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Sur8/Shoc2 Involves Both Inhibition of Differentiation and Maintenance of Self-Renewal of Neural Progenitor Cells via Modulation of Extracellular Signal-Regulated Kinase Signaling

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ABSTRACT

Sur8/Shoc2 is a scaffold protein that regulates the Ras-extracellular signal-regulated kinase (ERK) pathway. However, the roles of Sur8 in cellular physiologies are poorly understood. In this study, Sur8 was severely repressed in the course of neural progenitor cell (NPC) differentiation in the cerebral cortex of developing rat embryos. Similarly, Sur8 was also critically reduced in cultured NPCs, which were induced differentiation by removal of basic fibroblast growth factor (bFGF). Sur8 regulation occurs at the protein level rather than at the mRNA level as revealed by both in situ hybridization and reverse transcriptase polymerase chain reaction analyses. The role of Sur8 in NPC differentiation was confirmed by lentivirus-mediated *Sur8* knockdown, which resulted in increased differentiation, whereas exogenous expression of Sur8 inhibited differentiation. Contrastingly, NPC proliferation was promoted by overexpression, but was suppressed by *Sur8* knockdown. The role of Sur8 as an antidifferentiation factor in the developing rat brain was confirmed by an ex vivo embryo culture system combined with the lentivirus-mediated *Sur8* knockdown. The numbers and sizes of neurospheres were reduced, but neuronal outgrowth was enhanced by the *Sur8* knockdown. The Ras-ERK pathway is involved in Sur8-mediated regulations of differentiation, as the treatment of ERK kinase (MEK) inhibitors blocks the effects of Sur8. The regulations of NPCs' differentiation and proliferation by the Ras-ERK pathway were also shown by the rescues of the effects of bFGF depletion, neuronal differentiation, and antiproliferation by epidermal growth factor. In summary, Sur8 is an antidifferentiation factor that stimulates proliferation for maintenance of self-renewal in NPCs via modulation of the Ras-ERK pathway. STEM CELLS *2011; 29:320–331*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Scaffold proteins are important signal transducers that play specific roles by assembling multiple pathway members into functional modules [1, 2]. However, the physiological roles of scaffold proteins have been poorly investigated due to a lack of catalytic activity and similarities with known functional domains identified by amino acid sequences [2]. Scaffold proteins of the Ras-extracellular signal-regulated kinase (ERK) pathway, such as kinase suppressor of ras (KSR) or Sur8, play important roles in the differential regulation of the Ras-ERK pathway [1–3]. *Sur8* originally was identified as one of the genes that suppress the activated form of the EGL-15 fibroblast growth factor (FGF) receptor in *Caenorhabditis elegans* (*C. elegans*) [4]. Sur8 regulates *C. elegans* vulval development via Ras-mediated signaling [5]. *Sur8* is highly conserved in various eukaryotic organisms, and the *Sur8* mutant of *C. elegans* is complemented by the human homolog of *Sur8* [4]. Sur8 is involved in growth factor-mediated activation of the Ras-ERK pathway, acting as a scaffold for Ras and Raf [6–8]. Although these studies show that Sur8 is an activator of the Ras-ERK pathway, the physiological role(s) and regulatory mechanisms of Sur8 are poorly understood.

Interestingly, in this study, we observed a significant decrease in Sur8 protein levels in the course of neural progenitor cell (NPC) differentiation in the brain of developing rat embryo. Therefore, we investigated the roles of Sur8 in the differentiation and proliferation of NPCs that are known to be controlled via the Ras-ERK pathway [9–11]. We found that overexpression of Sur8 prevents NPC differentiation and increases self-renewal capacity, and that this is dependent on

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the Ras-ERK pathway activation. Importantly, the regulation of Sur8 was not attributed to transcriptional regulation but to the regulation of protein stability. The mRNA levels of *Sur8* did not change during NPC differentiation either in vivo or in vitro. Moreover, Sur8 protein was stabilized by treatment of the proteasome inhibitor, *N*-Acetyl-Leu-Leu-Nle-CHO (ALLN). The physiological role of Sur8 in the inhibition of NPC differentiation was determined by measuring the effects of lentivirus-mediated *Sur8* knockdown in the forebrains of rat embryos, which were cultured using an ex vivo system. Overexpression and knockdown of Sur8 both increased and decreased the levels of neural stem cell markers, Nestin [12] and CD133 [13], respectively. These results show that Sur8 is a potential stem cell marker for NPCs, involving both inhibition of differentiation and maintenance of self-renewal capacity.

Materials and Methods

Animals

E14 Sprague-Dawley (SD) rats were purchased from KOATECH (Gyeonggi do, Korea, http://www.koatech.co.kr). All animal procedures were approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. The rats were housed in filter-topped shoebox cages equipped with a computerized environmental control system from MJ LTD (Seoul, Korea, http://www.mjltd.co.kr). They were allowed free access to water and given a standard maintenance diet from Dae Han Bio Link (Daejeon, Korea, http://www.dhbiolink.net), and the housing facility was maintained at 26.5°C with a relative humidity of 40%–70%.

Preparation and Culture of Neuronal Progenitor Cells and Neurospheres

NPCs were isolated from the cerebral cortex of E14 SD rats (KOATECH), and isolated cortical cells were cultured as described previously [11]. To maintain stem cell characteristics, NPCs were cultured in N2 medium containing basic FGF (bFGF) for 4 days. The stemness of cultured NPCs was confirmed by expression of Nestin and CD133 staining (Supporting Information Fig. 1A, 1B) as well as by the observation of the high expression of *Sox2* and *Nestin* mRNAs, but not the *Oct-4* and *Nanog* mRNAs [14] (Supporting Information Fig. 1C). Neurospheres were established from suspended NPCs and were maintained in the N2 medium with bFGF (10 ng/ml) and epidermal growth factor (EGF; 20 ng/ml) in noncoated Petri dishes [15]. The neurosphere cells were dissociated with 0.25% trypsin-EDTA (Invitrogen, Grand Island, NY, http://www.invitrogen.com) for the purpose of immunocytochemistry analyses. For single-cell clonal assay [16], we serially diluted cell suspensions into each well of 96-well plates. We marked wells containing single cell after microscopic confirmation and analyzed neurospheres up to the third passage. The cultured cells and neurospheres were photographed using an ECLIPSE TE2000-U fluorescence microscope (Nikon, Tokyo, Japan, http://www.nikon.com) equipped with a digital CCD camera from Diagnostic Instruments, Inc. (Sterling Heights, MI, http://www.diaginc.com).

Plasmids and siRNA

The Sur8 expression vectors pGEX4T-1-hSur8 and pcDNA3.1-myc-hSur8 were generated by cloning the 560-bp full-length human *Sur8* cDNA fragment into the *Bam*HI and *Xho*I sites of pGEX4T-1 (Amersham Bioscience, Piscataway, NJ, http://www.amershambiosciences.com) and pcDNA3.1-myc-His (−) (Invitrogen), respectively. To generate a vector for in situ hybridization analysis of Sur8, polymerase chain reaction (PCR) amplification was carried out using HiPi Plus Taq DNA polymerase (Elpisbio, Daejeon, Korea, http://www.elpisbio.com) with 5′- TGCCGAGATCGGTGAACTGTGTAA-3′ (forward) and 5′- TAAATCGCCTTGGCCTGAGGTACA-3′ (reverse) primers. The PCR products were ligated into the pGEM-T vector (Promega, Madison, WI, http://www.promega.com) to generate the pGEM-T-Sur8 vector. The target sequence for knockdown of rat *Sur8* (Gene Bank accession number NM_001013155.1) was designed using the siRNA template design program (Ambion, Austin, TX, http://www.ambion.com). *Sur8* siRNAs were synthesized using the Silencer siRNA construction kit (Ambion). The *Sur8* siRNA sequences were 5′-AACGGGATCTTTGTGAGCTGACCTGTCTC -3′ and 5′-AATCAGCTCACAAAGATCCCGCCTGTCTC-3′, and 5′-A AGTTG GTTATCTGGGTGCAGCCTGTCTC-3′ and 5′-AACT GCACCCAGATAACC AACCCTGTCTC-3′. Vectors or siRNAs were transfected into NPCs using Lipofectamine 2000 (Invitrogen) in N2 medium without antibiotics, followed by growth in N2 medium containing penicillin/streptomycin (each 50 IU/ml) [17]. The transfection efficiency was as high as 32.4% with this approach (Supporting Information Fig. 2).

Production and Purification of Anti-Sur8 Antibody

Antibody production and purification methods used in this study are described in Supporting Information.

Production of Lentivirus-Containing *Sur8 shRNA*

A vector was constructed for production of *Sur8* shRNA, containing a U6 promoter-driven shRNA coding sequence, followed by a CMV-driven reporter and enhanced green fluorescent protein (EGFP). The oligonucleotide corresponding to the 510–528 positions of rat *Sur8* (sh Sur8) (5′-GCTGCG GATGCTTGATTTA-3′) was annealed and then ligated into the *Xba*I and *Eco*RI sites of pSHAG-1. The scramble oligonucleotide (sh Sc) (5′-TCGCATAGCGTATGCCGTT-3′) was inserted instead of sh *Sur8* to use as a control [18, 19]. The infected cell's EGFP expression was observed and photographed using the fluorescence microscope. The virus is usually used at a titer of approximately 10^6–10^7 transduction units (TU) per ml.

Immunoblot, Flow Cytometry, Reverse Transcriptase Polymerase Chain Reaction Analysis

Antibodies and primers used in this study are described in Supporting Information.

Immunofluorescence Staining and Quantification

Five-micrometer sections from the paraffin-embedded cortex were deparaffinized, treated for antigen retrieval, and incubated in 1% bovine serum albumin blocking solution at room temperature for 1 hour. The sections were then incubated with primary antibodies at 4°C for 18 hours. An immunocytochemical analysis was performed that involved fixing NPCs and neurospheres in 4% paraformaldehyde for 30 minutes, rinsing with phosphate-buffered saline (PBS), and rendering them permeable with 0.2% Triton X-100 in PBS for 20 minutes at room temperature. The antibodies employed were Tuj1 (1:500), EGFP (1:250; Clontech, Mountain View, CA, http://www.clontech.com), microtubule associated protein 2ab (MAP2ab) (1:500; Chemicon, Temecula, CA, http://www.chemicon.com), glial fibrillary acidic protein (GFAP; 1:300; Chemicon), proliferating cell nuclear antigen (PCNA) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), p-ERK (1:250; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), Myc-tag (1:200; Cell Signaling Technology), O4 (1:500; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), myelin basic

protein (MBP) (1:600; Abcam Ltd, Cambridge, U.K., http://www.abcam.com), Sur8 (1:200), Nestin (1:1,000; BD Pharmingen, San Diego, CA, http://www.bd.com), Oct3/4 (H-134) (1:500, Santa Cruz Biotechnology), and CD133/ Prominin one (1:500; BD Bioscience, San Diego, CA, http://www.bdbiosciences.com). The cells were washed three times in PBS and incubated with Alexa Fluor 488- or Alexa Fluor 555-conjugated IgG secondary antibody (1:250; Molecular Probes, Eugene, OR, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) at room temperature for 45 minutes. Cell nuclei were counterstained by incubating the cells in 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim, Mannheim, Germany, http://www.boehringer-ingelheim.com) for 10 minutes and washing them thoroughly in distilled water. The Radiance 2100 multiphoton (Bio-Rad, Wattford, U.K., http://www.bio-rad.com) or LSM510META (Carl Zeiss, Jena, Germany, http://www.zeiss.com) imaging system (confocal microscope) was used for fluorescent detection at excitation wavelengths of 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 555), and 405 nm (DAPI). The quantification of fluorescence intensity was performed as described previously [20] using WCIF ImageJ bundle software v1.37a (University Health Network Research, Toronto, Canada, http://www.uhnresearch.ca/facilities/wcif/fdownload.html).

In Situ Hybridization

In situ hybridization was performed as previously described [21]. Both antisense and sense riboprobes labeled with digoxigenin (DIG) were prepared with the pGEM-T Sur8 vector using a DIG RNA Labeling Kit (SP6/T7) and DIG-dUTP (Roche, Indianapolis, IN, http://www.roche-diagnostics.us) according to the manufacturer's instructions. The DIG-labeled riboprobe was detected with anti-DIG-alkaline phosphotase, Fab fragments (Roche), and nitrobluetetrazolium/bromochloroindolyl phosphate solution.

Whole Embryo Organ Culture

E12.5 SD rat whole embryos were cultured using the roller-bottle system [22] and isolated as described previously [23]. Either EGFP-sh *Sur8* or EGFP-sh *Sc* lentivirus was mixed with Fast Green dye (Sigma-Aldrich, St Louis, MO, http://www.sigmaaldrich.com) and injected into the ventricles of the embryo brains using fine glass needles. Virus-infected whole embryos were placed in culture bottles (NuAire Inc., Plymouth, MN, http://www.nuaire.com) containing 10 ml Dulbecco's modified Eagle's medium with 20% FBS, followed by wheel rotation (Wheaton Science Products, Millville, NJ, http://www.wheatonsci.com) at a speed of 20 rpm. The embryos were incubated at 37°C in 50% O_2, 45% N_2, and 5% CO_2. The media and gases were replaced every day during the 3 days of culture.

Statistical Analysis

Statistical differences among the groups were analyzed by Student's *t* test and are indicated in figures as follows: *, $p < .05$; **, $p < .005$; and ***, $p < .0005$. *, $p < .05$ was considered statistically significant.

Results

The Stability of Sur8 Protein Decreased During NPC Differentiation in Developing Rat Embryos

To investigate whether Sur8, the scaffold protein of the Ras-ERK pathway, plays any role in neuronal differentiation, we monitored its expression pattern in the cerebral cortex of E14.5 rat embryos. To monitor the Sur8 protein, we generated an anti-Sur8 polyclonal antibody that specifically recognizes both endogenous and exogenous Sur8 (Supporting Information Fig. 3). Immunohistochemical analysis showed Sur8 expression in the cerebral cortex of the developing E14.5 rat, predominantly in the ventricular zone and the subventricular zone (SVZ; Fig. 1A). These regions contained highly proliferative cells, as revealed by PCNA-positive NPCs (Fig. 1A, upper panels; Supporting Information Fig. 4A). Sur8 expression was gradually reduced along the migration direction of differentiating NPCs, and was almost completely repressed at the cortical plate (CP), where most differentiated neuronal cells reside (Fig. 1A; Supporting Information Fig. 4B, 4C). The neuronal differentiation of cells in the CP area was confirmed by Tuj1- and MAP2ab-positive staining of most of the cells (Fig. 1A, middle and lower panels; Supporting Information Fig. 4A–4C). It has been shown previously that NPCs isolated from rat embryo cortex (E14.5) cultured in bFGF-containing N2 medium did not differentiate but retained the self-renewal ability, when they are cultured in bFGF-containing N2 medium [17, 24]. Consistent with the in vivo results, Sur8 expression levels monitored in this in vitro system were reduced, and levels of Tuj1 and PCNA were increased and decreased, respectively, in NPCs induced to differentiate by the removal of bFGF (Fig. 1B). The mRNA levels of the *Tuj1* as well as other neural differentiation markers (*GFAP*, astrocytes; *MBP*, oligodendrocytes) were concomitantly increased during the differentiation of NPCs (Fig. 1C). However, interestingly, the mRNA level of *Sur8* did not significantly change during the differentiation of NPCs (Fig. 1C). The mRNA levels of *Sur8*, which were detected by in situ hybridization in the cerebral cortex of the developing E14.5 rat, did not significantly change along the direction of migration of the differentiating NPCs from the VZ to the CP (Fig. 1D). The decrease in Sur8 protein levels during NPC differentiation was primarily blocked by treatment with the proteasomal inhibitor, ALLN (Fig. 1E). Overall, the reduction in Sur8 levels during the neural differentiation of NPCs occurs by an alteration of protein stability involving proteasomal degradation rather than by transcriptional regulation.

The multipotency of the isolated NPCs was shown by the production of neurons, astrocytes, and oligodendrocytes in the differentiation assays, concurrent with the reduction of the neural stem cell markers, Nestin and CD133, by removal of bFGF (Supporting Information Fig. 1, 5A). The stemness of NPCs was also shown by neurosphere assays (Supporting Information Fig. 5B) determining self-renewal capacity [25]. Our results show that expressions of Tuj1, GFAP, MBP, and the early microglial cell marker, O4, were increased, whereas Nestin and CD133 expression were decreased during the differentiation of NPCs by removal of bFGF (Supporting Information Fig. 1A, 5A, 5B).

Sur8 Plays a Role in Inhibiting NPC Differentiation

To determine the role of Sur8 in NPC differentiation, we measured the effects of *Sur8* knockdown during this process. NPCs were infected with lentivirus-expressing EGFP-sh Sur8 or EGFP-sh Sc. The outgrowth of neurites increased significantly in cells expressing EGFP-sh *Sur8* compared with those expressing EGFP-sh *Sc* (Supporting Information Fig. 6A). The average length of the neurites was 30% longer in NPCs EGFP-sh Sur8 than in those expressing EGFP-sh Sc (Supporting Information Fig. 6B, left panel). The relative numbers of cells had been reduced approximately 50% at 4 days after infection with EGFP-sh *Sur8* lentivirus (Supporting Information Fig. 6B, right panel). Knockdown of *Sur8* in NPCs

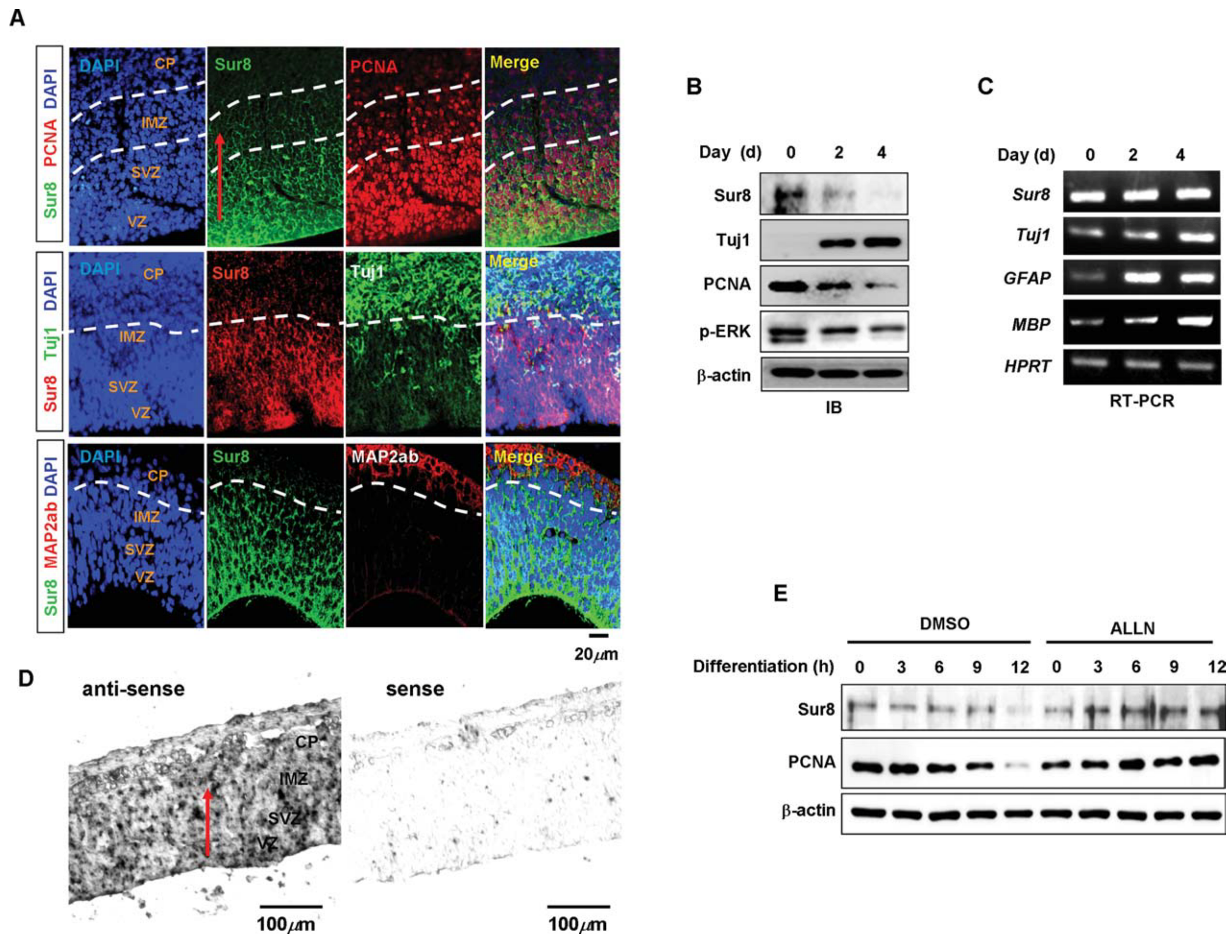

Figure 1. Sur8 expression in differentiating neural progenitor cells (NPCs) in developing rat embryos and in vitro culture system. **(A):** Sagittal sections of the paraffin-embedded E14.5 rat brain were immunohistologically stained for Sur8 (green or red), PCNA (red), Tuj1 (green), or MAP2ab (red). Nuclei were counterstained with DAPI (blue). Images were captured by a confocal microscope. The red arrow represents the migration direction of differentiating NPCs in the neural tube. **(B, C):** NPCs isolated from E14.5 rat brain were induced to differentiate by being grown in N2 media without basic fibroblast growth factor (bFGF) for 2 or 4 days. **(B):** Whole-cell lysates (WCLs) were subjected to IB analysis using anti-Sur8, -PCNA, -Tuj1, -p-ERK, or -β-actin antibody. **(C):** Quantitative RT-PCR analysis was performed with total RNA to detect mRNA level of *Sur8*, *Tuj1*, *GFAP*, *MBP*, or *HPRT*. **(D):** Sagittal sections of paraffin-embedded E14.5 rat brains were subjected to in situ hybridization with either anti-sense or sense probe to detect *Sur8* mRNA levels. **(E):** Effects of the proteasomal inhibitor ALLN on Sur8 protein stability during NPC differentiation. NPCs were grown in N2 medium without bFGF for the indicated times (0, 3, 6, 9, or 12 hours) with or without ALLN (5 μg/ml). WCLs were subjected to IB analyses using the indicated antibodies. Abbreviations: ALLN, *N*-Acetyl-Leu-Leu-Nle-CHO; CP, cortical plate; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; IB, immunoblot; IMZ, intermediate zone; MAP2ab, microtubule associated protein 2ab; MBP, myelin basic protein; PCNA, proliferating cell nuclear antigen; p-ERK, phosphorylated extracellular signal-regulated kinase; RT-PCR, reverse transcriptase polymerase chain reaction; SVZ, subventricular zone; VZ, ventricular zone.

resulted in differentiation into neurons, astrocytes, or oligodendrocytes, as determined by specific induction of Tuj1, GFAP, or O4, respectively (Fig. 2A–2C; left panels). A fluorescent-activated cell sorter (FACS) analysis showed that the *Sur8* knockdown resulted in differentiation into neurons (27%), astrocytes (11%), and oligodendrocytes (30%; Fig. 2A–2C; right panels). The role of Sur8 in the inhibition of differentiation was also shown by observing the effects of *Sur8* knockdown in the neurospheres (Supporting Information Fig. 6C). The number of NPCs in a neurosphere showing Tuj1-positive staining was critically increased by EGFP-sh Sur8 expression (Supporting Information Fig. 6C; left panel). Moreover, observation under higher magnification showed that specific induction of Tuj1 was observed only in cells expressing EGFP-sh Sur8, not in cells expressing EGFP-sh Sc (Supporting Information Fig. 6C; right panel).

The role of Sur8 in the inhibition of NPC differentiation was also shown by its exogenous expression of EGFP-tagged Sur8 (EGFP-Sur8). Differentiation of NPCs into neurons, astrocytes, and oligodendrocytes was blocked in the cells expressing EGFP-Sur8 but not in the cells expressing the EGFP control (Fig. 3A–3C, left panels). The ability of Sur8 to inhibit NPC differentiation was also shown by FACS analyses (Fig. 3A–3C, right panels). To further confirm that Sur8 inhibits differentiation of NPCs, we also monitored the differentiation markers by immunoblot (IB) analyses and reverse transcriptase polymerase chain reaction (RT-PCR) analyses. Both the protein and mRNA levels of *Tuj1*, *GFAP*, and *MBP* were increased and decreased by knockdown and exogenous expression of Sur8, respectively (Fig. 4A, 4B).

Sur8 Plays a Role in the Proliferation and Self-Renewal of NPCs

NPC differentiation and proliferation are generally considered as mutually exclusive states. The expression pattern of PCNA

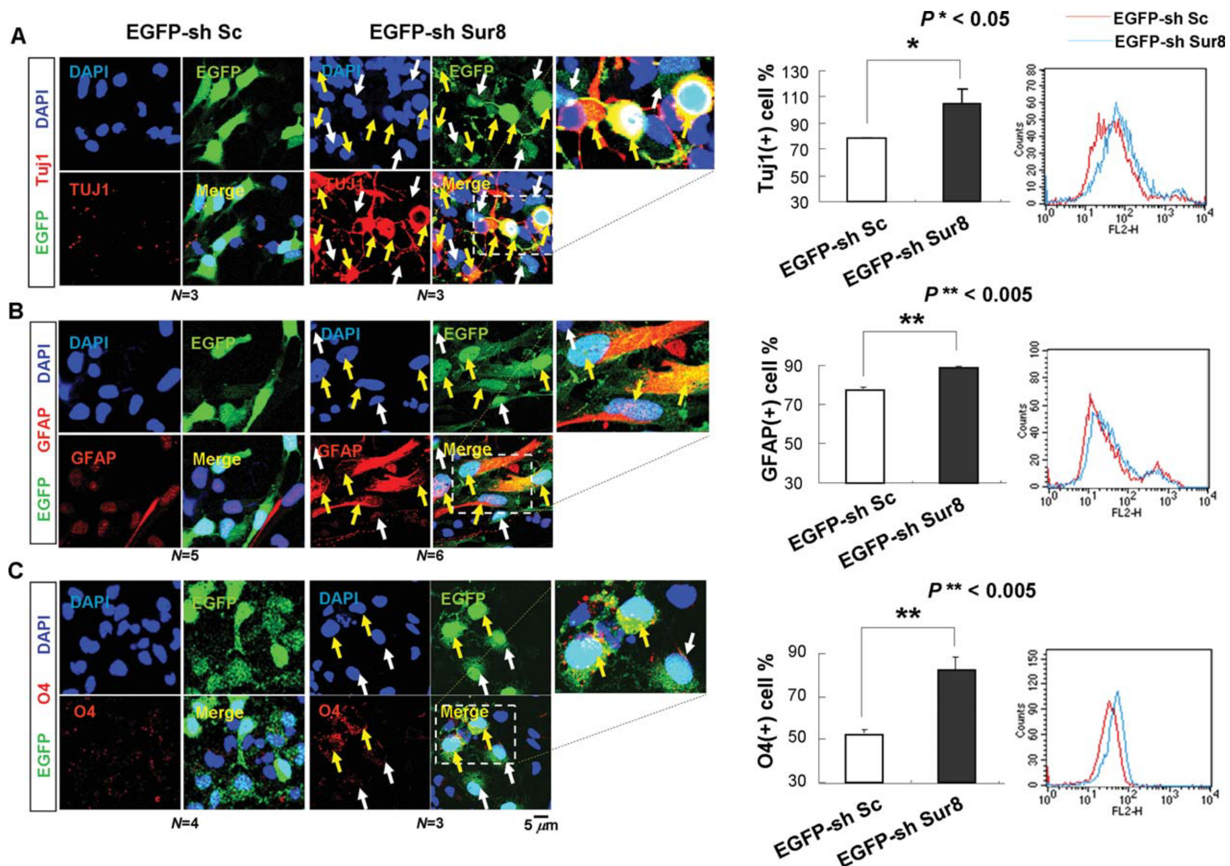

Figure 2. Effects of *Sur8* knockdown on differentiation of multipotent neural progenitor cells (NPCs). **(A–C):** NPCs, infected with EGFP-sh Sc or EGFP-sh Sur8 lentivirus, were grown for 96 hours with basic fibroblast growth factor (10 ng/ml). Immunofluorescent labeling (left panels) or fluorescent-activated cell sorter (FACS) analysis (right panels) were used to detect Tuj1 **(A)**, GFAP **(B)**, or **(C)** expression. Nuclei were counter-stained with DAPI. Cells expressing EGFP-sh Sc or EGFP-sh Sur8 RNA were detected by fluorescence using a confocal microscope (**[A]**, The microscopic fields/cell number; 3/100–120 or 3/80–160; **[B]**, 3/80–130 or 3/60–100; **[C]**, 4/80–160 or 3/40–100, of EGFP-sh Sc or EGFP-sh Sur8-expressing cell were estimated, respectively). The yellow and white arrows indicate cells showing positive and negative signals for differentiation, respectively. FACS analysis results for Tuj1, GFAP, or O4 signal were quantified. The error bars indicate the SD of three independent experiments. *, $p < .05$; **, $p < .005$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescence protein; GFAP, glial fibrillary acidic protein; Sc, scramble; sh, short hairpin.

was highly correlated with that of Sur8 in the cortex of the developing rat embryo (Fig. 1A). Moreover, the repression kinetics of Sur8 and PCNA during NPC differentiation induced by bFGF depletion were highly similar (Fig. 1B). The protein levels of PCNA were decreased and increased by knockdown and exogenous expression of Sur8, respectively (Fig. 4A, 4B). The inhibition of proliferation by *Sur8* knockdown was confirmed by inhibition of BrdU incorporation of specific cell expressing EGFP-sh Sur8, and percentage of BrdU-positive cells was reduced from 99.5% to 44.4% by EGFP-sh Sur8 (Fig. 4C). Inhibition and activation of proliferation by *Sur8* knockdown and overexpression was also confirmed by staining of the alternative proliferation markers Ki67 and PCNA, respectively (Supporting Information Fig. 7A, 7B).

The expression of Nestin was specifically inhibited in cells expressing EGFP-sh Sur8 (Fig. 5A, left panels), and the relative intensity of the Nestin signal was reduced by approximately 50% after *Sur8* knockdown (Fig. 5A, right panels). In contrast, Nestin expression was strongly enhanced by Sur8 overexpression (Fig. 5B). The mRNA level of *Nestin* was also decreased and increased by knockdown and overexpression of Sur8, respectively, as also monitored by RT-PCR analyses (Fig. 5C, 5D). The Nestin and PCNA increments by Sur8 overexpression were also shown by IB analysis (Fig. 5E, upper panel); an alternative neural stem cell marker, CD133, also increased by Sur8 overexpression as revealed by FACS analyses (Supporting Information Fig. 8A, 8B).

To further characterize Sur8 as a factor involved in the stemness of NPCs, we monitored the effects of *Sur8* knockdown on the formation of neurospheres that partly mimic the regional cellular composition of NPCs in vivo [15, 26]. The neurospheres formed from the NPCs either by EGFP-sh Sur8 or EGFP-sh Sc were grown up to the third passages, and both the number and size of neurospheres were measured. Interestingly, the relative numbers and sizes of neurospheres were reduced after infection with EGFP-sh Sur8 compared with those infected with EGFP-sh Sc control virus (Supporting Information Fig. 9A). The relative numbers of neurospheres were reduced to approximately 53% by the third passage of the EGFP-sh Sur8 lentivirus-infected cells (Fig. 5F and Supporting Information Fig. 9B). The relative numbers of neurospheres were reduced to approximately 42% by the third passage of the EGFP-sh Sur8 lentivirus-infected cells, especially those with a diameter longer than 50 μm (Fig. 5F). Nestin was significantly reduced in the neurosphere infected with the EGFP-sh Sur8 compared with those infected with EGFP-sh Sc (Supporting Information Fig. 9B). We further confirmed the role of Sur8 in maintenance of self-renewal by adapting the single-cell clonal assay, which provide better results than

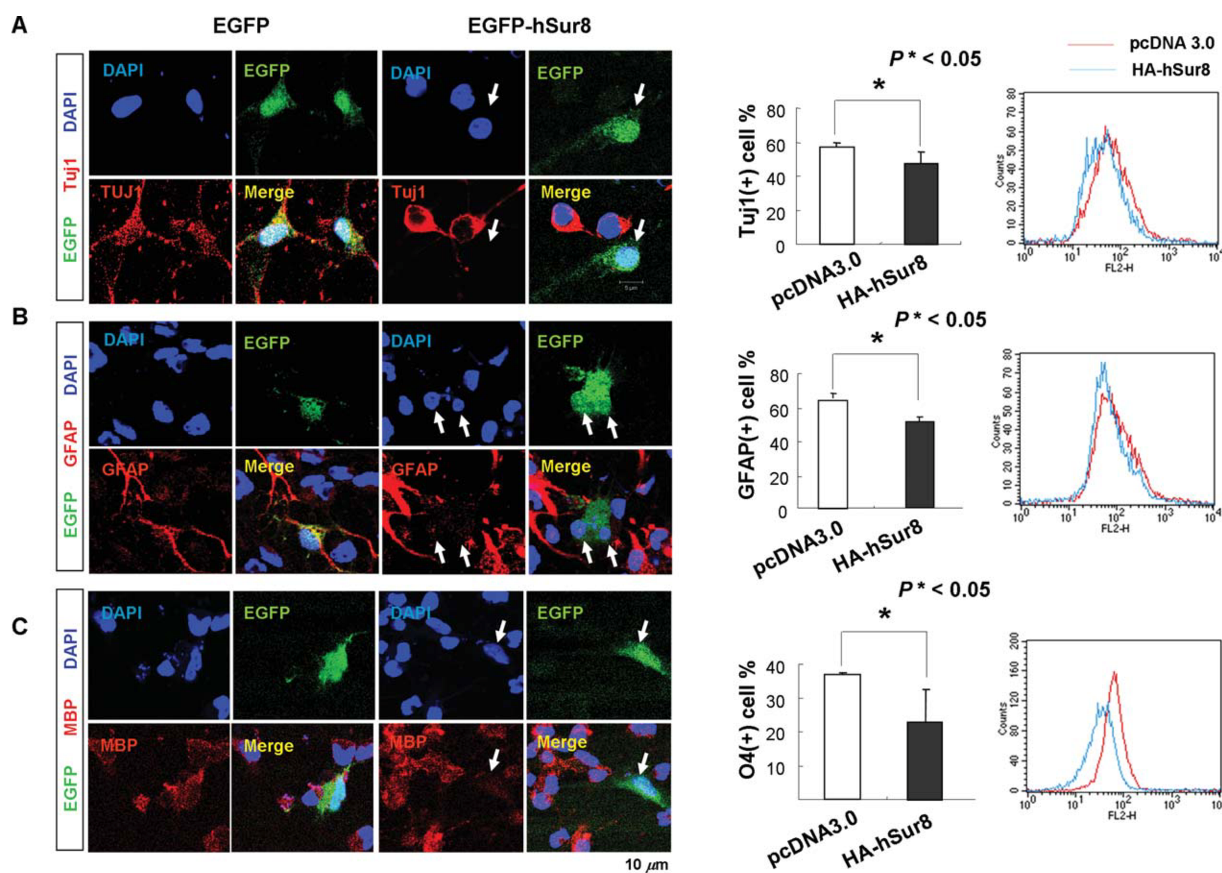

Figure 3. Effects of Sur8 overexpression on neural progenitor cell (NPC) differentiation. **(A–C):** NPCs were transfected with EGFP-empty vector or GFAP-human (h) Sur8 expression vector (for immunocytochemistry) or with pcDNA3.0 or pcDNA3.0-HA-hSur8 expression vector (for fluorescent-activated cell sorter [FACS] analysis) were grown for 48 hours in N2 medium without basic fibroblast growth factor. Immunofluorescent labeling (left panels) was used to detect Tuj1 **(A)**, GFAP **(B)**, or MBP **(C)**. Nuclei were counterstained with DAPI. Cells expressing GFP-Sur8 are marked by white arrows. The right panels show the results of quantitative and representative FACS analyses for expression of Tuj1, GFAP, or O4. The error bars indicate the SD of three independent experiments. *, $p < .05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescence protein; GFAP, glial fibrillary acidic protein; HA, hemagglutinin; MBP, myelin basic protein.

the traditional neurosphere assay [16]. The neurospheres which were formed from single cells were reduced in size around 40% by infection of *Sur8* knockdown lentivirus (Fig. 5G, 5H). Overall, Sur8 plays an important role in maintenance of NPC self-renewal.

The Ras-ERK Pathway Differentially Regulates NPC Differentiation and Proliferation Mediated by Sur8

Sur8 forms a complex with Ras and Raf-1 [8] and positively regulates Ras- and growth factor-mediated ERK pathway activation [6–8]. To determine whether the Ras-ERK pathway plays a role in the Sur8-mediated regulation of proliferation and differentiation in NPCs, we measured the effects of *Sur8* knockdown or overexpression on ERK activation. ERK activation, monitored with reference to the phosphorylation status (p-ERK), was significantly reduced after *Sur8* knockdown by either small interference RNA (siRNA) or lentiviral infection of EGFP-sh Sur8 (Fig. 6A). In contrast, ERK activity was increased by expression of Sur8 (Fig. 6B). The levels of p-ERK, especially in the nucleus, were critically decreased by viral infection of EGFP-sh Sur8 (Fig. 6C). To determine whether Sur8-mediated inhibition of NPC differentiation occurs through the Ras-ERK pathway, the effects of Ras-ERK pathway inhibition on the function of Sur8 in NPC differentiation were monitored. The effects of Sur8 overexpression, that is, Tuj1 reduction and increased PCNA, were abolished by inactivation of the ERK pathway via treatment with a MEK inhibitor, PD98059 (Fig. 6D) or U0126 (Supporting Information Fig. 10A). *Tuj1* mRNA levels were also reduced by Sur8 overexpression, and that effect was abolished by treatment of PD98059 (Fig. 6E). The role of the Ras-ERK pathway in inhibition of neuronal differentiation and induction of proliferation was confirmed by FACS analysis (Fig. 6F, 6G). To further confirm the role of the Ras-ERK pathway in Sur8-mediated inhibition of differentiation and activation of proliferation, it was determined whether expression of dominant negative MEK (dn-MEK) could rescue the effects of Sur8 expression (Supporting Information Fig. 10B). Overexpression of dn-MEK prevented Sur8-induced proliferation and inhibition of NPC differentiation, as determined by a FACS analyses for Tuj1 and PCNA (Supporting Information Fig. 10C, 10D).

To further understand the role of the Ras-ERK pathway in Sur8-mediated neuronal differentiation and proliferation, we monitored the effects of EGF, a ligand for the Ras-ERK pathway, in NPC differentiation. The activation of differentiation and inhibition of NPC proliferation by removal of bFGF were overcome by treatment with EGF (Fig. 6H; quantitative data for BrdU incorporation are shown in right panel). In fact, all of the biochemical effects of bFGF depletion were rescued by EGF treatment, including reduced Sur8 and PCNA, ERK inactivation,

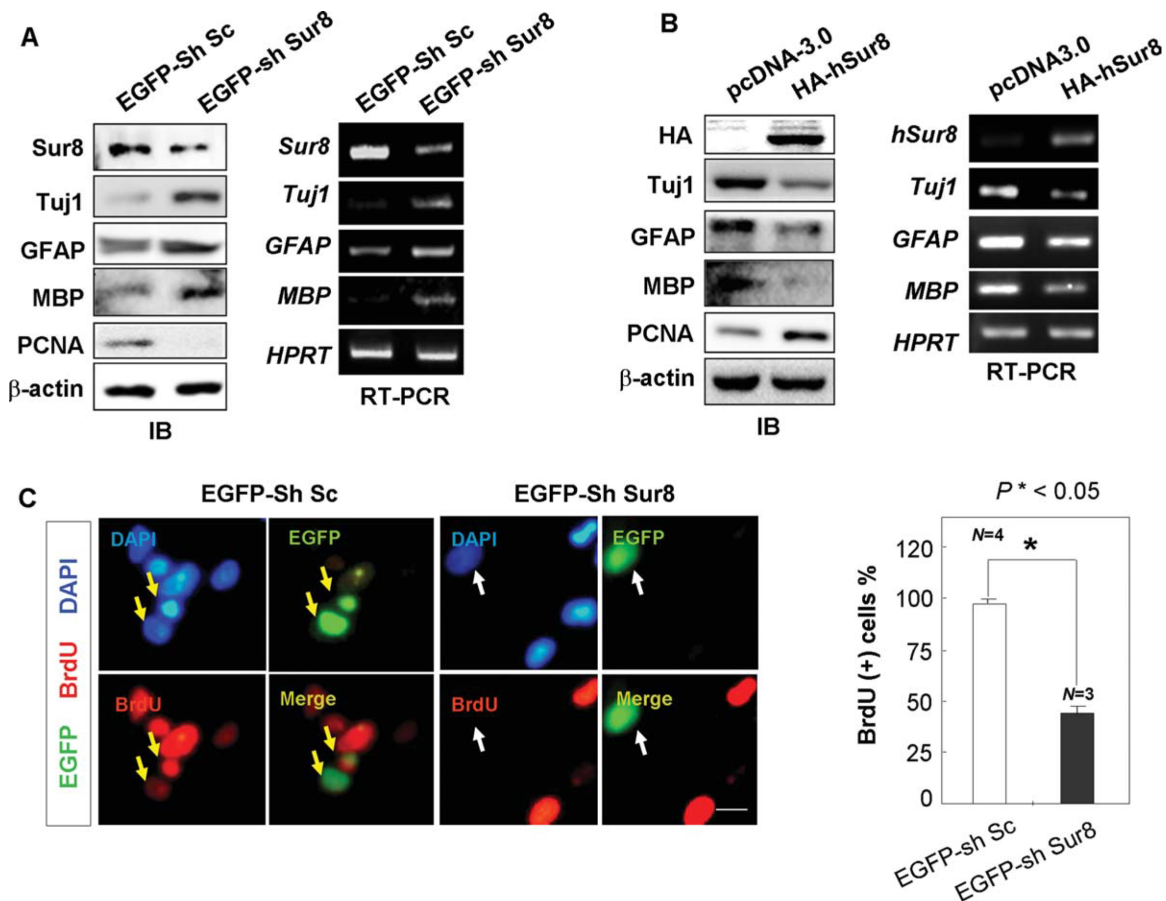

Figure 4. Effects of *Sur8* knockdown or overexpression on neural progenitor cell (NPC) differentiation and proliferation. **(A):** NPCs were infected with either EGFP-sh Sc or EGFP-sh Sur8, and were further grown for 72 hours in N2 medium with basic fibroblast growth factor (bFGF; 10 ng/ml). **(B):** NPCs were transfected with pcDNA3.0 or pcDNA3.0-HA-hSur8 expression vector, and were further grown for 48 hours in N2 medium without bFGF. **(C):** NPCs were infected with either EGFP-sh Sc or EGFP-sh Sur8 lentivirus. Proliferation was monitored by BrdU incorporation using immunocytochemical analysis. The microscopic fields per cell number (4/70–100 or 3/80–130) of EGFP-sh SC- or EGFP-sh Sur8-expressing cell were estimated, respectively, and represent the mean ± SD of three separate experiments. *, $p < .05$. **(A, B):** Left panels, IB analysis for Sur8, Tuj1, GFAP, MBP, PCNA, or β-actin. Right panels, RT-PCR analysis, for *Sur8*, *Tuj1*, *GFAP*, *MBP*, or *HPRT* mRNA. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescence protein; GFAP, glial fibrillary acidic protein; HA, hemagglutinin; IB, immunoblot; MBP, myelin basic protein; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction.

and increased Tuj1 (Fig. 6I). In contrast to the protein results, the mRNA levels of *Sur8* were not changed by EGF treatment during the differentiation by removal of bFGF (Fig. 6J).

Sur8 Inhibits Neuronal Differentiation of NPCs in the Neural Tube of Ex Vivo-Cultured Embryos

The role of Sur8 in neurogenesis was further verified using an ex vivo embryonic culture system. We intended to knockdown Sur8 in the cerebral cortices of E12.5 embryos by microinjection of concentrated EGFP-sh-Sur8 lentivirus into the forebrain followed by a 72-hour incubation of the whole embryo culture in roller bottles for expression of EGFP-sh Sur8 (Fig. 7A). The functionality of embryo culture was evidenced by further development of the embryos during the period of culture (Fig. 7B). The infected areas of brain by EGFP-sh Sur8 or EGFP-sh Sc lentivirus were identified by EGFP expression (Fig. 7B). Tuj1-positive signals were detected, but no significant signals for either GFAP or MBP were observed in the neural tube area of the embryos (Fig. 7C). We observed convincing, specific induction of Tuj1 in the cells in which Sur8 levels were reduced by lentivirus-mediated EGFP-sh Sur8 expression (Fig. 7D; upper panels). The correlation between Tuj1 induction and Sur8 levels in the cell of the neural tube area is shown under low magnification (Supporting Information Fig. 11A). The number of Tuj1-positive cells were increased from 25.79 (±14.09)% to 68.42 (±17.74)% by infection of EGFP-sh Sur8 compared with those infected with EGFP-sh Sc virus (Supporting Information Fig. 11A, right lower panel). We did not detect any increases in Tuj1-positive signals in neural tube cells expressing EGFP after EGFP-sh control virus infection (Fig. 7D; Supporting Information Fig. 11A). We also did not detect any significant GFAP- or MBP-positive cells, regardless of viral infection (Fig. 7D; Supporting Information Fig. 11B, 11C).

Discussion

Multipotency and self-renewal are important characteristics of stem cells [17, 27–29]. NPCs retain stem cell characteristics, including a high proliferative potential and the ability to self-renew [30]. In this study, we identified Sur8, the scaffold protein of the Ras-ERK pathway [6–8], as a factor that maintains

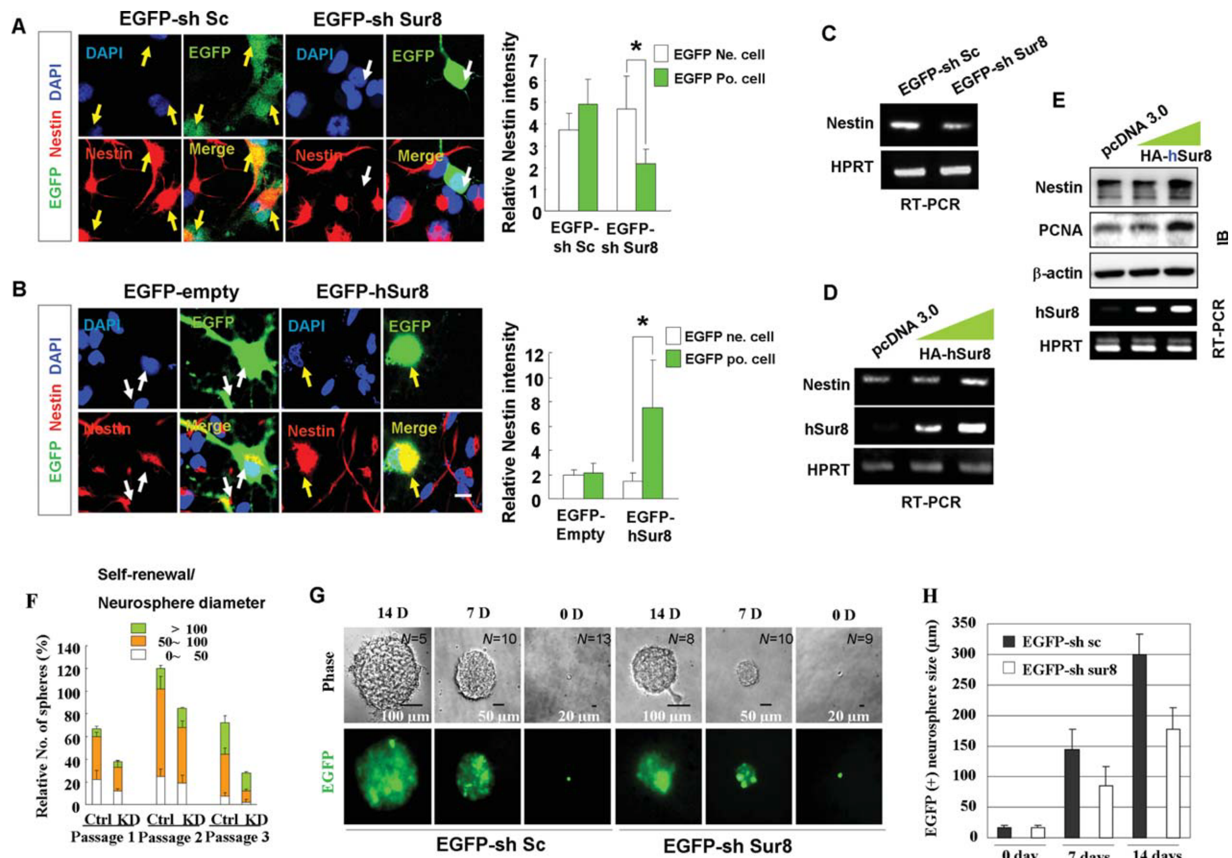

Figure 5. Effects of *Sur8* knockdown or overexpression on stemness of neural progenitor cells (NPCs). **(A, C):** NPCs were infected with either EGFP-sh Sc or EGFP-sh Sur8 lentivirus and were further grown for 72 hours in N2 medium with basic fibroblast growth factor (bFGF; 10 ng/ml). **(B, D, E):** NPCs were transfected with pcDNA3.0 or pcDNA3.0-HA-hSur8 vector and were further grown for 48 hours in N2 medium without bFGF. **(A, B):** Immunofluorescent labeling of Nestin. Nuclei were counterstained with DAPI. Immunofluorescent images of Nestin were obtained using a Zeiss confocal microscope, and the intensity of stained images (EGFP-sh Sc: three fields, EGFP positive/negative: 7/11 cells, EGFP-sh Sur8: three fields/EGFP positive/negative: 7/6 cells, EGFP-empty: four fields, EGFP positive/negative: 16/6 cells) using WCIF ImageJ bundle software as described in the "Materials and Methods." **(C, D):** RT-PCR analysis was performed to detect *Nestin*, *Sur8*, or *HPRT* mRNA. **(E):** IB analysis was performed to detect Nestin, PCNA, or β-actin. **(F, G, H):** NPCs **(F)** or single NPC **(G, H)** were infected with either EGFP-sh Sc or EGFP-sh Sur8 and grown in N2 medium containing bFGF (10 ng/ml) and EGF (20 ng/ml) for 72 hours in uncoated six-well plates for neurosphere formation [53]. **(F):** Relative numbers and sizes of neurospheres were measured after first, second, and third passage (the raw data are shown in Supporting Information Fig. 9A). **(G):** Neurospheres were captured as black and white (B/W) images (upper panels) or as fluorescent images (lower panels). **(H):** The neurosphere was grown up to 14 days and the size of neurospheres (EGFP-sh Sc: 0D/13 spheres, 7D/10 spheres, 14D/5 spheres; EGFP-sh Sur8: 0D/9 spheres, 7D/10 spheres, 14D/8 spheres) was quantified using *Ti* Nikon Fluorescence microscope software. The error bars indicate the SD of three independent experiments.*, $p < .05$. Abbreviations: Ctrl, control; KD, knock down; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescence protein; HA, hemagglutinin; IB, immunoblot; *N*, nuerosphere number; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction.

self-renewal capacity and the undifferentiated state in NPCs. We indicate that Sur8 is an intracellular biomarker for Nestin-positive undifferentiated NPCs. Sur8 was repressed during NPC differentiation in vitro and in the developing brain of rat embryos. Sur8 was expressed in undifferentiated proliferating cells. Conversely, Sur8 expression was decreased in cells that were nonproliferating and differentiated cells along the migration direction of the differentiating NPCs in the neural tube. The physiological roles of Sur8 in both the inhibition of differentiation and activation of proliferation were corroborated by costaining Tuj1 with EGFP-positive cells, which reduced Sur8 at the ventricular zone of the cerebral cortex infected with *Sur8* knockdown virus in ex vivo cultured embryos. We did not detect any significant signals for either GFAP or MBP in the neural tube of the (E12.5 + 3DIV) embryos. The importance of Sur8 in neuronal development may be related to the recent reports of Sur8 defects in Noonan syndrome, which accompanies mental retardation [31–32]. Sur8's potentiality as a neural stem cell factor is also shown in its role in inhibition of differentiation and activation of proliferation in the present study by both Sur8 overexpression and knockdown experiments using various experimental approaches. This role was further suggested by the increase and decrease of the neural stem cell markers, Nestin and CD133, by overexpression and knockdown of Sur8, respectively. The role of Sur8 as a factor required for the maintenance of self-renewal was further shown by reductions in both the numbers and sizes after lentivirus-mediated *Sur8* knockdown during serial passages of cultured neurosphere, which allows the maintenance of self-renewal. The antineurogenic effect of Sur8 also was revealed by activation of NPCs' neurite outgrowth and neuronal differentiation by *Sur8* knockdown in neurospheres. The role of Sur8 as an antidifferentiation factor may not be limited to the NPCs. We observed a decrease of Sur8 during the endothelial differentiation of mouse embryonic stem cells (mESCs) accompanying increment of the endothelial cell

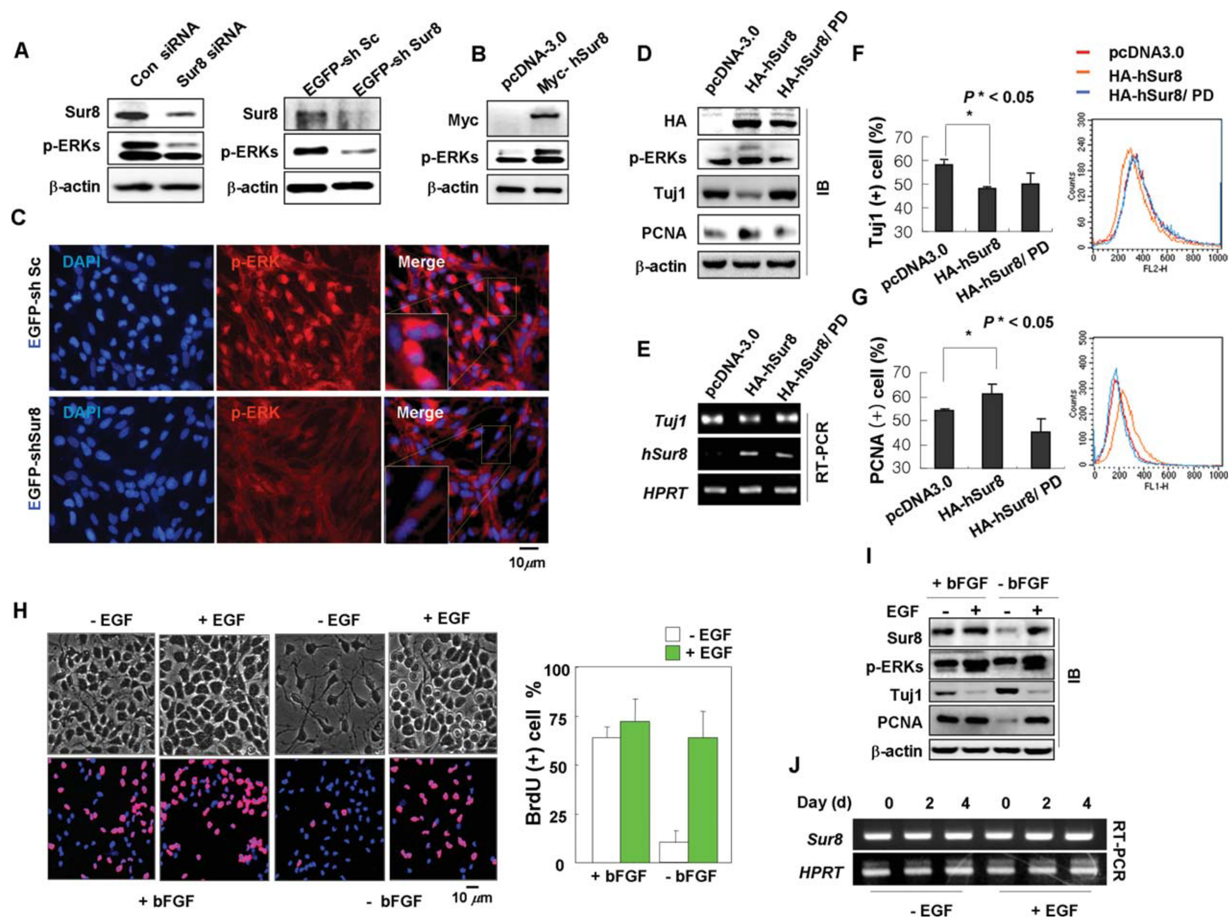

Figure 6. Roles of Ras-ERK pathway in Sur8-mediated effects on neural progenitor cell (NPC) differentiation and proliferation. **(A, C):** NPCs were transfected with 100 nM control or *Sur8* siRNA ([**A**], left panel), or infected with EGFP-sh Sc or EGFP-sh Sur8 ([**A**], right panel, [**C**]), and were further grown in N2 medium with bFGF (10 ng/ml) for 48 hours. **(B):** NPCs were transfected with pcDNA3.1 or pcDNA3.1-myc-hSur8, and the cells were further grown in N2 medium without bFGF for 48 hours. **(A, B):** Sur8, p-ERK, or β-actin was detected by IB. **(C):** p-ERK was detected by immunofluorescent labeling using p-ERK antibody. Nuclei were detected by DAPI staining. **(D, E):** NPCs were transfected with pcDNA3.0 or pcDNA3.0-HA-hSur8 and the cells were further grown for 48 hours with or without PD98059 (PD; 20 μM). HA-hSur8, p-ERKs, Tuj1, PCNA, or β-actin was detected by IB **(D)**. The mRNA level of *Tuj1*, *Sur8*, or *HPRT* was detected by RT-PCR **(E)**. **(F, G):** The relative numbers of Tuj1- or PCNA-positive cells were quantified by fluorescent-activated cell sorter analyses and representative results for expression of Tuj1 or PCNA are shown in the right panels. **(H, I):** NPCs were grown in medium in the presence or absence of bFGF (10 ng/ml) and grown for an additional 48 hours either in the presence or absence of EGF (20 ng/ml). **(H):** Cells were subjected to BrdU incorporation staining. Nuclei were counterstained with DAPI. Upper and lower panels represent B/W and merged images of BrdU and DAPI, respectively. Right panel represents percentages of relative numbers of BrdU-positive cells. Nuclei were counterstained with DAPI. The error bars indicate the SD of three independent experiments. *, $p < .05$. **(I):** IB analysis for Sur8, Tuj1, PCNA, p-ERKs, or β-actin. **(J):** Effects of EGF on regulation of *Sur8* mRNA levels during NPC differentiation. Abbreviations: bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; IB, immunoblot; PCNA, proliferating cell nuclear antigen; PD, PD98059; p-ERK, phosphorylated extracellular signal-regulated kinase; RT-PCR, reverse transcriptase polymerase chain reaction.

marker Flk-1 and a decrease of the stem cell marker Oct-4 (Supporting Information Fig. 12). Here, the indicators of proliferation, such as PCNA expression and BrdU incorporation were also reduced (Supporting Information Fig. 12B, 12C). We further showed the reduction of Oct-4 by lentivirus-mediated *Sur8* knockdown (Supporting Information Fig. 13A; successful knockdown of Sur8 was confirmed by immunoblotting, Supporting Information Fig. 13B). In addition, the alkaline phosphatase staining, which reveals undifferentiated ESCs, showed that differentiated mESCs were increased by EGFP-sh Sur8 lentivirus infection (Supporting Information Fig. 13C). The function of Sur8 in maintenance of self-renewal capacity of mesenchymal stem cell (MSC) was also reported [33]. Overall, we suggest that Sur8 is a potential intracellular factor for the maintenance of multipotency and self-renewal of neural stem cells and potentially other stem cells including ESCs and MSCs. We also showed that the Ras-ERK pathway is required for Sur8-induced differentiation inhibition and proliferation activation. It is known that the Ras-ERK pathway plays a role in both inhibition of differentiation and activation of proliferation [11, 34, 35]. The Ras-ERK pathway differentially regulates differentiation and proliferation of cells according to the intensity and duration of the signal [36–42]. The importance of the kinetics of ERK activation in the determination of neuronal cell fate is dependent on the physiological status of Ras-ERK pathway activation [43–45]. Currently, it is not understood how cellular functions, such as differentiation and proliferation, are differently regulated via the common Ras-ERK pathway in response to intrinsic and extrinsic signals. Our data suggest that Sur8 plays an important role in the regulation of NPC proliferation and differentiation through the Ras-ERK pathway in response to the growth factors bFGF or EGF.

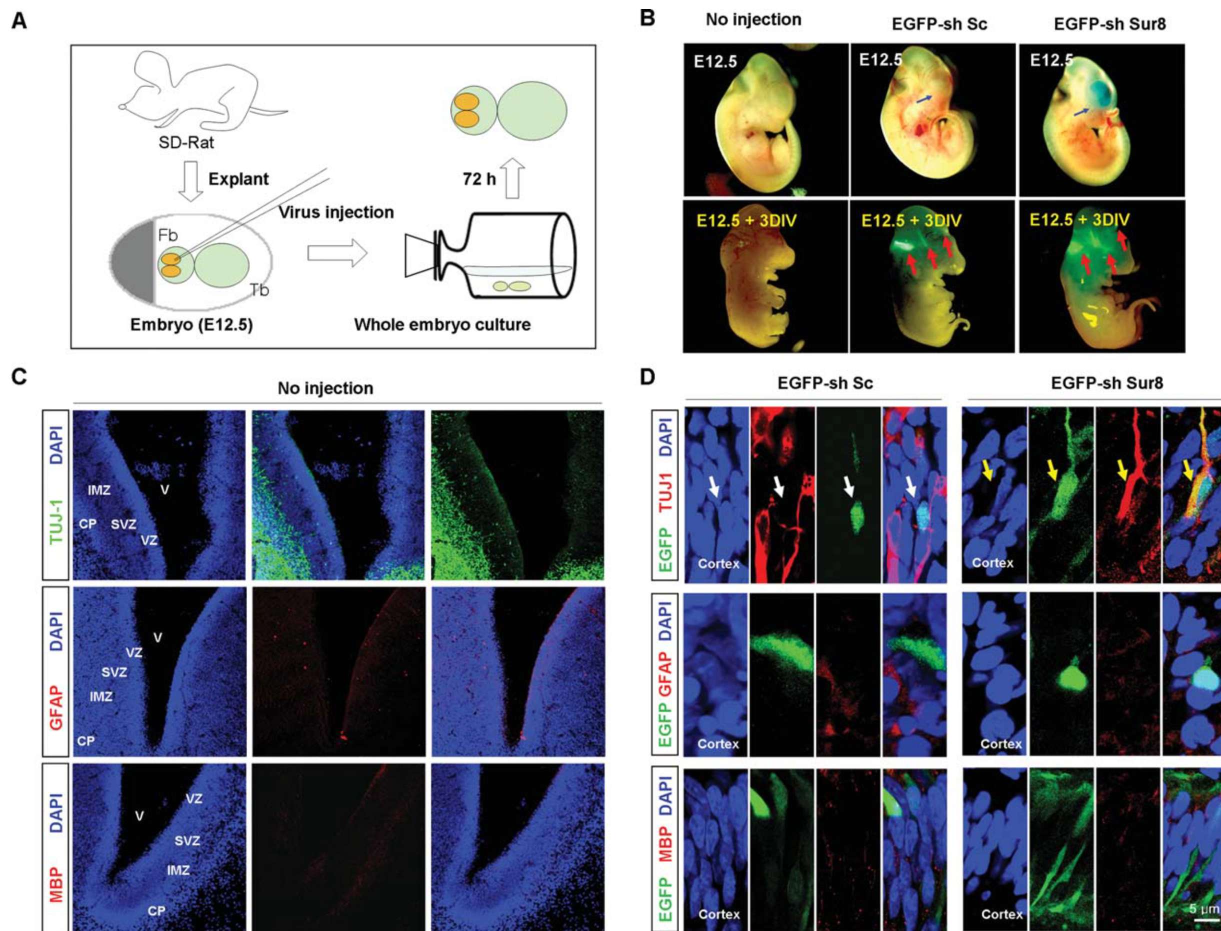

Figure 7. Effects of *Sur8* knockdown in the neural tubes of developing rat embryos. **(A):** Explanted E12.5 embryos were injected with a mixture of Fast green dye and either EGFP-sh Sc or EGFP-sh-Sur8 lentivirus, and then cultured in roller bottles containing Dulbecco's modified Eagle's medium with 20% FBS for 72 hours. **(B):** The forebrains of E12.5 embryos stained by Fast Green were injected with lentivirus (10^7–10^8 TU/ml) through the eye (marked by a blue arrow). Upper and lower panels represent embryo images before and after culture, respectively. **(C, D):** Immunofluorescent labeling of rat embryo neural tubes. Sagittal sections of paraffin-embedded cultured rat embryo brains, which were either noninfected **(C)** or infected with either EGFP-sh Sc or EGFP-sh Sur8 lentivirus **(D)**, were immunohistochemically stained for Tuj1 (green), GFAP (red), and MBP (red). Nuclei were counterstained with DAPI (blue). The embryonic cerebral cortex images were captured by a confocal microscope. **(D):** Upper panels show cells with Tuj1-negative and -positive signals after expression of EGFP-sh Sc and EGFP-sh Sur8 are marked by white and yellow arrows, respectively. Abbreviations: CP, cortical plate; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; Fb, forebrain; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IMZ, intermediate zone; MBP, myelin basic protein; SVZ, subventricular zone; Tb, trophoblast; VZ, ventricular zone.

The Scaffold proteins such as Sur8, KSR, and MP1 assemble intracellular signaling complexes and can play important roles in cellular responses [3, 46–48]. The scaffold proteins can regulate the efficiency of the signaling response by virtue of their ability to form different complexes with the signaling members of the Ras-ERK pathway such as Ras, Raf [7, 49], Erbin, and PP1 [6, 49]. Concentration of the scaffold proteins influences differential formation of the complexes, resulted in the output of different physiologies [50]. However, the mechanism for regulation of the scaffold proteins themselves has not been clearly illustrated. Interestingly, we found that Sur8 is not regulated at the mRNA level, as indicated by the lack of significant change in its mRNA level during NPC differentiation by removal of bFGF as well as during NPC stimulation with EGF. Furthermore, *Sur8* mRNA levels did not significantly change by NPC differentiation in the developing neural tubes of E14.5 rat embryos, where Sur8 protein levels gradually decreased along the direction of migration. The Sur8 protein reduction during the differentiation of NPCs was blocked by treatment of ALLN. In addition, we also observed stabilization of Sur8 by MG132 in the NIH3T3 fibroblasts (Supporting Information Fig. 14A). However, the stabilization of Sur8 did not occur by treatment of the lysosomal inhibitor leupeptin (Supporting Information Fig. 14B). All of these results indicate that Sur8 can be regulated at the level of protein stability involving proteasomal degradation machinery.

Although, the regulation of protein stability usually does not induce permanent effects on cellular physiologies, it can provide more rapid and efficient ways to regulate protein level than transcriptional regulation. Several stem cell markers, including MyoD [51] and Myc [52], were previously shown to be regulated by protein stability modulation during cellular differentiation. Regulation of the scaffold protein, Sur8, by protein stability can control assembly of the Ras-ERK pathway members in response to signals controlling NPC differentiation and proliferation. To our knowledge, the regulation of Sur8 by alterations in protein stability involving proteasomes is the first mechanism to be demonstrated among the scaffold proteins in the Ras-ERK pathway.

Conclusion

In conclusion, our results show that Sur-8, the scaffold protein of the Ras-ERK pathway which is required for self-renewal of NPCs, is a potential NPC bio-marker of stemness.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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