Induction of Pluripotency in Astrocytes through a Neural Stem Cell-like State

May Nakajima-Koyama, Joonseong Lee, Sho Ohta, Takuya Yamamoto, and Eisuke Nishida

From the Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, the Department of Reprogramming Science, Center for iPS Cell Research and Application, and Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Sakyo-ku, Kyoto 606-8507, and CREST, Japan Science and Technology Agency, Chiyoda-ku, Tokyo 102-0075, Japan

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) (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 epithelial 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,
NSC-like State in Astrocyte Reprogramming

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). Plat-E cells were maintained in DMEM containing 10% FBS. HEK293T cells were maintained in DMEM containing 10% KnockOut Serum Replacement (KSR) (Gibco) and 1500 units/ml leukaemia inhibitory factor.

MEFs or SNL cells in mouse ESC medium containing 15% ES-FBS (Thermo Scientific) and 1000 units/ml ESGRO leukemia inhibitory factor were cultured on feeder layers of mitomycin C-treated MEFs or SNL cells in mouse ESC medium containing 15% ES-FBS (Thermo Scientific) and 1000 units/ml ESGRO leukemia inhibitory factor. Mature iPSCs established from astrocytes, iPSCs established from MEFs or SNL cells in mouse ESC medium containing 15% ES-FBS (Thermo Scientific) and 1000 units/ml ESGRO leukemia inhibitory factor were cultured on feeder layers of mitomycin C-treated MEFs or SNL cells in mouse ESC medium containing 15% ES-FBS (Thermo Scientific) and 1000 units/ml ESGRO 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+/−) mice. To investigate Sox1 expression during reprogramming, we used heterozygous astrocytes or MEFs from Sox1-GFP mice (Sox1+/− mice) as a reporter of Sox1 expression. Homozygous Sox1 knock-out (Sox1−/−) 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 μg/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 Axiovert 2, Axiocam 200 M (Carl Zeiss), and SZX16 (Olympus). Primary antibodies used in this study are as follows: anti-GFAP (Dako, Z0334); anti-S100β (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-β-tubulin III (Sigma, T8660); anti-α-sarcosomic 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 × 10^3 cells/ml in ES medium containing 15% FBS. The cell suspension (100 μl) 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-μm nylon mesh (BD Biosciences) to obtain single-cell suspensions. Cells were analyzed on a FACSAria 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
NSC-like State in Astrocyte Reprogramming

Results

Generation of iPSCs from Mouse Astrocytes by Three (OKS) or Two (OKS) 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 S100β (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 the retroviruses encoding the reprogramming factors at the same time. Cell proliferation and cell morphology of the cultures were observed (Fig. 1B, Day 12). Then three-dimensional colonies, which are characteristic of iPSC colonies, appeared within 2 weeks (Fig. 1B, Day 14). At day 3, colonies became larger and GFAP-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...
without exogenous Sox2 because NSCs already express high levels of Sox2 (26–28). Because astrocytes expressed a relatively higher level of Sox2 (26–28), 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...
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 astrocyte reprogramming (Fig. 3A). The transient increase in Sox1 expression also occurred during astrocyte reprogramming using the OK method (Fig. 3B). Mature iPSCs established from astrocytes did not express Sox1 at all (Fig. 2E).

To further investigate whether Sox1 up-regulation during astrocyte reprogramming is transcriptionally regulated, we used Sox1-GFP mice (Sox1+/− mice) as a reporter of Sox1 expression (Fig. 3A, left panel) (17). The isolated astrocyte fractions contained both Sox1-GFP-positive and -negative cells (Fig. 3A, center and right panels). Sox1-GFP-negative cells expressed higher levels of Gfap as compared with Sox1-GFP-positive cells (Fig. 3B). Furthermore, about half of Sox1-GFP-positive cells became Sox1-GFP-negative after another 20 days in culture (Fig. 3C). 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. 3D). mCherry was used to trace the infected cells, and the percentages of Sox1-GFP-negative 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-positive and -negative cells (Fig. 3A). Smaller colonies

**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 μm.
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+/−) mice. Homozygous Sox1 knock-out (Sox1−/−) 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+/− cells and Sox1−/− cells in both OKSM- and OKS-induced reprogramming (Fig. 5, B and C). iPSCs established from Sox1−/− 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 in proportion to the reduced Sox1 expression levels in OK-induced reprogramming; there were very few AP- or Nanog-positive colonies in Sox1−/− 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+/+ MEFs, Sox1+/− MEFs, and Sox1−/− MEFs in OKS-induced reprogramming (Fig. 6D). In OK-induced MEF reprogramming, essentially no Nanog-positive colonies were generated from Sox1+/+ MEFs, as reported previously (1). Similarly, there were no Nanog-positive colonies in Sox1+/− MEFs and Sox1−/− 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
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 (Utf1 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
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 intermediate-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 5. **Transient expression of Sox1 is required for OK-induced reprogramming of astrocytes.** A, astrocytes (Sox1+/+, Sox1+/−, and Sox1−/−) were derived from littermates of Sox1-GFP heterozygous (Sox1+/−) 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 OKSM method were evaluated. The data are shown as means ± S.E. D, mature iPSCs established from Sox1−/− 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.
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 Hnnga2 (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 β-tubulin III-positive neurons; however, the length of their neurites was quite short, and the ratio of β-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 β-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 β-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
generated. Most (~97%) of these colonies did not differentiate into β-tubulin III-positive neurons or GFAP-positive astrocytes (Fig. 10I). A few colonies generated β-tubulin III-positive neurons, which contained only one or two neurons per colony (Fig. 10I). 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 differentiate 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
(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

| Term | Count | p value |
|------|-------|---------|
| cell motion | 23 | 6.23E-05 |
| neuron development | 18 | 5.86E-04 |
| neuron projection development | 15 | 6.85E-04 |
| cell morphogenesis involved in neuron differentiation | 13 | 9.75E-04 |
| neuron differentiation | 21 | 0.001304 |
| cell projection organization | 18 | 0.001551 |
| cell morphogenesis involved in differentiation | 14 | 0.001625 |
| axon guidance | 9 | 0.002216 |
| cell projection morphogenesis | 13 | 0.00319 |
| cell part morphogenesis | 13 | 0.004649 |
| cell morphogenesis | 16 | 0.006792 |
| cellular component morphogenesis | 17 | 0.009385 |
| enzyme binding | 15 | 0.00371 |
| kinase binding | 8 | 0.004436 |
| protein kinase binding | 6 | 0.028402 |
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).

**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 μm. C, Sox1-GFP-negative astrocytes obtained from Sox1-GFP heterozygous (Sox1+/−) 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.
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.

Acknowledgment—We are grateful to Dr. A. Smith for Sox1-GFP mice.

References

1. Takahashi, K., and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676
2. Buganim, Y., Faddah, D. A., and Jaenisch, R. (2013) Mechanisms and models of somatic cell reprogramming. Nat. Rev. Genet. 14, 427–439
3. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Sasaki, A., Yamamoto, M., Nakamura, M., Sutou, K., Osafune, K., and Yamanaka, S. (2014) Induction of pluripotency in human somatic cells via a transient state resembling primitive streak-like mesendoderm. Nat. Commun. 5, 3678
4. Mikkelsen, T. S., Hanna, J., Zhang, X., Xu, M., Wernig, M., Schorderet, P., Bernstein, B. E., Jaenisch, R., Lander, E. S., and Meissner, A. (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454, 49–55
5. Polo, J. M., Anderssen, E., Walsh, R. M., Schwarz, B. A., Nefzger, C. M., Lim, S. M., Borkent, M., Apostolou, E., Alaei, S., Cloutier, J., Bar-Nur, O., Cheloufi, S., Stadtfeld, M., Figueroa, M. E., Robinton, D., et al. (2012) A molecular roadmap of reprogramming somatic cells into iPSCs. Cell 151, 1617–1632
6. Koga, M., Matsuda, M., Kawamura, T., Sogo, T., Shigeno, A., Nishida, E., and Ebisuya, M. (2014) FoxD1 is a mediator and indicator of the cell reprogramming process. Nat. Commun. 5, 3197
7. Li, R., Liang, J., Ni, S., Zhu, T., Qing, X., Li, H., He, W., Chen, J., Li, F., Zhuang, Q., Qin, B., Xu, J., Li, W., Yang, J., Gan, Y., et al. (2010) A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell 7, 51–63
8. Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H. K., Beyer, T. A., Datti, A., Woltjen, K., Nagy, A., and Wrana, J. L. (2010) Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7, 64–77
9. O’Malley, J., Skylaki, S., Iwabuchi, K. A., Chantzourou, E., Ruetz, T., Johnsson, A., Tomlinson, S. R., Linnarsson, S., and Kaji, K. (2013) High-resolution analysis with novel cell-surface markers identifies routes to iPSCs. Nature 499, 88–91
10. Sridharan, R., Tchieu, J., Mason, M. J., Yachechko, R., Kuo, E., Horvath, S., Zhou, Q., and Plath, K. (2009) Role of the murine reprogramming factors in the induction of pluripotency. Cell 136, 364–377
11. Smith, Z. D., Nachman, I., Regew, A., and Meissner, A. (2010) Dynamic single-cell imaging of direct reprogramming reveals an early specifying event. Nat. Biotechnol. 28, 521–526
12. Buganim, Y., Faddah, D. A., Cheng, A. W., Itskovich, E., Markoulaki, S., Ganz, K., Klemm, S. L., van Oudenaarden, A., and Jaenisch, R. (2012) Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. Cell 150, 1209–1222
13. Golipour, A., David, L., Liu, Y., Jayakumaran, G., Hirsch, C. L., Trcka, D., and Wrana, J. L. (2012) A late transition in somatic cell reprogramming requires regulators distinct from the pluripotency network. Cell Stem Cell 11, 769–782
14. Yamanaka, S. (2009) A fresh look at iPSCs. Cell 137, 13–17
15. Imura, T., Kornblum, H. I., and Sofroniew, M. V. (2003) The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. Exp. Neurol. 23, 2824–2832
16. Okita, K., Ichisaka, T., and Yamanaka, S. (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448, 313–317
17. Aubert, J., Stavridis, M. P., Tweedie, S., O’Reilly, M., Vierlinger, K., Li, M., Ghazal, P., Pratt, T., Mason, J. O., Roy, D., and Smith, A. (2003) Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. Proc. Natl. Acad. Sci. U.S.A. 100, 11836–11841
18. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Komoda, Y., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872
19. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp. Hematol. 31, 1007–1014
20. Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J. F., Obaya, A. J., O’Connell, B. C., Mateyak, M. K., Tam, W., Kohlhuber, F., Dang, C. V., Sedivy, J. M., Eick, D., Vogelstein, B., and Kinzler, K. W. (2000) Identification of CDK4 as a target of c-MYC. Proc. Natl. Acad. Sci. U.S.A. 97, 2229–2234
21. Okamoto, K., Fujisawa, J., Reth, M., and Yonehara, S. (2006) Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kB. Genes Cells 11, 177–191
22. Ruiz, S., Brennand, K., Panopoulos, A. D., Herrerias, A., Gage, F. H., and Izpisua-Belmonte, J. C. (2010) High-efficient generation of induced pluripotent stem cells from human astrocytes. PLoS One 5, e15526
23. Tian, C., Wang, Y., Sun, L., Ma, K., and Zheng, J. C. (2011) Reprogrammed...
mouse astrocytes retain a “memory” of tissue origin and possess more tendencies for neuronal differentiation than reprogrammed mouse embryonic fibroblasts. Protein Cell 2, 128–140
24. Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mohiduki, Y., Takizawa, N., and Yamanaka, S. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. 26, 101–106
25. Wernig, M., Meissner, A., Cassidy, J. P., and Jaenisch, R. (2008) c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2, 10–12
26. Emini, S., Uitkul, J., Arnold, K., Jaenisch, R., and Hochedlinger, K. (2008) Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. Stem Cells 26, 2467–2474
27. Kim, J. B., Zehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M. J., Ruau, D., Han, D. W., Znek, M., and Scholer, H. R. (2008) Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454, 646–650
28. Shi, Y., Do, J. T., Desponts, C., Hahn, H. S., Schöler, H. R., and Ding, S. (2008) A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell 2, 525–528
29. Bylund, M., Andersson, E., Novitch, B. G., and Muhr, J. (2003) Vertebrate neurogenesis is counteracted by Sox1–3 activity. Nat. Neurosci. 6, 1162–1168
30. Graham, V., Khudyakov, J., Ellis, P., and Pevny, L. (2003) SOX2 functions required to maintain neural progenitor identity. Neuron 39, 749–765
31. Sarkar, A., and Hochedlinger, K. (2013) The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. Cell Stem Cell 12, 15–30
32. Raverot, G., Weiss, J., Park, S. Y., Hurley, L., and Jameson, I. L. (2005) Sox3 expression in undifferentiated spermatagonia is required for the progression of spermatogenesis. Dev. Biol. 283, 215–225
33. Pevny, L. H., Sockanathan, S., Placzek, M., and Lovell-Badge, R. (1998) A role for SOX1 in neural determination. Development 125, 1967–1978
34. Wood, H. B., and Episkopou, V. (1999) Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech. Dev. 86, 197–201
35. Ying, Q. L., Stavridis, M., Griffiths, D., Li, M., and Smith, A. (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat. Biotechnol. 21, 183–186
36. Chen, T., Yuan, D., Wei, B., Jiang, J., Kang, J., Ling, K., Gu, Y., Li, J., Xiao, L., and Pei, G. (2010) E-cadherin-mediated cell-cell contact is critical for induced pluripotent stem cell generation. Stem Cells 28, 1315–1325
37. Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F., and Morrison, S. J. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425, 962–967
38. Imura, T., Nakano, I., Kornblum, H. I., and Sofroniew, M. V. (2006) Phenotypic and functional heterogeneity of GFAP-expressing cells in vitro: differential expression of LeX/CD15 by GFAP-expressing multipotent neural stem cells and non-neurogenic astrocytes. Glia 53, 277–293
39. Bergland, M., Ramsköld, D., Zaouter, C., Klum, S., Sandberg, R., and Muhr, J. (2011) Sequentially activating Sox transcription factors in neural lineage development. Genes Dev. 25, 2453–2464
40. Wang, Y., Lin, L., Lai, H., Parada, L. F., and Lei, L. (2013) Transcription factor Sox1 is essential for both embryonic and adult neurogenesis. Dev. Dyn. 242, 638–653
41. Li, T., Yang, D., Li, J., Tang, Y., Yang, J., and Le, W. (2015) Critical role of Tet3 in neural progenitor cell maintenance and terminal differentiation. Mol. Neurobiol. 51, 142–154
42. Yagi, H., Saito, T., Yanagisawa, M., Yu, R. K., and Kato, K. (2012) Lewis X-carrying N-glycans regulate the proliferation of mouse embryonic neural stem cells via the Notch signaling pathway. J. Biol. Chem. 287, 24356–24364
43. Matsumata, M., Sakayori, N., Maekawa, M., Owada, Y., Yoshikawa, T., and Osumi, N. (2012) The effects of Fabp7 and Fabp5 on postnatal hippocampal neurogenesis in the mouse. Stem Cells 30, 1532–1543
44. Zhang, R. R., Cui, Q. Y., Murai, K., Lim, Y. C., Smith, Z. D., Jin, S., Ye, P., Rosa, L., Lee, Y. K., Wu, H. P., Liu, W., Xu, Z. M., Yang, L., Ding, Q., Yang, F., et al. (2013) Tet1 regulates adult hippocampal neurogenesis and cognition. Cell Stem Cell 13, 237–245
45. Knoop, P. S., Cheng, P. F., and Eisenman, R. N. (2002) Nmyc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev. 16, 2699–2712
46. Pereira, J. D., Sansom, S. N., Smith, J., Dobenecker, M. W., Tarakhovskaya, A., and Livesey, F. J. (2010) Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. Proc. Natl. Acad. Sci. U.S.A. 107, 15957–15962
47. Nishino, J., Kim, I., Chada, K., and Morrison, S. J. (2008) Hnrg2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf expression. Cell 135, 227–239
48. Kim, J., Efe, J. A., Zha, S., Talanta, M., Yuan, X., Wang, S., Lipton, S. A., Zhang, K., and Ding, S. (2011) Direct reprogramming of mouse fibroblasts to neural progenitors. Proc. Natl. Acad. Sci. U.S.A. 108, 7838–7843
49. Matsui, T., Takano, M., Yoshida, K., Ono, S., Fuijisa, C., Matsuoka, Y., Toyama, Y., Nakamura, M., Okano, H., and Akamatsu, W. (2012) Neural stem cells directly differentiated from partially reprogrammed fibroblasts rapidly acquire gliogenic competency. Stem Cells 30, 1109–1119
50. Okada, Y., Matsumoto, A., Shimazaki, T., Enoki, R., Koizumi, A., Ishii, S., Itoyama, Y., Sobue, G., and Okano, H. (2008) Spatiotemporal recapitulation of central nervous system development by murine embryonic stem cell-derived neural stem/progenitor cells. Stem Cells 26, 3086–3098
51. Kim, K., Doo, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M. J., Ji, H., Ehrlich, L. I., Yabuuchi, A., Takeuchi, A., Cunniff, K. C., Hongguang, H., McKinney-Freeman, S., et al. (2010) Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290
52. Polo, J. M., Liu, S., Figueroa, M. E., Kulaert, W., Eminli, S., Tan, K. Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., Natesan, S., Wagers, A. J., Melnick, A., Evans, T., and Hochedlinger, K. (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat. Biotechnol. 28, 848–855
53. Ele, J. A., Hilcove, S., Kim, J., Zhou, H., Ouyang, K., Wang, G., Chen, J., and Ding, S. (2011) Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. Nat. Cell Biol. 13, 215–222
54. Thier, M., Wörsdörfer, P., Lakés, Y. B., Gorris, R., Herms, S., Opitz, T., Seierling, D., Quandel, T., Hoffmann, P., Nothen, M. M., Brüstle, O., and Edenhoffer, F. (2012) Direct conversion of fibroblasts into stably expandable neural stem cells. Cell Stem Cell 10, 473–479
55. Noguchi, H., Saitoh, I., Tsugata, H., Kataoka, H., Watanabe, M., and Noguchi, Y. (2015) Induction of tissue-specific stem cells by reprogramming factors, and tissue-specific selection. Cell Death Differ. 22, 145–155