The effect of Ndrg2 expression on astroglial activation

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Abstract

N-myc downstream-regulated gene 2 (Ndrg2) is a differentiation- and stress-associated molecule predominantly expressed in astrocytes in the central nervous system (CNS). To study the expression and possible role of Ndrg2 in quiescent and activated astrocytes, mice were administrated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP), a Parkinson disease (PD)-related neurotoxin which causes both neurodegeneration and glial activation. Immunohistological analysis revealed that Ndrg2 was highly expressed in both types of astrocytes, but less so in astrocytes during the early process of activation. Ndrg2 was also expressed in astrocyte-like cells, but not in neurons, in human brains from PD and Cortico-basal degeneration (CBD) patients. In cultured astrocytes, gene silencing of Ndrg2 significantly enhanced the numbers of 5-bromo-2'-deoxy-uridine (BrdU)-incorporated and proliferating cell nuclear antigen (PCNA)-positive cells, and reduced the length of cell processes and the amount of F-actin. In contrast, adenovirus-mediated overexpression of Ndrg2 significantly reduced the numbers of BrdU-incorporated and PCNA-positive cells, and enhanced the amount of F-actin. Fractionation and immunocytochemical analysis further revealed that Ndrg2 was located in different cellular fractions including the cytosol and cell surface membranes. These results suggest that Ndrg2 may regulate astroglial activation through the suppression of cell proliferation and stabilization of cell morphology.

1. Introduction

Astrocytes are ubiquitous in the brain, and have long been considered as structural and nutritional supports for neurons. Recent evidence, however, has demonstrated that they also play pivotal roles in the maintenance of neuronal activity and survival (for review, Maragakis and Rothstein, 2006, Softroniew et al., 2009, Buffo et al., 2010). They modulate synaptic function by transporting the majority of glutamate from the synaptic cleft, balance ions and fluid in the extracellular spaces, and regulate blood flow through contact with the brain vasculature. In addition to these homeostatic functions in physiological conditions, astrocytes respond to all kinds of insults in the central nervous system (CNS), and undergo molecular and morphological changes, referred to as reactive astrogliosis or astroglial activation. This process involves cell hypertrophy, upregulation of intermediate filaments such as glial fibrillary acidic protein (GFAP) and vimentin, and cell proliferation. Activated astrocytes enhance the survival of neuronal cells by secreting neurotrophic factors (Maeda et al, 1994) and antioxidants (Maragakis and Rothstein, 2006), or by reducing glutamate levels in extracellular spaces. However, they also induce the inflammatory response, and release neurotoxic agents such as nitric oxide (NO) through up-regulated expression of the inducible NO synthase (iNOS). In severe astrogliosis, astrocytes migrate to the border between damaged and healthy areas, and form a tight barrier, termed the glial scar. Mature glial scar plays an important role in the restriction of inflammation and tissue remodeling in the damaged area, and saving the healthy area, although it has inhibitory effects on the axon regeneration and neovascularization (Gadea et al., 2008). It is, therefore, essential to properly regulate the level and duration of astroglial activation.

N-myc downstream-regulated gene 2 (Ndrg2) is a member of recently identified differentiation- and stress- associated genes, and normally expressed in the brain, heart, muscle, and to lesser extents in liver and kidney (Qu et al., 2002). An increasing number of reports has demonstrated that Ndrg2 had inhibitory effects on the proliferation (Deng et al., 2003, Kim YJ et al., 2009) and invasion (Kim A et al., 2009) of tumor cells including glioblastoma. It was also reported that Ndrg2 regulated the production of certain secretory proteins in dendritic cells (Choi et al., 2007, Choi et al., 2010). In the CNS, Ndrg2 has been reported to be predominantly expressed in astrocytes (Nichols, 2003, Okuda et al., 2008), and to a lesser degree in dystrophic neurons in Alzheimer's disease (AD) (Mitchelmore et al., 2004). Ndrg2 was upregulated by adrenal steroid hormones (Boulkroun et al., 2002, Nichols, 2003), while downregulated by antidepressant and electroconvulsive treatment (Takahashi et al., 2005a). These results suggest that Ndrg2 may be involved in the neurodegenerative and neuropsychiatric diseases. To understand the function of Ndrg2 in astrocytes, we studied its expression in both mouse and human brains, and the effect of expression in cultured astrocytes.

2. Materials and methods

2.1. Cell Cultures and animal experiments

Astrocytes were prepared from the cerebral cortex of 1-d old neonatal Wister rats and cultured in MEM containing 10% FBS as described previously (Yamaguchi et al, 1999). Rat C6 glioma cells were maintained in DMEM containing 10% FBS. Animal experimental protocols were approved by the Committee on Animal Experimentation at Kanazawa University. All animal experiments were performed using C57BL/6 mice (male, 8-12 week of age). A mouse model of Parkinson disease (PD) was created by four sequential intraperitoneal injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP; 20 mg/kg, Sigma, St. Louis, MO) at 2 h intervals.

2.2. Postmortem specimens

Informed consents of using postmortem specimens for biological study were obtained from all subjects with a letter of acceptance recognized in the ethics committee of Juntendo University School of Medicine. All postmortem specimens were fixed in 20% formalin and processed for paraffin-embedding and cut into 6-μm-thick sections. Samples were subjected to immunohistochmical anslysis as described below.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured astrocytes and striatum using TRIzol (Invitrogen, Carlsbad, CA). RT reactions containing 1 μg of total RNA were performed using PrimeScript (Takara, Shiga, Japan). The individual cDNA species were amplified in a reaction mixture containing 1 unit of Taq DNA polymerase (Takara) and specific primers for Ndrg2,

GFAP, S100β, CyclinB1, CyclinD1, CyclinE, p27 and β-actin. PCR products were separated on a 2% agarose gel. Densitometry was done with the individual PCR products, and standardized with β-actin.

2.4. Subcellular fractionation

The protein samples isolated from rat cultured astrocytes and mouse striatum were subfractionated into cytosol (C), membrane (M) and nuclear (N) fractions by solubility-based separation and sequential centrifugation using ProteoExtract subcellular fractionation kit (EMD Biosciences, Darmstadt, Germany) according to the manufacturer's instruction.

2.5. Western blotting

Cells were solubilized in RIPA buffer containing 10 mM Tris (pH7.6), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecylsulfate (SDS), 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml aprotinin, 10 mM NaF, 1 mM Na_3VO_4. Western blotting was performed using antibodies against Ndrg2 (Santa cruz, Santa cruz, CA), Glucose regulated protein (GRP) 78 (Stressgen, Victoria, BC, Canada), GRP75 (Stressgen), Cu/Zn superoxide dismutase (SOD) (Stressgen), Glutamate-Aspartate Transporter (GLAST) (Millipore, Billerica, MA) and β-actin (Sigma). Site of primary antibody binding were determined by alkaline phosphatase-conjugated secondary antibodies.

2.6. Immunostaining

Brains were removed from C57BL/6 mice (25 to 30 g) after perfusion with 4% paraformaldehyde, and cortical sections (10-μm) were cut on a cryostat. Sections were processed for immunostaining with antibodies against tyrosine hydroxylase (TH) (Sigma),

Ndrg2, GFAP (Daco, Glostrup, Denmark), proliferating cell nuclear antigen (PCNA) (Santa cruz) and S100β (Sigma). For human brains, paraffin-embedded sections were deparaffinized, and incubated in 3% hydrogen peroxide with methanol for 10minutes to remove endogenous peroxidase activity. After preincubation with 5% normal goat serum for 10 minutes, sections were incubated with anti-Ndrg2 antibody overnight at 4 ℃. Sections were then subjected to further incubation for 1h with biotinylated secondary antibody (DAKO), and for 45 minutes with avidin-biotin horseradish complex (ABC) (DAKO) at room tenperature. Finally, immunoreactivity was visualized with 0.5mg/ml 3.3.-diaminobenzidine tetrachloride (DAB) and 0.3%H_2O_2. The sections were lightly counterstained with hematoxylin.

For immunocytochemistry, cells were fixed with 4% paraformaldehyde and 0.2% NP-40, and immunostained using anti-PCNA antibody or anti-BrdU antibody (Daco). FITC- or Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) was used for visualization of the immunolabeling.

2.7. Recombinant adenovirus

A cDNA fragment spanning the entire coding regions of mouse Ndrg2 and the FLAG epitope was inserted into the pAxCALNLwtit2 cosmid vector. Recombinant adenovirus AxCALNLZ2 Ndrg2 (Ad-Ndrg2) was prepared using an adenovirus expression kit (Takara, Tokyo, Japan). Adenovirus was amplified in HEK293 cells. Viral titers were determined by a plaque-forming assay in HEK 293 cells. Viral stocks had titers of ~1 x 10^8 PFU/ml. Cre-recombinase expressing adenovirus AxCANCre for the regulation of Ndrg2 expression, and a control virus AxCALNLZ2 LacZ (β-Galactosidase) were similarly prepared.

2.8. RNA interference

Ndrg2-specific siRNAs, siNdrg2-1 (5’-UCAACAGGAGACUUCCAUGGUGUGC -3’and 5’-GCACACCAUGGAAGUCUCCUGUUGA-3’) and siNdrg2-2 (5’-UCAAUUCAGAAUUUCCAGAAAGCUC-3’, 5’-GAGCUUUCUGGAAAUUCUGAAUUGA-3’), and negative control siRNA (siControl) were all obtained from Invitrogen. Mixtures of Ndrg2-1 and Ndrg2-2 or control siRNA were transfected into astrocytes by Lipofectamine RNAiMAX (Invitrogen) under the reverse transfection protocol according to the manufacturer’s specification.

2.9. Plasmid construction and transfection of cDNA

Mouse Ndrg2 cDNA encoding the complete open reading frame was amplified by PCR and cloned into a pEGFP vector (Invitrogen). The integrity of the insert was verified by DNA sequencing. C6 cells were transfected with EGFP or EGFP-Ndrg2 expression vector using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

2.10. BrdU Labeling

Cells were incubated with 10 μM 5-bromo-2’-deoxy-uridine (BrdU) (Sigma) for 24 h, and then fixed with 4% paraformaldehyde and 0.2% NP-40. After washing twice with PBS, cells were treated with 2 N HCl to denature nuclear DNA for 20 min, and then incubated in 0.1 M sodium tetraborate for 20 min. The cells were subsequently subjected to immunostaining with anti-BrdU antibody. The numbers of BrdU-positive cells were normalized by the number of DAPI (Invitrogen)-positive cells.

2.11. Phalloidin staining

F-actin (Polymerized actin) content was determined as described previously (Ransom et al, 2005) with a slight modification. Adenovirus- and siRNA-treated cells were cultured in either the presence or absence of serum for 2 days after replating to 96 well plates. Cells were fixed with 4% paraformaldehyde and 0.2% NP-40, followed by washing twice with PBS, and subsequent staining with TRITC-phalloidin (Sigma) and DAPI for 1 h at room temperature. After washing twice with PBS, fluorescence intensity was measured with Fluoroskan Asent FL fluorescence microplate fluorometer (Thermo Fisher Scientific Inc., Waltham, MA) with excitation at 544 nm and emission at 590 nm. These values were then normalized to DAPI fluorescence to yield a measure of total F-actin per cell.

2.12. Evaluation of cell death

Cell death was evaluated using LIVE/DEAD assay (Invitrogen) as described before (Hori et al., 2004).

2.13. Statistical analysis

Results are all expressed as the mean ± SE and the statistical significance was determined by the two-tailed and unpaired Students' *t*-test.

3. Results

Expression of Ndrg2 in astrocytes

As Ndrg2 was reported to be expressed predominantly in astrocytes in the CNS (Nichols, 2003, Okuda et al., 2008), we first studied its expression in the quiescent astrocytes in both of the striatum (caudate putamen (CPu)) and the substantia nigra (SN). Ndrg2 was expressed in S100β-positive astrocytes in both regions (Fig.1 A, B I). The majority of S100β-positive, Ndrg2-positive astroyctes was GFAP-negative in this condition as previously described (Fig.1 A, arrow head, B I, MPTP 0h) (Himeda et al., 2006).

Once mice were administered with 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP), a Parkinson disease (PD)-related neurotoxin, the number of tyrosine hydroxylase (TH)-positive dopaminergic neurons was decreased (Fig.1 B 1), and that of GFAP-positive astrocytes was increased in both of the SN (Fig.1 B I) and the striatum (Fig.1 B II, C). The expression pattern of Ndrg2 was also changed. At early periods (8-24h) after MPTP administration, there were a lot of GFAP-positive astrocytes which had reduced levels of expression of Ndrg2 (Fig.1 B I, MPTP 1d, B II, MPTP 8h). At a later period (3 days), in contrast, the majority of GFAP-positive astrocytes had high levels of Ndrg2 expression (Fig.1 B I, MPTP 3d, B II, MPTP 3d). Interestingly, in the latter conditions, GFAP- and Ndrg2-positive astrocytes tended to accumulate in the substantia nigra pars compacta (SNpc), rather than distribute diffusely in the SN (Fig. 1 B I MPTP 3d). Furthermore, the proliferating cell nuclear antigen (PCNA)-positive cells which were observed around 8h-3 days after MPTP administration had low levels of Ndrg2 expression (Fig.1 B III). RT-PCR analysis with total RNA isolated from the striatum confirmed induction of GFAP as well as cell cycle-dependent molecules such as CyclinD1 (Fig.1 C) and PCNA (data not shown) at 8-24h after MPTP

administration. In contrast, expression of Ndrg2 tended to be slightly decreased at 24 h, and then increased at 3 days after MPTP administration (Fig.1 C).

As it was reported that Ndrg2 was also expressed in dystrophic neurons in Alzheimer's disease (AD) (Mitchelmore et al., 2004), its expression in other neurodegenerative diseases including PD was analyzed. Expression of Ndrg2 was not observed in the SNpc from control persons, but highly observed in astrocyte-like cells in the SNpc from both PD and Cortico-basal degeneration (CBD) patients (Fig. 1 D). There was no expression of Ndrg2 in the neurons including Lewy body (data not shown).

The effect of Ndrg2 expression on the proliferation of astrocytes

As Ndrg2 was reported to have growth-inhibitory and apoptosis-inducing effects on various types of tumor cells (Deng et al., 2003, Kim YJ et al., 2009) as well as on hepatocytes (Yang et al., 2010), the effect of Ndrg2 on astroglial proliferation and apoptosis was analyzed using cultured astrocytes isolated from neonatal rats. Transfection of Ndrg2 siRNA (siNdrg2-1, -2) effectively reduced the expressions of Ndrg2 protein (Fig. 2 A I) and mRNA (Fig. 2 D), when compared to those in the control siRNA-transfected cells (siControl). The mixture of siNdrg2-1 and -2 was used for further experiments (siNdrg2). In contrast, infection of adenoviruses bearing Cre recombinase (Ad-Cre) and LoxP-Ndrg2 (Ad-Ndrg2) cDNAs enhanced the expressions of Ndrg2 protein (Fig. 2 A II) and mRNA (Fig. 2 D) by approximately 8 and 6 fold, respectively, when compared to those in control cells (cells with infection of Ad-Ndrg2 alone, or cells with infection of both Ad-Cre and Ad-LacZ).

Gene silencing of Ndrg2 (siNdrg2) significantly enhanced the numbers of BrdU-incorporated and PCNA-positive cells (Fig.2 B, C). In contrast, adenovirus-mediated overexpression of Ndrg2 (Ad-Cre Ad-Ndrg2) significantly reduced those numbers, suggesting

that Ndrg2 may also have inhibitory effects on the proliferation of astrocytes. However, LIVE/DEAD assay revealed that overexpression of Ndrg2 did not cause cell death in cultured astrocytes (data not shown).

To dissect the mechanisms underlying the Ndrg2-mediated suppression of astroglial proliferation, expressions of cell cycle-associated molecules were analyzed by RT-PCR (Fig. 2 D). siNdrg2 significantly enhanced some of those transcripts such as CyclinB1 and CyclinE, while Ad-Cre Ad-Ndrg2 failed to reduce those transcripts in our system.

The effect of Ndrg2 expression on the morphology of astrocytes

As Ndrg2 was also reported to promote neurite outgrowth when overexpressed in PC12 cells (Takahashi et al., 2005b), the effect of Ndrg2 expression on the morphology of cultured astrocytes was analyzed. Gene silencing of Ndrg2 (siNdrg2) was performed as described above, and cells were replated on the culture plates at 48 h after transfection. Initial attachment of the cells to the plates was observed within 30 min in both siNdrg2 and siControl (Fig.3 A 0.5 h). At 24 h after plating, however, siNdrg2 showed shorter processes, compared to siControl (Fig.3 A 24h). Similar tendency was observed until 48 h after plating (data not shown). Consistent with these results, F-actin content, determined by phalloidin staining (Ransom et al, 2005), was significantly lower in siNdrg2 than in siControl, while enhanced levels of F-actin content were observed in Ndrg2-overexpressed cells (Ad-Cre Ad-Ndrg2) (Fig.3 B I). The enhancing effect of Ndrg2 on the amount of F-actin were observed both in serum-containing and serum-free conditions (Fig.3 B I, B II), suggesting that they may be independent of astroglial growth. To clarify whether these changes were associated with astroglial activation, the levels of GFAP expression were analyzed by RT-PCR. However, there were no significant differences in all the cell types (Fig.3 C).

Intracellular localization of Ndrg2 in astrocytes

Although Ndrg2 is considered to be a cytosolic protein, its localization in the nucleus (Zhang et al., 2007) and in the cell surface membranes (Takahashi et al., 2005b) was reported when overexpressed. To clarify this point in astrocytes, cell fractionation and immunocytochemical analysis were performed. The crude fractionation of the samples from cultured astrocytes and from mouse striatum revealed that Ndrg2 was located in both of the cytosol and the cell membranes (Fig.4 A). The location of Ndrg2 in the nucleus was weakly observed in mouse striatum but not in cultured astrycotes (Fig. 4 A). Immunostaining of cultured astrocytes with anti-Ndrg2 antibody revealed that Ndrg2 was located in both of the perinuclear and leading edge-containing cell surface membranes. In the latter cases, Ndrg2 was co-localized with F-actin (Fig.4 B). Similarly, expressions of transfected EGFP-NDRG2, but not EGFP, were observed in both perinuclear and leading edges of the cells (Fig.4 C I, C II).

4. Discussion

Reactive astrogliosis is observed in many neurodegenerative conditions including PD. Comparing to activated microglia and other inflammatory cells, activated astrocytes have been suggested to be more neuroprotective in PD as well as in the experimental models of PD (Teismann and Schulz, 2004). To accomplish their protective roles, however, astroglial activation should be tightly regulated to prevent toxic hyper-activation.

In the current study, we found that Ndrg2 had inhibitory effects on the proliferation of astrocytes. In a mouse PD model, reduced levels of expression of Ndrg2 (Fig.1 B I) were observed during the period when cell cycle-dependent molecules such as CyclinD1 (Fig.1 C) and PCNA (data not shown) were induced. Experiments using cultured astrocytes also revealed that the levels of expression of Ndrg2 were inversely associated with the numbers of BrdU-incorporated and PCNA-positive cells (Fig.2 B, C). These observations were consistent with previous studies in tumor cells (Deng et al., 2003, Kim et al., 2009) and in hepatocytes (Yang et al., 2010), although apoptosis was not observed in our system. It was reported that Ndrg2 was translocated from the cytosol to the nucleus, and transmitted signals in response to hypoxia (Wang et al., 2008), suggesting that Ndrg2 might be directly involved in the signal transduction. However, in astrocytes, the effect of Ndrg2 expression on cell cycle-associated molecules seemed to be partial (Fig. 2 D), and the location of Ndrg2 in the nucleus was weakly observed in mouse striatum, but not in cultured astroytes, suggesting that the underlying mechanisms may be more complex.

We also found that Ndrg2 had regulatory effects on the morphology of astrocytes. Gene silencing of Ndrg2 caused shorter processes (Fig.3 A I, A II) and lower amounts of F-actin (Fig.3 B I, B II), while overexpression of Ndrg2 enhanced the amount of F-actin (Fig.3 B I, B

II). These results were consistent with a previous study in PC 12 cells (Takahashi et al., 2005b), and led us to hypothesize that Ndrg2 might be involved in the morphological change (hypertrophy) and/or migration during astroglial activation. Our observation that Ndrg2 was at least partially located in the cell surface membranes including leading edges (Fig.4 B, C) supported this hypothesis. However, the levels of expression of GFAP were not altered by either gene silencing or overexpression of Ndrg2 (Fig.3 C), and our preliminary data in Scratch-induced migration assay (Etienne-Manneville, 2006) showed no significant difference of astroglial migration in control (wild type) cells, Ndrg2-overexpressed cells and Ndrg2-knockdown cells. These results suggest that Ndrg2 may not influence hypertrophy or migration during astroglial activation, but may rather play a role in the maintenance of astroglial morphology under more stable conditions (before and after activation).

It was also reported that Ndrg2 was expressed near the neurogenic regions such as the subgranular zone of the dentate gyrus and the subventricular zone of the lateral ventricles (Nichols, 2003). In these areas, Ndrg2 mRNA was reduced by day 3 after adrenalectomy and was restored to the sham-operated levels by corticosterone replacement (Nichols, 2003), suggesting that Ndrg2 may be involved in the adrenal steroid-mediated regulation of neurogenesis / gliogenesis. Interestingly, our preliminary experiments revealed expression of Ndrg2 in Nestin-positive, GFAP-positive progenitor cells in the striatum (Chen et al., 2004) after MPTP administration. One of our next goals, therefore, is to clarify the role of Ndrg2 in the regulation of neurogenesis / gliogenesis *in vivo*.

In conclusion, we have studied the expression and the effect of expression of Ndrg2 on astrocytes. Ndrg2 may regulate astroglial activation through the suppression of cell proliferation and stabilization of cell morphology.

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Figure Legends

Fig. 1. Expression of NDRG2 in a mouse model of PD and human neurodegenerative diseases. (A) Ndrg2 expression in quiescent astrocytes. Mouse brain sections including the striatum were subjected to immunostaining with anti-Ndrg2 antibody, anti-S100β antibody and anti-GFAP antibody. Arrows and arrowheads indicate the cells that do and do not express GFAP, respectively. (B) Neurodegeneration and astrogliosis after MPTP injection. Mice received four consecutive injections of MPTP (20mg/kg, i.p.) at two-hour intervals. Brain sections were prepared at indicated times after the first injection, and subjected to immunostaining with anti-Ndrg2 antibody, anti-TH antibody, anti-GFAP antibody and anti-PCNA antibody. B I: Substantia nigra, B II, B III: Striatum. In B I and B II, the arrows indicate the cells with high levels of expression of GFAP and low levels of expression of Ndrg2. In B III, the nucleus was stained with DAPI, and PCNA-positive cells are indicated by arrow. (C) Gene expression in the process of astrogliosis. Isolated striatum was subjected to RNA extraction, followed by RT-PCR with specific primers for Ndrg2, GFAP, S100β, CyclinD1 and β-actin. Values shown are the mean ± S.E. in three separate experiments. *$P<0.05$. (D) Ndrg2 expression in human brains. Post-mortem brain samples from patients with PD or CBD were subjected to immunostaining with anti-Ndrg2 antibody. Similar results were obtained in three separate sets of experiments.

Fig. 2. Effects of Ndrg2 on the proliferation of astrocytes.
(A) Gene silencing and overexpression Ndrg2. Cultured rat astrocytes were transfected with either control siRNA (siControl) or Ndrg2 siRNA (siNdrg2), or infected with or without Ndrg2-expressing adenovirus. Cells were cultured for 2 days after siRNA transfection or

adenovirus infection, and lysed in RIPA buffer. Cell lysates were then subjected to Western blotting with anti-Ndrg2 antibody. (B) BrdU incorporation. Cells were cultured for 2 days after siRNA transfection or adenovirus infection, and subjected to the BrdU labeling followed by the immunostaining with anti-BrdU antibody. (C) Determination of proliferating cells. Cells were cultured for 2 days after siRNA transfection or adenovirus infection, and subjected to immunostaining with anti-PCNA antibody. (D) Expressions of cell cycle-related genes. Cells were cultured for 2 days after siRNA transfection or adenovirus infection, and subjected to RNA extraction, followed by RT-PCR with specific primers for Ndrg2, CyclinB1, CyclinD1, CyclinE1, p27 and β-actin. Values shown are the mean ± S.E. in four separate experiments. *P<0.05, **P<0.01, significantly different from each control value obtained in cells transfected control siRNA or cells infected with Ad-Cre and Ad-LacZ.

Fig. 3. Effects of Ndrg2 on the morphology of astrocytes.

(A) Cellular morphology. Astrocytes were cultured for 2 days after siRNA transfection, followed by replating and subsequent observation by phase contrast microscope at indicated times. Cells were fixed, and subjected to TRITC-phalloidin staining after 24 h cultivation. (B) F-actin content. Astrocytes were replated 24 h after siRNA transfection or adenovirus infection. Cells were cultured for 2 days in either the presence or absence of serum, and subjected to staining with TRITC-phalloidin for F-actin and DAPI for nuclei. TRITC-phalloidin fluorescence was normalized by DAPI fluorescence to yield a measure of total F-actin per cell. (C) Expression of GFAP. Cells were subjected to RNA extraction after siRNA transfection or adenovirus infection, followed by RT-PCR with specific primers for GFAP and β-actin. Values shown are the mean ± S.E. in three separate experiments. *P<0.05, **P<0.01,

significantly different from each control value obtained in cells transfected with control siRNA or cells infected with Ad-Cre and Ad-LacZ.

Fig. 4. Subcellular localization of Ndrg2 in astrocytes.

(A) Subcellular fractionation. Cultured rat astrocytes or mouse striatum were subjected to subcellular fractionation, followed by the determination of protein expression levels for Ndrg2, GLAST, GRP75, GRP78, and SOD1 by Western blotting. C: cytosol fraction, M: membrane fraction, N: nuclear fraction. (B) Ndrg2 localization in astrocyte. Cultured rat astrocytes were subjected to immunostaining with anti-Ndrg2 antibody, and stained with TRITC-phalloidin. (C) Ndrg2 localization in Ndrg2-overexpressing C6 glioma cell. C6 cells were transiently transfected with EGFP or EGFP-Ndrg2 expression vector. Cells were subjected to Western blotting with anti-EGFP antibody (C I) or staining with TRITC-phalloidin, and subsequent observation by fluorescence microscope (C II). Similar results were obtained in three separate sets of experiments.

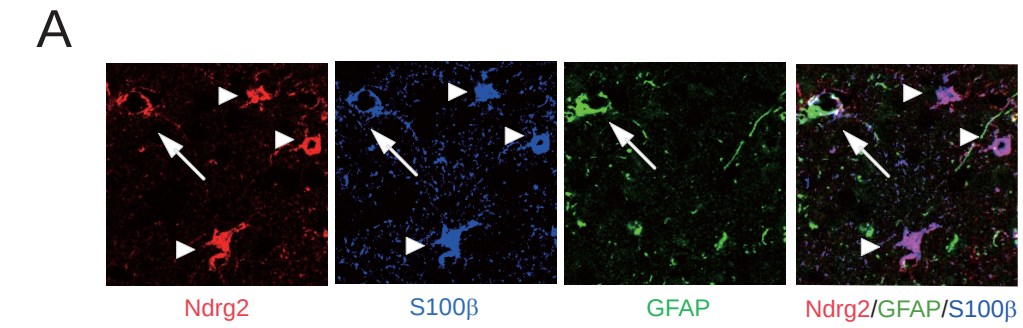

A
Ndrg2
S100β
GFAP
Ndrg2/GFAP/S100β
B I
MPTP 0h
MPTP 1d
MPTP 3d
TH
Ndrg2
/ GFAP
II
MPTP 0h
MPTP 8h
MPTP 3d
Ndrg2
/ GFAP
III
MPTP 8h
Ndrg2
/ PCNA
/ DAPI
C
MPTP
0h
8h
24h
3d
Ndrg2
S100β
GFAP
CyclinD1
β-actin
Relative intensity of Ndrg2
1.20
0.80
0.40
0.00
0h
8h
24h
72h
MPTP
D
Control
PD
high mag.
CBD
high mag.

Fig.1

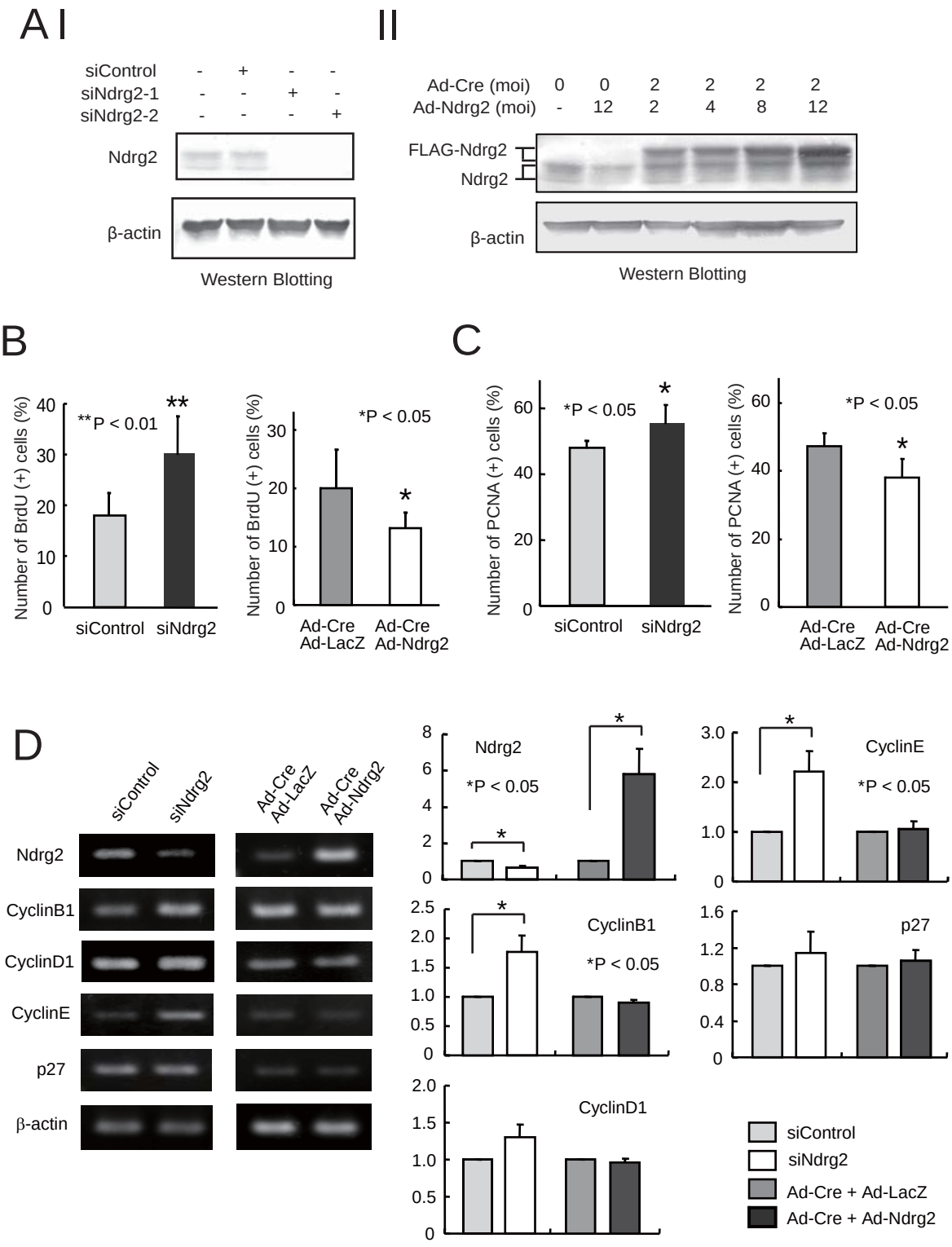
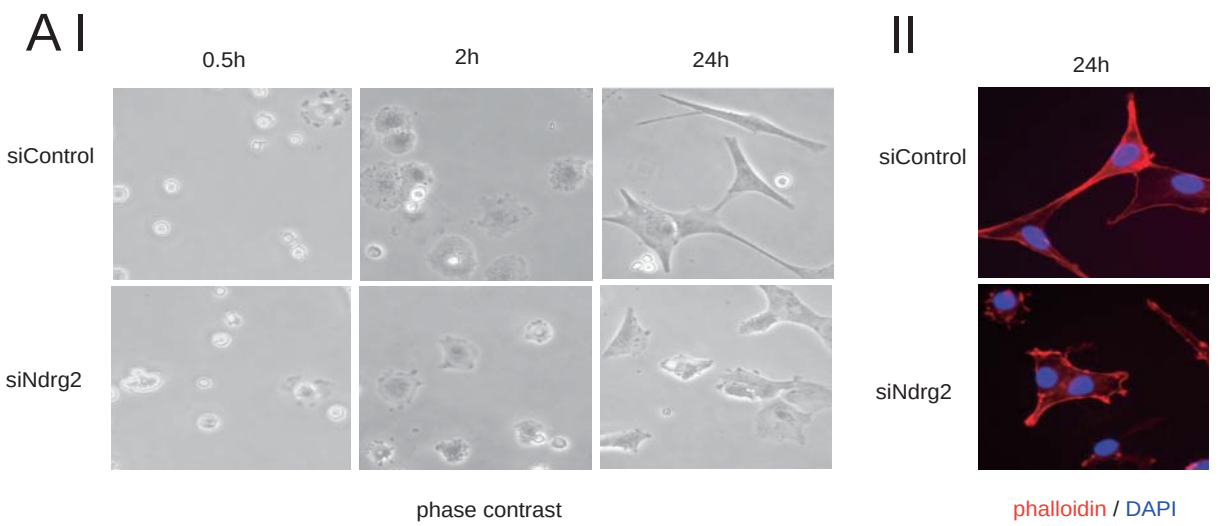
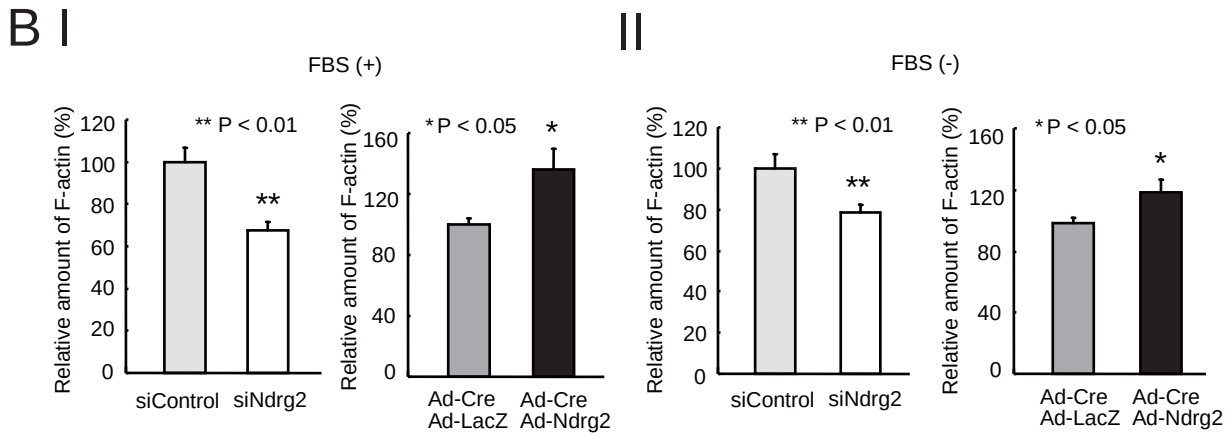

A I
siControl - + - -
siNdrg2-1 - - + -
siNdrg2-2 - - - +
Ndrg2
β-actin
Western Blotting
II
Ad-Cre (moi) 0 0 2 2 2 2
Ad-Ndrg2 (moi) - 12 2 4 8 12
FLAG-Ndrg2
Ndrg2
β-actin
Western Blotting
B
Number of BrdU (+) cells (%)
**P < 0.01
siControl siNdrg2
*P < 0.05
Ad-Cre Ad-LacZ
Ad-Cre Ad-Ndrg2
C
Number of PCNA (+) cells (%)
*P < 0.05
siControl siNdrg2
*P < 0.05
Ad-Cre Ad-LacZ
Ad-Cre Ad-Ndrg2
D
siControl
siNdrg2
Ad-Cre Ad-LacZ
Ad-Cre Ad-Ndrg2
Ndrg2
CyclinB1
CyclinD1
CyclinE
p27
β-actin
Ndrg2
CyclinE
CyclinB1
p27
CyclinD1
*P < 0.05
siControl
siNdrg2
Ad-Cre + Ad-LacZ
Ad-Cre + Ad-Ndrg2

Fig.2


A I
0.5h
2h
24h
siControl
siNdrg2
phase contrast
II
24h
siControl
siNdrg2
phalloidin / DAPI


B I
FBS (+)
Relative amount of F-actin (%)
** P < 0.01
**
siControl
siNdrg2
* P < 0.05
*
Ad-Cre
Ad-LacZ
Ad-Cre
Ad-Ndrg2
II
FBS (-)
** P < 0.01
**
siControl
siNdrg2
* P < 0.05
*
Ad-Cre
Ad-LacZ
Ad-Cre
Ad-Ndrg2

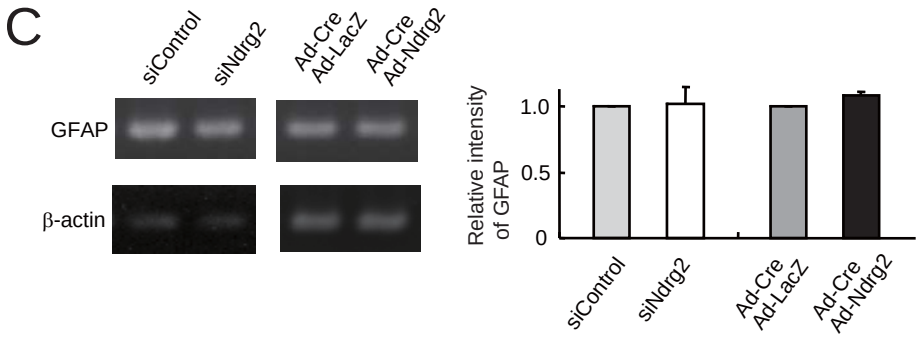
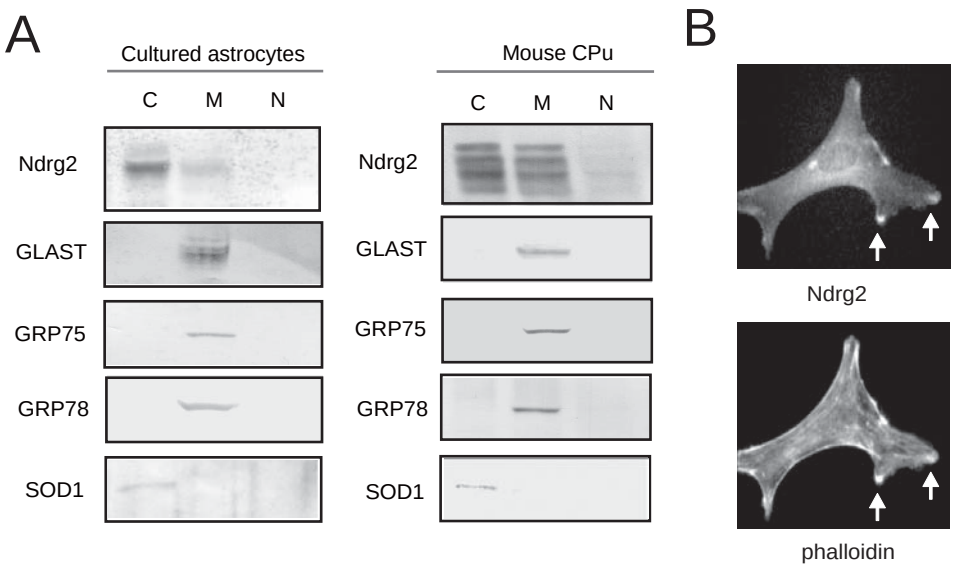

C
siControl
siNdrg2
Ad-Cre
Ad-LacZ
Ad-Cre
Ad-Ndrg2
GFAP
β-actin
Relative intensity
of GFAP
1.0
0.5
0
siControl
siNdrg2
Ad-Cre
Ad-LacZ
Ad-Cre
Ad-Ndrg2

Fig.3


A
Cultured astrocytes
C M N
Ndrg2
GLAST
GRP75
GRP78
SOD1
Mouse CPu
C M N
Ndrg2
GLAST
GRP75
GRP78
SOD1
B
Ndrg2
phalloidin

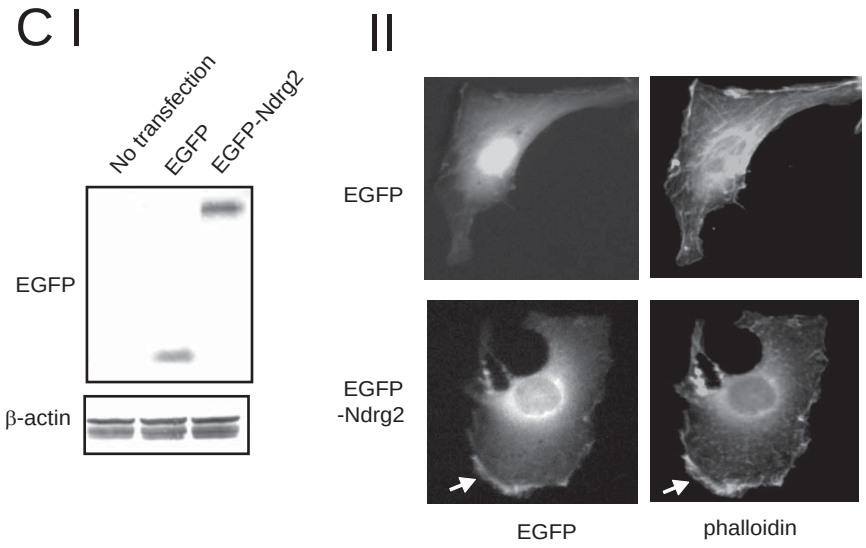

C I
No transfection
EGFP
EGFP-Ndrg2
EGFP
β-actin
II
EGFP
EGFP
-Ndrg2
EGFP
phalloidin

Fig..4