Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells

Ernesto Lujan[a,b], Soham Chanda[a,c], Henrik Ahlenius[a,d], Thomas C. Südhof[c,e,1], and Marius Wernig[a,d,1]

[a]Institute for Stem Cell Biology and Regenerative Medicine, Departments of [d]Pathology, [b]Genetics, and [c]Molecular and Cellular Physiology, and [e]Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305

Contributed by Thomas C. Südhof, December 21, 2011 (sent for review August 4, 2011)

We recently showed that defined sets of transcription factors are sufficient to convert mouse and human fibroblasts directly into cells resembling functional neurons, referred to as "induced neuronal" (iN) cells. For some applications however, it would be desirable to convert fibroblasts into proliferative neural precursor cells (NPCs) instead of neurons. We hypothesized that NPC-like cells may be induced using the same principal approach used for generating iN cells. Toward this goal, we infected mouse embryonic fibroblasts derived from *Sox2*-EGFP mice with a set of 11 transcription factors highly expressed in NPCs. Twenty-four days after transgene induction, *Sox2*-EGFP$^+$ colonies emerged that expressed NPC-specific genes and differentiated into neuronal and astrocytic cells. Using stepwise elimination, we found that *Sox2* and *FoxG1* are capable of generating clonal self-renewing, bipotent induced NPCs that gave rise to astrocytes and functional neurons. When we added the Pou and Homeobox domain-containing transcription factor *Brn2* to *Sox2* and *FoxG1*, we were able to induce tripotent NPCs that could be differentiated not only into neurons and astrocytes but also into oligodendrocytes. The transcription factors *FoxG1* and *Brn2* alone also were capable of inducing NPC-like cells; however, these cells generated less mature neurons, although they did produce astrocytes and even oligodendrocytes capable of integration into dysmyelinated *Shiverer* brain. Our data demonstrate that direct lineage reprogramming using target cell-type–specific transcription factors can be used to induce NPC-like cells that potentially could be used for autologous cell transplantation-based therapies in the brain or spinal cord.

induced neural precursor cells

During development, the creation of distinct cell types depends upon tightly regulated spatiotemporal expression of lineage-specific transcription factors. A key question is whether cells retain their competence to respond to such transcription factors even after differentiation and after their cell-type–specific phenotype has been stabilized by epigenetic mechanisms (1). A number of classic and recent studies have provided powerful evidence that the differentiated state of at least some somatic cells is more flexible than assumed. For instance, transfer of somatic nuclei into oocytes has been shown to impose an early embryonic program on somatic cells (2, 3). Similarly, aberrant cell-type–specific genes could be induced following cell fusion (4), and misexpression of defined transcription factors has been shown to induce conversion of cells in closely related cell types (5). For instance, the basic helix–loop–helix (bHLH) transcription factor *MyoD* has been shown to induce muscle-specific properties in fibroblasts but not in hepatocytes (6, 7); expression of *Cebpα* in B cells induces features of macrophages (8); loss of *Pax5* in B cells induces dedifferentiation to a common lymphoid progenitor (9); and the bHLH transcription factor *Ngn3* or *NeuroD1*, in combination with *Pdx1* and *MafA*, efficiently converts pancreatic exocrine cells or hepatic cells into functional B cells in vivo (10, 11).

More recently, we demonstrated that mouse and human fibroblasts can be converted into functional neurons by a defined set of transcription factors (12, 13). Moreover, we showed that defined factor-mediated reprogramming can be applied to distantly related lineages such as endodermal to ectodermal cells (14), raising the possibility that perhaps any cell might be converted into any other desired cell type if the right combination of reprogramming factors could be identified. Indeed, several papers subsequently have shown the induction of cardiomyocytes, hematopoietic cells, and hepatocytes from fibroblasts (15–18).

Another, conceptually different approach to direct lineage reprogramming is to dedifferentiate cells transiently into a pluripotent state and then allow spontaneous differentiation of the cells into the desired phenotype. Following the seminal work of induced pluripotent stem (iPS) cell reprogramming (19), it was shown that transient expression of the four iPS cell reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* followed by treatment with specific media succeeded in generating both cardiomyocytes and neural precursor cell (NPC) populations from fibroblasts (20, 21). However, in these experiments the NPCs were generated with low efficiency (presumably because of inefficient induction of pluripotency and the stochastic nature of differentiation), the described cells could not self-renew, and the NPCs apparently lacked the ability to differentiate into oligodendrocytes.

Here we investigated whether NPCs could be induced directly from fibroblasts using neural progenitor-specific transcription factors as reprogramming factors and thus bypassing a partial or complete pluripotent state. We show that specific combinations of factors can induce bi- or tripotent NPCs efficiently. These cells express an array of neural progenitor-specific genes and retain their potential for differentiation after prolonged clonal expansion, demonstrating a capacity for self-renewal.

Results

Induction of NPC-Like Cells from Mouse Embryonic Fibroblasts with a Pool of 11 Transcription Factors. We recently showed that three transcription factors directly and efficiently convert mouse fibroblasts into functional induced neuronal (iN) cells (12). Although this conversion has been demonstrated to be direct, with few or no cell divisions, we hypothesized that an intermediate NPC population also may be produced directly from mouse embryonic fibroblasts (MEFs) under appropriate conditions. In an attempt to achieve this goal, MEFs were derived from Sox2-internal ribosome entry site (IRES)-EGFP knockin mice expressing the reverse tetracycline transactivator (rtTA) under control of the Rosa26 locus. These MEFs were infected with a pool of 11 lineage-specific transcription factors (11F) under a tetO promoter (12, 22, 23). The 11 factors were chosen because of their demonstrated functions in neural development and their high expression levels in NPCs. After infection, cells were grown in EGF- and FGF2-containing media in the presence of doxycycline (24). Twenty-four days after transgene induction, *Sox2*-EGFP$^+$ cells were observed when MEFs were infected with the 11F pool. Some EGFP$^+$ cells formed colonies. Overall, 12.3% of

Author contributions: E.L., S.C., H.A., T.C.S., and M.W. designed research; E.L., S.C., H.A., and M.W. performed research; E.L., S.C., H.A., T.C.S., and M.W. analyzed data; and E.L., S.C., H.A., T.C.S., and M.W. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. E-mail: tcs1@stanford.edu or wernig@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121003109/-/DCSupplemental.

the total cell population expressed EGFP at day 25 (Fig. 1 *A* and *B* and Table S1). To determine whether this *Sox2*-EGFP$^+$ cell population exhibited a transcriptional profile similar to ES cell-derived NPCs, *Sox2*-EGFP$^+$ cells were purified by FACS 25 d after transgene induction (Fig. 1*B*). Endogenous expression levels of the NPC-associated genes *Sox1*, *Sox3*, *Olig2*, *Brn2*, *Ncan*, *Pax6*, *Nkx2.2*, *Gpm6a*, and *Tox3* were quantified by quantitative RT-PCR (qRT-PCR) (Fig. 1*C*). The *Sox2*-EGFP$^+$ population showed induction of these genes at levels similar to those seen in ES cell-derived NPCs, except for the region-specific gene *Nkx2.2*. A small number of cells with similar *Sox2*-EGFP expression levels also were detectable in control-treated MEFs after 25 d in culture (Fig. 1*B*). Expression analysis of this purified population revealed that *Sox1*, *Sox3*, *Olig2*, and *Pax6* levels were below detection limits, demonstrating that these cells were similar to uninfected MEFs rather than NPCs (Fig. 1*C*). Given the appearance of multiple independent NPC markers, we termed the converted MEFs "induced NPCs" (iNPCs).

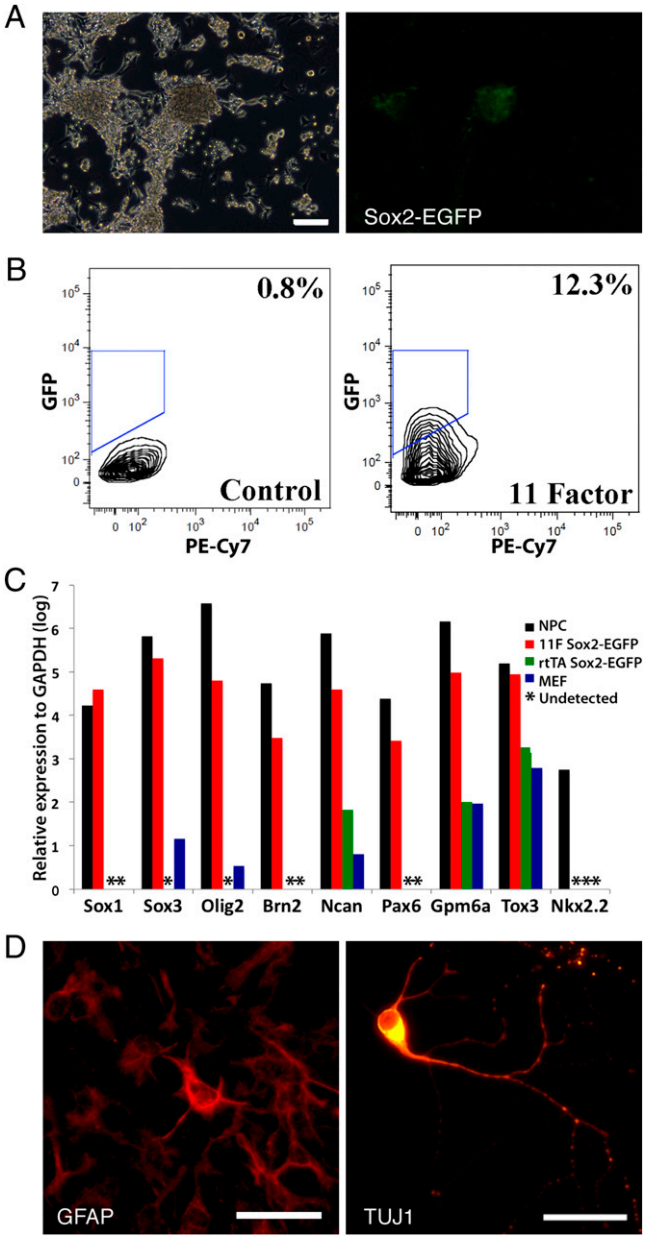

Fig. 1. Induction of a neural precursor-like population with 11 factors. (*A*) *Sox2*-EGFP$^+$ colonies 25 d after transgene induction by doxycycline. (*B*) *Sox2*-EGFP$^+$ cells from control (infected only with rtTA) and 11F infections were analyzed by FACS 25 d after transgene induction. (*C*) qPCR analysis of NPC-specific genes from passage 9 ES cell-derived NPCs (black), passage 4 MEFs (blue), 11F *Sox2*-EGFP$^+$ FACS-sorted cells (red), and negative control rtTA infections (green) 25 d after transgene induction ($n = 2$). (*D*) Differentiation of 11F pooled infections 25 d after induction into GFAP$^+$ astrocytes (*Left*) and Tuj1$^+$ neurons after 8 d (*Right*). (Scale bars: 50 mM.)

We next sought to determine whether 11F iNPCs also displayed functional neural precursor properties, such as the capacity to differentiate into neurons and glia under defined conditions. Indeed, upon withdrawal of growth factors and doxycycline from cultured iNPCs, Tuj1$^+$ cells with typical neuronal morphologies were detected (Fig. 1*D*). However, it is important to note that the 11 factors used also contained *Ascl1*, *Brn2*, and *Zic1,* which can induce neuronal cells directly from MEFs, and Tuj1$^+$ cells with neuronal morphologies were detectable before growth factor withdrawal (12). We next attempted to differentiate 11F iNPCs into astrocytes. The NPC growth medium was replaced 25 d after infection with medium containing 5% serum, and cells were cultured for another 8 d, a condition known to induce astrocyte differentiation (25, 26). Subsequent immunofluorescence detection of GFAP revealed distinct groups of GFAP$^+$ cells in this condition, but no such cells were seen in NPC growth medium (Fig. 1*D*). Thus, 11F iNPCs demonstrated the potential to differentiate into cells with neuronal and glial morphologies and marker expression under differentiation cues similar to those of regular NPCs.

Systematic Identification of the Critical Reprogramming Factors. To determine which transcription factors are necessary and sufficient to induce NPC-like cells, *Sox2*-EGFP MEFs were infected with nine- or 10-factor pools in which one or two genes were excluded at a time (11F − 2 pools). We ordered the 11 genes according to expression levels in NPCs to build these groups (27). Twenty-four days after transgene induction, a significant decrease in *Sox2*-EGFP$^+$ colony numbers was observed upon removal of (*i*) *FoxG1* and *Lhx2* or (*ii*) *Sox2*. This result suggested that *Sox2* and either *FoxG1* or *Lhx2* may be critical for the formation of iNPCs (Fig. 2*A* and Table S1). We therefore tested a pool of only the five most highly expressed transcription factors (5F pool) in NPCs that also contained these apparently critical three genes (27). Rewardingly, expression of the five factors *Rfx4, ID4*, *FoxG1*, *Lhx2*, and *Sox2* in MEFs was sufficient to induce *Sox2*-EGFP$^+$ colonies as assessed 24 d after infection (Fig. 2*B* and Table S2). Again, cells were tested for differentiation into neuronal and glial fates without further expansion. Distinct patches of Tuj1$^+$ and MAP2$^+$ neuronal cells and GFAP$^+$ astrocytic cells were readily identified 12 d after removal of growth factors (Fig. 2 *B* and *D*). Furthermore, qRT-PCR analysis of sorted *Sox2*-EGFP$^+$ cells 25 d after doxycycline addition showed induction of endogenous *Sox1*, *Sox3*, *Olig2*, *Ncan*, and *Pax6*. Of note, unlike the 11F pool, induction of *Brn2* was not detected (Fig. 2*C*).

Next, we asked whether any of the five factors was dispensable for NPC induction. We infected MEFs with pools of four factors, systematically omitting one factor at a time, and quantified *Sox2*-EGFP$^+$ colonies 24 d after transgene induction (Table S2). Consistent with the results of the 11F − 2 pools, a decrease in *Sox2*-EGFP$^+$ colonies was observed when *FoxG1* and *Sox2* were removed, suggesting that both factors are important for formation of *Sox2*-EGFP$^+$ colonies. When we tested the resulting putative NPCs for neuronal differentiation potential, we observed many Tuj1$^+$ neuronal cells with complex morphologies under all conditions (including omission of *Sox2*) except when *FoxG1* was removed, after which only a few cells with fibroblastic morphologies were labeled with Tuj1 antibodies (Fig. 2*D*). This observation highlighted *FoxG1* as perhaps the most critical factor for inducing NPC-like cells with neuronal differentiation potential (Fig. 2*D*). Similarly, *Sox1*, *Sox3*, *Olig2*, *Ncan*, and *Pax6* were induced in all conditions except when *FoxG1* or *Sox2* were

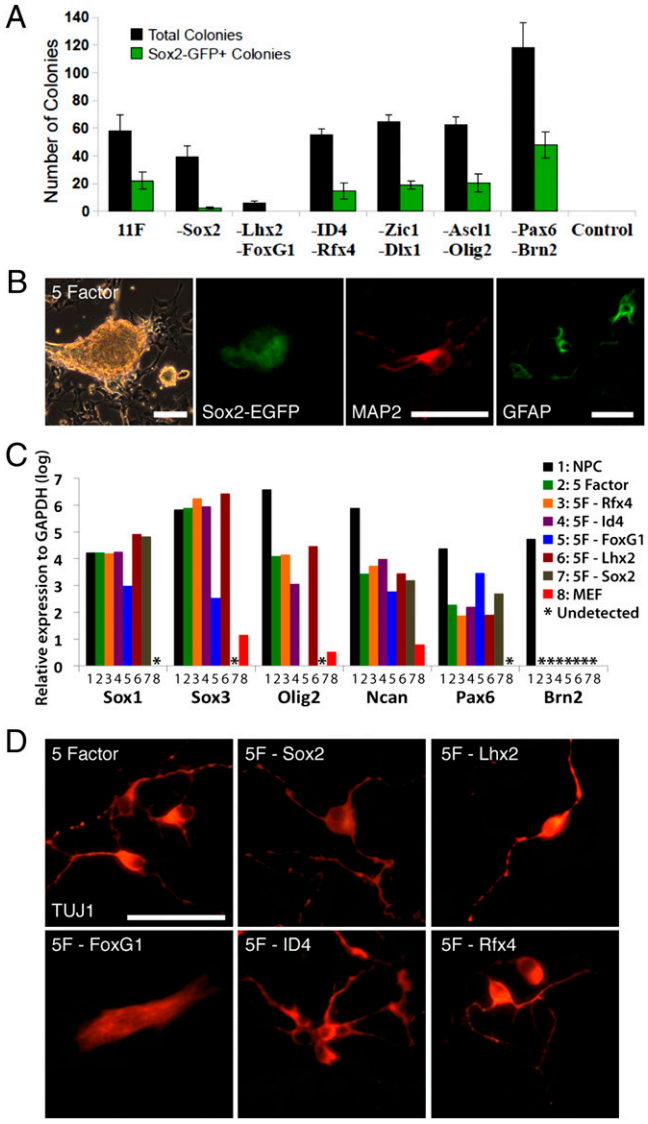

Fig. 2. Identification of critical NPC-inducing factors. (*A*) Quantification of total colonies (black) and *Sox2*-EGFP$^+$ colonies (green) from 11F, two pools 24 d after transgene induction. Control represents infection with rtTA virus alone. Error bars represent SDs of three experiments. (*B*) *Sox2*-EGFP$^+$ colony 25 d after 5F transgene induction (*Left*). MAP2$^+$ neurons and GFAP$^+$ astrocytes were differentiated from the 5F population by removal of mitogens and doxycycline and culturing for 12 d (*Right*). (*C*) qPCR analysis of NPC-specific gene induction for *Sox2*-EGFP$^+$ FACS-sorted populations from 5F and 5F−1 pools 25 d after transgene induction ($n = 2$). (*D*) When *FoxG1* was removed from the 5F pool, no Tuj1$^+$ cells with neuronal morphology could be derived. Neuronal cells were observed in all other conditions. Culture conditions are described in *B*. (Scale bars: 50 μm.)

omitted, suggesting that both genes are important for inducing an NPC profile (Fig. 2*C*).

***FoxG1* and *Sox2* Can Induce a Bipotent NPC Population.** Because *FoxG1* and *Sox2* were necessary for *Sox2*-EGFP$^+$ colony formation and NPC-specific gene induction, we investigated the possibility that these two factors alone may induce NPC-like cells. *Sox2*-EGFP MEFs infected with the two factors displayed morphological changes as early as 4 d after doxycycline addition (Fig. S1), and distinct elongated cell morphologies were detectable by 7 d. Colony formation was first observed 13 d after induction, and 5.0% of cells were EGFP$^+$ at day 25 (Fig. 3*B*). This cell population had the capacity to differentiate into Tuj1$^+$,

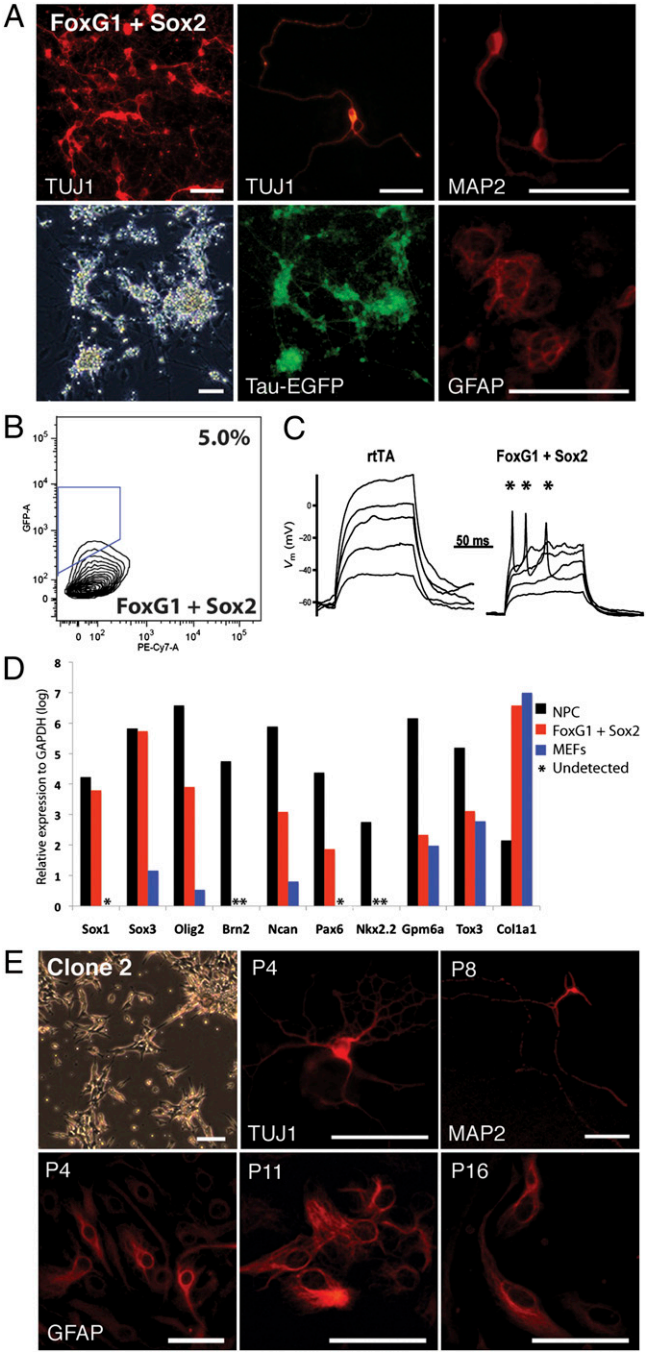

Fig. 3. *FoxG1+Sox2* can induce a self-renewing bipotent population from fibroblasts. (*A*) Sox2-EGFP MEF-derived iNPCs give rise to Tuj1$^+$ and MAP2$^+$ neurons (*Upper*) and GFAP$^+$ astrocytes (*Lower Right*). EGFP$^+$ neuronal cells (*Lower Left*) can be detected readily from Tau-EGFP MEF-derived iNPCs under the same conditions. Differentiation conditions are described in the text. (*B*) *Sox2*-EGFP$^+$ cells from *FoxG1+Sox2* infections were analyzed by FACS 25 d after infection. (*C*) Representative current-clamp traces in response to current pulses, in control condition (infection with rtTA virus only; *Left*) and after infection with *FoxG1+ Sox2* (*Right*). Neurons were differentiated 25 d after transgene induction. Asterisks indicate action potentials. (*D*) qPCR analysis reveals that *FoxG1* and *Sox2* can induce a subset of NPC-specific genes but not *Brn2* or the region-specific gene *Nkx2.2*. In addition, the fibroblast-specific gene *Col1a1* is not repressed ($n = 2$). (*E*) A clonal population derived from the *FoxG1+Sox2* condition can differentiate into Tuj1$^+$ and MAP2$^+$ neurons (*Upper*) and GFAP$^+$ astrocytes (*Lower*) at multiple passages. (Scale bars: 50 μm.)

MAP2$^+$ neurons, demonstrating that the two factors are sufficient to create a population with neurogenic potential (Fig. 3*A*). Patch-clamp recordings confirmed several functional neuronal properties with an average resting membrane potential of −57.0 ± 1.0 mV, an input resistance 1.2 ± 0.5 GΩ, and the capacity to generate action potentials (n = 4 of 7 cells; Fig. 3*C*).

FACS-purified *Sox2*-EGFP$^+$ cells displayed a morphologically homogeneous population and could be expanded in adherent conditions for many passages (up to 12 passages were tested), whereas *Sox2*-EGFP$^+$ cells sorted from MEFs infected with rtTA alone resembled fibroblasts with little proliferative capacity. After extensive expansion, these iNPCs maintained the ability to differentiate into both neuronal and astroglial cells (up to nine passages were tested) (Fig. 3*A*). Notably, when placed under astroglial differentiation conditions, GFAP$^+$ cells differentiated as patches, suggesting that only a subset of the cells had astroglial potential. Despite several attempts, no oligodendrocytes could be observed in these cultures as assessed by O4 immunostaining. These results were reproduced with MEFs derived from the *Tau*-EGFP knockin mice, which could also produce a proliferative population that could differentiate into *Tau*-EGFP$^+$, Tuj1$^+$, and MAP2$^+$ neurons and GFAP$^+$ astrocytes under differentiation conditions (Fig. 3*A*). Expression analysis of *FoxG1*/*Sox2*-infected *Sox2*-EGFP–sorted cells showed induction of the NPC markers *Sox1*, *Sox3*, *Olig2*, and *Pax6*, but, similar to results in 5F cells, *Brn2* was not induced (Fig. 3*D* and Fig. S2). Additionally, transcript levels of the fibroblast-specific genes *Col1a1*, *Col3a1*, *Twist2*, *Snail1*, and *Dkk3* were similar to those in fibroblasts, suggesting that this population had not silenced the fibroblast-specific transcriptional program (Fig. 3*D* and Fig. S3). In summary, these data suggest that *FoxG1* and *Sox2* are capable of inducing NPCs with bilineage-restricted differentiation potential but fail to induce the NPC marker *Brn2*.

We next explored the self-renewal potential of *FoxG1*/*Sox2* iNPCs. To this end, clonal analysis was performed. Three *Sox2*-EGFP$^+$ colonies and six colonies from *Tau*-EGFP knockin MEFs were picked manually and expanded. Differentiation was induced at multiple passages, and Tuj1$^+$ and MAP2$^+$ neurons were detected in all clones (Fig. 3*E*). In contrast, only one clone was able to differentiate also into GFAP$^+$ astrocytes (Fig. 3*E*). Efficient differentiation into GFAP$^+$ astrocytes was observed from this clone at passages 4, 11, and 17 when exposed to 5% serum (Fig. 3*E*). Thus, the *FoxG1*/*Sox2* iNPCs have self-renewal potential and consist mostly of neuron-restricted progenitor cells and some neuron/astroglial-restricted bipotent precursor cells, confirming our observations with the nonclonal iNPC cultures. No astroglial-restricted progenitors and no cells with oligodendroglial differentiation potential were observed.

Expression analysis of the bipotent and two unipotent clones showed that NPC markers were expressed at levels similar to those in the nonclonal iNPC population (Fig. S2).

Addition of *Brn2* Induces NPCs with Trilineage Differentiation Potential. As described above, *FoxG1*/*Sox2* iNPCs lacked oligodendrocyte differentiation potential and failed to activate the *Brn2* locus. We hypothesized that an additional factor might be able to induce a tripotent NPC population with a more complete NPC expression profile. To this end, we screened the remaining nine of our 11 candidate factors in combination with *FoxG1* and *Sox2* for their potential to induce NPC-like cells that could be differentiated into oligodendrocytes. Twenty-five days after transgene induction, *Sox2*-EGFP$^+$ cells were sorted by FACS, passaged twice, and exposed to oligodendrocyte differentiation media (*Materials and Methods*). Strikingly, O4$^+$ cells with a typical oligodendrocytic morphology could be produced only from the EGFP$^+$ population generated with the addition of *Brn2* (Fig. 4*A*). The addition of *Ascl1* also induced O4$^+$ cells, but these cells did not acquire a mature oligodendrocytic morphology. Characterization of the *FoxG1*/*Sox2*/*Brn2* *Sox2*-EGFP$^+$–sorted population showed that this population also had the potential to differentiate into GFAP$^+$ astrocytes and Tuj1$^+$/MAP2$^+$ neurons, suggesting that all three cell types of the central nervous system could be derived from this three-factor population (Fig. 4*A*).

Further experimentation showed that cells infected with *FoxG1* and *Brn2* alone could induce a *Sox2*-EGFP$^+$ population, suggesting that *Sox2* may be dispensable for the induction of NPC-like cells. We therefore characterized these potential *FoxG1*/*Brn2*-iNPCs in greater detail. Just like the cells infected with the three factors, the *FoxG1*/*Brn2*-infected cell population gave rise to cells with typical oligodendrocytic morphologies that could be labeled with antibodies against O4, Olig2, 2', 3′-cyclic nucleotide 3′-phosphodiesterase (CNP), and myelin basic protein (MBP) when exposed to oligodendrocyte differentiation media (Fig. 4*B*, *Top*). To investigate the potential of *FoxG1*/*Brn2* cells to differentiate into functional oligodendrocytes, passage 17 proliferating EGFP-infected iNPCs were injected into the neonatal brain of Shiverer mice, which are dysmyelinated and lack the wild-type form of MBP. Therefore, the presence of EGFP$^+$ and wild-type MBP$^+$ cells were assessed in Shiverer mice 6 and 10 wk after transplantation. Strikingly, 10 wk after transplantation EGFP$^+$ cells and MPB$^+$ myelin sheets were detected in white-matter tracts of the cerebellum (Fig. 4*H*). This result indicates that iNPC-derived oligodendrocytes behave similarly to oligodendrocytes derived from endogenous neural progenitor cells, differentiating into oligodendrocytes and appropriately myelinating axons of the developing brain. No MBP was detected at 6 wk, indicating that differentiation into fully mature oligodendrocytes is relatively slow in vivo.

When exposed to serum-containing media, the great majority of the *FoxG1*/*Brn2* iNPCs assumed a flat, astrocyte-like morphology very similar to the behavior of ES cell-derived NPCs. These astrocytic cells homogenously expressed GFAP, and the majority of these cells coexpressed S-100 protein (65.8 ± 3.5% at day 9 after addition of serum). The astrocyte-associated genes *Aldh1l1*, *S100b*, *Aqp4*, *Igfppb3*, and *GFAP* were expressed clearly, based on qRT-PCR (Fig. 4*G*, but note the moderate expression levels for both *Aldh1l1* and *Igfbp3* in MEFs). Moreover, characterization of the astrocytic population by patch-clamp recordings showed the development of an average resting membrane potential of −80.2 ± 3.9 mV with an input resistance of 1.2 ± 0.2 GΩ (n = 15 cells) and the lack of action potential generation upon depolarization (Fig. 4*F*). These properties were highly similar to those of astrocytes differentiated from ES cell-derived NPCs, which had an average resting membrane potential of −77.7 ± 4.4 mV and an input resistance of 1.3 ± 0.2 GΩ (n = 12 cells) (Fig. 4*F*).

Furthermore, upon withdrawal of growth factors and doxycycline, cells with typical neuronal morphologies and expression of Tuj1 and MAP2 could be identified readily (Fig. 4*B*). We performed patch-clamp recordings from neurons differentiated at passages 2, 4, and 20. These cells showed an average resting membrane potential of −47.9 ± 4.1 mV and an input resistance of 1.1 ± 0.8 GΩ (n = 7 of 39 cells) and had the capacity to generate action potential-like events, demonstrating the expression of functional voltage-gated ion channels (Fig. 4*E*). However, stereotypic action potentials could not be identified in this cell population, suggesting that *FoxG1*/*Brn2* iNPCs do not differentiate into mature neurons under standard conditions. On the other hand, when *FoxG1*/*Brn2*/*Sox2* iNPCs were differentiated, cells capable of action potential formation were readily detectable (n = 1 of 3 cells). This finding suggests that Sox2 is a critical factor in conferring a full neuronal differentiation potential to iNPCs.

After examining their capacity for differentiation, we further explored the characteristics of undifferentiated iNPCs. Both *FoxG1*/*Brn2* and *FoxG1*/*Brn2*/*Sox2* iNPC populations could be expanded easily under standard NPC growth conditions, and *FoxG1*/*Brn2* iNPCs exhibited a homogeneous population of Nestin$^+$, brain lipid-binding protein–positive (BLBP$^+$), Pax6$^+$, Olig1$^-$ cells (Fig. S4). Similar to *Sox2*/*FoxG1* iNPCs, *FoxG1*/*Sox2*/*Brn2* and *FoxG1*/*Brn2* iNPCs expressed *Sox1*, *Sox3*, *Olig2*, *Ncan*, and *Pax6* (Fig. S4). Importantly, iNPCs generated with pools containing *Brn2* also showed activation of the endogenous

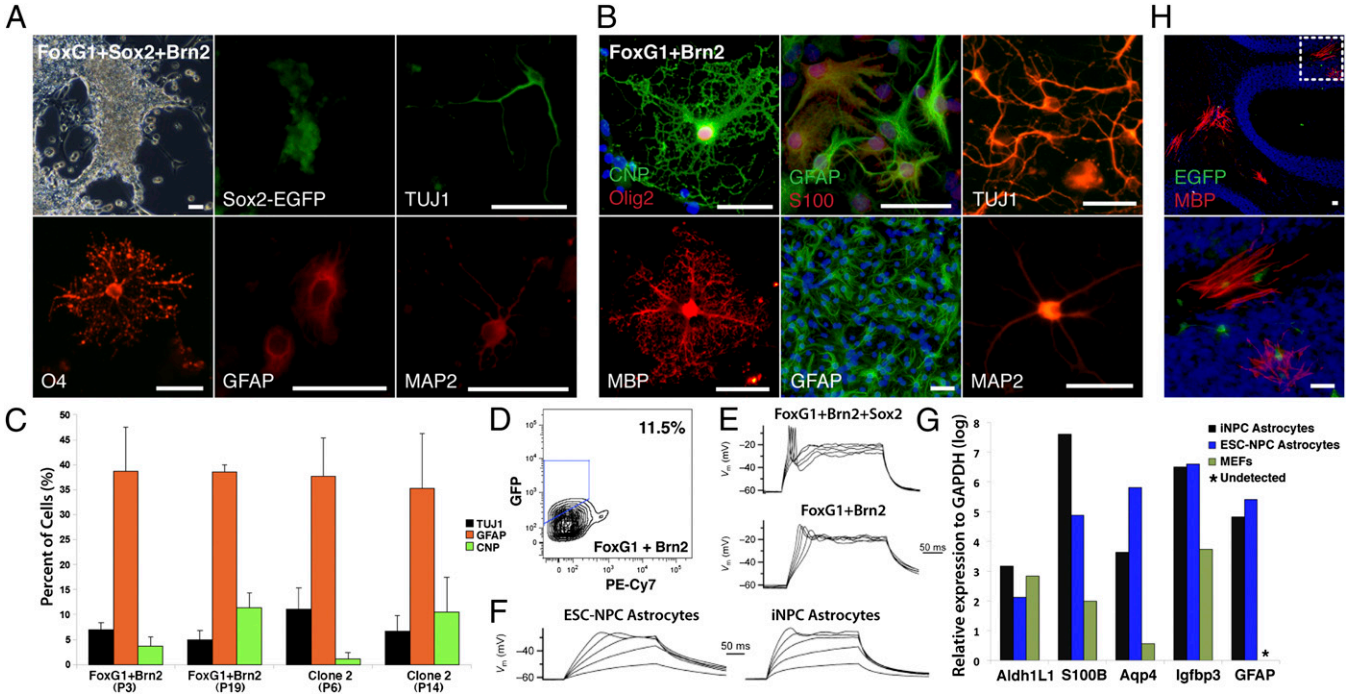

Fig. 4. Addition of *Brn2* induces a tripotent NPC population. (*A*) A *Sox2*-EGFP$^+$ population that gives rise to O4$^+$ oligodendrocytes, Tuj1$^+$ and MAP2$^+$ neurons, and GFAP$^+$ astrocytes can be induced from MEFs infected with *FoxG1*, *Sox2*, and *Brn2*. (*B*) *FoxG1* and *Brn2* alone induce a population that can give rise to mature CNP$^+$, Olig2$^+$, and MBP$^+$ oligodendrocytes, GFAP$^+$ and S100$^+$ astrocytes, and TUJ1$^+$ and MAP2$^+$ neurons. (*C*) Quantification of TUJ1$^+$, GFAP$^+$, and CNP$^+$ cells from a nonclonal (*Left*) and clonal (*Right*) *FoxG1+Brn2* iNPC population at multiple passages after 9 d in N3 and 3 d in N2B27 plus 1% serum. (*D*) *Sox2*-EGFP$^+$ cells from *FoxG1+Brn2* infections were analyzed by FACS 21 d after infection. (*E*) Representative current-clamp traces in response to current pulses. Neurons were differentiated from *FoxG1+Sox2+Brn2* (*Upper*) or *FoxG1+Brn2* (*Lower*) iNPCs 25 d after transgene induction and after 20 passages, respectively. (*F*) Electrophysiological characterization of *FoxG1/Brn2* iNPC-derived and ESC-derived astrocytes. Representative current-clamp traces recorded from iNPC-derived (*Left*) and ESC-derived (*Right*) astrocytes in response to current-pulses as in Fig. 3*C*. (*G*) *FoxG1/Brn2* iNPC-derived astrocytes express the astrocyte-associated markers *Aldh1L1*, *S100B*, *Aqp4*, *Igfbp3*, and *GFAP* ($n = 2$). (*H*) Passage 17 EGFP-labeled *FoxG1/Brn2* iNPCs were transplanted into P1 Shiverer mice targeted to the cerebellum. EGFP$^+$ transplanted cells and MBP$^+$ myelin tracks were detected 10 wk after transplantation. Right panel shows boxed area in left panel. (Scale bars: 50 μm.)

Brn2 gene and a reduction in the fibroblast-specific genes *Col1a1*, *Col3a1*, *Twist2*, *Snail1*, and *Dkk3* to levels similar to those in ES cell-derived NPCs (Figs. S3 and S4). Both findings contrast with the previously discussed 5F iNPCs and the *FoxG1/Sox2* iNPCs (compare with Figs. 2*C* and 3*D*). Thus, the addition of *Brn2* not only confers oligodendrocytic potential but also leads to repression of fibroblast-specific transcriptional characteristics.

Finally, to investigate the self-renewal potential of iNPCs, clonal analysis was performed. Four colonies were picked 29 d after infection of the fibroblasts with *FoxG1/Brn2*. After three passages, one of the four clonal populations showed the potential to differentiate into Tuj1$^+$, GFAP$^+$, and CNP$^+$ cells 12 d after removal of growth factors; the other three populations did not show NPC-like characteristics and could not differentiate into cells expressing any of these markers (Fig. S5). The differentiation potential of this clone was similar to that of the nonclonal iNPC population, and both clonal and nonclonal iNPCs remained tripotent over many passages when differentiated for 9 d in N3 medium alone and 3 d in N2B27 plus 1% serum (Fig. 4*C*).

Discussion

Here we show that specific NPC populations with defined differentiation potentials can be induced from mouse fibroblasts transduced with different combinations of lineage-specific transcription factors. Starting with a list of 11 candidate factors, we found that the combination of *FoxG1* and *Sox2* can induce NPCs (referred to as "iNPCs"), the majority of which exhibited a neuron-restricted differentiation potential and the minority a bilineage neuron/astroglial differentiation capacity. The addition of *Brn2* was sufficient to induce tripotent iNPCs from mouse fibroblasts, whereas transduction of *FoxG1* and *Brn2* alone produced iNPCs capable of differentiating into astro- and oligodendrocytes and only immature neurons. All tested bipotent and tripotent populations had the potential to self-renew on the single-cell level while maintaining their initial differentiation potential. Also, the tripotent iNPCs activated many genes selectively expressed in regular neural precursor populations and silenced a set of fibroblast-specific genes, suggesting the induction of NPC identity and elimination of at least some fibroblast-specific properties. Moreover, iNPCs responded to the same external stimuli as regular NPCs both for induction of self-renewal and differentiation into neurons, astrocytes, or oligodendrocytes (21). For example, when bi- or tripotent iNPCs were exposed to serum, astrocytic differentiation was induced very rapidly and efficiently, a phenomenon known to occur in primary or ES cell-derived NPCs and mediated by BMP and JAK/Stat signaling (25, 26, 28, 29). Thyroid hormone T3 induced the generation of oligodendrocytes, as described for other NPC populations (26, 30). These differentiated cell states are stable in the absence of the reprogramming factors, demonstrating that the transcriptional network maintaining these cell fates has been reactivated.

Recently, Kim et al. (21) described the generation of an iNPC population from mouse fibroblasts by transient expression of the four iPS cell-reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* for 6 d, followed by exposure to neural media. Following this protocol, NPC-like colonies appeared spontaneously at low frequencies (0.5–0.7%). After manual picking, these cultures gave rise to functional neurons and GFAP$^+$ astrocytes. However, the cells could not be maintained for more than three to five passages and apparently lacked the potential to differentiate into

oligodendrocytes. Conceptually, the approach of Kim et al. is fundamentally different from the approach taken in the present study. Although our underlying hypothesis is that lineage-determining transcription factors of the target cell type will directly induce the desired cell type, cell fate conversion using iPS cell transcription factors presumably first induces an unstable pluripotent state (perhaps without induction of endogenous pluripotency genes) followed by spontaneous differentiation into a neural lineage. In contrast, we believe that lineage reprogramming using target cell-type–specific transcription factors represents direct conversion (i.e., transdifferentiation) without involving an intermediate pluripotent state. Consistent with this hypothesis, *c-Myc* was required for the dedifferentiation approach (21), presumably because without *c-Myc* the induction of pluripotency is decreased by orders of magnitude (31, 32), and cell types representing a different germ layer, such as cardiomyocytes, can be achieved from a similar transient pluripotent state (20). Future studies will be required to determine whether specific NPC populations with defined differentiation potential, as shown here, can be generated using the transient dedifferentiation approach.

The generation of iNPCs from fibroblasts with subsequent differentiation to neurons may have several advantages over direct conversion of fibroblasts into postmitotic neuronal or glial cells. First, iNPCs can self-renew and can be expanded for many passages. This property should facilitate applications in which large cell numbers are needed, such as high-throughput drug screening or cell-transplantation therapy. Second, clonal populations can be generated that should lead to homogeneous cell populations more amenable to characterization than the conversion of mixed fibroblast populations. Additionally, direct iNPC generation may be more advantageous, at least for some applications, than NPC generation from iPS cells. For example, one of the major complications of potential pluripotent stem cell therapies is the risk of teratoma formation resulting from a lack of purity of the differentiated population or reactivation of the reprogramming factors. Unlike iPS cell or transient dedifferentiation, no overt oncogenes, such as *c-Myc*, or any other specific pluripotency factors are required to produce iNPCs.

However, many questions remain: Can similar cells be generated from human fibroblasts? Can iNPCs be patterned to generate any of the many neuronal and glial subtypes in the brain? Which regular NPC populations do iNPCs most resemble, and how complete is the reprogrammed NPC state? One of the key factors identified in this paper is *FoxG1*, which is expressed predominantly in the forebrain. It therefore is possible that the iNPCs described are broadly representative of rostral forebrain NPCs (which in turn are composed of many different types of NPCs). On the other hand, *FoxG1* expression also has been interpreted in the context of ES cell differentiation as a primitive (anterior) neuroectoderm capable of being patterned to many regional identities (33). These and other questions will need to be addressed before the therapeutic potential of iNPCs can be assessed.

Materials and Methods

Rosa-rtTA, Sox2-GFP MEFs were isolated from embryos on embryonic day 14.5. Internal organs, eyes, and spinal cord were carefully removed. Tau-GFP MEFs were isolated as previously described (12). cDNAs were cloned under the control of the tetracycline promoter, and lentiviral preparations were packaged in 293T cells as previously described (34). To induce lineage conversion, 200,000 cells from passage 4 MEFs were plated onto polyornithine-coated 6-cm plates and were infected the following day in MEF medium supplemented with Polybrene (8 μg/mL; Sigma). One day after infection, the medium was replaced with N3 medium (24) supplemented with FGF2 (10 ng/mL; Invitrogen), EGF (10 ng/mL; R&D Systems), and doxycycline (2 μg/mL) and was replaced every 3 d. Differentiation, immunofluorescence, FACS analysis, qRT-PCR, electrophysiology, and transplantation protocols are provided in *SI Materials and Methods*. Primers used in RT-PCR analysis are given in Table S3.

ACKNOWLEDGMENTS. We thank P. Lovelace and S. Marro for help with FACS sorting; T. Vierbuchen, Y. Kokubu, S. Mitra, and C. Khajvandi for reagents and experimental support; and N. Uchida for instrumental advice. E.L. was supported by California Institute for Regenerative Medicine Predoctoral Fellowship TG2-01159, and H.A. was supported by a postdoctoral fellowship from the Swedish Research Council. This work was enabled by a Robertson Investigator Award from New York Stem Cell Foundation; the Ellison Medical Foundation; the Stinehart-Reed Foundation; and National Institutes of Health Grants 1R01MH092931, AG010770-18A1, and RC4 NS073015.

1. Gurdon JB, Melton DA (2008) Nuclear reprogramming in cells. *Science* 322:1811–1815.
2. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810–813.
3. Gurdon JB, Byrne JA, Simonsson S (2003) Nuclear reprogramming and stem cell creation. *Proc Natl Acad Sci USA* 100(Suppl 1):11819–11822.
4. Blau HM (1989) How fixed is the differentiated state? Lessons from heterokaryons. *Trends Genet* 5:268–272.
5. Zhou Q, Melton DA (2008) Extreme makeover: Converting one cell into another. *Cell Stem Cell* 3:382–388.
6. Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51:987–1000.
7. Schäfer BW, Blakely BT, Darlington GJ, Blau HM (1990) Effect of cell history on response to helix-loop-helix family of myogenic regulators. *Nature* 344:454–458.
8. Graf T, McNagny K, Brady G, Frampton J (1992) Chicken "erythroid" cells transformed by the Gag-Myb-Ets-encoding E26 leukemia virus are multipotent. *Cell* 70:201–213.
9. Cobaleda C, Jochum W, Busslinger M (2007) Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 449:473–477.
10. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* 455:627–632.
11. Kaneto H, et al. (2005) PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. *Diabetes* 54:1009–1022.
12. Vierbuchen T, et al. (2010) Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463:1035–1041.
13. Pang ZP, et al. (2011) Induction of human neuronal cells by defined transcription factors. *Nature* 476:220–223.
14. Marro S, et al. (2011) Direct lineage conversion of terminally differentiated hepatocytes to functional neurons. *Cell Stem Cell* 9:374–382.
15. Ieda M, et al. (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142:375–386.
16. Szabo E, et al. (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* 468:521–526.
17. Sekiya S, Suzuki A. (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature* 475:390–393.
18. Huang P, et al. (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature* 475:386–389.
19. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
20. Efe JA, et al. (2011) Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. *Nat Cell Biol* 13:215–222.
21. Kim J, et al. (2011) Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci USA* 108:7838–7843.
22. Ellis P, et al. (2004) SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Dev Neurosci* 26:148–165.
23. Beard C, Hochedlinger K, Plath K, Wutz A, Jaenisch R (2006) Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. *Genesis* 44:23–28.
24. Wernig M, et al. (2002) Tau EGFP embryonic stem cells: An efficient tool for neuronal lineage selection and transplantation. *J Neurosci Res* 69:918–924.
25. Conti L, et al. (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3:e283.
26. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* 10:3129–3140.
27. Wu JQ, et al. (2010) Dynamic transcriptomes during neural differentiation of human embryonic stem cells revealed by short, long, and paired-end sequencing. *Proc Natl Acad Sci USA* 107:5254–5259.
28. Gross RE, et al. (1996) Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron* 17:595–606.
29. Mabie PC, et al. (1997) Bone morphogenetic proteins induce astroglial differentiation of oligodendroglial-astroglial progenitor cells. *J Neurosci* 17:4112–4120.
30. Glaser T, Perez-Bouza A, Klein K, Brüstle O (2005) Generation of purified oligodendrocyte progenitors from embryonic stem cells. *FASEB J* 19:112–114.
31. Nakagawa M, et al. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 26:101–106.
32. Wernig M, Meissner A, Cassady JP, Jaenisch R (2008) c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2:10–12.
33. Elkabetz Y, et al. (2008) Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes Dev* 22:152–165.
34. Wernig M, et al. (2008) A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat Biotechnol* 26:916–924.