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Short Article

Direct Reprogramming of Mouse and Human Fibroblasts into Multipotent Neural Stem Cells with a Single Factor

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SUMMARY

The generation of induced pluripotent stem cells (iPSCs) and induced neuronal cells (iNCs) from somatic cells provides new avenues for basic research and potential transplantation therapies for neurological diseases. However, clinical applications must consider the risk of tumor formation by iPSCs and the inability of iNCs to self-renew in culture. Here we report the generation of induced neural stem cells (iNSCs) from mouse and human fibroblasts by direct reprogramming with a single factor, Sox2. iNSCs express NSC markers and resemble wild-type NSCs in their morphology, self-renewal, ability to form neurospheres, and gene expression profiles. Cloned iNSCs differentiate into several types of mature neurons, as well as astrocytes and oligodendrocytes, indicating multipotency. Implanted iNSCs can survive and integrate in mouse brains and, unlike iPSC-derived NSCs, do not generate tumors. Thus, self-renewable and multipotent iNSCs without tumorigenic potential can be generated directly from fibroblasts by reprogramming.

INTRODUCTION

After seminal studies that succeeded in reprogramming mouse and human somatic cells to iPSCs (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007), researchers have taken great strides to improve reprogramming methods and to apply the technology to the understanding and potential future treatment of human diseases (Hanna et al., 2008; Hockemeyer et al., 2008; Kaji et al., 2009; Kim et al., 2009; Okita et al., 2008; Park et al., 2008a, 2008b; Shi et al., 2008; Stadtfeld et al., 2008; Wernig et al., 2008; Woltjen et al., 2009; Yu et al., 2009). A number of disease- and patient-specific iPSC lines have been established, including those from patients with amyotrophic lateral sclerosis (Dimos et al., 2008), spinal muscular atrophy (Ebert et al., 2009), Parkinson's disease (Park et al., 2008a; Soldner et al., 2011), schizophrenia (Brennand et al., 2011), Huntington's disease (Park et al., 2008a), and Alzheimer's disease (Israel et al., 2012). Furthermore, correction of genetic mutations in disease-specific iPSCs can rescue phenotypes in cultured cells (Soldner et al., 2011; Yusa et al., 2011) or in mouse models of human diseases, such as sickle cell anemia (Hanna et al., 2007). However, for successful therapeutic application, iPSCs need to be efficiently differentiated into the desired cell type. Moreover, pluripotent stem cells, including embryonic stem cells (ESCs) and iPSCs, can form teratomas in vivo, whereas multipotent, lineage-restricted stem cells, such as hematopoietic stem cells and NSCs, do not (Fong et al., 2010; Miura et al., 2009; Yamanaka, 2009). Thus, direct reprogramming of somatic cells into multipotent, lineage-restricted stem cells should complement iPSC technology and sidestep the difficulty of differentiating iPSCs. It would also lower the risk of immature teratoma formation after the transplantation of iPSC-derived multipotent stem cells or their progeny as a result of potential iPSC contamination or incomplete reprogramming.

Recently, transcription factors have been used to induce cell fate change from one type of somatic cell to another in cell cultures and in mice (Huang et al., 2011; Ieda et al., 2010; Sekiya and Suzuki, 2011; Zhou et al., 2008). Mouse and human fibroblasts and other types of cells have been transdifferentiated directly into postmitotic neurons with combinations of transcription factors (Ambasudhan et al., 2011; Caiazzo et al., 2011; Kim et al., 2011b; Marro et al., 2011; Pang et al., 2011; Qiang et al., 2011; Son et al., 2011; Vierbuchen et al., 2010; Yoo et al., 2011). iNCs have typical neuronal cell properties and exhibit proper electrical function in culture. Although iNCs can be generated with relatively high efficiency (5%–20%), current protocols generate a mixture of neuronal cells and other unknown types of cells, limiting the direct use of iNCs in transplantation therapy. The addition of neuronal fate-specifying factors to the reprogramming cocktail can influence the efficiency with which a specific neuronal subtype can be generated (Caiazzo et al., 2011; Son et al., 2011). However, this technology is limited by the fact that iNCs are terminally differentiated and cannot self-renew. Most recently, it has been reported that the combination of three or more factors can reprogram mouse fibroblasts into induced neural stem cells (iNSCs) with self-renewing ability (Han et al., 2012; Kim et al., 2011a; Lujan et al., 2012; Sheng

et al., 2012; Thier et al., 2012). Here, we present a novel method for generating self-renewable, multipotent, neural lineage-restricted, and nontumorigenic iNSCs from mouse and human fibroblasts by direct reprogramming with one factor.

RESULTS

Generation and Characterization of iNSCs from Mouse Fibroblasts

The protocol for generating iNSCs from mouse embryonic fibroblasts (MEFs) is shown in Figure S1A (available online). In choosing the reprogramming factors, we considered five key transcription factors that are important in NSC production, maintenance, and self-renewal: Sox2, Bmi-1, TLX, Hes1, and Oct1 (Graham et al., 2003; Jin et al., 2009; Kageyama et al., 2008; Molofsky et al., 2003; Shi et al., 2004; Williams et al., 2004). These factors were expressed individually or in different combinations in MEFs by retroviral-mediated gene transduction. In pilot studies, the morphology of MEFs cultured on gelatin-coated plastic in NSC medium supplemented with growth factors (epidermal growth factor [EGF] and fibroblast growth factor [FGF2]) was unchanged for up to 4 weeks after transduction (Figures S1A [Step 1] and S1D). However, when MEFs were cultured on glass coverslips coated with gelatin, their morphology was drastically altered by retroviral Sox2 alone (Figures 1A, 1B, S1A [Step 2], and S1B) or by Sox2 plus additional transcription factors. Because the combination of different transcription factors with Sox2 did not enhance the reprogramming efficiency, and in some cases yielded less encouraging results, we focused on using Sox2 alone.

By 2–10 days after transduction with Sox2, transformed cells had formed networks with colonies at the intersections of these networks (Figures 1B, S1A [Step 2], and S1B), many of which stained positive for Sox2 and Nestin (Figures 1C and S1C). The efficiency of generating Sox2 and Nestin double-positive colonies on gelatin-coated coverslips was 0.13% at day 8 postinfection and 0.52% at day 12 and was further enhanced to 0.96% at day 8 by culturing infected cells on coverslips coated with NSC-permissive substrates poly-L-ornithine and laminin (Table S1; Lee et al., 2007). Furthermore, immunostaining of Sox2-infected cells 14 days postinfection in NSC medium with growth factors revealed the lack of MAP2-positive neurons, GFAP-positive astrocytes, and O4-positive oligodendrocytes, indicating that Sox2 transduction does not generate differentiated neural cells directly (Table S2). Importantly, no morphological changes and no Nestin- or Sox2-positive cells were observed in MEFs not transduced with Sox2 (Figure 1D) or in Sox2-transduced MEFs cultured on gelatin-coated plastic for up to 4 weeks (Figure S1D). Furthermore, untransfected MEFs did not stain positive for the differentiated cell markers MAP2, GFAP, and O4 (Table S2).

Six to ten days after retroviral Sox2 transduction, cell mixtures containing multiple colonies were collected and recultured to promote cell proliferation and expansion (Figures 1E and S1A [Step 3]). Five days later, Sox2-infected cells were released for primary neurosphere culture in suspension to select for NSC-like cells (Figures S1A [Step 4] and S1E). The primary neurospheres were seeded, and cells with NSC-like morphology grew gradually from adhered neurospheres (Figures S1A [Step 5], S1F, and S1G). To further enrich reprogrammed NSC-like cells, we repeated the neurosphere culture procedures twice (Figure S1A [Steps 4 and 5]). NSC-like cells were then grown in a monolayer for many generations to generate a homogenous population of NSC-like cells (Figures 1F and S1A [Step 6]). Reprogrammed NSC-like cells at passages 8 (Figure S1H), 11 (Figure 1G), and 28 (Figure 1H) had morphologies very similar to those of wild-type mouse NSCs (Figures 1I and S1I). Similar results were obtained from independent reprogramming experiments with the same protocol (Figures S1J–S1L), demonstrating the repeatability of this reprogramming method.

The reprogrammed mouse NSC-like cells expressed NSC markers, including Sox2 and Nestin, similarly to the wild-type mouse NSC line SCR029 (Figures 1J–1M), as well as Pax6 and BLBP (Figures S2A–S2D). Quantitative real-time RT-PCR (qRT-PCR) confirmed that NSC-like cells expressed Sox2, Nestin, Sox1, and Zbtb16 (Figure 1N); however, they did not express pluripotency-related genes, such as *Oct4*, *Nanog*, and *Zfp42* (Figure 1O). In contrast, MEFs cultured in fibroblast or NSC medium for up to 4 weeks did not show significant expression of Sox2, Nestin, Pax6, Zbtb16, or Msi1 (Figures S2E–S2H). We next determined whether reprogrammed NSC-like cells had silenced exogenous Sox2 expression and turned on endogenous Sox2 expression by qRT-PCR (Figure S2I). Interestingly, endogenous Sox2-specific qRT-PCR detected low levels of endogenous Sox2 expression in reprogrammed NSC-like cells at passage 7 and significant levels of exogenous Sox2 expression compared to wild-type NSCs. In contrast, reprogrammed NSC-like cells at later passage had more comparable levels of endogenous Sox2 expression to wild-type NSCs (Figure S2I), suggesting that the endogenous Sox2 gene was gradually turned on in reprogrammed NSC-like cells over continuous passaging. Exogenous Sox2-specific qRT-PCR did not detect a significant signal in either reprogrammed NSC-like cells at passage 16 or in wild-type NSCs, consistent with silencing of the retroviral Sox2 transgene in reprogrammed NSC-like cells during continuous passaging (Figure S2I). Thus, the reprogrammed NSC-like cells do not require the expression of exogenous Sox2 to maintain their NSC identity at later passages.

Methylation patterns of NSC (*Sox2* and *Nestin*) and ESC (*Oct3/4*) gene promoters were next analyzed in reprogrammed NSC-like cells, wild-type NSCs, and MEFs at passages 12, 17, and 2, respectively (Han et al., 2009; Imamura et al., 2006; Western et al., 2010). Methylation analysis of bisulfite-treated DNA revealed that the *Oct3/4* promoter was hypermethylated in all three cell lines, indicating the transcriptional silencing of that gene (Figures S2J–S2L). In contrast, both the *Sox2* and *Nestin* promoters were hypomethylated in reprogrammed NSC-like cells similarly to wild-type NSCs, indicating that these genes are transcriptionally activated (Figures S2M–S2P).

Microarray studies demonstrated that the global gene expression pattern of the reprogrammed NSC-like cells was similar to that of wild-type mouse NSCs but different from that of MEFs (Figures S2Q and S2R). Furthermore, like wild-type mouse NSCs, the reprogrammed NSC-like cells formed neurospheres in suspension cultures and did so with similar efficiency (Figures 1P–1R). Taken together, these data strongly suggest that a single factor plus NSC-permissive culture conditions can reprogram MEFs into self-renewing NSCs that appear similar to wild-type

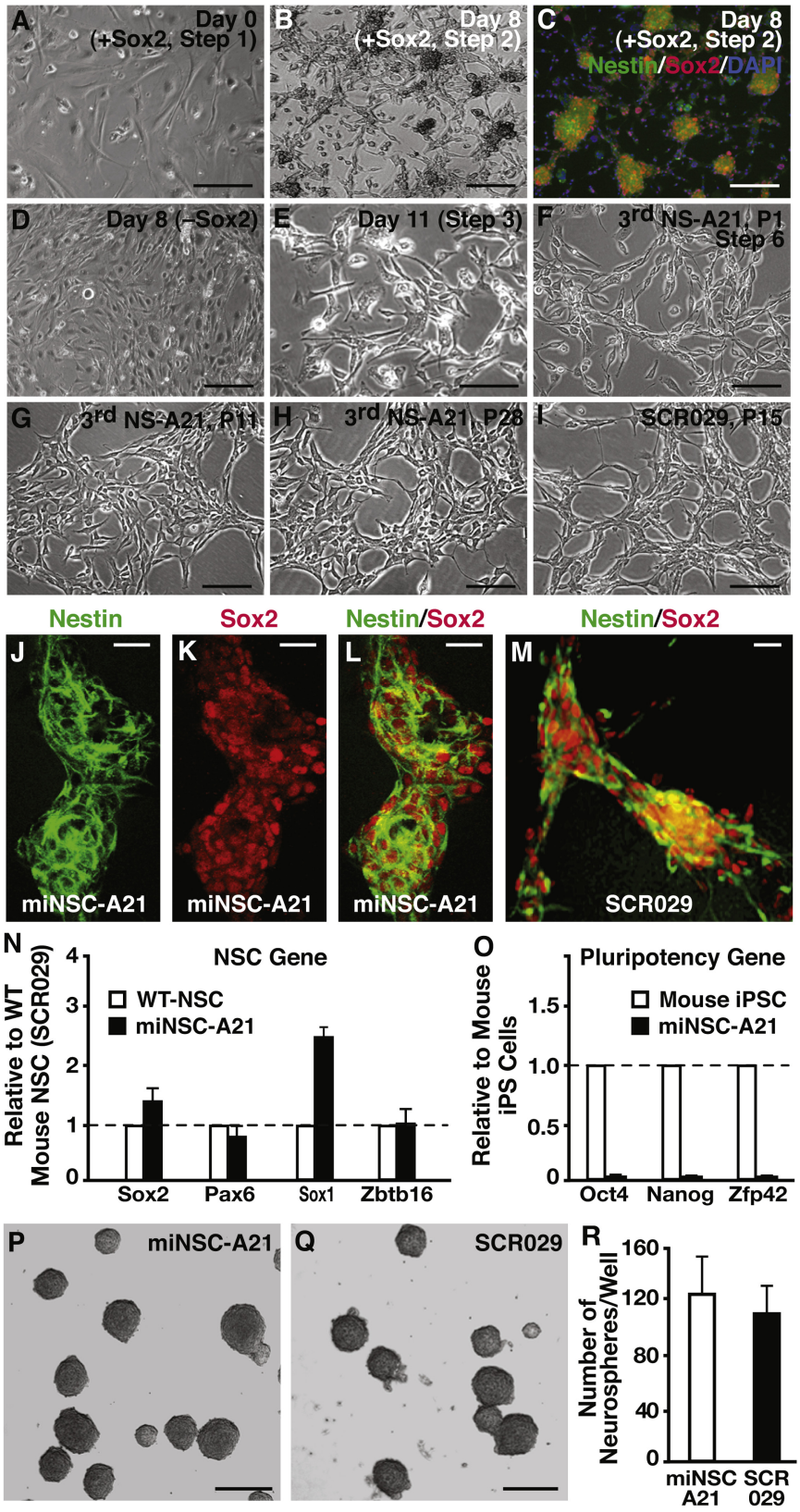

Figure 1. Generation and Characterization of iNSCs from Mouse Fibroblasts

(A) Phase-contrast image of MEFs after overnight treatment with Sox2 retrovirus in fibroblast medium.

(B) Sox2-infected cells in NSC medium with growth factors generate networks and colonies on gelatin-coated glass coverslips by 8 days after infection.

(C) Sox2-transformed colonies are positive for the NSC markers Nestin and Sox2.

(D) Fibroblasts cultured in NSC medium with growth factors but without Sox2 retroviral transduction do not generate colonies or networks.

(E) Sox2-transduced cells after 11 days have drastically different morphology from their fibroblast counterparts.

(F) After three rounds of neurosphere generation, reprogrammed cells take on the characteristic bipolar NSC morphology.

(G) After multiple passages as a monolayer, NSC-like cells are a morphologically homogenous population.

(H) Morphology of NSC-like cells stays the same over prolonged passaging, and reprogrammed cells can proliferate over 28 passages.

(I) The morphology of NSC-like cells is similar to that of wild-type cortical-derived NSCs such as the commercial cell line SCR029 (Millipore).

(J–M) For the miNSC-A21 cell line, expression of Nestin and Sox2 is similar to that of brain-derived wild-type NSCs as revealed by immunostaining.

(N and O) qRT-PCR reveals that miNSC-A21 express typical NSC markers (N) but do not express pluripotency-related genes (O). Error bars denote standard deviation of triplicate reactions.

(P–R) In suspension culture, miNSC-A21 generates neurospheres similar to wild-type NSCs and with similar efficiency (n = 3). Values are mean ± SD.

Scale bars represent 50 μm in (A), (D)–(I), and (J)–(M) and 100 μm in (B), (C), (P), and (Q).

See also Figures S1 and S2 and Tables S1, S2, and S4.

NSCs at the transcriptional level and in forming neurospheres. We therefore refer to them as mouse induced NSCs (miNSCs).

Multipotency of miNSCs in Culture

Under neuronal differentiation conditions involving removal of growth factors from the NSC medium, miNSCs differentiated into immature neurons (Tuj1-positive) at 1 week in culture similarly to wild-type NSCs (Figures 2A and 2B) and mature neurons (MAP2- and Tau-positive) at 2 weeks (Figures 2E–2H). At 4 weeks, miNSCs developed into MAP2-positive neurons with extensive and complex neurites similar to those of mouse primary neurons in culture (Figure 2I). At 14–28 days, miNSCs differentiated into vGluT1-positive excitatory neurons (Figures 2J and S3E) and GABA-positive inhibitory neurons (Figure 2K). Importantly, MAP2-positive mature neurons could be generated from miNSCs at passages P11–P28 (Figures S3A–S3D) and beyond. Thus, miNSCs have stable neurogenesis capability during continuous passaging.

Immunostaining revealed robust GFAP-positive astrocytes derived from both miNSCs and wild-type NSCs cultured for 7–22 days under various differentiation conditions (Figures 2C, 2D, and S3F). Importantly, the ability of miNSCs to generate neurons and astrocytes was confirmed in different batches of miNSCs (Figures S3G–S3L). Furthermore, miNSCs also developed into O4- and Olig2-positive oligodendrocytes (Figure 2L). Thus, miNSCs are multipotent, being able to differentiate into neurons, astrocytes, and oligodendrocytes.

To further confirm the multipotency of miNSCs, we subcloned miNSC-A21 line at passage 13 when we observed stable NSC gene expression and neuronal differentiation and tested the multipotency of each clone. All five clones tested could differentiate into MAP2-positive neurons, GFAP-positive astrocytes, and O4/Olig2 double-positive oligodendrocytes (Figures 2M–2U for three clones). Thus, miNSCs are a population of truly multipotent NSCs and are not a heterogenous population of different neural progenitor cells.

Lastly, we determined the efficiency of neuronal and glial differentiation in vitro at 14 days for two miNSC clones (A21-B8 and A21-C1). We found a similar yield in neurons (MAP2-positive cells normalized to DAPI-positive nuclei) between the two miNSC-A21 clones (clone B8, 67% ± 5%; clone C1, 59% ± 6%) and wild-type brain-derived NSCs (76% ± 6%). However, we saw a higher percentage in astrocytes (GFAP-positive cells normalized to DAPI-positive nuclei) generated from miNSC-A21 (clone B8, 25% ± 2%; clone C1, 18% ± 2%) compared to wild-type brain-derived NSCs (6% ± 4%).

Functional Neurons Derived from miNSCs

Neurons derived from miNSCs, under a condition conducive to primary neuron culture, expressed Synapsin with punctate distribution, suggesting synaptic formation in vitro (Figures 3A and 3B). Whole-cell patch-clamp recordings (Figure 3C) revealed that miNSC-derived neurons had hyperpolarized resting membrane potentials (–40 to –80 mV) (Figure 3D) and membrane resistance properties (Figure 3E). Action potentials could be elicited by depolarizing the membrane in current-clamp mode (Figure 3F). Furthermore, in voltage-clamp mode, both fast inactivating inward and outward currents, which correspond to opening of voltage-dependent Na^+ and K^+ channels, respectively, were recorded from miNSC-derived neurons (Figure 3G). Thus, miNSC-derived neurons appear to exhibit the functional membrane properties and activities of normal neurons.

miNSCs Can Survive, Integrate, and Differentiate In Vivo and Do Not Generate Tumors

We microinjected GFP-labeled miNSC neurospheres into the cortex of P2–P3 wild-type pups. Immunostaining revealed that miNSCs survived and differentiated into NeuN-positive neurons with mature-looking dendritic spines (Figures 3H–3J), GFAP-positive astrocytes (Figures 3K–3M), and Olig2-positive oligodendrocytes (Figures 3N–3P) 5 days posttransplantation. Thus, miNSCs are capable of differentiating into neurons, astrocytes, and oligodendrocytes in vivo.

Because transplantation of iPSC-derived neurospheres into mouse brains often results in teratoma formation, we also assessed the ability of miNSCs to generate tumors or teratomas in vivo (Yamanaka, 2009). Transplantation of miNSCs or wild-type brain-derived NSCs into mouse brains did not generate tumors; however, teratomas formed in more than 60% of mice transplanted with mouse iPSC-derived NSCs (Table S3). The observation that miNSCs did not form tumors in vivo in 28 separate hippocampal injections involving three different miNSC-A21 subclones suggests that miNSCs have little or no tumorigenic potential.

Generation and Characterization of iNSCs from Human Fetal Fibroblasts

We generated human iNSCs (hiNSCs) from human fetal foreskin fibroblasts (HFFs) by a similar protocol (Figure S1A), in which mouse Sox2 was replaced with human SOX2 and reprogrammed cells were cultured in human NSC (hNSC) culture medium supplemented with human EGF and FGF2. During reprogramming, the morphological changes in HFFs were similar to those in mouse fibroblasts (compare Figures S4A–S4G to Figures 1 and S1). Immunostaining revealed that within 5 days after SOX2 retroviral transduction, hiNSC colonies were positive for SOX2 and NESTIN (Figure S4E). After three or four rounds of neurosphere culture, hiNSCs had morphology different from the original HFFs (Figures S4F and S4A) and further passaging resulted in morphology similar to that of wild-type NSCs derived from human iPSCs (Figures S4G and S4H). hiNSCs did not express pluripotency-related genes as determined by qRT-PCR (Figure S4I) and had neurosphere-forming ability similar to that of NSCs derived from human iPSCs (Figure S4J).

Multipotency of hiNSCs in Culture

At 2–4 weeks in culture under conditions that favor neuronal differentiation (hNSC medium without growth factors in the presence of WNT5A (100 ng/ml) or retinoic acid (1 μM) plus forskolin (5 μM)), hiNSCs differentiated into immature neurons (TUJ1-positive) and mature neurons (MAP2-positive) (Figures 4A–4D). At 4 weeks, hiNSCs developed into MAP2-positive neurons with extensive and complex neurites (Figure 4E). Importantly, MAP2-positive neurons could be generated from hiNSCs at various passages from P8 to P22, suggesting stable neurogenic capacity of hiNSCs. Immunostaining revealed

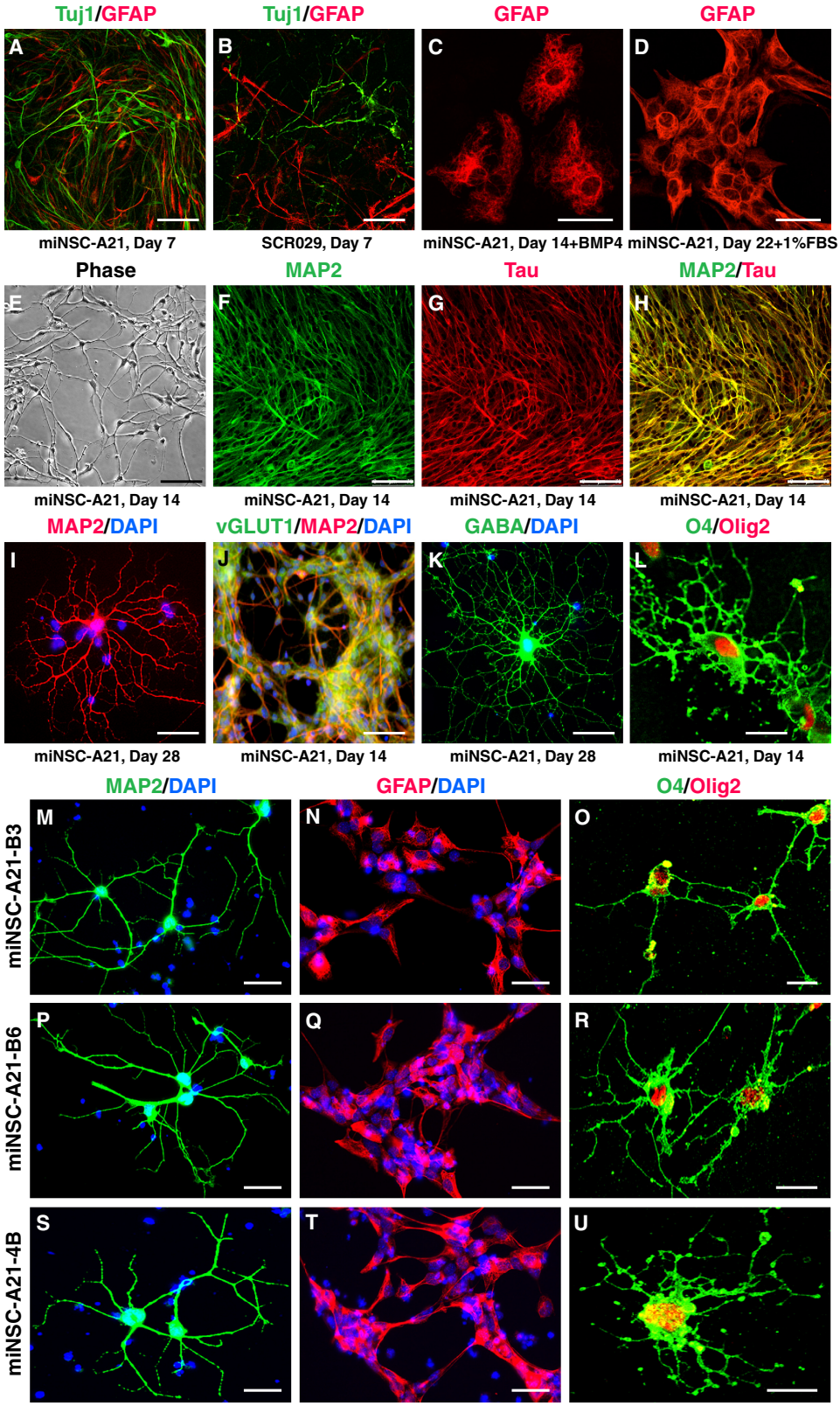

Figure 2. Multipotency of miNSCs In Vitro

(A and B) Like wild-type NSCs, miNSC-A21 can differentiate into Tuj1$^+$ neurons and GFAP$^+$ astrocytes by 7 days in culture after growth factor withdrawal.
(C and D) miNSC-A21 can robustly generate GFAP$^+$ astrocytes by 14 days in vitro in the presence of BMP4 or FBS.

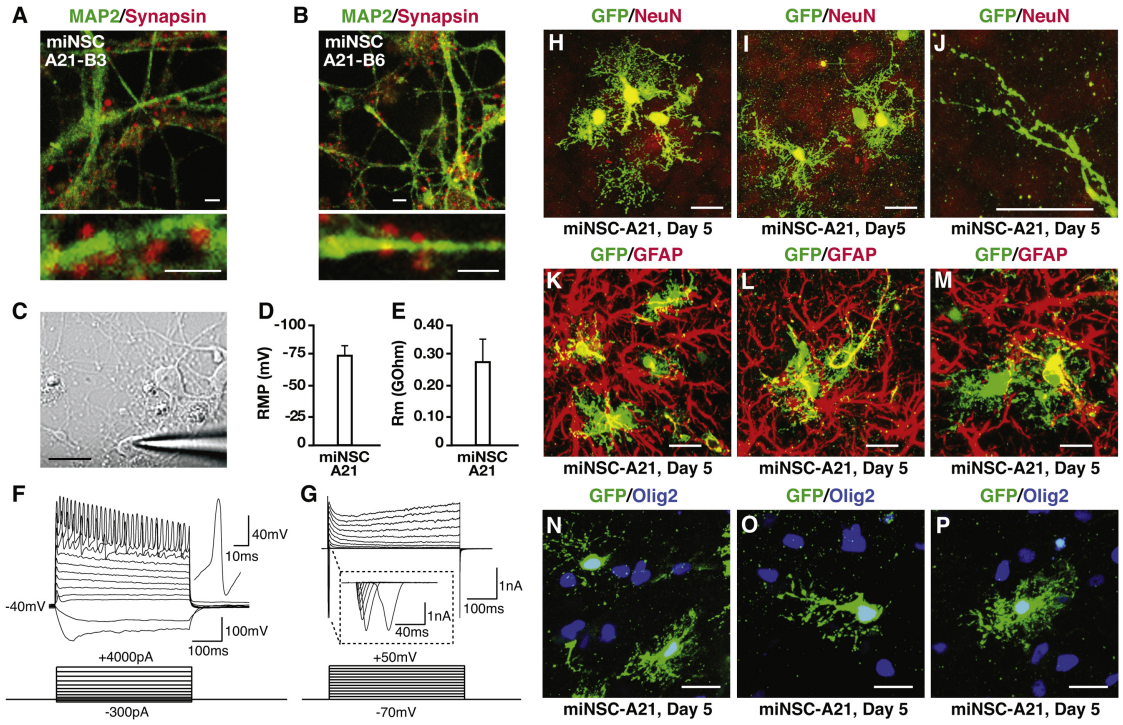

Figure 3. miNSC-Derived Functional Neurons In Vitro and Multipotency of miNSCs In Vivo

(A and B) Neurons derived from subclones miNSC-A21-B3 or miNSC-A21-B6 at 14 days in culture express MAP2 (green) and Synapsin (red), a presynaptic marker of mature neurons.

(C) A patched neuron derived from miNSC-A21-B3 at 17 days in culture.

(D and E) Whole-cell capacitance and membrane resistance of neurons derived from miNSC-A21 were determined from a transient 5 mV hyperpolarizing step from a holding potential of –70 mV. Values are mean ± SEM.

(F) Current-clamp recordings of neurons derived from miNSC-A21 at –40 mV reveal action potentials with stepwise current injection.

(G) Voltage-clamp recordings of neurons derived from miNSC-A21 reveal both fast inactivating inward and outward currents indicating functional voltage-dependent Na^+ and K^+ channels.

(H–P) GFP-labeled miNSC-A21 were grown in suspension cultures for 1 day to generate small neurospheres and then microinjected into the cortex of P2–P3 wild-type pups. Five days after transplantation, mouse brains were collected, fixed, sectioned, and immunostained.

(H–J) Immunostainings reveal that miNSC-A21 can differentiate into $NeuN^+$ neurons (H and I) with mature-looking dendritic spines (J) in vivo.

(K–M) miNSC-A21 can also differentiate into $GFAP^+$ astrocytes in vivo.

(N–P) miNSC-A21 can also differentiate into $Olig2^+$ oligodendrocytes in vivo.

Scale bars represent 2 μm in (A) and (B) and 10 μm in (C) and (H)–(P).

See also Table S3.

GFAP-positive astrocytes derived from two separate hiNSC lines cultured for 14 days in the presence of 50 ng/ml BMP4 (Figures 4F and 4G). Furthermore, hiNSCs also developed into O4- and OLIG2-positive oligodendrocytes (Figure 4H). Importantly, hiNSCs did not generate tumors upon transplantation into mouse brains (Table S3). Thus, hiNSCs are multipotent, being able to differentiate into neurons, astrocytes, and oligodendrocytes, and may not harbor any tumorigenic potential in vivo.

DISCUSSION

The ability to reprogram somatic cells into self-renewable iNSCs has major implications for regenerative medicine. iNSCs can serve as a model system for unveiling disease pathogenesis, for drug screening and toxicity tests, and ultimately for cell transplantation therapies. Many studies have focused on generating NSCs from pluripotent sources such as ESCs or iPSCs

(E) miNSC-A21 can generate mature-looking neurons and neuronal networks by 14 days in culture without growth factors.

(F–H) Neurons derived from miNSC-A21 stain positive for the mature neuronal markers MAP2 and Tau.

(I) miNSC-A21 can differentiate into mature arborized neurons by 28 days in vitro.

(J and K) miNSC-A21 can differentiate into subtypes of neurons, including excitatory $vGluT1^+$ neurons (J) and inhibitory $GABA^+$ neurons (K).

(L) miNSC-A21 can generate $O4^+$ and $Olig2^+$ oligodendrocytes by 14 days in culture.

(M–U) After 14 days in culture, subcloned lines B3 (M–O), B6 (P–R), and 4B (S–U) of miNSC-A21 can differentiate into $MAP2^+$ mature neurons (M, P, S), $GFAP^+$ astrocytes (N, Q, T), and $O4^+/Olig2^+$ oligodendrocytes (O, R, U).

Scale bars represent 50 μm in (A), (B), (E), and (J); 25 μm in (C) and (D); 75 μm in (F)–(H); and 10 μm in (I)–(U).

See also Figure S3.

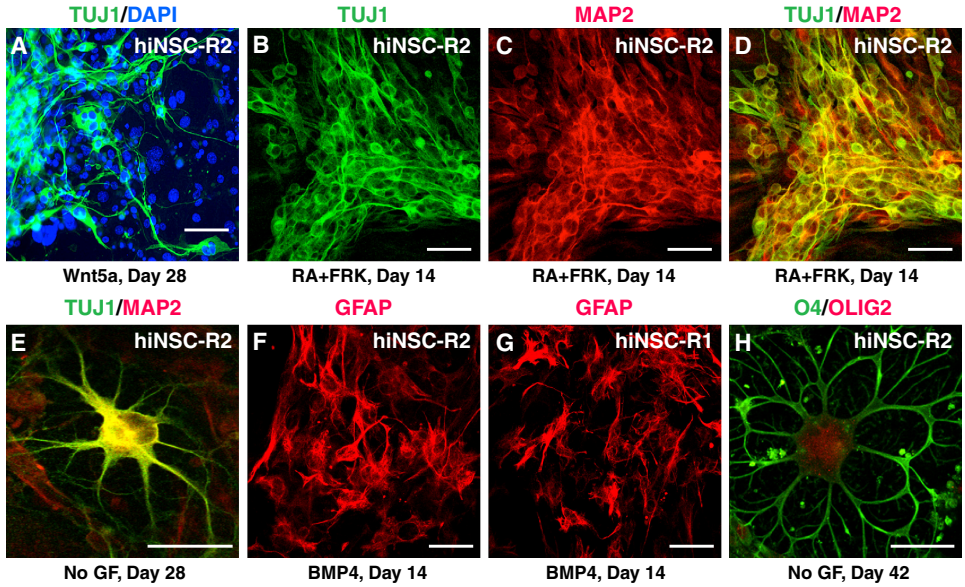

Figure 4. Multipotency of hiNSCs In Vitro

(A) hiNSCs can differentiate into TUJ1$^+$ immature neurons in the presence of the signaling protein WNT5A by 28 days in culture.

(B) The addition of retinoic acid (RA) and forskolin (FRK) to neuronal differentiation conditions pushed hiNSCs to differentiate into TUJ1$^+$ neurons by 14 days in vitro.

(C and D) hiNSCs can generate TUJ1$^+$/MAP2$^+$ neurons by 14 days in the presence of RA and FRK.

(E) hiNSCs can generate mature-looking neurons that are MAP2$^+$ by 28 days in vitro in hNSC medium without growth factors.

(F) hiNSCs can also generate GFAP$^+$ astrocytes in the presence of BMP4 by 14 days.

(G) A separate hiNSC line can also robustly generate GFAP$^+$ astrocytes at 14 days in vitro.

(H) hiNSCs can generate O4$^+$/OLIG2$^+$ oligodendrocytes by 40 days in culture in hNSC medium lacking growth factors.

Scale bars represent 20 μm in (A)–(D) and 10 μm in (E)–(H).

See also Figure S4.

(Hochedlinger and Plath, 2009; Yamanaka, 2009). However, these methods are plagued by ethical and practical issues, such as the origin of ESCs and the tendency for teratoma formation of cells derived from iPSCs (Fong et al., 2010; Miura et al., 2009; Yamanaka, 2009). Interestingly, transplantation of Sox2-reprogrammed iNSCs into mouse brains does not generate tumors, making iNSCs more attractive than NSCs derived from iPSCs. iNCs can be generated from fibroblasts and other somatic cell sources (Ambasudhan et al., 2011; Caiazzo et al., 2011; Kim et al., 2011b; Marro et al., 2011; Pang et al., 2011; Qiang et al., 2011; Son et al., 2011; Vierbuchen et al., 2010; Yoo et al., 2011), but iNCs are terminally differentiated and restricted to the subtypes of neurons they can generate. Having a patient-derived population of multipotent iNSCs would bypass some of the disadvantages of pluripotent and terminally differentiated cell populations. Thus, direct reprogramming of somatic cells into self-renewable and multipotent iNSCs should not only complement the iPSC and iNC technologies but also sidestep their shortcomings.

It has recently been reported that the four Yamanaka reprogramming factors in combination with NSC-permissive culture conditions can reprogram fibroblasts to induced neural progenitors (iNPCs) that can generate multiple neuronal cell types as well as astrocytes (Kim et al., 2011a). However, these iNPCs can self-renew for only 3–5 passages in culture and have not been shown to differentiate into oligodendrocytes. In a separate study, a combination of nine factors reprogramed Sertoli cells into iNSCs (Sheng et al., 2012). However, exogenous expression of eight out of the nine factors was not silenced even after multiple passages, raising the question of whether these iNSCs would revert back to their original state without constant overexpression of those factors. It also has been reported that three factors, Brn2, Sox2, and FoxG1, can reprogram mouse fibroblasts to tripotent, self-renewing iNPCs (Lujan et al., 2012). However, when the authors attempted to generate iNPCs with only two factors, they found that Sox2 and FoxG1 generated only bipotent iNPCs and that the combination of FoxG1- and Brn2-generated tripotent iNPCs were unable to form mature and functional neurons in vitro. Interestingly, the three-factor-reprogrammed iNPCs could generate oligodendrocytes in vivo although it was not tested for generation of neurons or astrocytes. Most recently, two studies have shown that the combinations of Sox2, Klf4, and c-Myc or Brn4, Sox2, Klf4, c-Myc, and E47/Tcf3 can reprogram mouse fibroblasts into iNSCs (Thier et al., 2012; Han et al., 2012). Although these studies do show that iNSCs can self-renew, generate functional neurons in vitro, and integrate in vivo, both reprogramming methods require overexpression of the potent c-Myc oncogene, which has been reported to be a cause of brain tumorigenesis from transplanted iPSC-derived NSCs (Okita et al., 2008).

The studies mentioned above are in line with our findings that mouse fibroblasts can be directly reprogrammed into iNSCs that exhibit typical NSC properties and differentiation abilities

in vitro and in vivo. However, our iNSC reprogramming protocol is advantageous because it requires a single factor to generate tripotent iNSCs from both mouse and human fibroblasts. The miNSCs described here can be passaged more than 40 times, can generate functional neurons with synaptic connections in vitro, and can survive, integrate, and be multipotent in vivo without tumor formation. Furthermore, our results show that our miNSCs are a homogeneous tripotent population, rather than a heterogenous population of different neural progenitor cells. Additionally, retroviral expression of Sox2 in miNSCs is silenced at later passage, suggesting that Sox2-reprogrammed miNSCs have turned on endogenous expression of NSC genes and can maintain a stable cell fate. Finally, our SOX2 reprogramming protocol can reprogram human fibroblasts into hiNSCs that express the typical NSC markers, can self-renew over 20 passages, can generate neurospheres comparable to NSCs derived from human iPSCs, and are tripotent in vitro.

Sox2 functions as a master regulator gene for NSC identity and maintenance, as shown by the fact that knocking down Sox2 expression leads to immediate cell cycle exit and terminal differentiation of NSCs (Bylund et al., 2003; Graham et al., 2003). Thus, it is conceivable that under conditions conducive to NSC expansion, including the presence of growth factors and proper surface and substrates, overexpression of Sox2 can reprogram fibroblasts to multipotent NSCs. If one factor can generate a multipotent population of NSCs from somatic cells, then certain combinations of more lineage-defined factors may generate subtype-specific NSCs, such as motor neuron, dopaminergic neuron, oligodendrocyte, or astrocyte progenitors. Overexpression of specific transcription factors such as Lmx1a in combination with extrinsic factors can bias NSCs toward differentiation into dopaminergic neurons that constitute 75%–90% of the total neuronal cell population (Panman et al., 2011). Thus, Sox2 might be used in combination with such factors to create neural progenitors that can develop into subtype-specific neurons, which would be invaluable for mechanistic studies, drug screening, and potential cell therapies for different neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Generation of iNSCs from Mouse and Human Fibroblasts

MEFs were isolated from wild-type E18 embryos, and 7.5×10^3 to 4×10^5 cells were transduced at passages 1–3 with pMX-Sox2 retrovirus to induce miNSC reprogramming. Sox2-transformed MEFs were cultured in NSC medium supplemented with EGF and FGF2, and NSC-like cells were selected during three rounds of neurosphere suspensions. NSC-like cells were further enriched by monolayer passaging and then characterized for their in vitro and in vivo properties. 7.5×10^3 HFFs at passages 5–8 were transduced with human SOX2 retrovirus to generate hiNSCs by a similar reprogramming procedure. All procedures were approved by the Gladstone Institutes and the University of California, San Francisco, Animal Care and Use Committees. Detailed methods, including iNSC reprogramming protocol, in vitro characterization and differentiation, immunostaining, qRT-PCR, microarray analysis, bisulfite sequencing, electrophysiology, transplantation, and tumorigenesis studies, can be found in Supplemental Information.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE37859.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at doi:10.1016/j.stem.2012.05.018.

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