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DIRECT REPROGRAMMING OF MOUSE AND HUMAN FIBROBLASTS INTO MULTIPOTENT NEURAL STEM CELLS WITH A SINGLE FACTOR

by

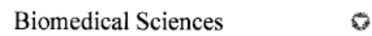
Karen Ring

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in



Biomedical Sciences

in the

GRADUATE DIVISION

of the

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I dedicate my thesis to my parents who were the ones who inspired me to become a scientist and have been my strongest cheerleaders throughout my life and especially during these past five years.

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CONTRIBUTIONS

Chapters 2-4, comprised of the materials and methods and the results sections, are taken directly from my first author publication Ring et al., 2012, which was published in Cell Stem Cell on July 6th (Cell Stem Cell, 2012, 11(1): 100-109). Portions of chapters 1, 5 and 6, which include the introduction, discussion, conclusion, and future directions have also been previously published.

Leslie Tong was the second author on my publication and contributed by helping generate the Sox2 reprogrammed human iNSC lines. Additionally she did the *in vivo* iNSC transplantation experiments into neonatal mice, which was a key experiment that proved that iNSCs were multipotent *in vivo*. Reeny Balestra dedicated a significant portion of her time to helping with the *in vitro* cultures of both our mouse and human iNSC lines. Robyn Javier was an important collaborator from Anatol Krietzer's lab at the Gladstone Institutes, and was responsible for proving the electrical function of our mouse iNSC-derived neurons. Yaisa Andrews-Zwilling helped with various experiments and also contributed intellectually to the success of our publication. Gang Li served as my mentor when I joined the lab and helped design the pilot experiments for the Sox2 reprogramming of mouse fibroblasts to iNSCs. David Walker was instrumental in helping with the molecular biology and epigenetic experiments involved in the paper. William Zhang, a summer undergraduate student, helped with various experiments and data collection. Lastly my mentor and the principle investigator on the publication, Dr. Yadong Huang, came up with the idea for direct reprogramming of fibroblasts to iNSCs, and helped me overcome the many obstacles that I faced when trying to complete this

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The published manuscript was a combined written effort by both Yadong and myself. Drs. Li Gan, Eric Huang, Deepak Srivastava, and Sheng Ding all contributed critical discussions towards the final publication. Finally Stephen Ordway and Gary Howard provided editorial assistance while Linda Turney prepared the manuscript.

ABSTRACT

Directly Reprogramming Mouse and Human Fibroblasts into Multipotent Neural Stem Cells With a Single Factor

Karen Ring

Seminal discoveries in the field of cellular reprogramming have demonstrated that human somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells and other somatic cell types such as induced neuronal (iN) cells by ectopically expressing different combinations of defined factors. The impact of these technologies is already being realized with the generation of patient- and disease-specific iPS and iN cells lines. These valuable tools provide new avenues for basic research and potential transplantation therapies for neurological diseases. However, clinical applications must consider the risk of tumor formation by iPS cells upon transplantation and the inability of iN cells 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 transcription factor, Sox2. iNSCs express neural stem cell (NSC) markers such as Nestin, Sox2, Pax6, and BLBP. They also 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 that Sox2 reprogrammed iNSCs are a homogenous population of multipotent NSCs. Implanted iNSCs can survive and integrate in mouse brains and, unlike iPS cell-derived

NSCs, do not generate tumors. Thus, self-renewable and multipotent iNSCs without tumorigenic potential can be generated directly from both mouse and human fibroblasts by direct reprogramming with a single factor.

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CHAPTER 1

Introduction

Pluripotent Stem Cells and Cellular Reprogramming

Embryonic stem (ES) cells originate from the inner cell mass of the mammalian blastocyst during early embryonic development and represent a pluripotent population of cells that can differentiate into the three germ layers, the endoderm, ectoderm, and mesoderm, and further differentiate into any cell type in the body (Thomson et al., 1998). ES cells are an important source of donor cells for organ regeneration, mechanistic studies and treatment of human diseases, and therapeutic drug testing. They also are used for *in vitro* modeling of developmental processes and for generating cellular and mouse models of human diseases. However, use of ES cells faces substantial obstacles involving ethical concerns about their embryonic origin and immune rejection resulting from allogenic transplantation (Pera, 2008).

In response to concerns about ES cell research, scientists have pursued other sources of pluripotent stem cells. Somatic cell nuclear transfer generates pluripotent stem cells by transplantation of a somatic cell nucleus into an unfertilized, enucleated oocyte (Gurdon, 1962). The donor nucleus is reprogrammed by the oocyte environment back to a pluripotent state, and the egg cell can be induced to divide and generate a blastocyst. The resulting pluripotent stem cells derived from the blastocyst contain the same genetic material as the donor nucleus. Dolly the sheep was the first successfully cloned mammal using this nuclear transfer method (Wilmut et al., 1997). Since then, numerous other mammals have been successfully cloned, however the risk of phenotypic defects is still high due to the observation of chromosomal and other abnormalities in cloned animals

(Thuan et al., 2010). Cell fusion is a similar technique that involves the fusion of a pluripotent stem cell and a somatic cell to generate a pluripotent cell hybrid (Yu et al., 2006a). However this fusion technique has been shown to be highly inefficient and the resulting cells are tetraploid which limits their usefulness in studying pluripotency and cellular reprogramming (Cowan et al., 2005; Hochedlinger & Jaenisch, 2006; Tada et al., 2001). The generation of induced pluripotent stem (iPS) cells from mouse and human somatic cells by ectopic expression of defined factors is the most recent technique for generating pluripotent stem cells (Takahashi & Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). iPS cells are an attractive alternative to ES cells. Like their ES cell counterparts, iPS cells can differentiate into all cell types of the three germ layers *in vitro*, can form teratomas when injected into mice, and can contribute to generation of mouse chimeras (Takahashi & Yamanaka, 2006).

Current Status of Generating iPS Cells

Seminal studies conducted by Shinya Yamanaka and his associates using mouse embryonic and adult fibroblasts demonstrated that somatic cells can be reprogrammed into pluripotent stem cells by ectopic expression of only four factors—Oct4, Sox2, Klf4, and c-Myc (Takahashi & Yamanaka, 2006). Soon after, two different groups (Yamanaka and Thomson) demonstrated that similar approaches worked in human somatic cells using a similar combination of four factors, including Oct4, Sox2, Klf4, c-Myc, Nanog and Lin28 (Takahashi et al., 2007; Yu et al., 2007). Since then, reprogramming technology has been improved in numerous ways, including the use of small molecules and microRNAs to increase reprogramming efficiency (Huangfu et al., 2008; Judson et al., 2009), drug-inducible systems to generate genetically homogeneous iPS cell lines

(Brambrink et al., 2008), and non-integrative methods such as adenoviral vectors, transient plasmid vectors, episomal vectors, synthesized mRNAs, microRNAs, and recombinant proteins (Stadtfeld et al., 2008; Okita et al., 2008; Okita et al., 2011; Anokye-Danso et al., 2011; Warren et al., 2010; Kim et al., 2009; Zhou et al., 2009).

While these techniques can reproducibly generate stable sources of iPS cells, the efficiency of reprogramming still remains quite low at around 1% or below of transfected cells. Originally concerns were raised about the possible presence of stem or progenitor cells in fibroblasts cultures as the contaminating iPS cells but further studies showed that terminally differentiated somatic cells such as B lymphocytes (Loh et al., 2009) and neurons (Kim et al., 2011a) can be reprogrammed to iPS cells. Another hurdle currently faced by the iPS cell field is the differences between iPS cells and their ES cell counterparts. Studies comparing global gene expression and differentiation abilities of iPS cells to ES cells have revealed major differences. Some iPS cell lines carry signature gene expression patterns from the host cell they were derived from (Ghosh et al., 2010; Marchetto et al., 2009). Additionally iPS cell clones derived from the same cell source or patient can have different abilities to differentiate into certain cell lineages (Hu et al., 2010). Thus the field of iPS cell reprogramming is still evolving as scientists attempt to find more efficient and effective ways to generate iPS cells that are indistinguishable from ES cells with the end goal of using these cell lines to study mechanisms of disease and eventually for cell transplantation therapies.

Mechanisms of Cellular Reprogramming

Much effort has focused on understanding the mechanisms involved in somatic cell reprogramming. Oct4, Sox2, Klf4, and c-Myc—the four factors that dedifferentiate

somatic cells into iPS cells—may reactivate pluripotency specific genes while suppressing somatic tissue-specific genes (Jaenisch & Young, 2008). Oct4 and Sox2 are involved in an auto-regulatory loop that includes Nanog, another critical transcription factor that regulates self-renewal of ES cells. These three transcription factors can bind to their own and each other's promoters to activate the pluripotency program (Jaenisch & Young, 2008). These processes are made possible by stochastic epigenetic remodeling events that enable reactivation of silenced pluripotency genes. During development, genes responsible for pluripotency are heavily silenced with suppressive DNA methylation marks and histone modifications to prevent disastrous oncogenic activity in the adult (Reik, 2007). During reprogramming, epigenetic remodeling is required to switch from the somatic cell epigenome to a pluripotent stem cell epigenome. Thus, proper reprogramming requires a combination of exogenous expression of pluripotency promoting transcription factors and stochastic epigenetic remodeling events (Maherali et al., 2007).

Applications of iPS Cells for Regenerative Medicine

Numerous studies have demonstrated the usefulness of human iPS cells for modeling human diseases, testing drugs, and developing cell-based therapies (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008; Soldner et al., 2009; Wernig et al., 2008). Furthermore, iPS technology allows for the generation of patient-specific cell lines—an important step toward personalized regenerative medicine and treatment. A number of disease- and patient-specific iPS cell lines have been established, including those from patients with platelet deficiency (Takayama et al., 2010), spinal cord injury (Nori et al., 2011), macular degeneration (Okamoto and Takahashi, 2011), amyotrophic lateral

sclerosis (Dimos et al., 2008), spinal muscular atrophy (Ebert et al., 2009), Parkinson's disease (Park et al., 2008; Soldner et al., 2011), schizophrenia (Brennand et al., 2011), Huntington's disease (Park et al., 2008), and Alzheimer's disease (Israel et al., 2012). Furthermore, correction of genetic mutations in disease-specific iPS cells 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 and Fanconi anemia (Hanna et al., 2007; Raya et al., 2009).

Besides generating patient and disease-specific cell lines, iPS cells can be used to generate whole organs in different mammals. Kobayashi et al., showed that rat iPS cells transplanted into transgenic mouse blastocysts engineered to lack a gene necessary for pancreatic development were able to differentiate into a functional pancreas (Kobayashi et al., 2010). These types of developments foreshadow a future where iPS cell-derived human organs could be generated in other mammals for transplantation.

Direct Conversion of Somatic Cells to Other Types of Somatic Cells

Exogenous expression of specific developmental regulators can reprogram somatic cells directly into stem cells or can transdifferentiate somatic cells into other somatic cell types. 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). One of the first master regulator genes identified was MyoD, which alone can convert fibroblasts directly into myocytes (Davis et al., 1987). Since then, finding single factors that can convert somatic cells to different lineages has proven to be quite difficult. As a result, scientists have pursued combinations of factors to mediate transdifferentiation and reprogramming of one

differentiated cell type to another. For example, three pancreatic transcription factors, Mafa, Pdx1, and Ngn3, can transdifferentiate mouse pancreatic exocrine cells into insulin-secreting beta cells *in vivo* (Zhou et al., 2008). Mouse mesoderm can be transdifferentiated into cardiac myocytes by ectopic expression of the cardiac transcription factors Gata4 and Tbx5 and the BAF complex subunit Baf60c (Takeuchi & Bruneau, 2009). Similar studies subsequently revealed that three factors including Gata4 and Tbx5 with the addition of Mef2C can reprogram fibroblasts into functional cardiac myocytes *in vitro* (Ieda et al., 2010) and can reprogram cardiac fibroblasts into functional cardiac myocytes *in vivo* (Qian et al., 2012). Several other papers have shown that fibroblasts can be directly converted into hepatocytes (Sekiya and Suzuki, 2011; Huang et al., 2011), hematopoeitic progenitor cells (Szabo et al., 2010), and neurons (Vierbuchen et al., 2010). Lastly, direct reprogramming of somatic cells to the neural lineage is a technology that has received much attention in the past few years as a new way to answer questions about neurodegenerative disease pathogenesis and to generate possible cures for such diseases.

Transdifferentiation of Somatic Cells to Neurons

Initial work by the lab of Marius Wernig revealed that a combination of three factors, Brn2, Ascl1, and Myt1l, can transdifferentiate mouse fibroblasts directly into induced neurons (iNs) (Vierbuchen et al., 2010). Since then, mouse and human fibroblasts and other types of somatic cells such as hepatocytes have been transdifferentiated directly into postmitotic neurons with various 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). iNs have

typical neuronal cell properties and exhibit proper electrical function in culture. Although iNs 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 iNs 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). For example, the combination of Nurr1, Lmx1a, and Ascl1, can reprogram mouse and human fibroblasts directly into functional dopaminergic neurons that release dopamine (Caiazzo et al., 2011). Spinal motor neurons can be generated by expressing the BAM cocktail and four additional factors, Lhx3, Hb9, Isl1, and Ngn2 (Son et al., 2011). In the near future, it can be expected that different combinations of factors will be identified that will generate other subtypes of induced neurons such as forebrain neurons or inhibitory interneurons. However, iN technology is still limited by the fact that iNs are terminally differentiated and cannot self-renew.

Reprogramming somatic cells to neural stem cells

To address some of the obstacles face by iN technology, scientists have attempted to reprogram somatic cells to a self-renewing and multipotent neural progenitor state. The first study to do so was by Kim et al. in 2011. The authors 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., 2011c). However, these iNPCs could only self-renew for 3–5 passages in culture and were not shown to differentiate into oligodendrocytes. In a separate study, a combination of nine factors

reprogramed Sertoli cells into iNSCs (Sheng et al., 2011). 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 that 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, c-Myc, and limited Oct3/4 expression, or Brn4, Sox2, Klf4, c-Myc, and E47/Tcf3 can reprogram mouse fibroblasts into iNSCs (Thier et al., 2012; Han et al., 2012). While 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 iPS cell-derived NSCs (Okita et al., 2008).

The studies mentioned above firmly establish the fact that somatic cells can be reprogrammed directly into induced neural cells using various combinations of factors. However, no published study has shown that a single factor can reprogram somatic cells into NSCs. Thus, the generation of iNSCs from MEFs by ectopic expression of Sox2 alone, the findings of which I detail in this dissertation, is an important discovery to the field of iNSC reprogramming.

NSC Biology and Neurogenesis

NSCs are multipotent stem cells that reside in the developing brain and in two main neurogenic niches of the adult brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Miller & Gauthier-Fisher, 2009). NSCs can proliferate and self-renew and can generate all three neural lineage cells—neurons, astrocytes, and oligodendrocytes. Certain transcription factors are essential for NSC identity, including Sox2, Bmi1, TLX, and Hes1. Sox2, a SRY-related HMG-box transcription factor, is highly expressed in NSCs and is required for NSC self-renewal, maintenance, and inhibition of neuronal differentiation (Episkopou, 2005; Ferri et al., 2004). Knock down of Sox2 expression in NSCs and neural progenitor cells causes immediate cell cycle exit and terminal differentiation (Bylund et al., 2003; Graham et al., 2003). Bmi1, a polycomb transcriptional repressor, is required for NSC proliferation, self-renewal, and postnatal maintenance of NSCs in the central and peripheral nervous systems; it prevents premature senescence of NSCs by repressing the $p16^{Ink4a}$ and $p19^{Arf}$ senescence pathways (Bruggeman et al., 2005; Molofsky et al., 2005). TLX is an orphan nuclear receptor whose NSC maintenance and self-renewal properties resemble those of Sox2 and Bmi1; it also inhibits neuronal differentiation by regulating cell-cycle progression and exit (Shi et al., 2004). Finally, Hes1 is a repressor-type bHLH transcription factor that regulates NSC maintenance and directly inhibits Mash1, a gene that promotes neuronal differentiation (Kageyama et al., 2005). Loss of any of these transcription factors results in impaired NSC maintenance and self-renewal, cell-cycle exit, and premature differentiation of NSCs—events that contribute to a decrease in neurogenesis (Shi et al., 2008).

NSCs can be isolated from mouse and human brains and expanded in suspension cultures as neurospheres in the presence of growth factors, including epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2) (Gage, 2000; Reynolds & Weiss, 1992; Gritti et al., 1996). Adherent monolayer cultures of NSCs can also be generated by dissociating neurospheres to single cells (Wachs et al., 2003). NSCs cultured *in vitro* express specific markers such as Sox2, Nestin, and Musashi1 (Graham et al., 2003; Lendahl et al., 1990; Sakakibara et al., 1996). Like their endogenous counterparts, they can proliferate and self-renew (in the presence of growth factors) and differentiate into all three neural lineages (upon growth factor withdrawal).

NSC fate specification is a subject of great interest that is not fully understood. Development and plasticity of NSCs are largely influenced by intrinsic and extrinsic factors that control gene expression programs and therefore cell fate and function (Ma et al., 2009). Chromatin remodeling and epigenetic modifications, including DNA methylation and histone modifications, are intrinsic events that play major roles in NSC fate specification by regulating at the level of gene transcription (Namihira et al., 2008; Hsieh & Gage, 2005). Both the SVZ and the SGZ neurogenic niches harbor a plethora of extrinsic cues that promote NSC maintenance and self-renewal. Signaling molecules such as Wnt, sonic hedgehog, bone morphogenic proteins and their antagonists, Notch, transforming growth factor-alpha, leukemia inhibitory factor (LIF), and cytokines as well as growth factors such as FGFs and neurotrophins all have direct or indirect effects on NSC fate specification in developing brains and in the SVZ and the SGZ of adult brains (Ma et al., 2009).

Significance of Directly Reprogramming Somatic Cells into NSCs

Direct reprogramming of somatic cells into multipotent stem cells will complement current iPS cell technology and sidestep the difficulties associated with differentiating iPS cells *in* vitro and potential teratoma formation *in vivo*. Establishing lineage-specific somatic stem cell lines directly from human somatic cells will be useful for the treatment of many serious diseases. In the case of neurodegenerative disorders, patients suffer from loss of function or even death of a specific subset of neural cells. For example, patients with PD suffer from loss of dopaminergic neurons in the substantia nigra, and patients with ALS suffer from motor neuron degeneration in the central nervous system. At present, these diseases are incurable, and treatments are palliative. NSC transplantation is a promising stem cell therapy that aims to replace diseased or dying cell populations in the brain (Gage, 2000). It is also being pursued to treat acute neurological diseases, such as stroke and traumatic brain injury (Jeong et al., 2003; Lim et al., 2002). Thus developing patient-specific iNSC lines would improve current transplant therapies by circumventing immunological transplant rejection. Furthermore, in the case of genetic diseases, patient-specific iNSCs could be corrected for disease-causing mutations and transplanted back as healthy cells that could repopulate damaged areas of the brain.

CHAPTER 2

Materials and Methods

Reagents and Cell Culture

All cells were cultured on tissue culture dishes or plates coated with 0.1% gelatin, 1 μg/ml laminin, or a combination of both substrates. Mouse iNSCs and wild-type NSCs were cultured in NSC basal medium (Millipore) supplemented with 20 ng/ml FGF-2, 20 ng/ml EGF, and 2 μg/ml heparin (NSC-BM+++). NSC basal medium contains Dulbecco's modified Eagle's medium (DMEM)/F12 with 1X B27 CHEM, 2 mM L-glutamine, and 1X penicillin/streptomycin without HEPES. Human iNSCs and NPCs derived from human iPS cells were culture in ReNcell medium (Millipore) supplemented with 20 ng/ml human FGF-2 and 20 ng/ml human EGF (ReNcell++). ReNcell medium consists of DMEM/F12 w/o HEPES, w/ L-Glutamine, L-glutamine, human serum albumin, human transferrin, putrescine dihydrochloride, human recombinant insulin, L-thyroxine, tri-iodo-thyronine, progesterone, sodium selenite, heparin, and corticosterone. Mouse and human iNSCs and wild-type NSCs were passaged with Accutase (Chemicon). Mitotically inactive mitomycin C-treated mouse STO feeder cells, MEFs, and HFFs (System Biosciences), were cultured in DMEM with high glucose (Gibco) containing 10% heat-inactivated FBS (Life Technologies), 50 U penicillin, and 50 mg/ml streptomycin. Plat-E packaging cells were cultured in the same medium with puromycin (1 μg/ml) and blasticidin S (10 μg/ml).

Three wild-type mouse NSC lines were used in this study. The SCR029 cell line, which are cortical NSCs derived from E14-18 embryos, were purchased from Chemicon and have been well characterized. Wild-type brain-derived NSCs were generated in our

lab from E14.5 cortical primary neuron preps, as reported (Pacey et al., 2006). This cell line was characterized in our lab and found to express proper NSC markers such as Sox2 and Nestin and differentiate into all three neural lineages *in vitro*. Lastly, wild-type NSCs derived from mouse iPS cells (Clone-WT.9 and Clone-411) were generated and characterized in our lab using previously established protocols (Conti et al., 2005). Wild-type human NSCs were derived from human iPS cells in our lab using previously established protocols (Conti et al., 2005).

Sox2 Retroviral Reprogramming of Mouse and Human Fibroblasts

Mouse iNSCs were generated from wild-type MEFs (at passages 1–3) by retroviral transduction of the transcription factor Sox2. MEFs were isolated from E18 wild-type mouse embryos. Glass coverslips (Fisher Scientific) were placed in wells of a 24-well culture plate and coated with 0.1% gelatin (Sigma) for 30 minutes at 37°C. Extra gelatin was removed, and mitomycin C–treated mouse STO feeder cells were plated at $1.25x10^5$ cells/well into all wells in DMEM +10% FBS. The next day, wild-type MEFs were plated at $7.5x10^3$ to $4x10^5$ cells/well onto the layer of mitomycin C–treated mouse STO feeder cells. One day later, Sox2 retroviral medium was collected and filtered through a 0.45-μm filter (Millipore), and Polybrene (Sigma) was added (final concentration, 4 μg/ml). The pMX-Sox2 retroviral plasmid was a gift from the Yamanaka lab and Sox2 retroviral medium was generated as described (Takahashi et al., 2007). Sox2 retroviral medium was mixed with an equal volume of regular DMEM + 10% FBS. MEFs were then transduced with 500 μl of Sox2 retrovirus for 24 hours and then cultured in NSC-BM+++. The culture medium was changed fully every day. Within 3–4 days after transduction, fibroblast morphology had changed considerably. By six days after transduction, mature

networks consisting of thin chains of elongated cells were observed along with small colonies of round, bright cells at the intersections of these networks. These colonies grew and expanded rapidly during days 7–10. The cells were then collected with Accutase (Millipore) and transferred to a fresh six-well plate. At confluency, cells were resuspended in 60-mm Petri dishes with NSC-BM+++ for primary neurosphere formation. After culturing for 5–7 days, spheres were collected by gravity and plated onto gelatin-coated six-well plates for monolayer expansion. At confluency, cells were harvested and resuspended for a second round of neurosphere formation. After three rounds of neurosphere formation, the cells were passaged in monolayer cultures in tissue culture coated plates in NSC-BM+++ every 3–5 days.

For reprogramming of HFFs, human SOX2 (hSOX2) retrovirus was prepared similarly to mouse retrovirus. Fugene transfection was conducted with 8 μg of pMX-hSOX2 and 1 μg of pCMV-VSVG plasmids. STO feeder cells were plated at $1.25x10^5$ cells/well in 24-well plates. The next day, HFF cells (at passages 5-8) were seeded at $7.5x10^3$ cells/well in DMEM + 10% FBS. Twenty-four hours later, the medium was replaced with 250 μl of hSOX2 viral medium and 250 μl of DMEM + 10% FBS. The following day, the viral medium was removed and replaced with DMEM +10% FBS. On Day 5, the medium was changed to ReNcell with human recombinant basic FGF2 and EGF, both at 20 ng/ml. Over the next 7 days, reprogrammed cells began generating floating spheres, which were collected and plated in Petri dishes with growth factor medium. These spheres were cultured in ReNcell++ for at least 5 days and transferred to laminin-coated plates for monolayer outgrowth. Reprogrammed cells were subjected to

three rounds of neurosphere formation and monolayer passaging, as described for the miNSC cell lines.

Determining miNSC Reprogramming Efficiency

MEFs were seeded on feeder cell layers on either gelatin-coated glass coverslips at $1.25x10^4$ cells/well or poly-L-ornithine/laminin-coated glass coverslips at $7.5x10^3$ cells/well, infected with Sox2 retrovirus for 24 hours as described above, cultured in NSC-BM+++, and fixed with paraformaldehyde at days 8 and 12. The cells were immunostained for Nestin and Sox2, and the number of double-positive colonies was counted (five coverslips per day). The efficiency of colony formation was determined by dividing the average number of double-positive colonies per coverslip by the total starting number of MEFs per well.

NSC Neurosphere Formation

Neurosphere formation was assayed for iNSCs and wild-type NSCs by resuspending $2.0x10^4$ cells in growth medium (NSC-BM+++ or ReNcell++) in a 60-mm bacterial culture dish (non-tissue culture coated). Fresh medium was added each day to the suspension cultures. Six days after suspension, neurospheres were collected and counted with a light microscope.

Bisulfite Sequencing and DNA Methylation Analysis

Genomic DNA was isolated from the following cell lines, miNSC-A21, wild-type SCR029 NSC, and wild-type MEFs, using standard procedures. Bisulfite treatment was conducted for all three samples using the EpiTect Bisulfite Kit (Qiagen). Previously published nested primer sets were used to amplify the promoter regions of the following

genes: Sox2, Nestin, and Oct3/4 (Han et al, 2009; Imamura et al., 2006; Western et al., 2010). PCR products were subcloned into the PCR 2.1 vector (Invitrogen) and clones that contained inserts were purified using the QIAprep Spin Miniprep kit (Qiagen). Individual clones (10 or more) were sequenced (Elim Biosciences) and only clones with over 90% bisulfite conversion were accepted. Bisulfite conversion of CpG was scored using the online software QUMA or Quantification tool for Methylation Analysis (Kumaki et al., 2008).

Gene Expression Microarray Analysis of iNSCs

Mouse iNSCs and wild-type NSCs were cultured in NSC basal medium containing EGF (20 ng/ml), FGF-2 (20 ng/ml), and heparin (2 μg/ml). MEFs were cultured in DMEM+10% FBS. All cells were washed with serum-free medium before collection. Trizol extraction of total RNA was performed according to the manufacturer's instructions. The extracted RNA was further purified with a Qiagen RNeasy Mini Kit. Biotinylated cRNA was prepared according to the standard Affymetrix protocol of 300 ng total RNA according to the Expression Analysis Technical Manual of Affymetrix. Following fragmentation, 5.5 μg of cRNA were hybridized for 18 hr at 45°C on an Affymetrix Mouse Gene 1.0 ST Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 400, scanned using the GeneArray Scanner GCS3000, and processed using GCOS1.4. Raw intensities from the CEL files were analyzed using Affymetrix Power Tools (APT, version 1.10.1) to generate a robust multi-array average (RMA) intensity on a log2 scale for each probe set and various quality metrics. The perfect match (PM) intensities per probe set were background corrected, quantile-

normalized to make the distribution of intensities the same for all arrays, and summarized for each probe set using a robust fit of linear models.

Neuronal, Astrocytic, and Oligodendrocytic Differentiation of iNSCs.

Mouse neurons, astrocytes, and oligodendrocytes were generated by plating $5x10^3$ to $1.5x10^4$ miNSCs onto laminin/gelatin-coated glass coverslips in 24-wells containing NSC-BM+++. After 24 hours, the medium was switched to NSC-BM without growth factors to induce differentiation of NSCs into all three types of neural cells by 2–4 weeks after plating (Hsieh et al., 2004). Robust astrogenesis was induced by adding BMP4 (50 ng/ml; R&D Biosystems) or 1% FBS to NSC-BM without growth factors (Gross et al., 1996).

Human iNSCs were differentiated in ReNcell medium without growth factors. hiNSCs were seeded at $1.0–1.5x10^3$ cells/well on laminin/gelatin-coated coverslips in ReNcell++. Two days later, the medium was switched to non-growth factor medium, which was changed every 3–4 days. Between 1 and 2 months of differentiation, the cultures were immunostained to identify neurons, astrocytes, and oligodendrocytes. Specific differentiation conditions included neuronal induction with retinoic acid (1 µM) plus forskolin (5 µM) (Hsieh et al., 2004) or WNT5A (100 ng/ml; R&D) (Yu et al., 2006b) and astrocyte induction with BMP4 (50 ng/ml) (Gross et al., 1996).

Immunocytochemistry and Image Collection

Cells on glass coverslips were fixed in 4% paraformaldehyde for 15 minutes at room temperature and washed with PBS. Nonspecific antibody binding was blocked, and cells were permeabilized with 10% normal donkey serum (Jackson ImmunoResearch)

containing 0.2% Triton X-100 (Sigma) in PBS (PBS-T) for 1 hour at room temperature. Cells were rinsed and then incubated in primary antibody containing 3% normal donkey serum in PBS-T overnight at 4°C. After washing in PBS, cells were incubated in secondary antibody containing 3% normal donkey serum in PBS-T for 1 hour at room temperature. Cells were immunostained with the following primary antibodies: monoclonal mouse anti-Nestin (1:200; Chemicon), monoclonal mouse anti-Nestin human specific (1:200, Chemicon), polyclonal goat anti-Sox2 (1:400; Santa Cruz Biotechnology), monoclonal mouse anti-Pax6 (1:100, DSHB), polyclonal rabbit anti-BLBP (1:300, Chemicon), monoclonal mouse anti-βIII tubulin (1:800; Promega), polyclonal rabbit anti-MAP2 (1:250; Chemicon), monoclonal mouse anti-MAP2 (1:100; Chemicon), polyclonal rabbit anti-Tau (1:1000, Sigma), monoclonal mouse anti-vGlut1 (1:100; Chemicon), polyclonal rabbit anti-GABA (1:1000, Sigma), polyclonal rabbit anti-Synapsin (1:1000; Chemicon); polyclonal rabbit anti-Olig2 (1:500, Chemicon), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP, 1:400; DakoCytomation), and monoclonal mouse anti-O4 (1:50; Chemicon). Primary antibodies were detected with the following fluorescently tagged secondary antibodies: donkey anti-rabbit, donkey anti-goat, or donkey anti-mouse Alexa488 IgG, Alexa594 IgG, Alexa647 IgG, or Alexa488 IgM (1:1000; Invitrogen). Coverslips with stained cells were mounted on glass slides in VectaShield mounting medium that contained DAPI. Stained cells were examined with a Radiance 2000 laser-scanning confocal system (Bio-Rad) mounted on a Nikon Optiphot-2 microscope. Images were processed with Photoshop CS (Adobe Systems).

RNA Isolation and Real-Time qRT-PCR Analysis

Total RNA was isolated from mouse and human cell lines with the RNeasy Mini Kit (Qiagen) with DNase I digestion (Sigma). Complementary DNA was generated from 1–3 μg of total RNA with the RT^2 First Strand Kit (Qiagen). Real-time qPCR was performed with custom mouse and human RT^2 Profiler 96-well PCR arrays (SABiosciences) and SYBR Green. See Table 1 for PCR Array gene lists. For qPCR of endogenous and exogenous Sox2 expression, previously published primer sets were used (Takahashi and Yamanaka, 2006).

Table 1. Customized quantitative RT-PCR 96-well array gene list.

Custom Mouse RT^2Profiler PCR Array

Symbol	GenBank	Description
Pou5f1	NM_013633	POU domain, class 5, transcription factor 1
Nanog	NM_028016	Nanog homeobox
Zfp42	NM_009556	Zing finger protein 42
Sox2	NM_011443	SRY box containing gene 2
Nes	NM_016701	Nestin
Pax6	NM_013627	Paired box gene 6
Sox1	NM_009233	SRY-box containing gene 1
Zbtb16	NM_001033324	Zinc finger and BTB domain containing 16
Msi1	NM_008629	Musashi homolog 1 (Drosophila)
Gfap	NM_010277	Glial fibrillary acidic protein
Mtap2	NM_001039934	Microtubule-associated protein 2
Neurod1	NM_010894	Neurogenic differentiation 1
Gapdh	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase
MGDC	SA_00106	Mouse Genomic DNA Contamination
RTC	SA_00104	Reverse Transcription Control
PPC	SA_00103	Positive PCR Control

Custom Human RT^2Profiler PCR Array

Symbol	GenBank	Description
POU5F1	NM_002701	POU class 5 homeobox 1
NANOG	NM_024865	Nanog homeobox
ZFP42	NM_174900	Zing finger protein 42 homolog (mouse)
SOX2	NM_003106	SRY (sex determining region Y)-box 2
NES	NM_006617	Nestin
PAX6	NM_000280	Paired box 6
SOX1	NM_005986	SRY (sex determining region Y)-box 1
ZBTB16	NM_006006	Zinc finger and BTB domain containing 16
MSI1	NM_002442	Musashi homolog 1 (Drosophila)
GFAP	NM_002055	Glial fibrillary acidic protein
MAP2	NM_002374	Microtubule-associated protein 2
NEUROD1	NM_002500	Neurogenic differentiation 1
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase
HGDC	SA_00105	Human Genomic DNA Contamination
RTC	SA_00104	Reverse Transcription Control
PPC	SA_00103	Positive PCR Control

Electrophysiology Studies

miNSCs were differentiated for 2–4 weeks on laminin-coated coverslips in NSC-BM without EGF or FGF2 and switched to primary neuron media 24 hours before being transferred to a fixed stage for electrophysiology experiments. Primary neuron medium consists of Neural Basal medium (Gibco), 1X B27 CHEM, 1% Pen/Strep, and 1X Glutamax. Whole-cell patch clamp recordings were obtained from visually identified cells with neuronal-like processes. Coverslips were immersed in a HEPES-buffered saline solution containing (in mM) 115 NaCl, 2 KCl, 10 HEPES, 1.5 MgCl2, 3 CaCl, and 10 glucose. All experiments were performed at room temperature. Resistance of borosilicate glass micropipettes was 2-4 MOhm when filled with the following (in mM): 130 KMeSO3, 10 NaCl, 2 MgCl2, 0.16 CaCl2, 10 HEPES, and 0.5 EGTA. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices), filtered at 2 kHz, and digitized at 10 kHz. Whole-cell capacitance and membrane resistance were determined from a transient 5mV hyperpolarizing step from a holding potential of –70mV. Data were acquired and analyzed online with custom Igor Pro software.

In Vivo iNSC Studies

For *in vivo* integration and differentiation studies, GFP-labeled miNSC-A21 were grown in suspension dishes in NSC-BM plus growth factors for 24 hours to generate small neurospheres. Neurospheres were collected and microinjected into the cortices of P2–3 pups (CD1 genetic background). Pups were anesthetized on ice and placed in a customized head mold. ~$3x10^4$ cells in 50 nl volumes were injected into 4 sites on a single hemisphere between bregma and lambda at a 25 degree angle towards the midline at a depth of 0.26 mm using a Nanoject by Drummond on a Kopf stereotaxic frame.

Brains were collected at 1, 2, and 5 days post injection following a saline perfusion, fixed in 4% paraformaldehyde for 24 hours, washed with PBS, soaked in 30% sucrose for 48 hours and sectioned into 50-µm coronal sections with a sliding microtome. Brain sections were immunostained for NeuN (neurons), GFAP (astrocytes), and Olig2 (oligodendrocytes) using methods described previously.

Tumorigenesis studies were conducted as follows. miNSC, wild-type NSC, and iPS cell-derived NSC lines, as well as hiNSC lines were cultured simultaneously and harvested for stereotaxic injection. Cells were resuspended at 1.0×10^3 cells/µl in PBS and incubated on ice. Mice were anesthetized using standard procedure. Cell injections were targeted to the hippocampus of 3–12-month-old NOD/SCID mice (Jackson Laboratory) (x: ± 1.5 mm, y: 2.1 mm, z: 2.1 mm). Side-by side-injections into the hippocampus were performed with implanted bilateral cannulas held in place by an adaptor. Cells were delivered to the hippocampal area via an injector that was inserted into the cannula (0.1 µl/min, 1 µl volume per side). After transplantation, the cannula was slowly and carefully removed, and the heads were closed up. Injected mice were euthanized at 4–6 weeks and perfused with saline. Brains were fixed in 4% paraformaldehyde for 48 hours, washed with PBS, incubated in 30% sucrose for 48 hours, and cut with a sliding microtome into 30-µm coronal sections. The slices were then immunostained for neural stem cell, neuronal, and glial markers to determine the presence of tumors.

Statistical Analyses

Values are expressed as mean ± SD. Differences between means were assessed by *t* test or analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

CHAPTER 3

Generating and Characterizing Mouse Induced Neural Stem Cells

Generation of iNSCs from Mouse Fibroblasts

The protocol for generating iNSCs from mouse embryonic fibroblasts (MEFs) is shown in Figure 1A. In choosing the reprogramming factors, we considered five key transcription factors—Sox2, Bmi-1, TLX, Hes1, and Oct1. Sox2, Bmi-1, TLX and Hes1 are key transcription factors that are highly expressed in NSCs and are important in NSC production, maintenance, self-renewal, and proliferation (Graham et al., 2003; Jin et al., 2009; Kageyama et al., 2008; Molofsky et al., 2003; Shi et al., 2004; Suh et al., 2007). Oct1 is a POU class 2 transcription factor that is expressed in the developing neural tube, binds to the Nestin enhancer during embryonic development, and directly interacts with Sox2 on the transcriptional level (Jin et al., 2009; Williams et al., 2004). These transcription factors were expressed individually or in different combinations in MEFs by retrovirus-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 (Figure 1A-Step 1 and Figure S1C). However, when MEFs were cultured on glass coverslips coated with gelatin, their morphology was drastically altered by retroviral Sox2 alone (Figures 1A-Step 2, 1B, 1C and Figure S1A) or by Sox2 plus additional transcription factors. Since 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 and established colony-like cell clusters at the intersections of these networks (Figure 1A-Step 2, Figure 1C, and Figure S1A). Many of these clusters were positive for Sox2 and Nestin, as shown by immunofluorescence staining (Figure 1D and Figure S1B), suggesting that they expressed Sox2 and began to express the NSC marker Nestin. The efficiency of generating Sox2 and Nestin double-positive colony-like clusters on gelatin-coated coverslips was 0.13% at day 8 and 0.52% at day 12 (Table 2). However, most cells along the networks were negative for Sox2 and Nestin (Figure 1D and Figure S1B). Additionally, the efficiency of generating Sox2 and Nestin double-positive colony-like clusters was enhanced to 0.96% at 8 days post infection by culturing infected cells on Poly-L-Ornithine- and Laminin-coated coverslips (Table 2). This alternate coating condition is known to be more conducive to the growth and passage of NSCs *in vitro* (Lee et al., 2007). Furthermore, immunostaining of Sox2-infected cells 14 days post infection in NSC-media 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 3). Importantly, no morphological changes and no Nestin- or Sox2-positive cells were observed in MEFs not transduced with Sox2 (Figure 1E) or MEFs cultured on gelatin-coated plastic up to 4 weeks after retroviral Sox2 transduction (Figure S1C). Furthermore, untransfected MEFs did not stain positive for the differentiated cell markers MAP2, GFAP, and O4 (Table 3). We therefore concluded that there were no contaminating neural progenitor, neural crest, neuronal or glial cells in our MEF cell populations.

Six to ten days after retroviral Sox2 transduction, cell mixtures containing multiple colony-like clusters were collected and re-cultured in gelatin-coated six-well plates without glass coverslips to promote cell proliferation and expansion (Figure 1A-Step 3). At this stage, the cells represented a mixture with different morphologies and were thought to contain untransformed MEFs, partially reprogrammed cells, and potentially fully reprogrammed NSCs (Figure 1F). Five days later, Sox2-infected cells were released for primary neurosphere culture in suspension to select for NSC-like cells (Figure 1A-Step 4 and Figure S1D). The primary neurospheres were seeded into gelatin-coated six-well plates (Figure 1A-Step 5), and cells with NSC-like morphology grew gradually from adhered neurospheres (Figures S1E and S1F). To further enrich and purify potentially reprogrammed NSC-like cells, we repeated the neurosphere culture procedures twice (second and third neurospheres, Figure 1A-Steps 4 and 5). After the third neurosphere culture, the NSC-like cells were grown in monolayer culture for many generations (Figure 1A-Step 6 and 1G). During the first few monolayer passages, there was a mixture of NSC-like cells (majority) and cells with various other morphologies (minority). However, by five passages, most cells with varying morphologies were lost, and NSC-like cells with bipolar morphology were predominant. Further passaging as a monolayer in NSC medium with growth factors resulted in a homogenous population of NSC-like cells. The reprogrammed NSC-like cells at passages 8 (Figure S1G), 11 (Figure 1H), and 28 (Figure 1I) had morphologies very similar to those of wild-type mouse NSCs (Figure 1J and Figure S1H). Similar results were obtained from independent reprogramming experiments using the same protocol (Figures S1I–S1K), demonstrating the repeatability of this reprogramming method.

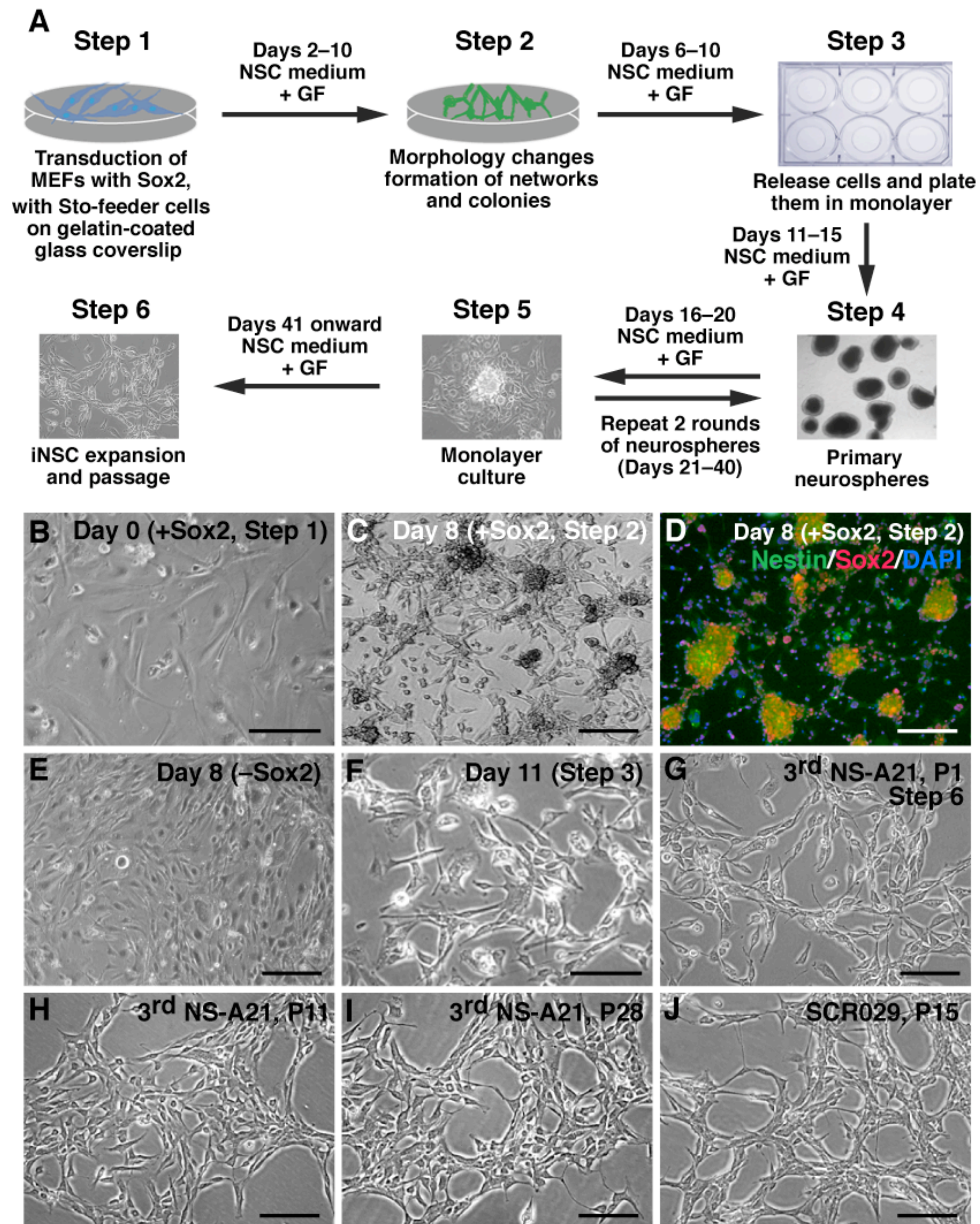

Figure 1. Generation of iNSCs directly from mouse fibroblasts by retroviral expression of Sox2.

(A) Schematic describing the retroviral Sox2 reprogramming protocol. MEFs were infected with Sox2 retrovirus and cultured in NSC medium with growth factors on gelatin-coated glass coverslips for 6–10 days and subjected to three rounds of neurosphere suspension to isolate and enrich reprogrammed NSC-like cells. NSC-like

cells were enriched by further passaging in monolayer culture in NSC medium with growth factors. **(B)** Phase-contrast image of MEFs after overnight treatment with Sox2 retrovirus in fibroblast medium. **(C)** Sox2-infected cells in NSC medium with growth factors generate networks and colonies on gelatin-coated glass coverslips by 8 days after infection. **(D)** Sox2-transformed colonies are positive for the NSC markers Nestin and Sox2. **(E)** Fibroblasts cultured in NSC medium with growth factors but without Sox2 retroviral transduction do not generate colonies or networks. **(F)** Sox2-transduced cells after 11 days have drastically different morphology from their fibroblast counterparts. **(G)** After three rounds of neurosphere generation, reprogrammed cells take on the characteristic bipolar NSC morphology. **(H)** After multiple passages as a monolayer, NSC-like cells are a morphologically homogenous population. **(I)** Morphology of NSC-like cells stays the same over prolonged passaging, and reprogrammed cells can proliferate over 28 passages. **(J)** The morphology of NSC-like cells is similar to that of wild-type cortical-derived NSCs such as the commercial cell line SCR029 (Millipore).
Scale bars = 50 μm in B and E–J; scale bars = 100 μm in C and D.

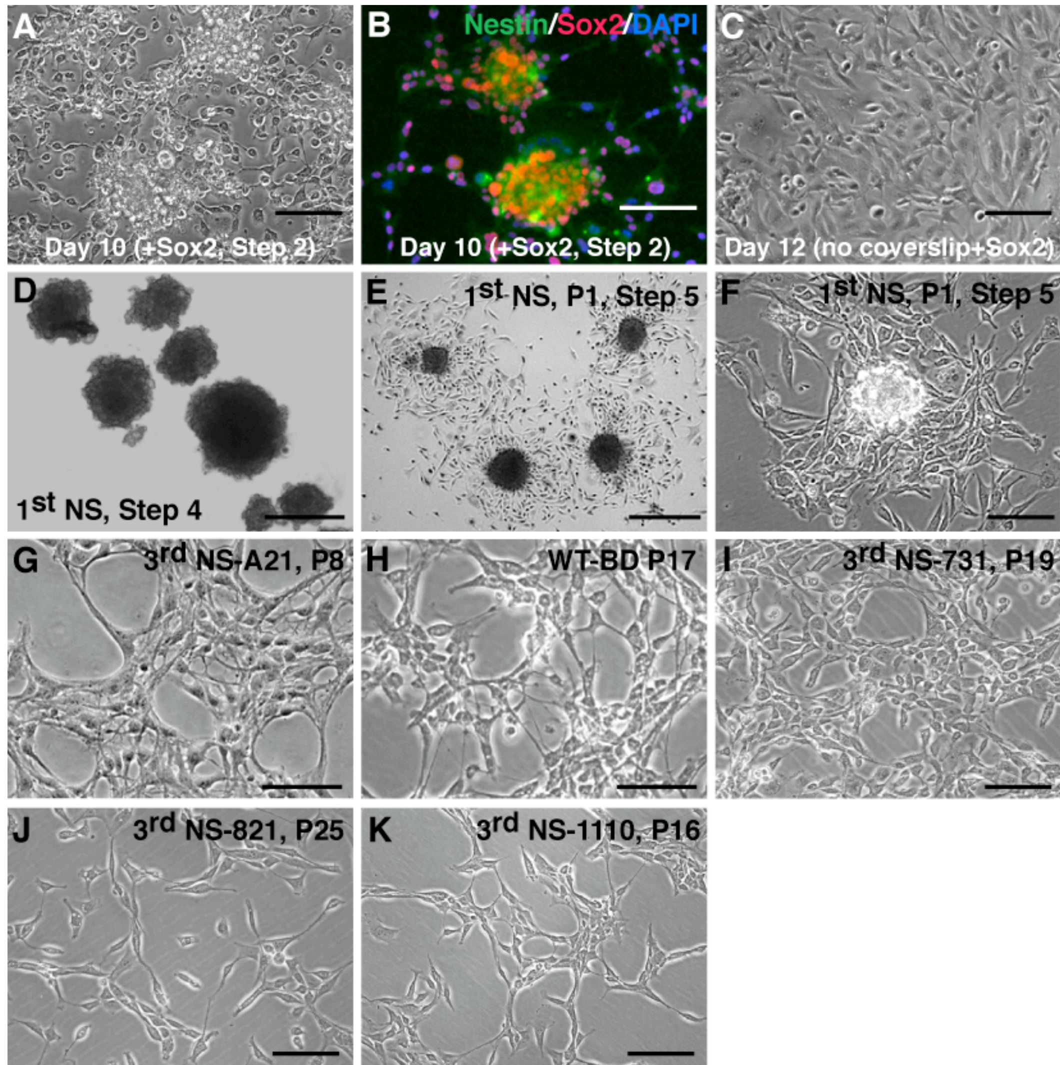

Figure 2. Sox2 reprogrammed mouse iNSCs from mouse fibroblasts at different stages of reprogramming.

(A) Sox2-infected cells in NSC medium with growth factors generate colonies on gelatin-coated glass coverslips by 10 days post infection. **(B)** Zoomed in image of Nestin- and Sox2-positive colonies that are infected with Sox2 retrovirus at 10 days in NSC medium with growth factors. **(C)** Fibroblasts in NSC medium with growth factors for 12 days infected with Sox2 retrovirus but lacking coverslips do not generate networks or colonies and therefore do not generate NSC-like cells. **(D)** NSC-like cells are isolated and

expanded by culturing Sox2-infected cells in suspension dishes to generate primary neurospheres. **(E and F)** Outgrowth of NSC-like cells from neurospheres in gelatin-coated tissue culture dishes is characteristic of NSC outgrowth from wild-type neurospheres. **(G)** One batch of NSC-like cells, A21, after three rounds of neurosphere formation and cultured to 8 passages as a monolayer. **(H)** NSCs derived from wild-type mouse brains at E14.5 mice are shown at passage 17 in a monolayer culture. **(I–K)** Morphology of different batches of reprogrammed NSC lines at various passages. Scale bars = 50 μm in A–C and F–K; scale bars = 100 μm in D and E.

Table 2. Efficiency of generating Nestin$^+$/Sox2$^+$ colonies from MEFs using retroviral Sox2 on different substrate-coated glass coverslips.

Substrate	Day	Coverslips Counted	Average # of Nestin$^+$/Sox2$^+$ Colonies	Starting MEFs Per Well	Efficiency of Colony Formation
Gelatin	8	5	16	1.25×10^4	0.13%
Gelatin	12	5	65.2	1.25×10^4	0.52%
Poly-L-Ornithine/Laminin	8	5	72.2	7.5×10^3	0.96%

Table 3. Neural marker immunostaining of MEFs with or without Sox2 retroviral infection in NSC media with growth factors.

Condition	**Days *in vitro***	**Map2**	**GFAP**	**O4**	**Nestin**	**Sox2**
MEFs + Sox2 retrovirus	14	–	–	–	+	+
MEFs – Sox2 retrovirus	14–28	–	–	–	–	–

Characterization of iNSCs from Mouse Fibroblasts

The reprogrammed mouse NSC-like cells expressed NSC markers, including Sox2 and Nestin, similarly to the wild-type cortical mouse NSC line, SCR029 (Chemicon) (Figures 2A-2D) as well as Pax6, and BLBP, as shown by immunostaining (Figures S2A–S2D). Quantitative real-time RT-PCR (qRT-PCR) confirmed that NSC-like cells express the NSC marker genes Sox2 and Nestin, and additionally expressed Sox1 and Zbtb16 (Figure 1E); however, even at low passages, they did not express pluripotency-related genes, such as Oct4, Nanog, and Zfp42 (Figure 1F), nor did they express Oct4 or Nanog during the early reprogramming stages. 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), suggesting that simply culturing MEFs in NSC medium does not automatically turn on NSC-related gene expression. We next determined whether reprogrammed NSC-like cells had silenced retroviral exogenous Sox2 expression and turned on endogenous Sox2 expression similarly to the wild-type NSCs. We conducted qRT-PCR on reprogrammed cells at early passage (P7) and later passage (P16) and compared the exogenous and endogenous Sox2 expression to wild-type NSCs (Figure S2I). Interestingly, endogenous Sox2-specific qRT-PCR detected low level 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 at passage 16 (Figure S2I), suggesting that the endogenous Sox2 gene was gradually turned on in Sox2-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 have true endogenous expression of NSC genes and 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 to determine whether miNSCs had properly activated NSC genes, as indicated by hypomethylated CpG, and kept pluripotency genes silenced, as indicated by hypermethylated CpG. miNSCs at passage 12 were compared to wild-type NSCs at passage 17 and MEFs at passage 2 (Han et al., 2009; Imamura et al., 2006, Western et al., 2010). Methylation analysis of bisulfite-treated DNA from miNSCs, wild-type NSCs, and MEFs revealed that the Oct3/4 promoter was hypermethylated, indicating the transcriptional silencing of that gene (Figure S2J–S2L). In contrast, both the Sox2 and Nestin promoters were hypomethylated in miNSCs similarly to wild-type NSCs, indicating that these genes are transcriptionally activated (Figure S2M–S2P). Thus, miNSCs appropriately activated the transcription of NSC-related genes and keep pluripotency genes, such as Oct3/4, silenced.

Microarray studies demonstrated that the global gene expression pattern of the reprogrammed mouse 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 mouse NSC-like cells formed neurospheres in suspension cultures and do so with similar efficiency (Figures 2G–2I). Taken together, these data strongly suggest that a single factor can reprogram MEFs into self-renewing NSCs that

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

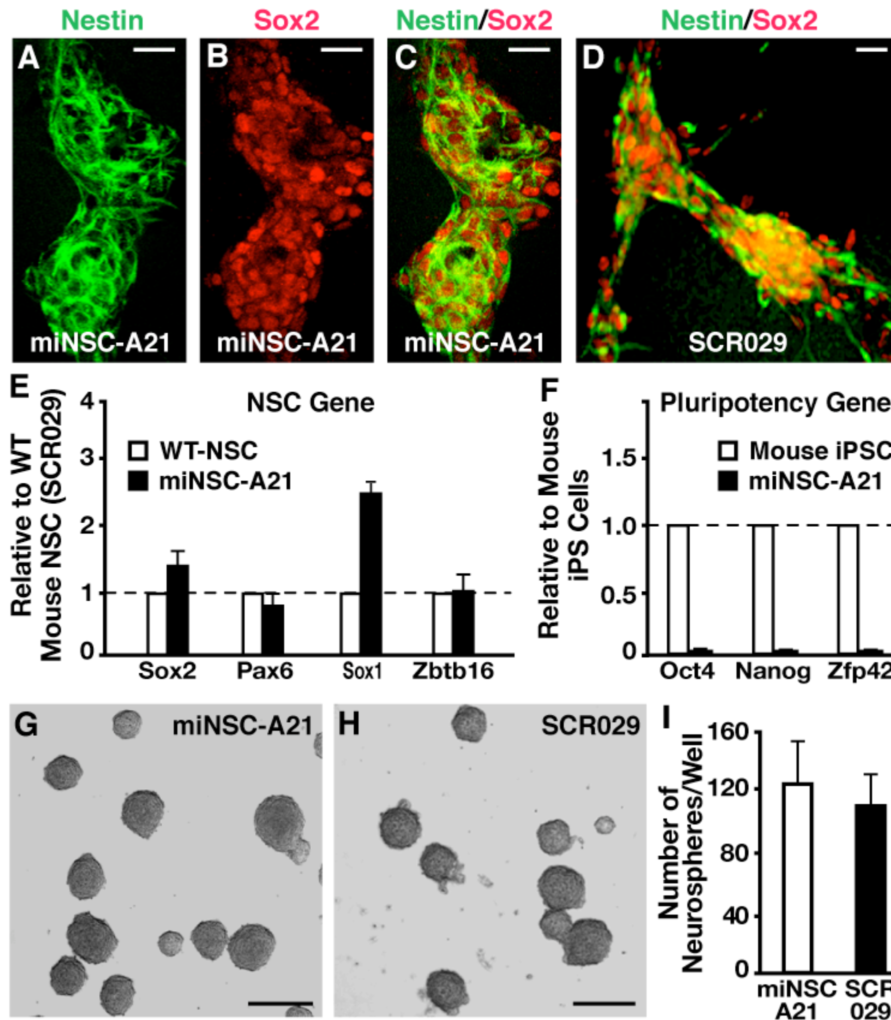

Figure 3. Characterization of miNSCs *in vitro.*

(A–D) 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. **(E)** qRT-PCR reveals that miNSC-A21 express typical NSC markers. Error bars denote standard deviation of triplicate reactions. **(F)** qRT-PCR indicates that miNSC-A21 do not express pluripotency related genes. Error bars denote standard deviation of triplicate reactions. **(G–I)** In suspension culture, miNSC-A21 generates neurospheres similar to wild-type NSCs and with similar efficiency (n=3). Scale bars = 50 μm in A–D; scale bars = 100 μm in G and H.

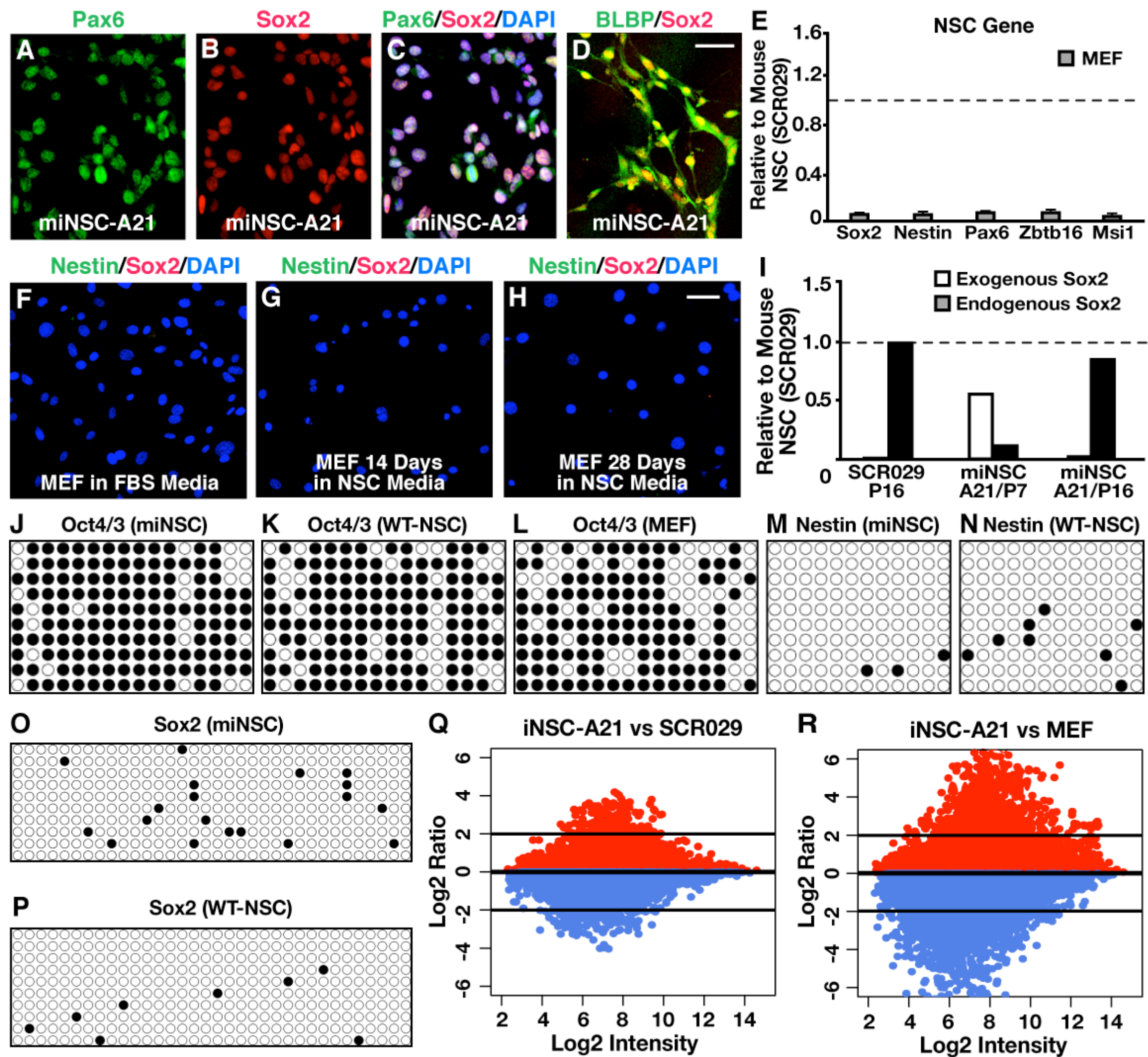

Figure 4. Additional *in vitro* characterization of miNSCs.

(A–C) miNSC-A21 expresses the NSC transcription factors Pax6 and Sox2. **(D)** miNSC-A21 also expresses the radial glial marker BLBP in combination with Sox2. **(E)** qRT-PCR comparing MEFs to wild-type NSCs indicates that the MEF population does not express NSC markers and is not contaminated with NSCs. Dotted line indicates 100% expression compared to control wild-type NSCs. Error bars indicate standard deviation of triplicate reactions. **(F)** MEFs cultured in FBS-containing medium do not stain positive for Nestin or Sox2. **(G and H)** MEFs cultured in NSC permissive culture conditions do

not turn on expression of Nestin or Sox2 as evidenced by immunostaining. **(I)** qPCR of endogenous and exogenous Sox2 expression levels reveals that miNSC-A21 at low passage has low levels of endogenous Sox2 expression and significant levels of exogenous Sox2 expression compared to wild-type NSCs (SCR029). However, later passage miNSC-A21 has more comparable endogenous Sox2 expression and low levels of exogenous Sox2 expression compared to wild-type NSCs. Dotted line indicates 100% expression compared to control wild-type NSCs. **(J–L)** Methylation patterns of the Oct4/3 gene promoter were analyzed using bisulfite treated DNA from ten clones each of miNSCs, wild-type NSCs, and MEFs. Black circles indicate methylated CpG and open circles indicate unmethylated CpG. **(M–P)** Methylation patterns of the Sox2 and Nestin gene promoters were analyzed using bisulfite treated DNA from miNSCs and wild-type NSCs. **(Q and R)** Transcriptional microarray data reveal that the global gene expression profile of miNSC-A21 is more similar to wild-type NSCs than to the original MEFs. Scale bars = 50 μm.

Multipotency of miNSCs in Culture

To assess the neural developmental potential (multipotency) of miNSCs, we tested their ability to differentiate into the three major neural cell types (neurons, astrocytes, and oligodendrocytes) *in vitro*. 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 3A-3B) and mature neurons (MAP2- and Tau-positive) at 2 weeks (Figures 3E–3H). At 4 weeks, iNSCs developed into MAP2-positive neurons with extensive and complex neurites similar to those of mouse primary neurons in culture (Figure 3I). At 14–28 days, miNSCs differentiated into vGluT1-positive excitatory neurons (Figure 3J and Figure S3E) and GABA-positive inhibitory neurons (Figure 3K). Importantly, MAP2-positive mature neurons could be generated from miNSCs at passages P11–P28 (Figures S3A–S3D). Thus, miNSCs have stable neurogenesis capability and their neural fates are not restricted at later passage.

Immunostaining revealed GFAP-positive astrocytes for both miNSCs and wild-type NSCs at 7 days *in vitro* after the removal of growth factor from the culturing medium (Figures 3A-3B). Additionally, a robust population of GFAP-positive astrocytes were derived from miNSCs cultured for 24–22 days under various differentiation conditions, including culturing cells with 50 ng/ml BMP4 or 1% fetal bovine serum (FBS) (Figures 3C, 3D and Figure S3F). miNSCs also generated astrocytes at early and late passages (data not shown). Importantly, the ability of miNSCs to generate neurons and astrocytes was confirmed in different batches of miNSCs (Figures S3G–S3L). Furthermore, under oligodendrocyte differentiation conditions involving the removal of growth factors from

NSC medium and culturing on gelatin-coated glass coverslips for 2 weeks, miNSCs also developed into oligodendrocytes, as indicated by staining positive for the markers O4 and Olig2 (Figure 3L). These findings suggest that miNSCs are multipotent, being able to differentiate into all three neural cell populations—neurons, astrocytes, and oligodendrocytes.

To further confirm the multipotency of miNSCs, we subcloned miNSC-A21 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. Representative data from three clones are shown in Figures 4A–4I. 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* for two miNSC clones (A21-B8 and A21-C1). Under a typical neuronal differentiation condition involving the removal of FGF2 and EGF growth factors from NSC media and differentiation on laminin/gelatin-coated coverslips, the percentages of neurons and astrocytes were determined by counting the numbers of MAP2-positive and GFAP-positive cells, respectively, which were normalized to the total numbers of DAPI-positive nuclei. We found a similar yield in neurons 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 generated from miNSC-A21 (Clone B8: 25 ± 2%; Clone C1: 18 ± 2%) as compared to wild-type brain-derived NSCs (6 ±

4%). Under this typical neuronal differentiation condition, very few oligodendrocytes were generated.

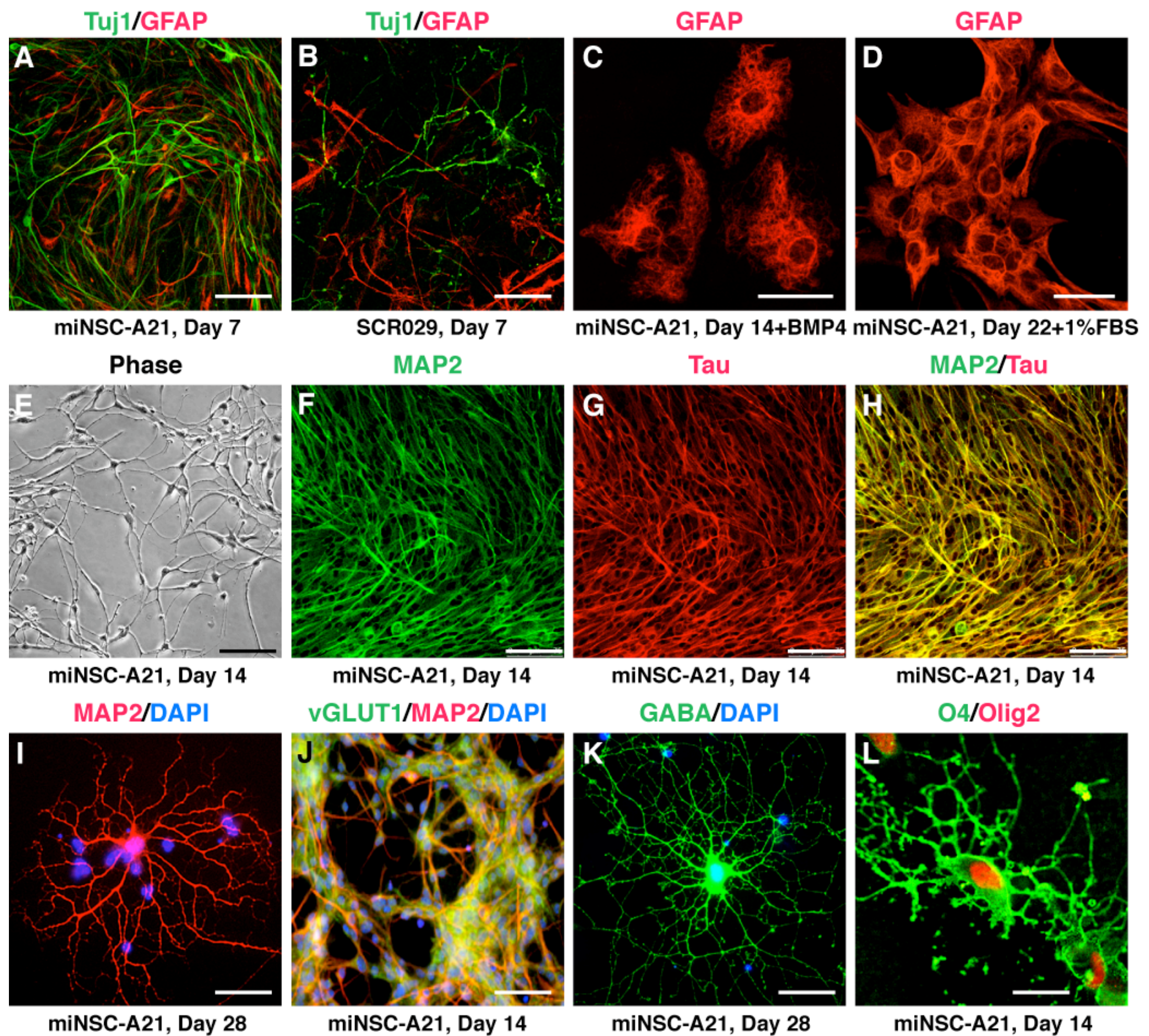

Figure 5. Multipotency of miNSC-A21 *in vitro*.

(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. **(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. Scale bars = 50 μm in A, B, E, and J; scale bars = 25 μm in C and D; scale bars = 75 μm in F–H; scale bars = 10 μm in I, K, and L.

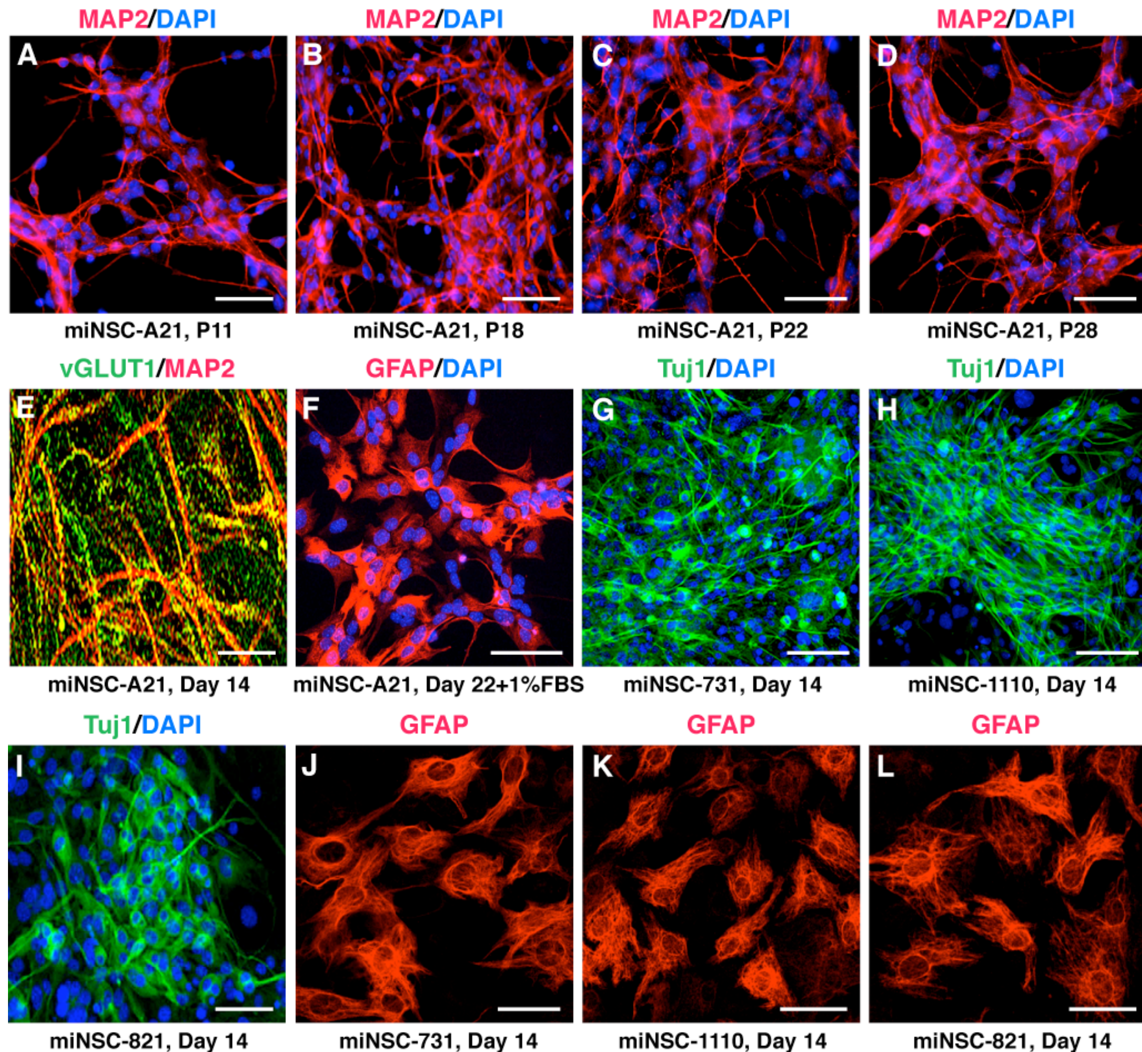

Figure 6. Multipotency of different miNSC lines *in vitro*.

(A–D) miNSC-A21 can differentiate into MAP2-positive neurons at early and later passages *in vitro*. **(E)** vGLUT1 colocalizes with MAP2 in neurons derived from miNSC-A21. **(F)** miNSC-A21 generates robust astrocyte networks in the presence of FBS over 22 days in culture. **(G–I)** Additional miNSC lines can generate TUJ1-positive neurons by 14 days in culture. **(J–L)** Additional miNSC lines can also generate mature looking GFAP-positive astrocytes. Scale bars = 50 μm in A–D and G–I; scale bar = 10 μm in E; scale bar = 25 μm in F; scale bars = 25 μm in J–L.

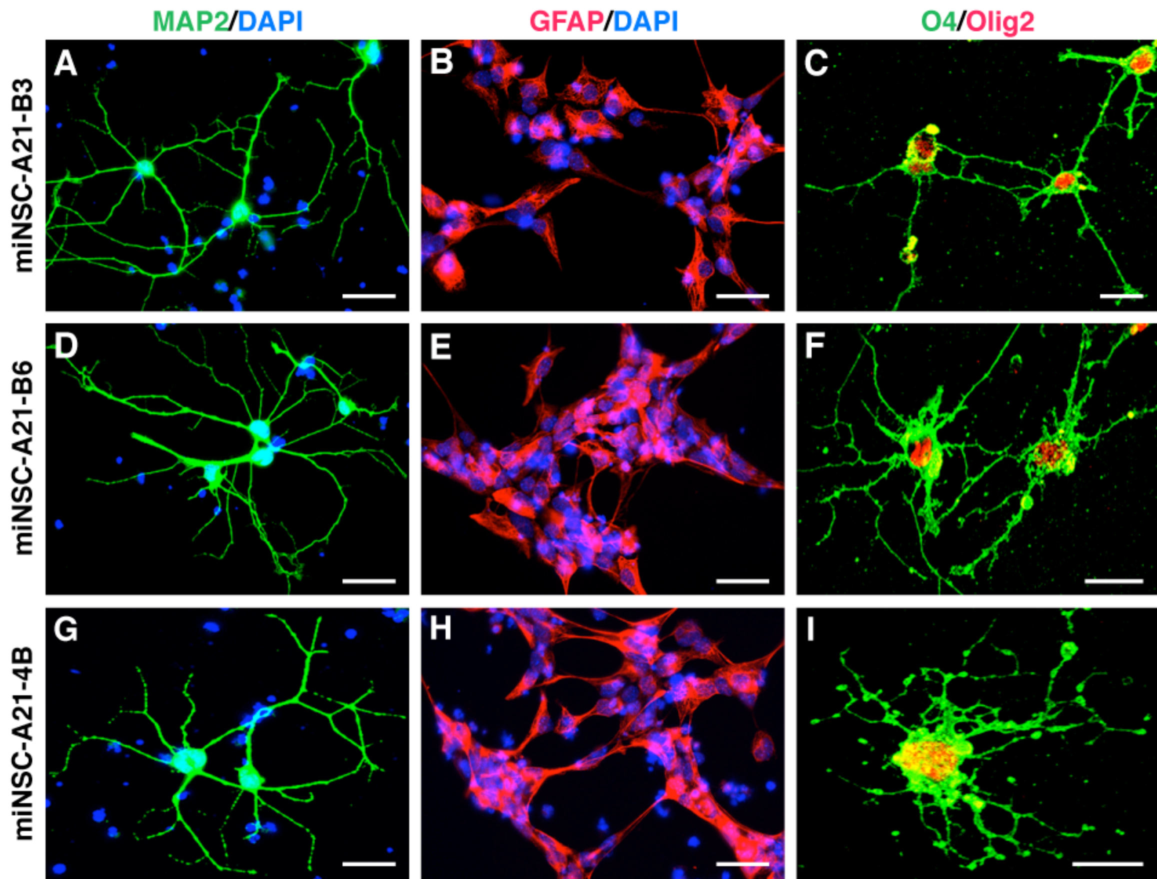

Figure 7. Multipotency of cloned miNSC lines.

(A–I) After 14 days in culture, subcloned lines B3 **(A–C)**, B6 **(D–F)**, and 4B **(G–I)** of miNSC-A21 can differentiate into MAP2$^+$ mature neurons **(A, D, G)**, GFAP$^+$ astrocytes **(B, E, H)**, and O4$^+$/Olig2$^+$ oligodendrocytes **(C, F, I)**. Scale bars = 10 μm in all panels.

Functional Neurons Derived from miNSCs

Mature looking neurons were generated with a protocol that mimics conditions conducive to primary neuron culture. The miNSC-derived neurons developed with this protocol expressed Synapsin, a synaptic marker that is concentrated at the nerve terminals of mature neurons, indicating that miNSC-derived neurons seem to form synaptic connections *in vitro* (Figures 5A and 5B). To test the functionality of miNSC-derived neurons, we characterized their electrophysiological properties by whole-cell patch-clamp recordings (Figure 5C). At 3 weeks in culture, the neurons had hyperpolarized resting membrane potentials (–40 to –80 mV) (Figure 5D), and membrane resistance properties (Figure 5E). Action potentials could be elicited by depolarizing the membrane in current-clamp mode (Figure 5F). 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 5G). Thus, miNSC-derived neurons appear to exhibit the functional membrane properties and activities of normal neurons.

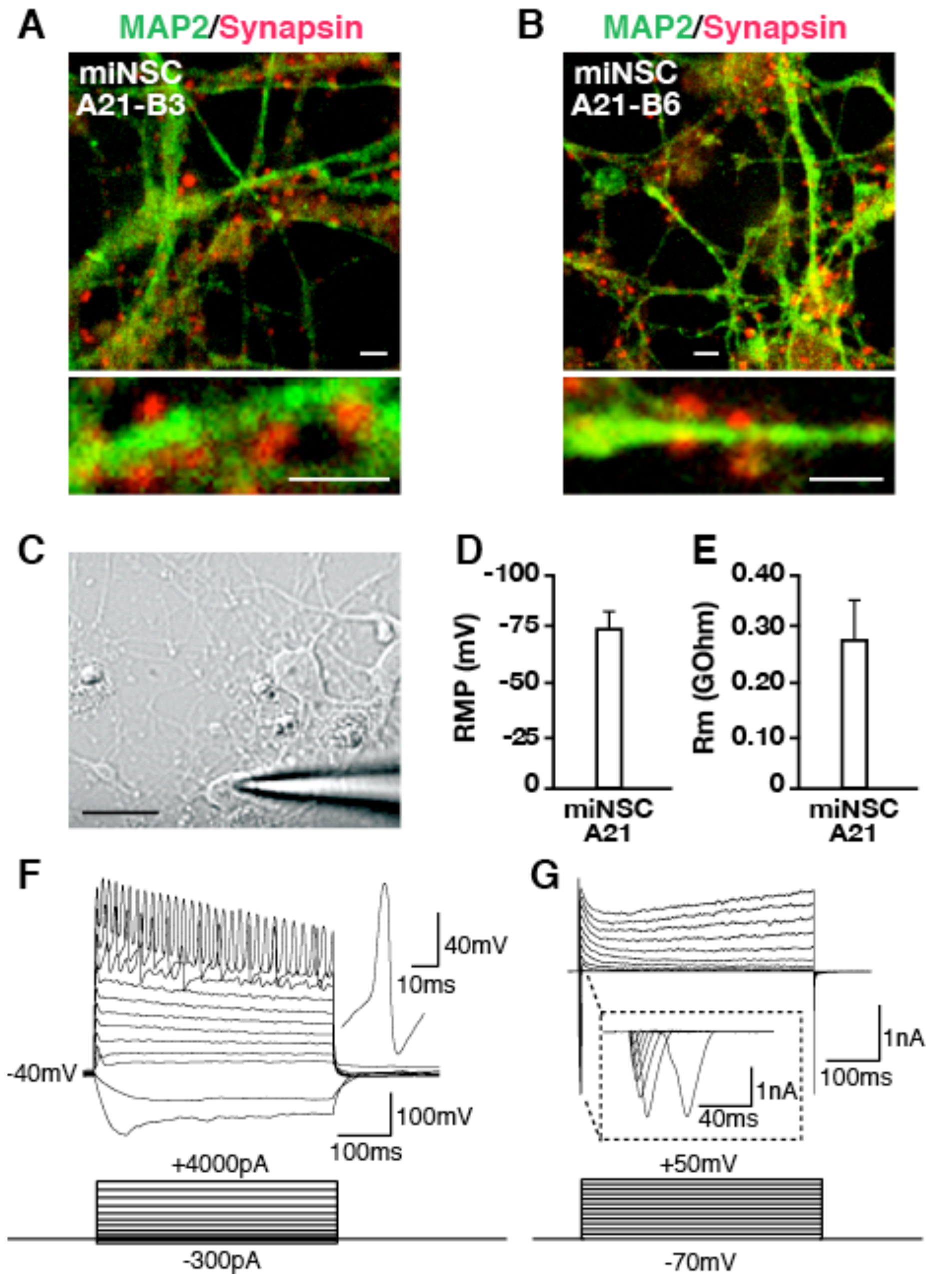

Figure 8. Functional neurons derived from miNSCs.

(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. **(F)** Current-clamp recordings of neurons derived from miNSC-A21 at –40mV 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. Scale bars = 2 μm in A and B; scale bar = 10 μm in C.

miNSCs can Survive, Integrate, and Differentiate *In Vivo*

We next determined whether miNSCs could survive and integrate into the mouse brain. We transplanted GFP-labeled miNSCs that were growing 24 hours in suspension cultures in order to generate small neurospheres. We found that transplanting miNSCs as neurospheres rather than single cells from monolayer cultures resulted in better survival and integration in neonatal brains. miNSCs were micro-injected into the cortex of P2–3 wild-type pups and survival and integration was assessed at 1, 2, and 5 days post transplantation. Immunostaining revealed that miNSCs can differentiate into NeuN-positive neurons with mature-looking dendritic spines (Figures 6A−6C), GFAP-positive astrocytes (Figures 6D−6F), and Olig2-positive oligodendrocytes, (Figures 6G−6I) between 1 and 5 days post transplantation. Thus, miNSCs are capable of differentiating into neurons, astrocytes, and oligodendrocytes both *in vitro* and *in vivo*.

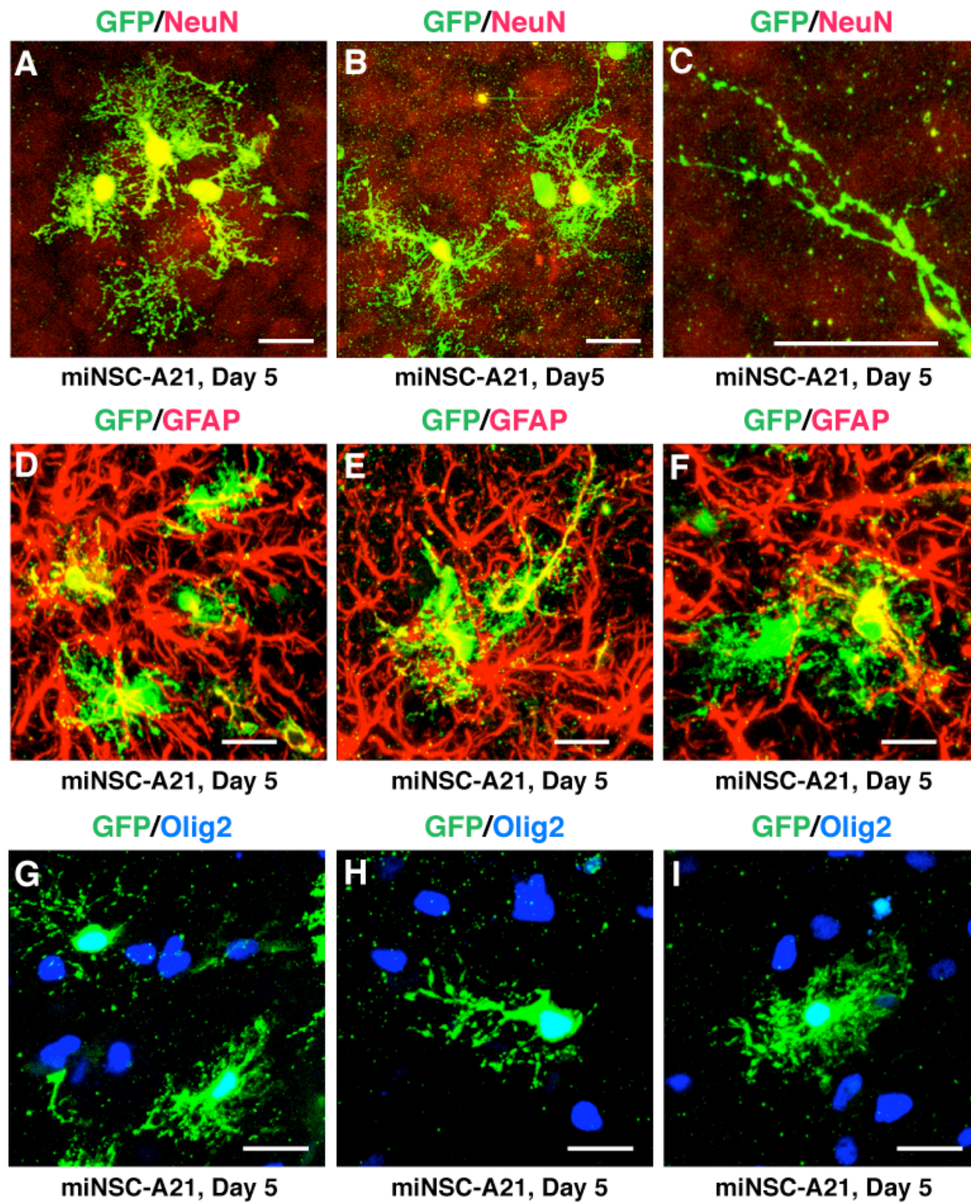

Figure 9. Multipotency of miNSCs *in vivo*.

GFP-labeled miNSC-A21 were grown in suspension cultures for one day to generate small neurospheres and then microinjected into the cortex of P2-3 wild-type pups. Five days after transplantation, mouse brains were collected, fixed, and sectioned. **(A–C)** Immunostainings reveal that miNSC-A21 can differentiate into NeuN-positive neurons (A and B) with mature looking dendritic spines (C) *in vivo*. **(D–F)** miNSC-A21 can also

differentiate into GFAP-positive astrocytes *in vivo*. **(G–I)** miNSC-A21 can also differentiate into Olig2-positive oligodendrocytes *in vivo* Scale bars = 10 μm in all panels.

Transplantation of miNSCs into Mouse Brains Does not Generate Teratomas

Transplantation of iPS cell-derived neurospheres into mouse brains often results in teratoma formation, while transplantation of multipotent, lineage-restricted brain-derived NSCs does not (Yamanaka, 2009). We compared the rate of teratoma formation among miNSCs, mouse iPS cell-derived NSCs, and brain-derived wild-type NSCs. Transplantation of miNSCs or control brain-derived wild-type NSCs into mouse brains did not generate teratomas; however, teratomas formed in over 60% of mice transplanted with mouse iPS cell-derived NSCs (Table 4). The observation that miNSCs did not form teratomas *in vivo* in 28 separate hippocampal injections involving three different miNSC-A21 subclones suggests that miNSCs have little or no tumorigenic potential.

Table 4. Assessing teratoma formation after transplantation of different NSC cell lines into NOD/SCID mouse brains.

Cell line	Passage Number	Number of Hippocampi injected	# of Tumors formed	Deaths	% Tumor Formation
NSCs from mouse iPSC-411	P10, P28	21	15	0	71.4%
NSCs from mouse iPSC-WT.9	P6	8	5	2	62.5%
iNSC-A21 (Subclones 4B, B3, B6)	P5, P8, P19	28	0	0	0%
WT mouse NSCs (SCR029)	P 17	8	0	0	0%
hiNSC-R2	P 10	8	0	0	0%

CHAPTER 4

Human iNSC Generation and Characterization

Generation and Characterization of iNSCs from Human Fetal Fibroblasts

We generated human iNSCs (hiNSCs) from human fetal foreskin fibroblasts (HFFs) using a similar protocol (Figure 1), 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 these cells were similar to those in mouse fibroblasts (Figure 1) (Figures S4A– S4D). Immunostaining revealed that within 5 days after SOX2 retroviral transduction, hiNSC colonies were positive for SOX2 and NESTIN, as shown by immunostaining (Figure S4E). After three or four rounds of neurosphere culture, hiNSCs had morphology similar to that of wild-type human NSCs (Figure S4F) and could be passaged over 20 generations in culture (Figures S4G), like NSCs derived from mouse iPS cells (Figure 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 mouse iPS cells (Figure S4J).

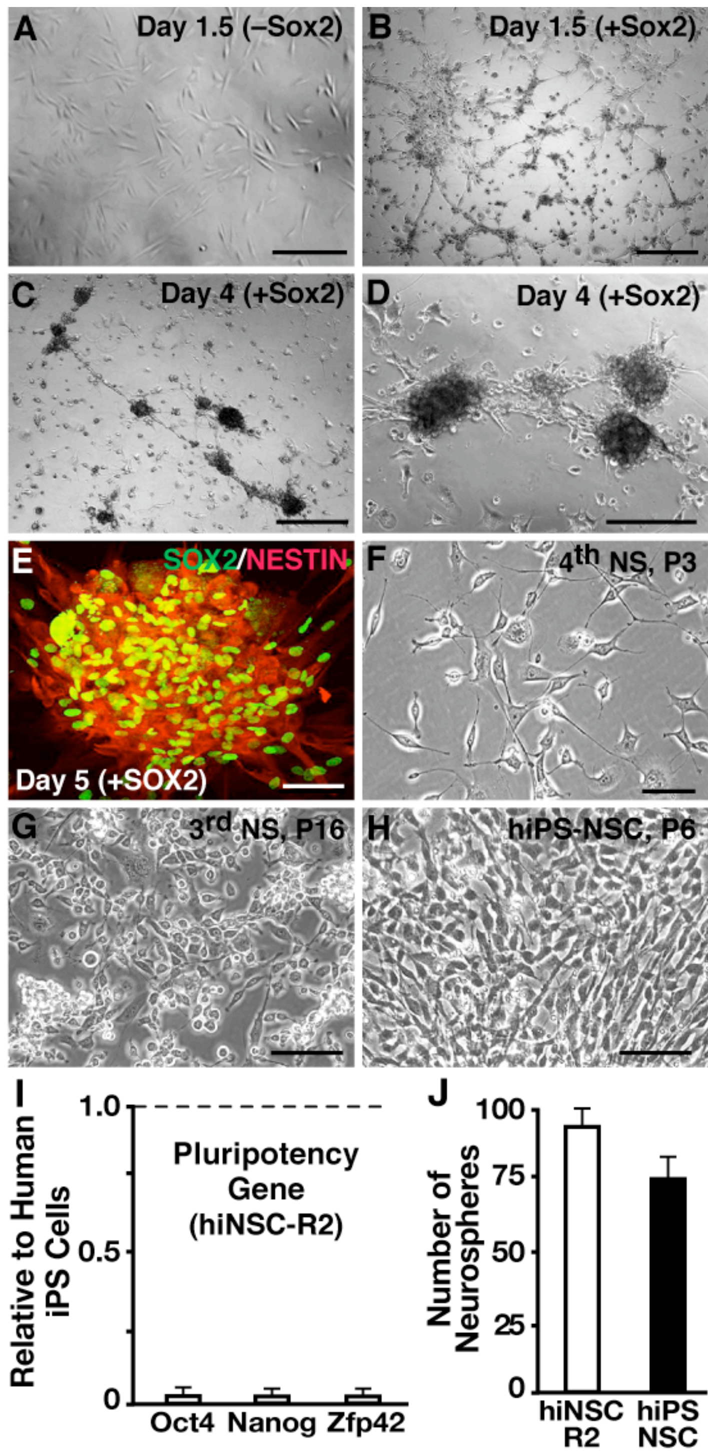

Figure 10. Generation of human iNSCs from human fibroblasts.

(A) Human fetal foreskin fibroblasts (HFF) plated on gelatin coated glass coverslips before SOX2 retroviral infection. **(B)** At 1.5 days after SOX2 infection, HFFs begin to

form colonies and networks in hNSC medium supplemented with growth factors. **(C and D)** Four days after SOX2 infection, colonies have increased in size. **(E)** Reprogrammed colonies are positive for both NESTIN and SOX2 at 5 days after SOX2 infection. **(F)** SOX2 reprogrammed cells have denser, more bipolar morphology than their HFF counterparts after three rounds of neurosphere culturing. **(G)** hiNSCs acquire a homogenous morphology after multiple monolayer passages and maintain their morphology and proliferation capacity to later passages. **(H)** hiNSCs have similar morphology to NSCs derived from human iPS cells (hiPS-NSC). **(I)** qRT-PCR indicates that hiNSCs do not express pluripotency-related genes. Dotted line indicates 100% expression compared to control human iPS cells. Error bars indicate standard deviation of triplicate reactions. **(J)** hiNSCs and hiPS-NSCs have a similar capacity to form neurospheres in suspension culture (n=3). Scale bars = 50 μm in A, D, and F–H; scale bars = 100 μm in B and C; scale bar = 10 μm in E.

Multipotency of hiNSCs in Culture

To assess the multipotency of hiNSCs, we tested the ability of hiNSCs to differentiate into neurons, astrocytes, and oligodendrocytes 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 7A–7D). At 4 weeks, hiNSCs developed into MAP2-positive neurons with extensive and complex neurites (Figure 7E). Importantly, MAP2-positive mature neurons could be generated from hiNSCs at various passages from P8 to P22, suggesting stable neurogenic capacity of hiNSCs (data not shown). Anti-GFAP immunostaining revealed GFAP-positive astrocytes derived from two separate hiNSC lines cultured for 14 days in the presence of 50 ng/ml BMP4 (Figures 7F and 7G). Furthermore, under an oligodendrocyte differentiation condition, hiNSCs also developed into oligodendrocytes, as indicated by positive staining for O4 and OLIG2 (Figure 7H). We also confirmed that hiNSCs did not generate teratomas upon transplantation into mouse brains (Table S2). These data suggest that hiNSCs are multipotent, being able to differentiate into all three neural cell populations—neurons, astrocytes, and oligodendrocytes, and may not harbor any tumorigenic potential *in vivo*.

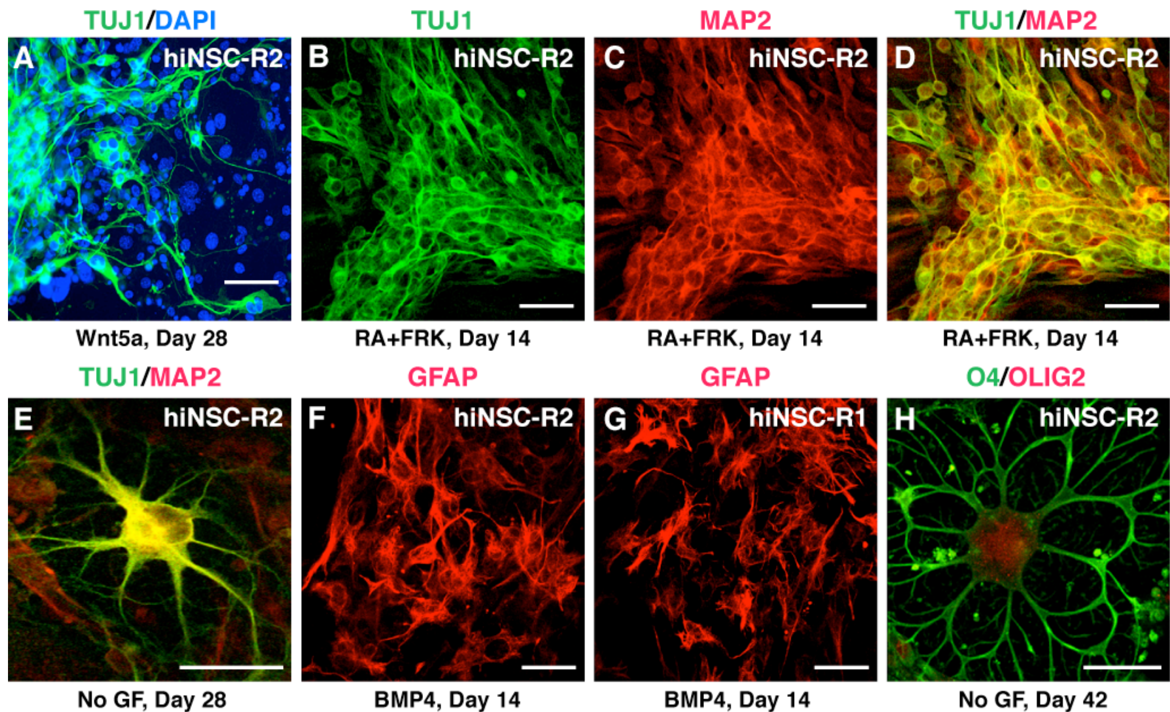

Figure 11. Multipotency of hiNSCs *in vitro*.

(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)** hiNSC 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-positive astrocytes in the presence of BMP4 by 14 days. **(G)** A separate hiNSC line can also robustly generate GFAP-positive astrocytes at 14 days *in vitro*. **(H)** hiNSCs can generate O4/OLIG2 double-positive oligodendrocytes by 40 days in culture in hNSC medium lacking growth factors. Scale bars = 20 μm in A–D; scale bars = 10 μm in E–H.

CHAPTER 5

Discussion and Conclusion

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 ES cells or iPS cells (Hochedlinger and Plath, 2009; Yamanaka, 2009). However, these methods are plagued by ethical and practical issues, such as the origin of ES cells and the tendency for teratoma formation of cells derived from iPS cells (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 iPS cells (Fong et al., 2010; Miura et al., 2009; Yamanaka, 2009). iN cells can be generated from fibroblasts and other somatic cell sources (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 these iN cells 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 iPS cell and iN technologies but also sidestep their shortcomings.

A handful of studies were published previously showing that various combinations of transcription factors can reprogram mouse fibroblasts into iNSCs or iNPCs (Kim et al.,

2011a; Han et al., 2012; Lujan et al., 2012; Sheng et al., 2012; Thier et al., 2012). The reprogrammed cells generated in these studies varied in their ability to self-renew, terminally differentiate, generate functional neurons, and integrate *in vivo* into mouse brains. However all these studies showed that reprogramming to directly the neural lineage avoided the generation of a transient iPS cell state. The results 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*. Our iNSC reprogramming protocol is advantageous, however, because it requires ectopic expression of a single factor to generate tripotent iNSCs from both mouse and human fibroblasts. Prior to our work, no study showed that reprogramming fibroblasts to iNSCs could be accomplished with a single factor or that human fibroblasts could be reprogrammed to iNSCs.

The miNSCs we developed can be passaged more than 40 times, are multipotent, have similar gene expression profiles compared to wild type NSCs, and can generate functional neurons with synaptic connections *in vitro*. When transplanted into neonatal mouse brains, miNSCs can survive, integrate, and differentiate into neurons, astrocytes, and oligodendrocytes without tumor formation. Furthermore, as evidenced by five cloned miNSC lines, our results uniquely show that miNSCs are a homogeneous population of tripotent NSCs, 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. In our human studies, we showed that ectopic expression of human SOX2 reprograms human foreskin fibroblasts into hiNSCs

that express typical NSC markers, self-renew over 20 passages, generate neurospheres comparable to NSCs derived from human iPS cells, and are tripotent *in vitro*.

Our discoveries regarding Sox2's ability to reprogram both mouse and human fibroblasts into iNSCs have been supported by a recent study in which human CD133+ cord blood cells were converted directly into neuronal-like cells by retroviral expression of Sox2 in human ES/iPS cell culture conditions (Giorgetti et al., 2012). The efficiency of Sox2 conversion was very low at 0.004-0.008% and was significantly increased with the addition of c-Myc, which resulted in a 15- to 25-fold increase in the conversion rate (0.06-0.1%). The cord blood derived induced neuronal cells (CB-iNCs) can self-renew in culture, but require an irradiated human foreskin fibroblast feeder layer and the growth factor bFGF to maintain propagation. Interestingly, the authors found that CB-iNCs predominantly differentiated into the neuronal lineage rather than the glial lineage and that CB-iNCs are a heterogeneous population of neural cells containing a small population of self-renewing progenitor like cells. Neurons derived from CB-iNCs were functional *in vitro* and CB-iNCs were able to differentiate *in vivo* to generate mature neurons that could generate action potentials. Our results are somewhat different than Giorgetti et al. as our Sox2 reprogramming protocol generates hiNSCs that are multipotent *in vitro* and are not restricted to the neuronal lineage. We will need to conduct further studies to determine the functionality of our Sox2 reprogrammed hiNSC-derived neurons both *in vitro* and *in vivo*.

Sox2 can function as a master regulator gene for NSC identity and maintenance, as 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 NSC proliferation conditions, including the presence of growth factors EGF and FGF2 and the proper surface and substrates, overexpression of Sox2 can reprogram fibroblasts to multipotent NSCs that can generate all neural cell types. 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. Exogenous expression 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 with high efficiency. In conclusion, we have developed a protocol that directly reprograms both mouse and human fibroblasts into self-renewing, multipotent iNSCs by ectopic expression of a single factor, Sox2. Our findings open up new doors for generating patient specific iNSC lines that will be invaluable for mechanistic studies, drug screening, and potential cell therapies for neurodegenerative diseases.

CHAPTER 6

Future Directions

Further Characterization of Human Sox2 iNSCs

In our preliminary human studies, we were able to show that Sox2 is sufficient to reprogram human fetal fibroblasts directly into hiNSCs that can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes. We will continue further characterization of our current hiNSC lines by assessing their ability to differentiate into subtypes of neurons such as excitatory, inhibitory, motor, and dopaminergic neurons *in vitro*. Additional experiments include global gene expression analysis of hiNSCs compared to human fetal NSCs and hES cell-derived NSCs, qPCR analysis of NSC and ES cell gene expression, epigenetic analysis of NSC and ES cell gene promoter methylation, electrophysiology to determine the functionality of neurons derived from hiNSCs, and finally, *in vivo* integration and differentiation of hiNSCs in mouse brains. We will also generate hiNSCs from adult human fibroblasts including those from patients with different neurodegenerative disorders. When generating patient-specific hiNSC lines, we will use a Sox2 episomal reprogramming vector instead of a Sox2 retroviral vector in order to avoid potential viral integration into the host genome.

Determine the Mechanism of Sox2 iNSC Reprogramming

Sox2 is important to both ES cell and NSC maintenance and self-renewal. The mechanism by which the Yamanaka four factors reprogram somatic cells to iPS cells has been studied in detail. What is known currently is that Sox2, Oct3/4, and Nanog are involved in an auto-regulatory loop that promotes iPS and ES cell proliferation, maintains the pluripotency state, and inhibits differentiation (Jaenisch & Young, 2008). Thus far we

do not know the exact mechanism by which ectopic expression of Sox2 can reprogram fibroblasts directly into NSCs. We hypothesize that Sox2 is involved in a separate auto-regulatory loop along with other important NSC transcription factors that promotes NSC proliferation and maintenance and inhibits neural and glial differentiation. It is possible that Sox2 binds to the promoters of NSC-specific genes to upregulate their expression. To determine what factors Sox2 might interact with, we could do a pull down assay during different states of iNSC reprogramming. If we identify potential Sox2 target proteins, we then can conduct knockdown experiments of those specific genes to determine whether silencing part of the auto-regulatory loop reduces efficiency or abolishes iNSC reprogramming. We could also identify potential Sox2 interacting genes during the reprogramming process using qPCR to highlight NSC genes that are immediately turned on after exogenous Sox2 expression.

Another area to focus on is post-translational modification of Sox2 during iNSC reprogramming. Jeong et al. discovered that phosphorylation of Sox2 at Threonine 118 by Akt stabilizes Sox2 and enhances its transcription, which subsequently improves the efficiency of generating iPS cells (Jeong et al., 2010). It would be interesting to determine whether this same amino acid is phosphorylated on Sox2 during iNSC reprogramming. Another modification that we could pursue is *O*-linked-N-acetylglucosamine (*O*-GlcNAc), which modifies serine and threonine residues in nucleocytoplasmic proteins. A recently published study by Jang et al. discovered that *O*-GlcNAc modification of Threonine 288 in the Oct3/4 protein is essential for maintaining ESC self-renewal and iPS cell reprogramming (Jang et al., 2012). When the authors attempted reprogramming with an Oct3/4 T288A point mutant, iPS cell reprogramming

efficiency was dramatically reduced, thus proving that *O*-GlcNAc modification regulates key players of the pluripotency and reprogramming networks. It would be interesting to determine whether *O*-GlcNAc modifications of Sox2 effects iNSC reprogramming in a similar manner. We will pursue this question through a collaboration with a UCSF lab that has given us a mutant Sox2 retroviral pMX vector that prevents *O*-GlcNAc modification at a specific amino acid within the Sox2 protein.

Identify Factors that can Reprogram Fibroblasts to iNSC Subtypes

As mentioned in the Discussion and Conclusion section, Sox2 could be used in combination with other neural specifying factors to generate subtype specific iNSCs. One of the current interests in our lab is whether we can generate iNSCs that differentiate into GABAergic inhibitory interneurons. Currently another graduate student in the lab is attempting to reprogram human skin cells to hiNSCs using Sox2 and plans to add two additional factors involved in interneuron specification to direct differentiation of Sox2 reprogrammed iNSCs into inhibitory neurons. We will also attempt to use the three factors in combination to determine whether we can generate inhibitory neuron iNSCs.

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