres generated. Since Rbpj is also a downstream integrator of the Notch signaling pathway, we determined whether the interaction between Ptf1a and Rbpj relies on Notch signaling. The W298A mutation of Ptf1a interrupts its interaction with Rbpj but keeps the Notch pathway intact. We found that Ptf1aW298A lost the ability to reprogram fibroblasts into iNSCs, demonstrating that the reprogramming activity of Ptf1a depends on its Notch-independent interaction with Rbpj.
Second, upon association with Rbpj, Ptf1a activates a few key families of downstream TF genes involved in NSC specification and homeostasis including proliferation, self-renewal, and prevention of quiescence and neuronal differentiation. RNA-seq and ATAC-seq data show that at least six families of TF genes were activated upon transdifferentiation from MEFs to iNSCs. These include Sox, bHLH, homeobox, and POU domain TF family members that have demonstrated functions in NSC generation (Fig. 8h). For instance, several Sox TFs, in particular, Sox2 were upregulated in Ptf1a-reprogrammed iNSCs. Given the ability for Sox2 alone to reprogram fibroblasts into iNSCs, the reprogramming activity of Ptf1a may be mediated in part by Sox2 through direct or indirect activation by Ptf1a. All four members of the class 3 POU domain TFs were also upregulated, which presumably bind in cooperation with Sox2 to distant enhancers to regulate the specification, proliferation, and neuronal differentiation of iNSCs44-46. Olig2 is an upregulated bHLH TF, which has been shown to promote proliferation but prevent neuronal differentiation and quiescence of NSCs40. The upregulated homeobox TFs Pax6 and Lhx2 are required for the determination, proliferation, multipotency, and neurogenosis of NSCs41-43.

Third, Ptf1a activates DLL1-Notch signaling and perhaps other signaling pathways for efficient iNSC reprogramming. As a crucial pathway to regulate NSC proliferation and self-renewal, Notch signaling is indispensable for iNSC maintenance. Thus, neurosphere reprogrammed from MEFs by Ptf1a were decreased in the presence of Notch signaling inhibitor DAPT. Similar to its mechanism in pancreas progenitor cell expansion52, the Notch pathway is likely to promote iNSC proliferation in a paracrine manner. In this model (Supplementary Fig. 13k), the Ptf1a and Rbpj complex binds to DLL1 promoter and activates its expression inside an iNSC cell; DLL1 binds to Notch receptors on a neighbor iNSC cell and activates Hes1, Hey1, and other downstream effectors. At the same time, Hes1 and Hey1 activate downstream genes to promote cell proliferation. This positive feedforward loop acts in a paracrine way to promote iNSC proliferation and expansion. Consistent with this model, our RNA-seq data reveal a dramatic upregulation of Notch1, DLL1, Hes1, and Hey1 genes in iNSCs compared with MEFs. Apart from the Notch pathway, Ptf1a may activate other cell signaling pathways to promote iNSC reprogramming. For instance, many important molecules in the Notch pathway, including Notch receptors and their ligands, are upregulated in Ptf1a-reprogrammed iNSCs (Supplementary Fig. 16c). This cluster is located on chromosome 12 in mouse and accounts for approximately 10% of total microRNAs. Its expression is activated in fully pluripotent mouse stem cells but repressed in partially pluripotent cells; however, its role in iNSCs remains to be determined.

In summary, iNSCs have great values in patient-specific cell replacement therapies, in vitro modeling of disease pathogenesis, drug screening, and basic neurobiological studies. In this study, we have demonstrated that mouse and human fibroblasts can be successfully reprogrammed into iNSCs with a single, non-neural progenitor TF Ptf1a. The Ptf1a-derived iNSCs are clonogenic and self-renewable. They are tripotent and capable of differentiating into functional neurons, astrocytes and oligodendrocytes with high efficiency. When transplanted into the damaged hippocampus, they can significantly improve cognitive impairment of AD mouse models. The reprogramming activity of Ptf1a depends on its Notch-independent interaction with Rbpj which leads to subsequent activation of expression of TF genes and Notch and other signaling pathways involved in NSC specification, self-renewal, and maintenance.

Methods

Animals. Protocols and experiments in animals were approved by the Institutional Animal Care and Use Committees (IACUC) of the Zhejiang Ophthalmic Center of Sun Yat-sen University, Third Military Medical University and Rutgers University. All animals were maintained and bred in the animal facilities at these universities. Mice were fed with normal diet. The C57BL/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River (Beijing, China). The APP/PS1 mice were also obtained from the Jackson Laboratory. The Aβ40-42-injured mice were prepared as described previously. Experiments with animals were performed strictly following the approved protocols.

Cell culture. HEK293T cells (ATCC, CRL-3216) and human newborn foreskin fibroblasts (HFFs, from ATCC, SCR4-1041) were purchased from ATCC. The SCR029 cells (Chemicon, SCR029) are well-characterized mouse cortical NSGs obtained from Chemicon. These cells were tested for mycoplasma contamination before experiments and they are negative. HEK293T cells, HFFs, and MEFs were all expanded in the MEF medium [Dulbecco’s modified Eagle’s medium (DMEM), Hyclone), 10% fetal bovine serum (FBS) (Gibco), 1× Pen/Strep. All animals were maintained and bred in the animal facilities at these universities. Mice were fed with normal diet. The C57BL/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River (Beijing, China). The APP/PS1 mice were also obtained from the Jackson Laboratory. The Aβ40-42-injured mice were prepared as described previously. Experiments with animals were performed strictly following the approved protocols.

Viral plasmid construction and preparation of lentiviruses. The FUW-TetO vector was reported previously. It is a lentiviral vector containing the tetracycline operator (TetO), a minimal cytomegalovirus (CMV) promoter and FUW backbone. The full-length open reading frames of Ptf1a, Ptf1a, and GFP were cloned into the EcoRI sites of FUW-Teto vector. The reverse tetracycline transactivator M2rtTA was cloned into the EcoRI sites of the FUW backbone containing a constitutively active human ubiquitin C promoter. Lentiviruses were prepared as previously described. Briefly, 293T cells were transfected with a mixture of viral plasmids and lentiviral packaging plasmids using the Lipotransfecta- min 2000 reagent (Life Technologies) according to the manufacturer’s instruction. After 48 h of transfection, medium was replaced by 4 ml fresh medium and viral supernatants were collected at 48 and 72 h. After filtration through a 100 μm filter, viral supernatants were concentrated using an ultracentrifuge (Beckman, Optima L-100XP).

Preparation of MEFs. E13.5–E16.5 C57BL/6j mouse embryos were collected in a 10 cm tissue culture dish containing Hank’s balanced salt solution (HBSS; Gibco), washed briefly with HBSS, and transferred to a fresh 10 cm culture dish. The arms, legs, spinal cord, and internal organs of the embryos were removed using a pair of fine-tip forceps under a dissection microscope and any tissues above the shoulder and below hip joints were discarded. The remaining tissues were transferred to a fresh 10 cm culture plate containing 1 ml 0.25% trypsin, thoroughly minced into
small pieces using a pair of surgical scissors and forceps, and incubated for 15 min at 37 °C in a CO₂ incubator. Following the addition of 10 ml MEF medium to the plate, the digested tissues were triturated to dissociate cells by pipetting up and down several times with a 10 ml pipette. The dissociated cells were transferred into a 15 ml tube, centrifuged at 1000 rpm for 5 min, and resuspended with fresh MEF media. Cells derived from three to four embryos were pooled into one 10 cm dish, cultured at 37 °C in a CO₂ incubator, and expanded by no more than three passages.

**Generation and isolation of miNSCs and hiNSCs.** To generate miNSCs, 3–4 × 10⁵ MEF cells (at passage 3) were plated on each well (12-well plate) pre-coated with poly-ornithine and laminin, and cultured with 1 ml MEF medium for 24 h at 37 °C in CO₂ incubator. After removal of the MEF medium next day, 1 ml of lentinusives and MEF medium mixture containing polyethylene (100 μg/ml) was added into each well. The medium consisted of poly-L-ornithine and laminin (M2rtTA) viruses in a volume ratio of 1:1. To achieve optimal reprogramming efficiency, virus titration was tested for each batch of prepared viruses. Following infection of MEFs by the desired viruses for 16 h, the virus and medium mixture was replaced by neurobasal-a medium (Life Technologies) supplemented with 2 mM glutamax (Gibco), and 1 × Pen/Strep for 2 days, then the conditioned medium and virus mixture was removed and replaced with the NSC medium appropriate for the stage of differentiation. miNSCs were cultured in N3 medium supplemented with 2% B27 (Sigma), 20 ng/ml ciliary neurotrophic factor (CNTF) (Pepro Tech), 10 μM forskolin (Sigma), 25 mM L-glutamic acid, 200 mM L-glutamine, 1% B27, 1% N2 (Life Technologies), and 1 × Pen/Strp. The cells were cultured for 12 more days before analysis. For generation of astrocytes, miNSCs were cultured in DMEM supplemented with 1% N2, 2 mM glutamax, and 1% FBS for 14 days. Oligodendrocyte differentiation was carried out as previously described. Pre-treatment of miNSCs with the DNA methylation inhibitor 5-azacytidine was performed for 2 days in serum-free medium before inducing differentiation into oligodendrocytes. miNSCs were then cultured in astrocyte medium for additional 10 days, after which the cells were harvested for RNA extraction and immunocytochemistry.

**Immunohistochemistry and immunocytochemistry.** Immunostaining of mouse embryonic tissue sections was carried out as previously described. For immunostaining transplanted mouse brain tissues, the sections were transferred using a soft brush into wells of a 24-well plate containing 500 μl of phosphate-buffered saline (PBS) in each well, washed with 0.3% Triton-100 in PBS (PBST), blocked with 10% normal donkey serum in PBST for 1 h at room temperature, followed by a 1 h incubation with primary antibodies in 2% normal donkey serum at 4°C overnight. After washing with PBST, the sections were incubated with secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) in PBST for 1 h at room temperature, then washed with PBST, transferred onto glass slides using a soft brush, and mounted with mounting medium (Polysciences). Images were captured by a laser scanning confocal microscope (Carl Zeiss, LSM700).

For immunocytochemical staining, cells grown on glass coverslips pre-coated with poly-ornithine and laminin were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, washed three times with PBS, permeabilized with 0.1% Triton-100 for 10 min, blocked in 10% donkey serum for 1 h at room temperature, and then incubated at 4 °C overnight with primary antibodies diluted in 0.1% Triton-100 and 2% donkey serum. Following three rinses with PBS, the cells were incubated in secondary antibodies diluted in 2% donkey serum for 1 h at room temperature. Images were captured by a laser scanning confocal microscope (Carl Zeiss, LSM700).

The primary antibodies used in this study were as follows: chicken anti-GFP (Abcam, ab13970, 1:2000), mouse anti-Ki67 (BD PharmMingen, 550609 1:20), mouse anti-vGLUT3 (Sigma, SAB5200312, 1:500), rabbit anti-synaptophysin (Abcam, ab66581, 1:500), rabbit anti-Ptf1a (Beta Cell Biology Consortium, AB2153, 1:2000), miR-203 shRBPj (Santa Cruz, sc-371679, 1:200), rabbit anti-MAP2 (Boster Wuhan, BM1243, 1:200), chicken anti-MAP2 (Millipore, AB5543, 1:3000), mouse anti-vGLUT3 (Millipore, AB78N, 1:2000), mouse anti-NeuN (Millipore, MAB377, 1:2000), mouse anti-Tau (Santa Cruz, sc-21761-5300), rabbit anti-GABA (Sigma, A2052, 1:3000), goat anti-Dcx (Santa Cruz, sc-8066, 1:500), rabbit anti-peripherin (Millipore, AB1530, 1:1000), rabbit anti-GFAP (DAKO, Z0334, 1:2000), mouse anti-O1 (Millipore, MAB344, 1:1000), mouse anti-MBP (Biogenoid, 836502, 1:500), and mouse anti-CNP (Sigma, c5922, 1:500). The secondary antibodies used included donkey anti-rabbit, donkey anti-goat, donkey anti-mouse, and donkey anti-chicken Alexa488 IgG, Alexa594 IgG, or Alexa594 IgG 1:1000; Invitrogen). DAPI (Invitrogen) was used for nuclear counterstaining.

**Co-immunoprecipitation assay.** MEFs infected with GFP or Ptf1a-Flag (flag-tagged Ptf1a) lentiviruses were collected at day 12 and lysed with the Celllytic MT Cell Lysis Reagent (Sigma, C3228) containing protease inhibitor cocktail (Roche), followed by centrifugation in a microfuge at 12,000 rpm for 20 min. The protein concentration was determined by a standard bichinchoninic acid kit (Beyo- time). Immunoprecipitation was carried out by incubating the cell lysates with anti-Flag resin (Sigma, A2230) at 4 °C overnight. After the resins were washed three times with Tris-buffered saline. The precipitates were incubated with 3 × Flag peptide elution solution with gentle shaking for 30 min at 4 °C. The eluates and whole-cell extracts (input) were separated on an 8% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Western blotting was performed using the following primary antibodies: mouse anti-Rbpj (Santa Cruz, sc-71218A, 1:500), mouse anti-Flag (Sigma, F1804, 1:5000), rabbit anti-GFP (MBL, 598, 1:2000), mouse anti-β-actin (Sigma, A5316, 1:5000), and secondary antibodies: goat anti-mouse or rabbit IgG horseradish peroxidase (KangChen, KC-MM-035, KC-RR-035, 1:5000). The membranes were incubated with enhanced chemiluminescent reagent (Pierce Biotechnology, 34093) and imaged with a digital imager (FluorChem E System, ProteinSimple).

**BDU staining.** The proliferation rate of miNSC and SCR029 cells was assessed by BDU (5-ethyl-2'-deoxyuridine, Life Technologies) labeling as described previously. In brief, 1 × 10⁵ miNSC or SCR029 cells were plated on a 6 cm tissue culture dish containing a number of glass coverslips pre-coated with poly-ornithine and laminin, and incubated for 12 h at 37 °C in a cell culture incubator. BDU was then added into the culture medium to a final concentration of 10 μM and the cells were incubated for two more hours. The ensuing staining procedure was performed according to the manufacturer’s instructions. Images were captured with a confocal microscope.

**Bisulfite sequencing and DNA methylation analysis.** Genomic DNA was isolated from MEF, miNSC, and SCR029 cells using the DNA extraction kit (Solarbio). Unmethylated cytosines were then converted to thymines using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Previously reported primers were used to amplify the promoter regions of the bisulfite-converted genes: Nestin, Oct4, Nanog, Bmi1, Notch1, and Hes1. Purified PCR products were subcloned into the pMD18 vector (Sangon Biotech) and then transformed into Escherichia coli cells (DH5a, Takara). No fewer than 10 clones were picked for each gene and sequenced. Statistical analysis of the methylation result was performed by the BiQ Analyzer software.

**Real-time qRT-PCR analysis.** Total RNA was extracted from cells or tissues using the TriZol Reagent (Life Technologies) and genomic DNA contamination was removed by DNaseI (New England BioLabs). Complementary DNA was then prepared libraries were sequenced using an Illumina HiSeq 4000 sequencer.
following: 125 mM NaCl, 2.5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 1.25 mM HEPES, and 2 mM ascorbate (pH 7.2). Coverslips with adhered cells were transferred to the recording chamber, a 2-ml volume filled with an internal solution consisted of the following: 105 mM K-glucuronate, 5 mM KCl, 5 mM NaOH, 15 mM KOH, 0.5 mM CaCl2, 2 mM MgCl2, 5 mM EGTA, 2 mM adenosine 5'-triphosphate (disodium salt), 0.5 mM guanosine 5'-triphosphate (trisodium salt), 10 mM HEPES, and 2 mM ascorbate (pH 7.2). Cover slips with adhered cells were transferred into the recording chamber and bathed in an internal solution containing the following: 125 mM NaCl, 2.5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 1.25 mM NaH2PO4, 26 mM NaHCO3, 20 mM glucose, bubbled with 95% O2 and 5% CO2. The chamber was mounted on an upright microscope equipped with a ×40 water-immersion objective and differential interference contrast (DIC) optics. The cells and recording pipettes were viewed on a monitor that coupled to a charge-coupled device camera (Evolve, Photometrics, Tucson, USA) mounted on the microscope. Oxygenated external solution was continuously perfused into the recording chamber at a flow rate of 1.5–2 ml/min by a peristaltic pump (Leader-2, LongErPump, Hebei, China). Capacitive transients were compensated via the PatchMaster 500 software (HEKA) and the series resistance was compensated by ~50%. For current-clamp recording, we set the initial resting membrane potential (Vrest) to ~70 mV using a small, constant holding current and applied current pulses with a step size of 10 pA to test the ability to generate action potentials. Voltage-clamp recordings were performed directly following current-clamp recordings on the same cell. A simple step protocol from -90 mV to +30 mV for 200 ms was applied to assess the voltage-gated sodium channels and voltage-gated potassium channels.

Transplantation of miNSCs and immunohistochemistry. miNSCs and hiNSCs infected with the pLenti-CMV-GFP-Neo (Addgene, 17447) lentiviruses were cultured on a 10 cm tissue culture dish in the NSC medium in the presence of 4% G418 (500 μg/ml). After 48 h in culture, GFP-tagged miNSCs survived and produced small neurospheres. These neurospheres were digested with Accutase and centrifuged, and the dissociated cells were resuspended and diluted to a final concentration of 1 × 105 cells/ml. Two microliters of cell suspension were injected into the hippocampal region (anterior-posterior, 2 mm; left-right, lateral, 1.5 mm; dorsal-ventral, 2 mm) of an anesthetized C57Bl/6J mouse (8 weeks old, male or female) using a stereotaxic apparatus (RWD Life Science). The injected mouse was placed on an electric blanket until it fully awoke.

One to 1.5 months after transplantation, the animals were anesthetized and perfused with cardiac puncture with PBS followed by 4% paraformaldehyde. The brains were collected, fixed for 4 h at 4°C with slow shaking and dehydrated in 30% sucrose for 48 h. They were then enucleated in tissue-freezing medium (Leica) and stored at ~80°C, followed by sectioning into 20 μm coronal sections using a cryostat (CM1950, Leica) for immunohistochemistry.

Behavioral tests. The APP/PS1 mice (B6.Cg-Tg(APPsw,PSEN1D958S69o/ Mmjax; Jackson Laboratory, Bar Harbor, ME) utilized in this study were male and at the age of 11–12 months. Two-month-old male C57Bl/6J mice were used for Ap1-40-injury as described previously25. The APP/PS1 and Ap1-40 injured animals were transplanted with GFP-tagged miNSCs into the hippocampus as described35. Before the behavioral tests began, mice were placed in the experimental room for at least 30 min for acclimatization. All mice were kept in their home cages and were handled by the base of their tails at all times. The less stressful tests (nest-building test, open-field test, and novel object recognition test) were conducted before the more stressful tests (Y-maze test and Morris water maze test). So behavioral tests were conducted sequentially in the following order: nest-building test, open-field test, novel object recognition test, Y-maze test, and Morris water maze test.

Nest-building test. The nest-building behavior of the mice was assessed by assessing the nest quality after 24 h exposure to the sheet of tissue cotton. Mice were placed into a new cage with thin padding and a sheet of tissue cotton (5 × 5 cm, mesh weight 2.5 g) for 24 h36,38. Nest-building ability was assessed after 24 h according to a 5-point rating scale81.

Open-field test. The open-field locomotion in a novel environment was evaluated as previously described54. The open-field apparatus was constructed of grey plywood and measured 40 × 40 cm with 30 cm-high walls. Mice were placed into the center of the open field and the movements of the mice were recorded for 10 or 30 min using a video camera secured to the top of the apparatus and analyzed using Euthionio 11.0 (Noldus). The test apparatus was cleaned with 70% ethanol between two subjects and wiped out with clear paper towels.

Novel object recognition test. Short-term spatial memory of mice was assessed using a novel object location test as described previously26. In the same apparatus as the open-field test, mice were placed for 10 min into the same quadrant of the open field with two identical objects located diagonally. After 90 min, one object was replaced by a novel one and the mice were allowed to explore for another 10 min and the time spent by the animals exploring the novel and old objects was recorded and the test was clearly ended for 10 or 30 min using a video camera secured to the top of the apparatus and analyzed using Euthionio 11.0 (Noldus). To analyze the cognitive performance, a location index was calculated as (T novel) / (T novel + T old), where T novel and T old are the time spent exploring the novel and old objects, respectively.

Y-maze test. SAP behavior (SAB) was evaluated in a symmetrical Y maze (3 arms, 40 × 9 cm with 16 cm-high walls). Arm choices (all four paws entering one arm) were recorded during an 8 min exploration of the mice in the Y-shaped maze. Alternation was defined by recording the order of the visited arms (A, B, or C). Overlapping triplets of 3-arm visits was counted as one complete SAP. The SAP score was calculated according to the following formula: (number of SAP - total number of arm visits - 2) / 300. The test apparatus was cleaned with 70% ethanol and wiped out with clean paper towels between two subjects. Videos were recorded and analyzed using Euthionio 11.0 (Noldus).

Morris water maze test. The Morris water maze, which was widely used to test the spatial learning and memory, was conducted in a round white pool 150 cm in diameter and 50 cm deep87,88. The pool was filled up to a depth of 30 cm. The pool temperature was maintained at 25 ± 0.5°C. The escape platform was a 25 cm2 plexiglass square, placed in the center of one quadrant of the pool, 15 cm from the pool’s edge and submerged 1 cm beneath the water surface. The platform remained in the same position throughout the entire test and was removed from the pool during the probe test.

On the first day (visible platform), mice were placed into the water facing the visible platform. If the mouse reached the platform before a 60 s cut-off, it was allowed to stay on the platform for 5 s then returned to the home cage. If the mouse did not find the platform within 60 s, it was gently guided onto the platform and allowed to sit on it for 20 s before returning to the home cage. This procedure was repeated for three more trials, each starting in a different quadrant. Once the animal had completed every trial, it was dried off with a clean paper towel. The four-trial training procedure was repeated for all the mice. On the next 4 days (hidden platform), the mice were trained as on the first day but with the platform submerged. After 5 consecutive days of pre-training, the animals were tested with the platform removed. During the test, mice were placed into the water from the opposite quadrant where the platform used to be, and were tested for 60 s. Videos were recorded and analyzed using Euthionio 11.0 (Noldus). The behavioral data were statistically analyzed as described below.

Statistics. In general, each result is obtained from three or more biological samples. Statistical analysis was performed using the GraphPad Prism 6.0 and Microsoft Excel computer programs. The results are expressed as mean ± SD for experiments conducted at least in triplicates. Unpaired two-tailed Student’s t-test.
of variance (ANOVA) test and/or Mann–Whitney U-test were used to assess differences between two groups, and a value of P < 0.05 was considered statistically significant. Replicated measures ANOVA was used to analyze the di-
ference of escape latency between groups in the Morris water maze test. One-way ANOVA was used to assess the difference between groups in other behavioral tests.

Data availability. The RNA-seq and ATAC-seq data that support the findings of this study have been deposited in the NCBI Gene Expression Omnibus database under accession codes SRP135657 and SRP136063, respectively.

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Authors contribution
D.X., K.J., H.X. and M.X. conceived and designed the research. D.X., K.J., H.X. and M.X. performed the experiments and interpreted the data and wrote the manuscript. All authors contributed to critical reading of the manuscript.

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