

# Induction of Pluripotency in Astrocytes through a Neural Stem Cell-like State<sup>\*[5]</sup>

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It remains controversial whether the routes from somatic cells to induced pluripotent stem cells (iPSCs) are related to the reverse order of normal developmental processes. Specifically, it remains unaddressed whether or not the differentiated cells become iPSCs through their original tissue stem cell-like state. Previous studies analyzing the reprogramming process mostly used fibroblasts; however, the stem cell characteristics of fibroblasts made it difficult to address this. Here, we generated iPSCs from mouse astrocytes, a type of glial cells, by three (OCT3/4, KLF4, and SOX2), two (OCT3/4 and KLF4), or four (OCT3/4, KLF4, and SOX2 plus c-MYC) factors. Sox1, a neural stem cell (NSC)-specific transcription factor, is transiently up-regulated during reprogramming, and Sox1-positive cells become iPSCs. The up-regulation of Sox1 is essential for OCT3/4- and KLF4-induced reprogramming. Genome-wide analysis revealed that the gene expression profile of Sox1-expressing intermediate-state cells resembles that of NSCs. Furthermore, the intermediate-state cells are able to generate neurospheres, which can differentiate into both neurons and glial cells. Remarkably, during fibroblast reprogramming, neither Sox1 up-regulation nor an increase in neurogenic potential occurs. Our results thus demonstrate that astrocytes are reprogrammed through an NSC-like state.

Somatic cells can be reprogrammed into a pluripotent state through ectopic expression of defined transcription factors, such as Oct3/4, Klf4, Sox2, and c-Myc (OKSM)<sup>4</sup> (1). Since the generation of induced pluripotent stem cells (iPSCs), many studies have been done to elucidate the mechanism and nature of molecular changes underlying the reprogramming process

(2). However, many questions still remain unanswered. One of the fundamental questions to be answered is whether reprogramming reverses normal developmental processes or not. A recent report showed that human somatic cells are reprogrammed through a mesendoderm-like state (3). In contrast, several reports demonstrated that cells in the intermediate state of fibroblast reprogramming express developmental regulators and cell type-specific genes that are not involved in the fibroblast lineage (4–6). In addition, several reports have shown transient expression of epidermis genes during fibroblast reprogramming (7–9). These results suggest that the reprogramming process is not simply the reversal of normal developmental processes. However, it remains unclear whether the routes to iPSCs are related to the reverse order of normal developmental processes, and thus, it also remains unknown whether differentiated cells become iPSCs through their original tissue stem cell-like state or not.

Most previous studies dealing with the reprogramming process used mouse embryonic fibroblasts (MEFs) (10–13), which are heterogeneous and contain both mesenchymal and non-mesenchymal cells. Moreover, MEFs themselves are a type of tissue stem cell, and the cell lineage of MEFs is largely unknown. Therefore, the use of MEFs as donor cells may not be suitable for the study addressing the question of whether or not differentiated cells become iPSCs through their original tissue stem cell-like state.

It has been reported that various types of cells can be reprogrammed to iPSCs (14). However, the characteristics of intermediate-state cells during reprogramming from donor cell types other than MEFs have been poorly understood. In this study, we used mouse astrocytes. Astrocytes are a type of glial cells, which are differentiated from neural stem cells (NSCs), and embryonic stem cells (ESCs) differentiate into astrocytes through NSCs. Thus, if the route to iPSCs from astrocytes follows the reverse order of the normal developmental process, astrocytes may go through an NSC-like state during reprogramming. To test this, we generated iPSCs from mouse astrocytes by three (OCT3/4, KLF4 and SOX2 (OKS)) or two (OCT3/4 and KLF4 (OK)) factors as well as by four (OCT3/4, KLF4, SOX2, and c-MYC (OKSM)) factors and investigated the reprogramming process. Our results show that the expression level of Sox1, an NSC-specific transcription factor, is transiently increased in the intermediate state of astrocyte reprogramming and that the increased Sox1 expression is an indicator of successful progression of reprogramming. Remarkably,

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<sup>4</sup> The abbreviations used are: OKSM, Oct3/4, Klf4, Sox2 and c-Myc; OKS, Oct3/4, Klf4, and Sox2; OK, Oct3/4, Klf4; iPSC, induced pluripotent stem cell; NSC, neural stem cell; MEF, mouse embryonic fibroblast; ESC, embryonic stem cell; qRT, quantitative RT; AP, alkaline phosphatase; GFAP, glial fibrillary acidic protein; bFGF, basic FGF.

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the transient up-regulation of Sox1 is essential for OK-induced reprogramming. Moreover, genome-wide gene expression analysis revealed that the gene expression profile of intermediate-state cells resembles that of NSCs. We also find that intermediate-state cells are able to generate neurospheres, which can differentiate into both neurons and glial cells. Interestingly, during MEF reprogramming, neither Sox1 up-regulation nor neurogenic potential occurs. Thus, our results demonstrate that astrocytes are reprogrammed through a transient Sox1-positive state that exhibits some NSC characteristics.

### Experimental Procedures

**Cell Culture Protocols**—Primary astrocyte cultures and NSC cultures were prepared as described previously (15) with some modifications. For astrocyte cultures, postnatal 3-day mouse cortex was dissected, and cells were dissociated by mechanical titration and seeded in T75 tissue culture flasks in DMEM/F-12 (Life Technologies, Inc.) with 10% fetal bovine serum (FBS). Once confluence was attained (7–10 days), cells were shaken at 200 rpm overnight to remove nonadherent cells. Next, cells were treated with 10  $\mu$ M Ara-C (Sigma) for 2 days to remove rapidly dividing cells, and the remaining cells were then trypsinized and replated. After they reached confluence, the cells were trypsinized and replated and used for subsequent experiments. More than 90% of the resultant cells were astrocyte marker-positive, as confirmed by immunostaining for GFAP and S100 $\beta$ . Neither  $\beta$ -tubulin III-positive neurons nor O4-positive oligodendrocytes were detected. Neural stem cells were prepared from telencephalons of embryonic day 12.5–14.5 embryos. Briefly, the telencephalons were triturated by mild pipetting, and dissociated cells were seeded on non-coating culture dishes to generate neurospheres. Cells were maintained in DMEM/F-12 medium supplemented with N2 (25  $\mu$ g/ml insulin (Sigma), 100  $\mu$ g/ml apo-transferrin (Nacalai Tesque), 20 nM progesterone (Sigma), 100  $\mu$ M putrescine (Sigma), and 30 nM sodium selenite (Sigma)), 20 ng/ml basic FGF (bFGF) (R&D Systems), and 20 ng/ml EGF (R&D Systems). MEFs were isolated from embryonic day 14.5 embryos. MEFs and HEK293T cells were maintained in DMEM containing 10% FBS. Plat-E cells were maintained in DMEM containing 10% FBS, puromycin (1  $\mu$ g/ml), and blasticidin S (10  $\mu$ g/ml). The cells undergoing reprogramming were cultured in standard mouse ESC medium containing 15% ES-FBS (Thermo Scientific) and 1000 units/ml ESGRO leukemia inhibitory factor (Millipore). Mature iPSCs established from astrocytes, iPSCs established from MEFs (iPS-MEF-Ng-20D-17) (16), and reprogrammed astrocytes after cell sorting were cultured on feeder layers of mitomycin C-treated MEFs or SNL cells in mouse ESC medium containing 15% ES-FBS or 15% KnockOut Serum Replacement (KSR) (Gibco) and 1500 units/ml leukemia inhibitory factor.

**Mice**—Astrocytes and MEFs were derived from ICR mice or Sox1-GFP mice (provided by Dr. A. Smith). Sox1-GFP mice were generated by inserting enhanced GFP reporter into the Sox1 open reading frame (17), and thus Sox1-GFP mice are heterozygous Sox1 knock-out (Sox1<sup>+/-</sup>) mice. To investigate Sox1 expression during reprogramming, we used heterozygous astrocytes or MEFs from Sox1-GFP mice (Sox1<sup>+/-</sup> mice) as a reporter of Sox1 expression. Homozygous Sox1 knock-out

(Sox1<sup>-/-</sup>) mice were derived from littermates of Sox1-GFP heterozygous mating pairs. All mouse experiments were conducted in accordance with the Regulation on Animal Experimentation at Kyoto University and approved by the Animal Experimentation Committee of Kyoto University.

**Plasmid Vectors**—pMXs vectors (pMXs-hOCT4, -hSOX2, -hKLF4, -hc-MYC, and -GFP) were obtained from Addgene (Addgene plasmids 17217, 17218, 17219, and 17220) (18) or Cell Biolabs Inc. (19). MadMyc-HA was a gift from Bert Vogelstein (Addgene plasmid 16557) (20). The MadMyc-HA sequences were subcloned into a pMXs vector.

**iPSC Generation**—The generation of iPSCs by retroviruses was performed as described previously (1) with some modifications. For production of retroviruses, pMXs vectors encoding the reprogramming factors were transfected into Plat-E cells using FuGENE HD transfection reagents (Promega). Culture supernatants containing the viruses were collected 48 h after transfection. Astrocytes or MEFs were infected with the retroviruses (day 0) in the presence of 6  $\mu$ g/ml Polybrene, and the medium was replaced with the ESC medium 24 h after infection. The medium was changed every 2 days. For MEF reprogramming, the cells were plated onto new gelatin-coated plates at day 3. Neither astrocyte reprogramming nor MEF reprogramming required feeder cells.

**Cell Staining**—Cells were fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. After blocking with 3% bovine serum albumin in PBS, cells were incubated with primary antibodies in blocking buffer. After washing with PBS, cells were incubated with secondary antibodies. Images were acquired with Axiophot 2, Axiovert 200 M (Carl Zeiss), and SZX16 (Olympus). Primary antibodies used in this study are as follows: anti-GFAP (Dako, Z0334); anti-S100 $\beta$  (Sigma, S2532); anti-Oct3/4 (Santa Cruz Biotechnology, sc-9081); anti-Nanog (Calbiochem, sc1000); anti-Nanog (BD Biosciences, M55-312); anti-SSEA1 (Santa Cruz Biotechnology, sc-21702); anti- $\beta$ -tubulin III (Sigma, T8660); anti- $\alpha$ -sarcomeric actinin (Sigma, A7811); anti-Afp (Dako, A000829); anti-Sox1 (Santa Cruz Biotechnology, sc-17318); anti-E-cadherin (Cell Signaling, 3195); anti-GFP (Life Technologies, Inc., A1112); anti-O4 (Millipore, MAB345); and anti-HA (Covance, 16B12).

**Alkaline Phosphatase Staining**—Alkaline phosphatase (AP) staining was performed with the leukocyte alkaline phosphatase kit according to the manufacturer's protocol (Sigma).

**In Vitro Differentiation**—The iPSCs were isolated and suspended at  $7.5 \times 10^3$  cells/ml in ES medium containing 15% FBS. The cell suspension (100  $\mu$ l) was transferred into Ultra Low Attachment 96-plates and cultured for 5 or 6 days. The aggregated cells were plated onto gelatin-coated dishes and cultured for another 10 days. The cells were analyzed by immunostaining.

**Flow Cytometry**—Cells were dissociated using StemPro Accutase (Gibco) and passed through 35- $\mu$ m nylon mesh (BD Biosciences) to obtain single-cell suspensions. Cells were analyzed on a FACS Aria II instrument (BD Biosciences). Dead cells were excluded by staining with DAPI. In some experiments (Figs. 3, C, right panel, and E, and 6B), dissociated cells were fixed with 4% paraformaldehyde in PBS and analyzed on a BD

LSRFortessa instrument (BD Biosciences). Cutoffs were set using WT astrocytes, OKSM-introduced WT astrocytes, OKS-introduced WT astrocytes, WT MEFs, and OKS-introduced WT MEFs, respectively. The data were analyzed using FCS express4 software (De Novo software).

**qRT-PCR**—Total RNA was extracted with an RNeasy kit (Qiagen) and reverse-transcribed with a QuantiTect reverse transcription kit (Qiagen). PCR was performed with a LightCycler (Roche Applied Science). The measured value was normalized to  $\beta$ -actin. The primers used for the PCR analysis are as follows:  $\beta$ -actin, 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGATGACCTGGCCGT-3'; *Sox2*, 5'-ATGGCC-CAGCACTACCAG-3' and 5'-CCTCCCAATTCCCTTGT-ATC-3'; GFP, 5'-GACGACGGCAACTACAAGAC-3' and 5'-TCGGCCATGATATAGACGTT-3'; *Gfap*, 5'-AGGTTGAA-TCGCTGGAGGAG-3' and 5'-GCTTGGCCACATCCAT-CTC-3'; *Sox1*, 5'-TGCAGGAGGCACAGCTGGCCTAC-3' and 5'-TGCCGCCACCGCCGAGTTCTGG-3'; *Bmi1*, 5'-AAGAAATGGCCCACTACCTTTG-3' and 5'-CCAGCATT-CGTCACTCCATC-3'; *Nestin*, 5'-GCAACTGGCACACCTC-AAGA-3' and 5'-CCCAAGGAAATGCAGCTTCA-3'; *Myc*, 5'-TGTCATTCAAGCAGACGAG-3' and 5'-CACGAGAG-ATTCCAGCTCCT-3'; *Mycn*, 5'-AGTGTGTCTGTTCCAG-CTACTGC-3' and 5'-TCATCTTCCTCCTCGTCATCCT-3'; and *Nanog*, 5'-CCTCCATTCTGAACCTGAGC-3' and 5'-GGGATACTCCACTGGTGCTG-3'.

**Microarray Analysis**—Two (NSCs, iPSCs-A, and iPSCs-M) or three (astrocytes and intermediate-state cells) biological replicates were prepared for microarray samples. Total RNA was extracted with an RNeasy kit (Qiagen). cDNA synthesis and transcriptional amplification were performed using 50–100 ng of total RNA with the GeneChip WT PLUS reagent kit (Affymetrix). Fragmented and biotin-labeled cDNA targets were hybridized to GeneChip Mouse Gene 1.0 ST arrays (Affymetrix) according to the manufacturer's protocol. Hybridized arrays were scanned using an Affymetrix GeneChip Scanner. The probe set signals were calculated with the robust multiarray average algorithm implemented in GeneSpring GX 12.6 software (Agilent Technologies). Unsupervised hierarchical clustering analysis was done with Euclidean as the distance metric and average linkage as the cluster method by using GeneSpring GX. Up-regulated genes and down-regulated genes during astrocyte reprogramming were identified by statistical analysis and fold changes by using GeneSpring GX. Statistical analysis was performed by one-way analysis of variance with a Benjamini and Hochberg false discovery rate ( $= 0.05$ ) multiple testing corrections followed by Tukey post hoc tests. Tissue expression analysis and GO term analysis were performed using the DAVID Bioinformatics tool from National Institutes of Health ([david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)). Note that we refer to probe sets or entities as "genes" in the text. The genes that had low signals ( $<100$ ) are considered not to be expressed. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under the accession number GSE69237.

**Lentivirus Production**—Constructs expressing shRNAs against *Mycn* (N-Myc) were made by subcloning the following oligonucleotides into a CSII-U6-MCS-EGFP vector using the

Apal/EcoRI sites (21). The shRNA sequences were as follows: Control sense, 5'-AAGGCCAGACGCGAATTATTTCAAGA-GAATAATTCGCGTCTGGCCTTTTTTTT-3', and Control antisense, 5'-AATTAAAAAAGGCCAGACGCGAATTA-TTCTCTTGAAATAATTCGCGTCTGGCCTTGGCC-3'; *Mycn*#1-sense, 5'-TCACTAGTGTGTCTGTTCCCTTCAAG-AGAGGAACAGACACACTAGTGACCTTTTTTT-3', and *Mycn*#1-antisense, 5'-AATTAAAAAAGGTCAGTGTG-TCTGTTCCCTCTCTTGAAGGAACAGACACACTAGTGA-GGCC-3'; and *Mycn*#2-sense, 5'-CAGCAGCAGTTGCTAA-AGATTCAAGAGATCTTTAGCAACTGCTGCTGCCTTT-TTT-3', and *Mycn*#2-antisense, 5'-AATTAAAAAAGG-CAGCAGCAGTTGCTAAAGATCTCTTGAATCTTTAGC-AACTGCTGCTGGGCC-3'. To produce the recombinant lentiviruses, each shRNA vector was co-transfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into HEK293T cells using the FuGENE HD transfection reagent. Culture supernatants containing the viruses were collected 48 h after transfection and concentrated with PEG-it (System Biosciences), and the titer was calculated. Astrocytes were infected with the lentiviruses and the retroviruses encoding the reprogramming factors at the same time.

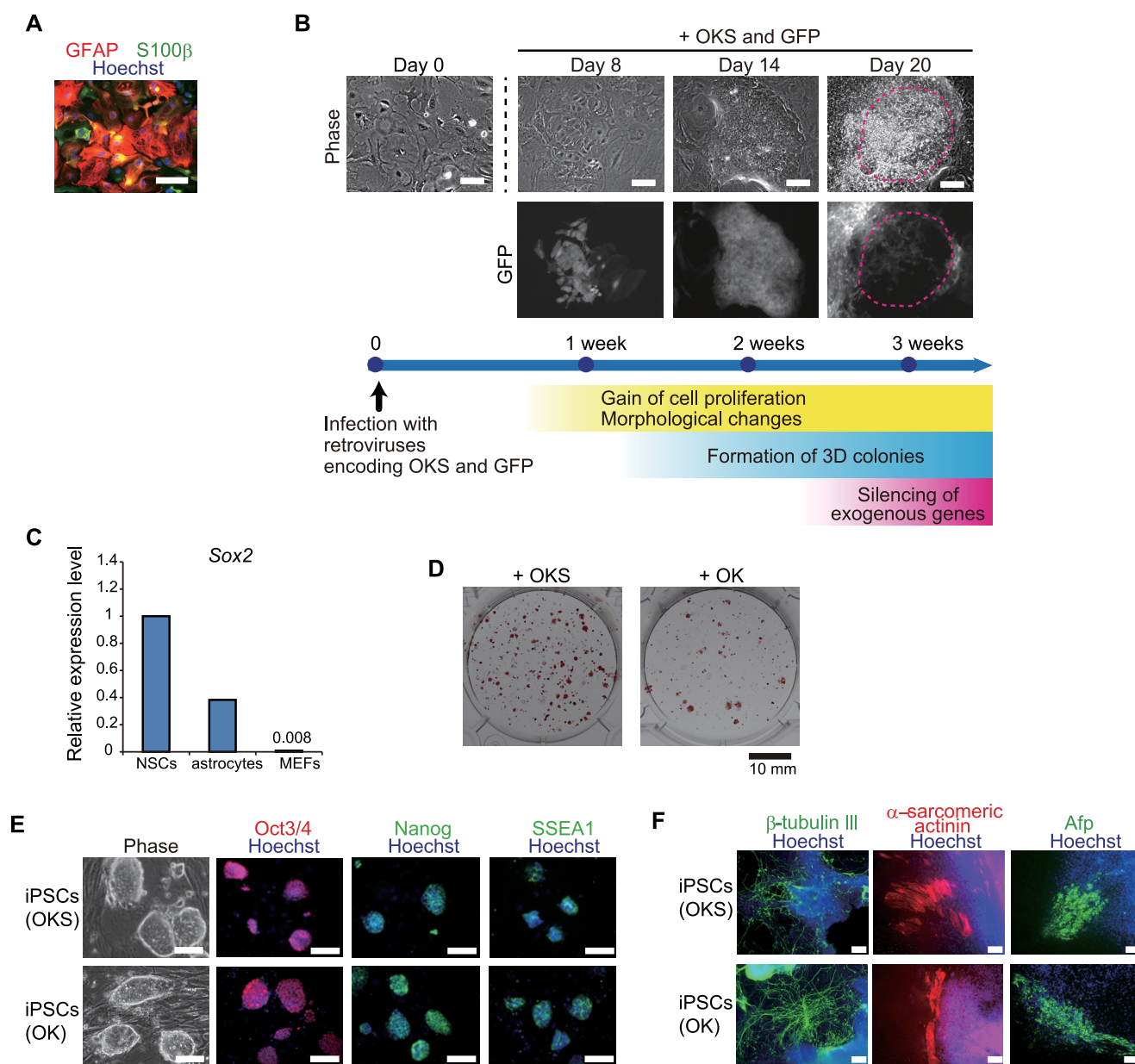
**Sphere Formation and Differentiation Assay**—Cells were dissociated using StemPro Accutase (Gibco), passed through 35- $\mu$ m nylon mesh (BD Biosciences), and resuspended at a density of 40,000 cells/ml in sphere formation medium (DMEM/F-12 supplemented with N2, 20 ng/ml bFGF, and 20 ng/ml EGF) for about 10 days. One-third of the volume of the medium was replaced, and bFGF and EGF were added every 2 days. To assay the differentiation potentials of cells, spheres or colonies were plated on poly-L-ornithine (Sigma)/fibronectin (Life Technologies, Inc.)-coated chambers and allowed to differentiate without growth factors for 5 days.

## Results

**Generation of iPSCs from Mouse Astrocytes by Three (OKS) or Two (OK) Factors**—Previous reports have demonstrated that mouse and human astrocytes can be reprogrammed to iPSCs by four factors (OKSM) (22, 23). Because this OKSM method produces numerous partially reprogrammed cells (24, 25), an OKS method is more suitable for tracing the cells undergoing successful reprogramming. Therefore, we attempted to induce reprogramming in astrocytes using the OKS method. Astrocytes were prepared from neonatal mice, and these cells expressed astrocyte marker genes, GFAP and S100 $\beta$  (Fig. 1A). At day 0, astrocytes were infected with retroviruses encoding OKS and cultured in ESC medium containing serum and leukemia inhibitory factor. At the same time, cells were infected with a retrovirus encoding GFP to visualize exogenous factor expression. In astrocytes infected with OKS, cell proliferation activation and cell morphological changes were detected within 10 days (Fig. 1B, Day 8). Then three-dimensional colonies, which are characteristics of iPSC colonies, appeared within 2 weeks (Fig. 1B, Day 14). At  $\sim 3$  weeks post infection, three-dimensional colonies became large and GFP-negative (Fig. 1B, Day 20), implying that exogenous factor expression had been silenced, and reprogramming had been completed. Previous studies have shown that NSCs can be reprogrammed into iPSCs



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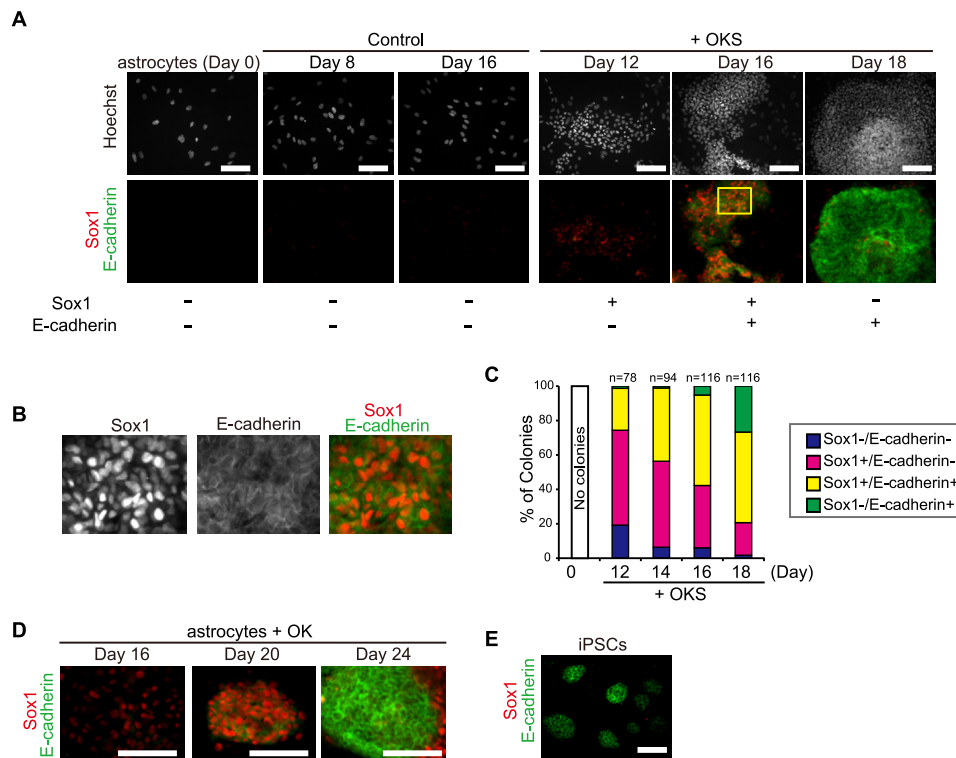


**FIGURE 1. Generation of iPSCs from mouse astrocytes by three (OKS) or two (OK) factors.** *A*, astrocyte cultures expressed astrocyte marker genes, GFAP and S100β. *B*, astrocytes were infected with retroviruses encoding OKS plus GFP at day 0 and cultured in ESC medium. Shown are phase contrast images (upper panels) and GFP expression images (lower panels) at the indicated time points. The dotted lines at day 20 indicate the outline of the colony. A scheme summarizing major changes observed during OKS-induced astrocyte reprogramming is shown in the lower part. *C*, qRT-PCR analysis of Sox2 expression in NSCs, astrocytes, and MEFs. *D*, colonies derived from OKS-introduced astrocytes and OK-introduced astrocytes were fixed at day 24 and stained with AP. *E*, phase contrast images and immunostaining analysis of pluripotency marker gene products, Oct3/4, Nanog, and SSEA1, of iPSCs established from mouse astrocytes using the OKS method or OK method are shown. *F*, embryoid bodies after 5 or 6 days of differentiation were transferred to gelatinized dishes for further differentiation. 10 days later, embryoid bodies were immunostained for β-tubulin III (ectoderm), α-sarcomeric actinin (mesoderm), and α-fetoprotein (Afp) (endoderm). Scale bars, 100 μm.

without exogenous Sox2 because NSCs already express high levels of Sox2 (26–28). Because astrocytes expressed a relatively higher level of Sox2, although to a level lower than NSCs (Fig. 1C), we expected that OCT3/4 plus KLF4 without SOX2 would be able to induce pluripotency from astrocytes. We therefore infected astrocytes with retroviruses encoding OCT3/4 and KLF4 (OK). Although the number of alkaline phosphatase (AP)-positive colonies was smaller compared with the OKS method, iPSC colonies were generated with the OK method (Fig. 1D). The iPSCs established from astrocytes using the OKS

or OK method showed ESC-like morphology and expressed pluripotent marker genes such as Oct3/4, Nanog, and SSEA1 (Fig. 1E). These iPSCs were able to differentiate into all three germ layers *in vitro* (Fig. 1F).

**Transient Increase in Sox1 Expression during Astrocyte Reprogramming**—To investigate whether astrocytes are reprogrammed into iPSCs through an NSC-like state, we first focused on Sox1 expression. SoxB1 (Sox1, Sox2, and Sox3) transcription factors are important for NSC maintenance because they inhibit differentiation (29, 30). Although Sox2 is expressed



**FIGURE 2. Transient increase in Sox1 expression during astrocyte reprogramming.** *A*, control astrocytes or OKS-introduced astrocytes were fixed and immunostained for Sox1 (lower panels, red) and E-cadherin (lower panels, green) at the indicated time points. *B*, higher magnification images of the region outlined in yellow in *A* are shown. Strong Sox1 expression was observed in E-cadherin-positive cells. *C*, quantitative analysis of the results in *A*. Representative data from two independent experiments are shown. The colony was identified as an area showing higher densities of nuclei compared with surrounding areas. *D*, OK-introduced astrocytes were immunostained for Sox1 (red) and E-cadherin (green) at the indicated time points. *E*, mature iPSCs established from astrocytes were immunostained for Sox1 (red) and E-cadherin (green). Scale bars, 100  $\mu$ m.

in various types of tissue stem cells (31) and Sox3 is expressed in the gonad (32), expression of Sox1 is limited to NSCs. During normal development, Sox1 is first expressed in the neural plate and subsequently in neuroepithelial cells, which are NSCs, and then becomes down-regulated during neuronal and glial differentiation (33, 34). During ESC differentiation into neurons and glial cells *in vitro*, Sox1 is also transiently expressed during the NSC stage (35).

To assess whether cells undergoing reprogramming express Sox1, we investigated Sox1 expression at the single cell level using immunostaining. We also investigated E-cadherin expression to identify cells undergoing reprogramming, because previous studies have shown that E-cadherin is up-regulated during the early stage of reprogramming and is required to form ESC-like compact colonies (7, 8, 36). Astrocytes in the starting population expressed neither Sox1 nor E-cadherin (Fig. 2*A*, Day 0). In control samples, which were infected with a retrovirus encoding an empty vector and cultured in ESC medium, Sox1 was not expressed or very faintly expressed (Fig. 2*A*, Control). In marked contrast, Sox1 was strongly expressed in OKS samples during astrocyte reprogramming. At day 12, Sox1 began to be expressed in the proliferating cells (Fig. 2*A*, OKS Day 12). At day 16, Sox1 expression became very strong in E-cadherin-positive colonies (Fig. 2, *A*, OKS Day 16, and *B*). At day 18, a number of large colonies with stronger E-cadherin expression showed greatly reduced expression levels of Sox1 (Fig. 2*A*, OKS Day 18). Quantitative analysis clearly demonstrated the transient expression of Sox1, which is followed by

E-cadherin expression, during astrocyte reprogramming (Fig. 2*C*). The transient increase in Sox1 expression also occurred during astrocyte reprogramming using the OK method (Fig. 2*D*). Mature iPSCs established from astrocytes did not express Sox1 at all (Fig. 2*E*).

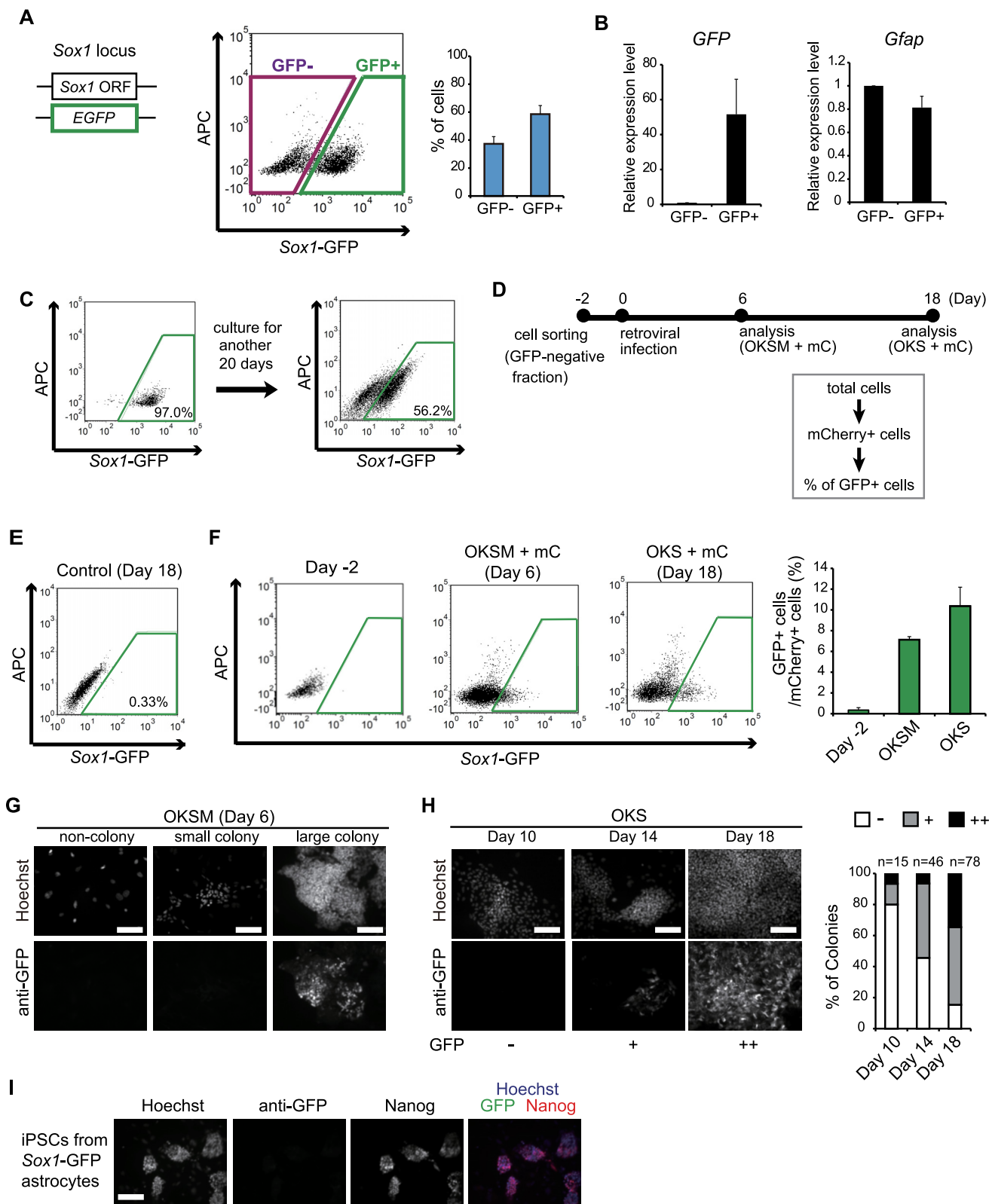
To further investigate whether Sox1 up-regulation during astrocyte reprogramming is transcriptionally regulated, we used Sox1-GFP mice (Sox1<sup>+/+</sup> mice) as a reporter of Sox1 expression (Fig. 3*A*, left panel) (17). The isolated astrocyte fractions contained both Sox1-GFP-positive and -negative cells (Fig. 3*A*, center and right panels). Sox1-GFP-negative cells expressed higher levels of *Gfap* as compared with Sox1-GFP-positive cells (Fig. 3*B*). Furthermore, about half of Sox1-GFP-positive cells became Sox1-GFP-negative after another 20 days in culture (Fig. 3*C*). Thus, we concluded that Sox1-GFP-negative cells were mature astrocytes. Therefore, we collected Sox1-GFP-negative cells by FACS (BD Biosciences) 2 days before the infection with retroviruses encoding reprogramming factors and *mCherry* (Fig. 3*D*). *mCherry* was used to trace the infected cells, and the percentages of Sox1-GFP-positive cells were determined during reprogramming. In control samples, which were infected with a retrovirus encoding *mCherry* and cultured in ESC medium for 18 days, cells remained Sox1-GFP-negative (Fig. 3*E*). In contrast, a fraction (~10%) of the *mCherry*-positive cells became Sox1-GFP-positive cells during OKSM- or OKS-induced reprogramming (Fig. 3*F*). Immunostaining analysis using the antibody against GFP showed that Sox1-GFP-positive cells existed only in large colonies (Fig. 3*G*). Smaller colonies

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did not express *Sox1*-GFP, suggesting that promotion of cell proliferation precedes the *Sox1* up-regulation. The number and the proportion of *Sox1*-GFP-positive colonies increased in later stages (Fig. 3H). Finally, iPSCs generated from *Sox1*-GFP astrocytes did not express *Sox1*-GFP at all (Fig. 3I). Collectively,

these data suggest that *Sox1* is an intermediate state marker during astrocyte reprogramming.

**Transient Expression of *Sox1* Represents Successful Reprogramming of Astrocytes**—Although E-cadherin expression can be used as an early marker of reprogramming, it is not a sufficient





marker of successful reprogramming. To investigate whether transient expression of Sox1 could be an indicator of successful reprogramming of astrocytes, we examined the relationship between Sox1 and Nanog expression using immunostaining (Fig. 4A). Nanog-positive cells first appeared in Sox1-positive colonies around days 14–16 (Fig. 4A, *left panel*). Then in later stages, the proportion of Nanog-positive cells in the colony gradually increased, and concurrently, the proportion of Sox1-positive cells decreased (Fig. 4A, *center panels*). At the single cell level, strong expression of Nanog did not coexist with strong expression of Sox1, although cells that weakly expressed Nanog simultaneously expressed Sox1 (Fig. 4B). These observations suggest that Sox1-positive cells change into Nanog-positive cells.

Next, we used Sox1-GFP reporter astrocytes to investigate whether Sox1-GFP-positive cells in the intermediate state of astrocyte reprogramming become iPSCs. We isolated Sox1-GFP-positive cells or -negative cells during astrocyte reprogramming by FACS and evaluated the reprogramming efficiencies in their later stages (Fig. 4C). More AP-positive colonies were generated from Sox1-GFP-positive cells (Fig. 4D). In addition, more Nanog-positive colonies were generated from Sox1-GFP-positive cells (Fig. 4E). Taken together, our results suggest that transient expression of Sox1 represents successful reprogramming of astrocytes. Some colonies generated from Sox1-GFP-negative fractions expressed Sox1-GFP 6 days after cell sorting, suggesting that timing of Sox1 up-regulation differs among cells (Fig. 4F).

**Transient Up-regulation of Sox1 Is Required for OK-induced Reprogramming**—Next, we investigated the role of the transient increase in Sox1 in astrocyte reprogramming. Because Sox1-GFP mice were generated by inserting enhanced GFP reporter into the Sox1 open reading frame (ORF), Sox1-GFP mice are heterozygous Sox1 knock-out (Sox1<sup>+/-</sup>) mice. Homozygous Sox1 knock-out (Sox1<sup>-/-</sup>) mice were derived from littermates of Sox1-GFP heterozygous mating pairs. We prepared astrocytes from these mice and evaluated the reprogramming efficiency (Fig. 5A). There was essentially no difference in the numbers of AP-positive colonies and Nanog-positive colonies between Sox1<sup>+/-</sup> cells and Sox1<sup>-/-</sup> cells in both OKSM- and OKS-induced reprogramming (Fig. 5, B and C). iPSCs established from Sox1<sup>-/-</sup> astrocytes expressed iPSC marker genes, such as Nanog, Oct3/4, SSEA1, and E-cadherin (Fig. 5D). In contrast, the numbers of AP-positive colonies and Nanog-positive colonies were dramatically reduced roughly in proportion to the reduced Sox1

expression levels in OK-induced reprogramming; there were very few AP- or Nanog-positive colonies in Sox1<sup>-/-</sup> cells (Fig. 5E). These results indicate that the transient Sox1 up-regulation plays an essential role in astrocyte reprogramming in the absence of exogenous SOX2.

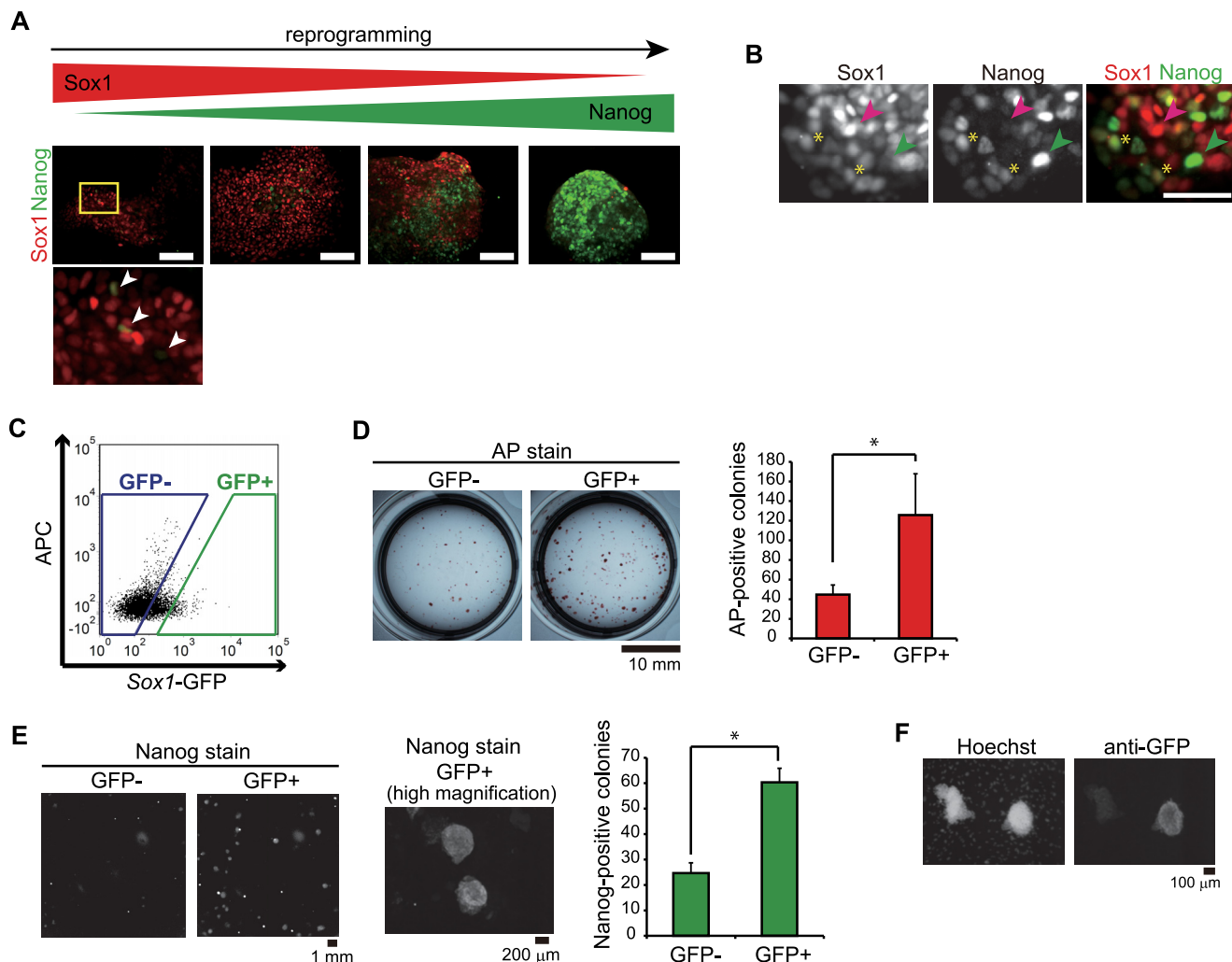
**Transient Expression of Sox1 Does Not Take Place during Fibroblast Reprogramming**—To investigate whether transient expression of Sox1 is a general phenomenon during reprogramming or specific to astrocyte reprogramming, we examined Sox1 expression during fibroblast reprogramming. MEFs were infected with retroviruses encoding OKS and subjected to immunostaining analysis at several time points. E-cadherin-positive colonies were first detected around days 12–14, and the expression level of E-cadherin was increased in later stages of reprogramming (Fig. 6A). E-cadherin-positive colonies did not express Sox1 throughout MEF reprogramming. In reprogramming of MEFs from Sox1-GFP reporter mice, FACS analysis demonstrated that Sox1-GFP-positive cells did not emerge during the reprogramming process (Fig. 6B). A number of Nanog-positive colonies were detected at day 18, suggesting that MEFs were successfully reprogrammed into iPSCs (Fig. 6C).

Next, we investigated the effect of Sox1 knock-out on MEF reprogramming. There was essentially no difference in the number of Nanog-positive colonies among Sox1<sup>+/+</sup> MEFs, Sox1<sup>+/-</sup> MEFs, and Sox1<sup>-/-</sup> MEFs in OKS-induced reprogramming (Fig. 6D). In OK-induced MEF reprogramming, essentially no Nanog-positive colonies were generated from Sox1<sup>+/+</sup> MEFs, as reported previously (1). Similarly, there were no Nanog-positive colonies in Sox1<sup>+/-</sup> MEFs and Sox1<sup>-/-</sup> MEFs. These results indicate that Sox1 does not have a role in MEF reprogramming. Taken together, our results show that transient expression of Sox1 is not a general event during somatic cell reprogramming.

**Cells in the Intermediate State of Astrocyte Reprogramming Show a Gene Expression Profile Similar to That of NSCs**—To examine the gene expression changes during astrocyte reprogramming, we performed qRT-PCR analysis in five kinds of samples as follows: starting astrocytes; intermediate-state cells expressing Sox1-GFP; NSCs; iPSCs established from Sox1-GFP astrocytes (iPSC-A); and iPSCs established from MEFs (iPSCs-M) that had been reported previously (Fig. 7A) (16). Intermediate-state cells expressed Sox1-GFP and Sox1, whose expression levels were similar to those in NSCs (Fig. 7B, Sox1-GFP and Sox1). We also found that expression of Bmi1, which is

**FIGURE 3. Sox1 is transcriptionally up-regulated during astrocyte reprogramming.** A, astrocytes from Sox1-GFP heterozygous mice (Sox1<sup>+/-</sup> mice) were used to investigate Sox1 expression during reprogramming (*left panel*). FACS analysis of Sox1-GFP expression. Astrocyte cultures contained both Sox1-GFP-positive and -negative cells (*center panel*). A cutoff was set using WT astrocytes. Percentages of Sox1-GFP-positive and -negative cells are shown (*right panel*). The data are shown as means  $\pm$  S.E. ( $n = 5$ ). B, Sox1-GFP-positive and -negative cells were sorted by FACS, and the expression levels of GFP and Gfap were analyzed by qRT-PCR. The data are shown as means  $\pm$  S.E. ( $n = 3$ ). C, FACS plot of Sox1-GFP-positive fractions is shown in the *left panel*. Sox1-GFP-positive cells were cultured for another 20 days, fixed, and subjected to FACS analysis (*right panel*). Sox1-GFP-positive cells are outlined in green. A cutoff was set using WT astrocytes, as in E. D, scheme for experiments using Sox1-GFP astrocytes. mC, mCherry. E, control Sox1-GFP astrocytes, which were infected with a retrovirus encoding mCherry, were fixed at day 18 and subjected to FACS analysis. Sox1-GFP-positive cells are outlined in green. A cutoff was set using WT astrocytes that were cultured in the same condition as control Sox1-GFP astrocytes. F, Sox1-GFP-negative astrocytes were reprogrammed with the OKSM and OKS methods. FACS plots of Sox1-GFP expression are shown. Sox1-GFP-positive cells are outlined in green. Cutoffs were set using WT astrocytes, OKSM-introduced WT astrocytes, and OKS-introduced WT astrocytes, respectively. The percentages of Sox1-GFP-positive cells are shown in the *right graph*. The data are shown as means  $\pm$  S.E. (day -2,  $n = 4$ ; OKSM,  $n = 4$ ; and OKS,  $n = 3$ ). G, OKSM-introduced Sox1-GFP astrocytes were fixed at day 6 and immunostained for GFP (*lower panels*). Anti-GFP antibody was used to visualize GFP expression, because fluorescence intensity of Sox1-GFP is quite weak. Typical images of non-colony area, small colonies, and large colonies are shown. H, OKS-introduced Sox1-GFP astrocytes were fixed and immunostained for GFP (*lower panels*) at indicated time points. Representative images of Sox1-GFP expression of the colonies (little or no GFP expression (-), weak GFP expression (+), and strong GFP expression (++)) are shown. Percentages of Sox1-GFP-expressing colonies were determined. Representative data from two independent experiments are shown. I, mature iPSCs established from Sox1-GFP astrocytes were immunostained for GFP (green) and Nanog (red). Scale bars, 100  $\mu$ m.

## NSC-like State in Astrocyte Reprogramming



**FIGURE 4. Transient expression of Sox1 represents successful reprogramming.** *A*, OKS-introduced astrocytes were fixed and immunostained for Sox1 (red) and Nanog (green). Typical images of colonies containing Nanog-positive cells are shown. A higher magnification image of the region outlined in yellow is shown in the lower panel. Nanog-positive cells are indicated by white arrowheads. Scale bars, 100  $\mu$ m. *B*, green arrowheads indicate a cell with strong Nanog expression. Magenta arrowheads indicate a cell with strong Sox1 expression. Yellow asterisks indicate cells that weakly express both Nanog and Sox1. Scale bar, 50  $\mu$ m. *C*, Sox1-GFP-positive cells outlined in green and Sox1-GFP-negative cells outlined in blue were sorted by FACS at day 6 of OKSM-induced astrocyte reprogramming. *D*, Sox1-GFP-positive and -negative cells were sorted, and 2000 cells were plated on new feeder layer-coated plates at day 6. Representative images of AP staining are shown. The number of AP-positive colonies was evaluated by AP staining at day 12. The data are shown as means  $\pm$  S.E. ( $n = 4$ ). \*,  $p < 0.05$  for paired t test. *E*, Sox1-GFP-positive and -negative cells were sorted, and 2000 cells were plated on new feeder layer-coated plates at day 6. Representative images of Nanog staining are shown. A high magnification image of Nanog-positive colonies from Sox1-GFP-positive fraction is shown in the right panel. The number of Nanog-positive colonies was evaluated by anti-Nanog staining at day 13. The data are shown as means  $\pm$  S.E. ( $n = 3$ ). \*,  $p < 0.05$  for paired t test. *F*, sorted Sox1-GFP-negative cells were plated on feeder layer-coated plates and cultured for another 6 days, and then fixed and immunostained for GFP. The right colony expressed GFP.

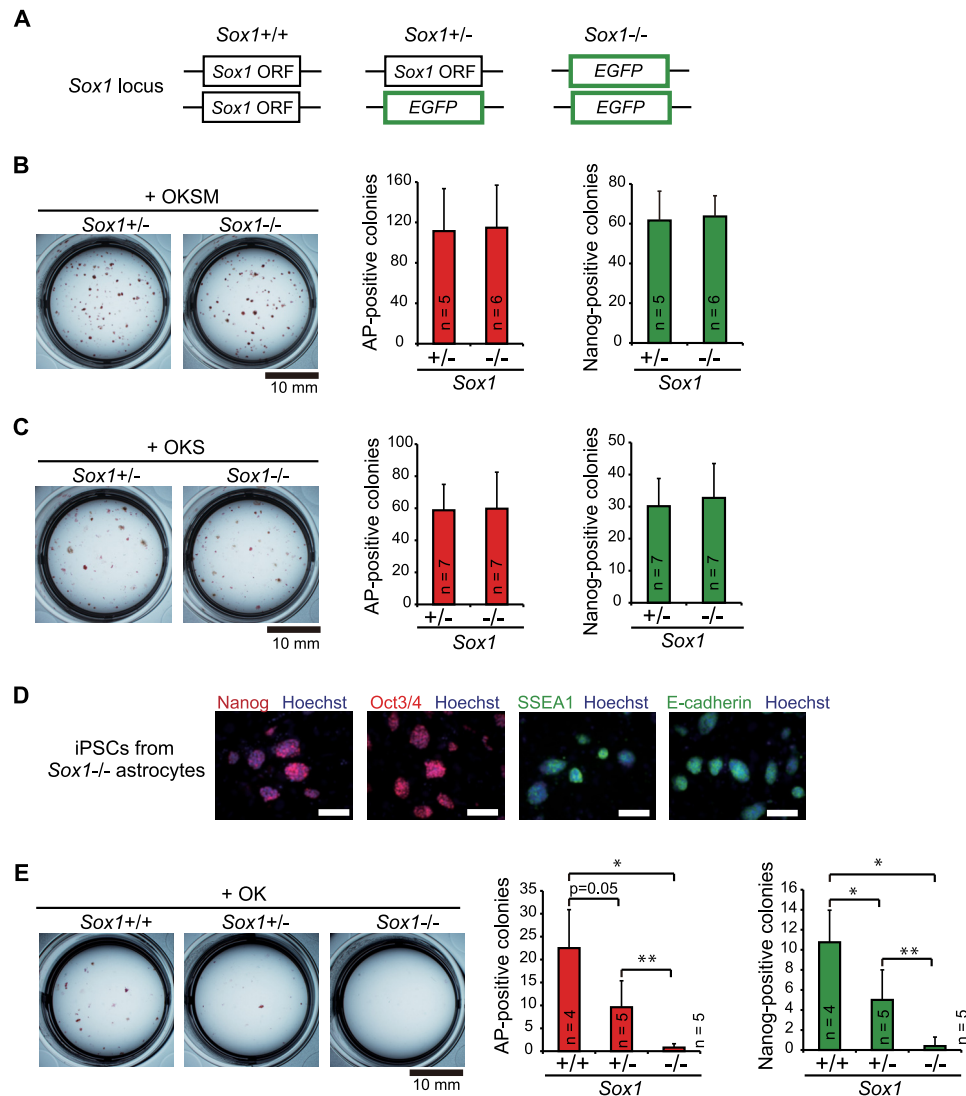
essential for the self-renewal and differentiation potential of NSC (37), was also transiently up-regulated during OKSM- and OKS-induced reprogramming (Fig. 7B, *Bmi1*). Although *Nestin* is well known as an NSC-specific gene, astrocytes also expressed *Nestin* when cultured *in vitro*, as reported previously (38). Expression of *Nestin* was down-regulated during astrocyte reprogramming (Fig. 7B, *Nestin*).

To further investigate the gene expression profile of intermediate-state cells, we performed genome-wide gene expression analysis in the five samples mentioned above. We first examined the expression of marker genes in early, middle, and late stages of reprogramming, which were identified in the previous report using MEFs (Fig. 7C) (8). Several epithelial genes (*Cdh1* (E-cadherin) and *Epcam*) and pluripotency genes (*Sall4* and *Nanog*), which are up-regulated in the early and middle stages of MEF reprogramming, respectively,

began to be up-regulated in the Sox1-positive cells during astrocyte reprogramming. However, pluripotency marker genes, which are up-regulated in the late stage of MEF reprogramming (*Ulf1* and *Dnmt3l*), were not expressed at all in the Sox1-positive cells during astrocyte reprogramming. These results suggest that the Sox1-positive intermediate state during astrocyte reprogramming may correspond to the middle stage of MEF reprogramming (8). Furthermore, astrocyte marker genes were completely down-regulated in the intermediate-state cells (Fig. 7D), which is consistent with the previous observation that MEF identity is lost in the early stage of MEF reprogramming.

We then asked which cell lineage is most similar to the intermediate-state cells. We focused on 501 genes whose expression level was reduced in iPSCs compared with that in intermediate-state cells (Fig. 7E and supplemental Table S1), because cell



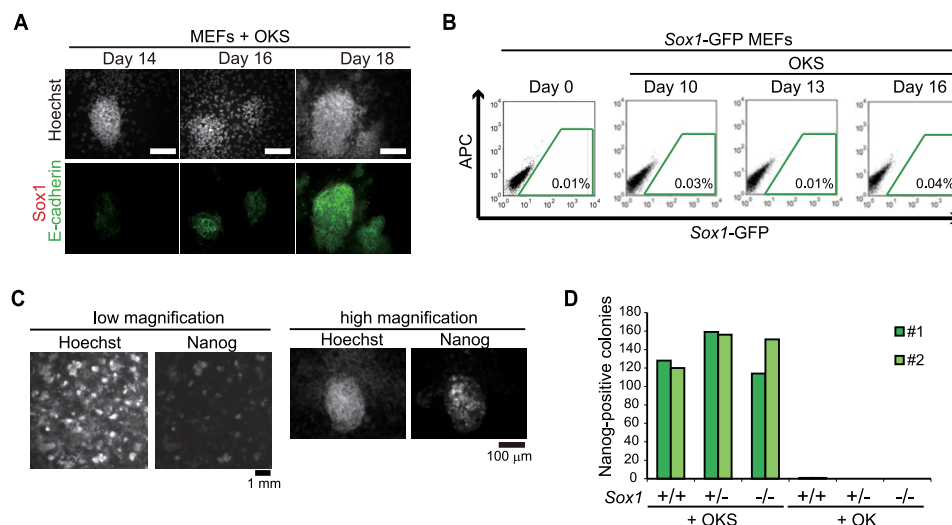


**FIGURE 5. Transient expression of Sox1 is required for OK-induced reprogramming of astrocytes.** *A*, astrocytes (Sox1<sup>+/+</sup>, Sox1<sup>+/-</sup>, and Sox1<sup>-/-</sup>) were derived from littermates of Sox1-GFP heterozygous (Sox1<sup>+/-</sup>) mating pairs. *B*, *C*, and *E*, “n” indicates the number of mice used for astrocyte cultures. *B*, numbers of AP-positive and Nanog-positive colonies generated with the OKSM method were evaluated. The data are shown as means ± S.E. *C*, numbers of AP-positive and Nanog-positive colonies generated with the OKS method were evaluated. The data are shown as means ± S.E. *D*, mature iPSCs established from Sox1<sup>-/-</sup> astrocytes with the OKS method were immunostained for iPSC marker genes, Nanog, Oct3/4, SSEA1, and E-cadherin. Scale bars, 100 μm. *E*, numbers of AP-positive and Nanog-positive colonies generated with the OK method were evaluated. The data are shown as means ± S.E. \*, *p* < 0.05, and \*\*, *p* < 0.01 for Mann-Whitney *U* test.

lineage-specific genes expressed in intermediate-state cells should be down-regulated in the fully reprogrammed state. Comparison with expression profiles of various tissues on the database revealed that genes, which were highly expressed in cerebellum and brain, were enriched in the 501 genes (Fig. 7F). Furthermore, GO analysis showed that genes, which were related to “neuron migration,” “axogenesis,” and “neuron development,” were concentrated (Table 1). We also checked the expression levels of marker genes for various developmental lineages (Fig. 7G). Neuroectoderm markers were expressed in astrocytes and NSCs but not in iPSCs. Notably, the expression levels of several neuroectoderm markers such as *Pax6*, *Zic1*, *Pou3f2*, *Pou3f4*, *Foxg1*, and *Otx1* remained high until the intermediate state of reprogramming. Moreover, marker genes for mesoderm and endoderm, which were not expressed in astrocytes and NSCs, were also not expressed in the intermediate-state cells. Collectively, our analysis suggests that the interme-

diate-state cells express a number of neural genes and thus have the characteristics of neural lineage.

Unsupervised hierarchical clustering analysis showed that the intermediate-state cells were categorized between NSCs and iPSCs (Fig. 8A). Comparison of the intermediate-state cells with the starting astrocyte population revealed that 1922 genes were up-regulated more than 2-fold in the intermediate state (Fig. 8B and supplemental Table S2). About half of the up-regulated genes in the intermediate state (903 out of 1922 genes, 47%) showed higher expression in NSCs than in astrocytes, suggesting that the gene expression profile of the intermediate-state cells resembles that of NSCs. The 903 genes can be divided into two groups as follows: genes whose expression was also up-regulated in iPSCs (767 genes, group B) or not up-regulated in iPSCs (136 genes, group A). Group A contained genes that are known to play a role in NSCs such as *Sox11*, *Fabp5*, *Tet3*, and *Fut9* (the fucosyltransferase responsible for producing



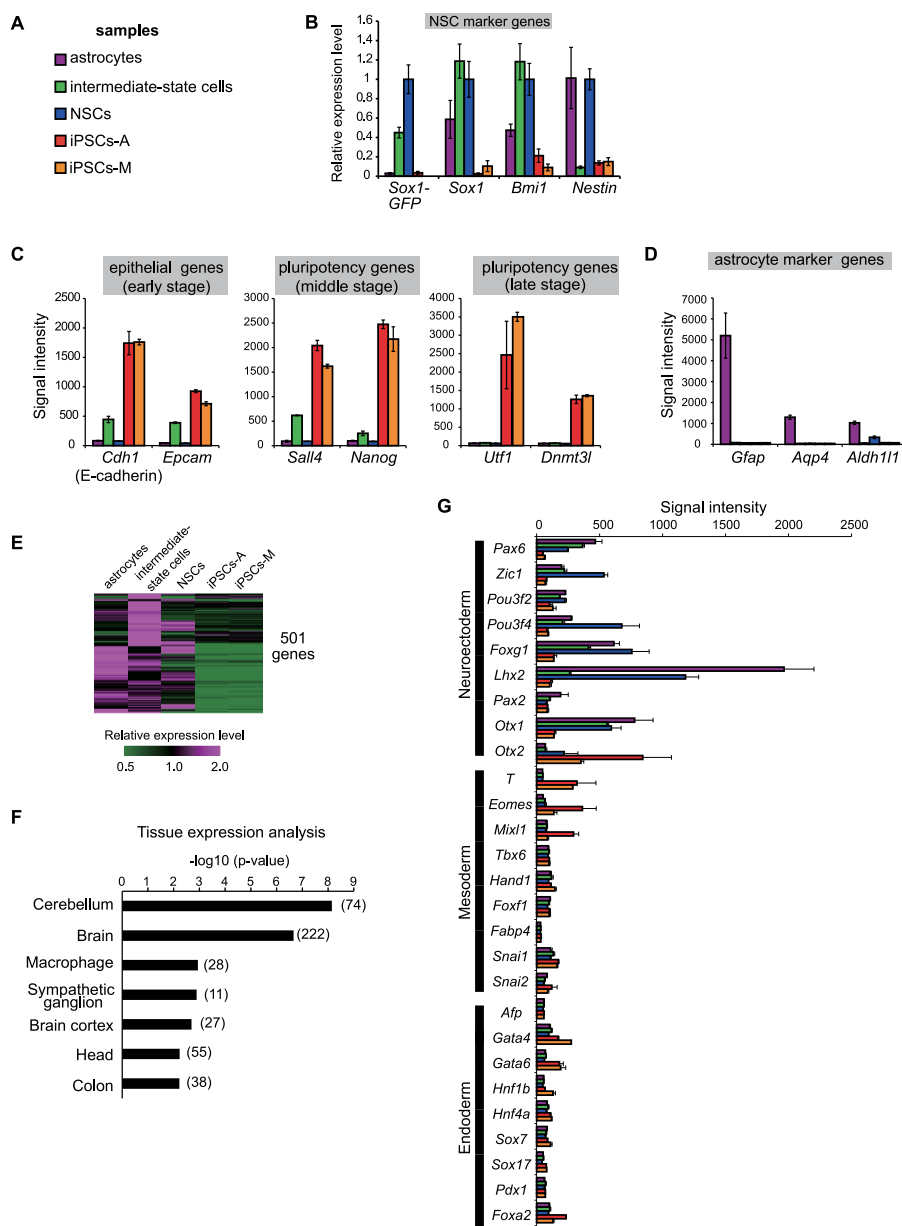
**FIGURE 6. Transient expression of Sox1 does not take place during fibroblast reprogramming.** A, OKS-introduced MEFs were immunostained for Sox1 (lower panels, red) and E-cadherin (lower panels, green). Typical images of E-cadherin-positive colonies, which do not express Sox1, are shown. Scale bars, 100  $\mu$ m. B, FACS plots of Sox1-GFP expression at indicated conditions are shown. Cutoffs were set using WT MEFs and OKS-introduced WT MEFs. C, OKS-introduced Sox1-GFP MEFs were fixed and immunostained for Nanog at day 18. A number of Nanog-positive colonies were generated. Higher magnification images of Nanog-positive colony are shown in the right part. D, MEFs (Sox1<sup>+/+</sup>, Sox1<sup>+/-</sup>, and Sox1<sup>-/-</sup>) were derived from littermates of Sox1 heterozygous (Sox1<sup>+/-</sup>) mating pairs. The number of Nanog-positive colonies generated with the OKS or OK method was evaluated. Two mice from each genotype were investigated and shown in the graph.

SSEA1) (39–43), and the expression pattern of these genes in astrocytes, intermediate-state cells, NSCs, and iPSCs was similar to that of Sox1 (Fig. 8C). Group B contained many genes that are related to cell cycle, suggesting that the intermediate-state cells possess stem cell characteristics. Group B also contained stemness genes, which are essential for the self-renewal and differentiation potential of NSCs such as *Tet1*, *Mycn* (N-Myc), *Ezh2*, and *Hmga2* (44–47), and these genes were up-regulated in the intermediate-state to the same extent as in NSCs (Fig. 8D). The up-regulation of stemness genes may correspond to the expression of c-MYC. Interestingly, endogenous *Myc* (c-Myc) and *Mycn* (N-Myc) were up-regulated during OKS-induced reprogramming, and the increases in c-Myc and N-Myc expression preceded that of *Nanog* (Fig. 9A). The inhibition of Myc activity by MadMyc expression (20) suppressed the appearance of Sox1-GFP-positive cells during reprogramming (Fig. 9, B and C). Moreover, MadMyc expression or the shRNA-mediated N-Myc knockdown markedly reduced the efficiency of OKS-induced reprogramming (Fig. 9, D–F). These results suggest that the up-regulation of Myc proto-oncogenes may be involved in both NSC-like characteristics in the intermediate-state cells during astrocyte reprogramming and the progression of reprogramming.

**Cells in the Intermediate State of Astrocyte Reprogramming Can Generate Multipotential Neurospheres**—We then examined whether intermediate-state cells have the potential to generate neurospheres that differentiate into neurons and glial cells. Control astrocytes or OKS-introduced astrocytes were dissociated into single cells at several time points and cultured for about 10 days in a serum-free medium containing bFGF and EGF (Fig. 10A). Floating spheres were then subjected to adherence culture without growth factors for 5 days to induce neuronal and glial differentiation. Although spheres were generated from control astrocytes (Fig. 10B, left panel), most (>95%) of them only differentiated into GFAP-positive astrocytes (Fig.

10C, Control left panels). A few spheres differentiated into  $\beta$ -tubulin III-positive neurons; however, the length of their neurites was quite short, and the ratio of  $\beta$ -tubulin III-positive neurons to GFAP-positive astrocytes was quite low (Fig. 10, C, Control right panels, and D). This result suggests that control astrocyte cultures have some astroglia-restricted progenitor potential but not neurogenic potential. Moreover, the spheres derived from astrocytes infected with a single OKS factor also showed very low, if any, neurogenic potential (Fig. 10D). In marked contrast, the spheres generated from cells in the intermediate state of astrocyte reprogramming (Fig. 10B, right panel) showed high neurogenic potential (Fig. 10, C, OKS, and D). Cells from the middle stage (days 12–14) of reprogramming could most efficiently generate spheres that gave rise to both neurons and astrocytes. Spheres derived from intermediate-state cells could also differentiate into O4-positive oligodendrocytes (Fig. 10E). Spheres derived from intermediate-state cells during OKSM-induced reprogramming could also differentiate into both  $\beta$ -tubulin III-positive neurons and GFAP-positive astrocytes (Fig. 10F). We then generated spheres from mature iPSCs by the same protocol. Most of the iPSCs could not survive in the serum-free medium containing bFGF and EGF, but some cells survived and generated spheres. However, these iPSC-derived spheres only differentiated into  $\beta$ -tubulin III-positive neurons (Fig. 10G). Taken together, our results show that cells in the intermediate state of astrocyte reprogramming can generate multipotential neurospheres and thus are similar to NSCs in terms of differentiation potential.

To test whether cells in the intermediate state of MEF reprogramming acquire neurogenic potential, we tried to generate spheres in the same way mentioned above. When intermediate-state cells were dissociated and cultured in serum-free medium containing bFGF and EGF, the cells attached to the surface of culture dishes, and some colonies were generated on the top of cells attached to the plate (Fig. 10H). Floating spheres were not



**FIGURE 7. Cells in the intermediate state of astrocyte reprogramming is similar to neural lineage.** *A*, five samples used for qRT-PCR analysis and microarray analysis are shown as follows: astrocytes (*Sox1*-GFP-negative cells); intermediate-state cells (*Sox1*-GFP-positive cells at day 6 of OKSM-induced reprogramming); NSCs from *Sox1*-GFP reporter mice; mature iPSCs established from *Sox1*-GFP astrocytes (iPSC-A); and iPSCs established from MEFs (iPSC-M). *B*, qRT-PCR analysis of NSC marker genes. The data are shown as means  $\pm$  S.E. ( $n = 3$ ). *C*, expression analysis of stage-specific marker genes. *D*, expression analysis of astrocyte marker genes. *E*, expression profiles of 501 genes whose expression level was reduced in iPSCs compared with that in intermediate-state cells. *F*, tissue expression analysis of 501 genes shown in *E*. The numbers of genes in indicated categories are shown at right. Analysis was performed using the NIH DAVID Bioinformatics tool. *G*, expression analysis of marker genes for neuroectoderm, mesoderm, and endoderm lineages. Signal intensity in microarray analysis is shown. *C*, *D*, and *G*, signal intensity in microarray analysis is shown. The data are shown as means  $\pm$  S.E. ( $n = 3$  for astrocytes and intermediate-state cells,  $n = 2$  for NSCs, iPSCs-A, and iPSCs-M).

generated. Most (~97%) of these colonies did not differentiate into  $\beta$ -tubulin III-positive neurons or GFAP-positive astrocytes (Fig. 10*I*). A few colonies generated  $\beta$ -tubulin III-positive neurons, which contained only one or two neurons per colony (Fig. 10*J*). The ratio of the generation of neurogenic colonies was quite low throughout the entire MEF reprogramming, and none of the colonies differentiated into GFAP-positive astrocytes. These results suggest that cells in the intermediate state of MEF reprogramming exhibit low neurogenic potential and are unable to generate multipotential neurospheres. Previous reports have shown that OKSM-introduced fibroblasts can dif-

ferentiate into multipotential NSCs (48, 49). As OKSM-induced reprogramming generates numerous partially reprogrammed cells, NSCs may arise from partially reprogrammed cells. The OKS-induced reprogramming generates fewer partially reprogrammed cells, and thus OKS-introduced MEFs may not be able to generate multipotential NSCs.

## Discussion

In this study, we have demonstrated that mouse astrocytes are reprogrammed into iPSCs through an NSC-like state. We generated iPSCs from mouse astrocytes by three (OKS) or two



TABLE 1  
GO analysis of the down-regulated genes in iPSCs compared with intermediate-state cells

Term	Count	p value
Annotation cluster 1, enrichment score 3.3313017147757016		
GO:0006928~cell motion	23	6.23E-05
GO:0016477~cell migration	17	1.90E-04
GO:0048870~cell motility	17	0.001202
GO:0051674~localization of cell	17	0.001202
GO:0001764~neuron migration	8	0.001289
Annotation cluster 2, enrichment score 2.855423016763187		
GO:0006928~cell motion	23	6.23E-05
GO:0007409~axonogenesis	13	4.94E-04
GO:0048666~neuron development	18	5.86E-04
GO:0031175~neuron projection development	15	6.85E-04
GO:0048812~neuron projection morphogenesis	13	9.75E-04
GO:0048667~cell morphogenesis involved in neuron differentiation	13	0.001304
GO:0030182~neuron differentiation	21	0.001304
GO:0030030~cell projection organization	18	0.001551
GO:0000904~cell morphogenesis involved in differentiation	14	0.001625
GO:0007411~axon guidance	9	0.002216
GO:0048858~cell projection morphogenesis	13	0.003119
GO:0032990~cell part morphogenesis	13	0.004649
GO:0000902~cell morphogenesis	16	0.006792
GO:0032989~cellular component morphogenesis	17	0.009385
Annotation cluster 3, enrichment score 2.254233766585647		
GO:0019899~enzyme binding	15	0.001371
GO:0019900~kinase binding	8	0.004436
GO:0019901~protein kinase binding	6	0.028402

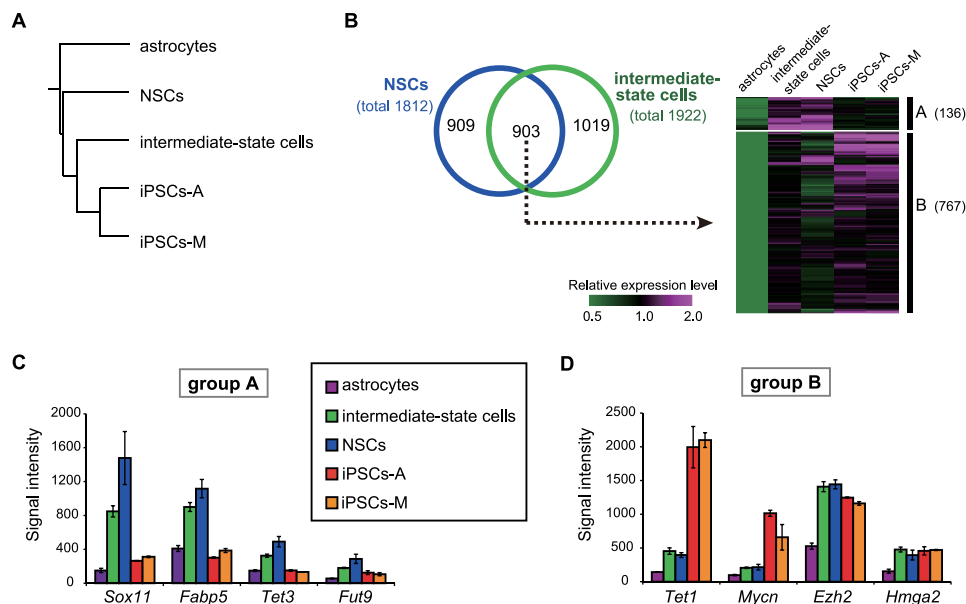
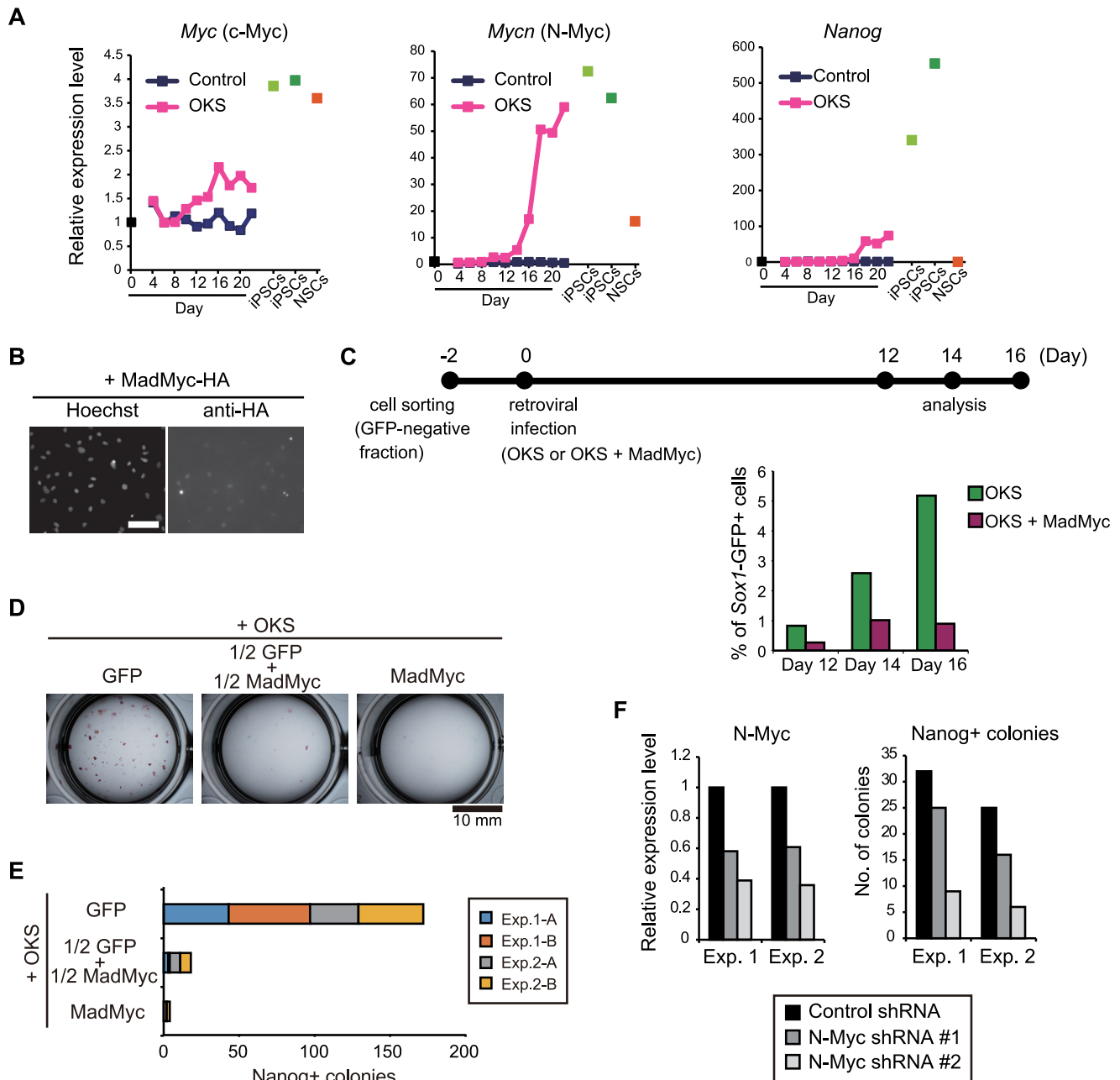


FIGURE 8. Cells in the intermediate state of astrocyte reprogramming exhibit a gene expression profile similar to that of NSCs. A, unsupervised hierarchical clustering analysis of gene expression profiles. B, Venn diagram for genes whose expression level was higher in NSCs or intermediate-state cells than in astrocytes more than 2-fold (left). A heatmap for the 903 genes whose expression was commonly higher in intermediate-state cells and NSCs (right). These 903 genes can be divided into two groups as follows: genes whose expression was also up-regulated in iPSCs (767 genes, group B) or not up-regulated in iPSCs (136 genes, group A). C, expression analysis of genes in group A. D, expression analysis of genes in group B. C and D, signal intensity in microarray analysis is shown. The data are shown as means  $\pm$  S.E. ( $n = 3$  for astrocytes and intermediate-state cells and  $n = 2$  for NSCs, iPSCs-A, and iPSCs-M).

(OK) reprogramming factors as well as by four (OKSM) factors, and we found that Sox1 is an intermediate state marker of astrocyte reprogramming. Because Sox1-positive cells become iPSC colonies in later stages, Sox1 can be used as an indicator of successful progression of reprogramming. Although Sox1 knock-out did not significantly influence the reprogramming efficiency in the OKSM and OKS methods, it dramatically reduced iPSC generation in the OK method. Because Sox1 and Sox2 have redundant function (30), Sox1 may act in place of Sox2 in OK-induced reprogramming. Moreover, our genome-wide analysis revealed that Sox1-positive intermediate-state

cells are most similar to neural lineage and exhibit an NSC-like gene expression profile. Our results also showed that many NSC-related stemness genes were up-regulated in the intermediate state prior to the activation of the pluripotent gene network. Expression of both neural lineage genes and stemness genes in the intermediate state may underlie the transient up-regulation of NSC-specific genes. Furthermore, our results show that cells in the intermediate state of astrocyte reprogramming are able to generate neurospheres that can differentiate into both neurons and astrocytes, similar to NSCs. In the case of ESC differentiation, neurogenic NSCs appear first, and



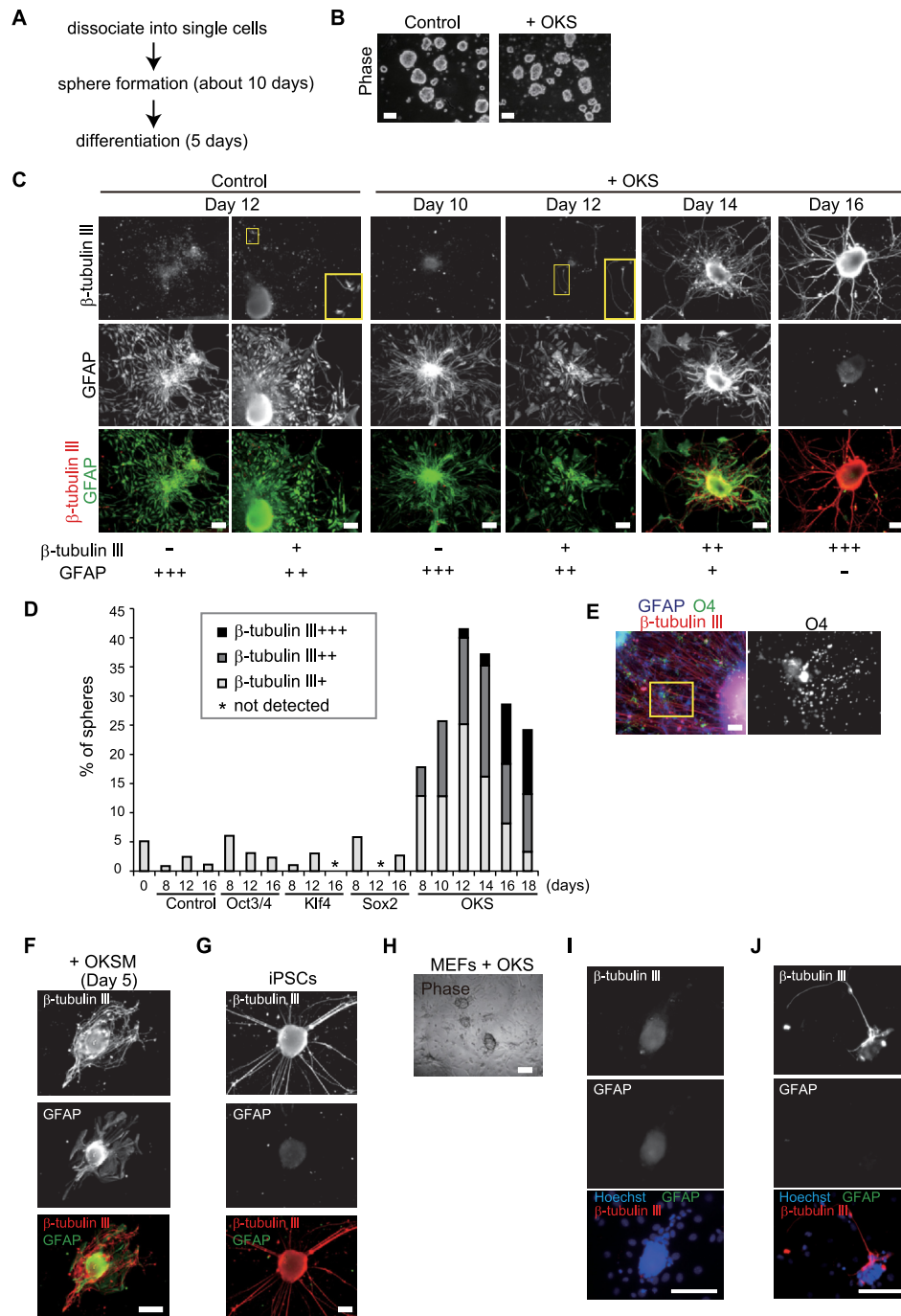
**FIGURE 9. Up-regulation of c-Myc and N-Myc during OKS-induced astrocyte reprogramming.** *A*, expression levels of *Myc* (c-Myc), *Mycn* (N-Myc), and *Nanog* in control astrocytes, OKS-introduced astrocytes, two iPSC lines, which have been established from astrocytes and MEFs respectively, and NSCs were evaluated by qRT-PCR. Representative data from two independent experiments are shown. The increase in the expression levels of c-Myc and N-Myc preceded that of *Nanog*. *B*, MadMyc is a fusion protein and functions as a dominant negative mutant of c-Myc. HA tag was added to the C terminus of MadMyc. Astrocytes were infected with retroviruses encoding MadMyc-HA. Expression of MadMyc-HA was confirmed by immunostaining against anti-HA antibody. Scale bar, 100  $\mu$ m. *C*, Sox1-GFP-negative astrocytes obtained from Sox1-GFP heterozygous (*Sox1*<sup>+/-</sup>) mice (see Fig. 3D) were infected with retrovirus encoding OKS or OKS plus MadMyc. The percentages of Sox1-GFP-positive cells at days 12, 14, and 16 were evaluated by FACS analysis. Representative data from two independent experiments are shown. *D*, astrocytes were infected with retroviruses encoding OKS plus GFP or MadMyc and subjected to AP staining analysis. *E*, numbers of Nanog-positive colonies from two independent experiments (Exp. 1 and Exp. 2), each with two technical replicates (A and B), are shown. *F*, astrocytes were infected with lentiviruses encoding N-Myc-specific shRNAs (N-Myc shRNA #1 or N-Myc shRNA #2) or the control shRNA. qRT-PCR analysis for knockdown efficiencies from two independent experiments is presented in the left graph. The numbers of Nanog-positive colonies from two independent experiments are shown in the right graph.

then neurogenic potentials are gradually decreased although gliogenic potentials are increased (50). Our results show that during astrocyte reprogramming, differentiation potentials of spheres change from gliogenic to neurogenic, which is the reverse order of normal neural lineage development. Although cells in the intermediate state of astrocyte reprogramming exhibit characteristics similar to those of NSCs, they do not

express some NSC marker genes (including Nestin). This suggests that intermediate-state cells are not identical to native NSCs, and thus astrocyte reprogramming is not simply the reversal of normal neural lineage developmental processes.

It has previously been reported that iPSCs established from mouse astrocytes using the OKSM method possess higher neurogenic potential than iPSCs established from MEFs (23).

### NSC-like State in Astrocyte Reprogramming



**FIGURE 10. Cells in the intermediate state of astrocyte reprogramming can generate multipotential neurospheres.** *A*, scheme for the sphere formation and differentiation assay is shown. *B*, phase contrast images of floating spheres generated from control astrocytes or OKS-introduced astrocytes are shown. *C*, differentiation potentials of spheres derived from indicated conditions were evaluated by immunostaining for  $\beta$ -tubulin III (red) and GFAP (green). Representative images of differentiated spheres are shown. *Insets*, higher magnification images of the regions outlined in yellow. *D*, quantitative analysis of neurogenic potential is shown. Representative data of two independent experiments are shown. The level of neurogenic potential was evaluated as shown in *C* and presented as the percentage of total spheres. More than 80 spheres per condition were examined. *E*, spheres derived from OKS-introduced astrocytes were immunostained for GFAP (blue),  $\beta$ -tubulin III (red), and O4 (green). A higher magnification image of the region outlined in yellow is shown in the right panel. *F*, OKSM-introduced astrocytes at day 5 were subjected to the experiments outlined in *A*. Representative images of the colonies, which differentiated into  $\beta$ -tubulin III-positive neurons and GFAP-positive astrocytes, are shown. *G*, mature iPSCs established from astrocytes were subjected to the experiments outlined in *A*. Representative images of the colonies that differentiated into only  $\beta$ -tubulin III-positive neurons. *H–J*, OKS-introduced MEFs were subjected to the experiments outlined in *A*. *H*, phase contrast image of colonies that were generated on the top of cells attached to the plate is shown. *I*, representative images of the colonies that did not differentiate into  $\beta$ -tubulin III-positive neurons or GFAP-positive astrocytes. *J*, typical images of colonies that generated  $\beta$ -tubulin III-positive neurons. Scale bars, 100  $\mu$ m.

Other studies have demonstrated that early passage-iPSCs retain an epigenetic memory of their cells of origin, which influences the differentiation potential of iPSCs (51, 52). Therefore,

our finding that cells in the intermediate state of astrocyte reprogramming transiently show some NSC-like characteristics may suggest that intermediate-state cells also acquire an



NSC-like epigenetic status and retain its memory until the generation of iPSCs, which contributes to the high neurogenic potential of iPSCs established from astrocytes. Further analyses of the epigenetic status during astrocyte reprogramming are required to elucidate the molecular mechanism underlying differentiation potentials of intermediate-state cells and established iPSCs.

Various types of cells can be reprogrammed into iPSCs. However, common and different characteristics among intermediate-state cells derived from different donor cell types have been poorly understood. In this study, we compared cells in the intermediate state of astrocyte and fibroblast reprogramming. Notably, unlike astrocyte reprogramming, neither increased expression of Sox1 nor neurogenic potential occurred during MEF reprogramming, indicating that cells in the intermediate state of astrocyte reprogramming are more similar to NSCs than those of MEF reprogramming. Previous reports have shown the direct reprogramming from fibroblasts toward specific cell types using the combination of conventional iPSC-inducing methods and specific lineage-inducing methods (48, 49, 53, 54). Our results suggest that understanding the intermediate state of reprogramming from various cell types would be helpful to choose the appropriate donor cell types for efficient direct reprogramming.

Our results suggest the possibility that differentiated cells are reprogrammed through an intermediate state that exhibits some tissue stem cell-like characteristics, and thus somatic reprogramming into iPSCs partially follows the retrograde pathway of normal developmental processes. A recent report has shown that transient expression of the reprogramming factors is able to generate tissue-specific stem cells from pancreatic cells and liver cells (55), implying that the occurrence of the intermediate state with tissue stem cell-like characteristics may be a common phenomenon during somatic cell reprogramming. Further characterization of intermediate-state cells from various types of donor cells should be performed in future studies.

**Author Contributions**—M. N.-K. conceived the study, designed and performed most of experiments, and analyzed the data. J. L. established protocols for astrocyte culture and astrocyte reprogramming, together with M. N.-K. S. O. and T. Y. performed FACS analysis and iPSC culture together with M. N.-K. E. N. supervised and coordinated the project. M. N.-K. and E. N. wrote the manuscript.

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