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Research article

Arhgef1 is expressed in cortical neural progenitor cells and regulates neurite outgrowth of newly differentiated neurons

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HIGHLIGHTS

- The RhoGEF Arhgef1 is highly expressed in developing cortex and cortical neural progenitor cells.
- Arhgef1 is expressed in newly differentiated cortical neurons but not astrocytes.
- Arhgef1 expression is downregulated during neuronal differentiation.
- Arhgef1 inhibits neurite outgrowth of newly differentiated neurons.

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ABSTRACT

Neurite outgrowth is crucial for the maturation of neurons and the establishment of anatomical connections during development of the nervous system. We report here that Arhgef1, a RhoA guanine nucleotide exchange factor previously found expressed in the early stages of neuronal development to regulate neurite outgrowth, is also highly expressed in cortical neural progenitor cells (NPCs). To better dissect its role in NPCs, we knocked down Arhgef1 expression in these cells and induced differentiated of them into neurons. Notably, silencing of Arhgef1 markedly enhanced neurite outgrowth in neurons derived from NPCs. Furthermore, we showed that Arhgef1 silencing inhibited the activity of RhoA, and pharmacological blockade of RhoA activity promoted neurite outgrowth in NPC-derived neurons. These findings reveal that Arhgef1 controls the process of neurite formation in newborn cortical neurons derived from NPCs.

1. Introduction

In the developing cerebral cortex, neural stem cells (NSCs) divide asymmetrically to self-renew and generate neural progenitor cells (NPCs) in the lateral ventricle [1]. The NPCs differentiate into immature neurons which migrate to the cortical plate (CP) and terminally differentiate into mature neurons [2]. In this process, neurite outgrowth is an essential differentiation event for neuronal patterning and connections, playing crucial role for the development of the nervous system [3]. Recent studies have implicated that neurite outgrowth was regulated spatially and temporally by several extracelluar and intracellular signals [4]. The Rho family GTPases, such as RhoA, Rac1 and Cdc42 have been already known to be involved in various aspects of neuronal differentiation, including neurite outgrowth, neuronal migration, and synapse development, by activating multiple effector pathways that affect actin and microtubule dynamics [5–7]. Previous work has demonstrated that RhoA activation is responsible for the regulation of the formation of focal adhesions and the assembly of actin stress fibers, and is able to inhibit the formation of neurite outgrowth in some neuronal cell types [8,9]. Rac1 promotes the formation of membrane lamellae, whereas Cdc42 regulates the outgrowth of filopodia [10]. Both Rac1 and Cdc42 positively regulate neurite outgrowth [11]. Rho GTPases are activated by large family of Rho guanine nucleotide exchange factors (RhoGEFs) [12,13], and several RhoGEFs are known to regulate neuronal morphogenesis during neural development [14–16]. Currently, more than 80 RhoGEFs have been identified in the human genome [13,14]. Considering the existence of a large number of

Abbreviations: Arhgef1, Rho guanine nucleotide exchange factor 1; GEF, guanine nucleotide exchange factor; RhoA, Ras homolog gene family, member A; Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, cell division control protein 42; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Rho GTPase, Ras homology family GTPase; shRNA, short hairpin RNA; PFA, paraformaldehyde; DIV, days in vitro; RT, room temperature; PP, preplate; MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; NSC, neural stem cell; NPC, neural progenitor cell.

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RhoGEFs in mammalian, the spatio-temporal regulation of RhoGTPase during neuronal development still needs more supporting evidence.

The Arhgef1 protein, also known as Lsc or p115RhoGEF, acts as a RhoA-specific GEF that is highly expressed in hematopoietic system, including the spleen, thymus and lymph nodes [17,18]. Therefore, most previous studies in Arhgef1 have focused on the hematopoietic system. Arhgef1-deficient neutrophils and lymphocytes have been shown migration and adhesion defects [19,20], and Arhgef1$^{-/-}$ mice have impaired thymus-dependent and type 2 thymus-independent immune responses [18]. In addition, we have previously shown that Arhgef1 is enriched in early stages of neuronal development, and functions as a negative regulator of neurite outgrowth in Neuro-2a cells and primary cortical neurons through regulating RhoA-cofilin pathway and actin dynamics [21]. However, the expression and function of Arhgef1 in NPCs remains largely unknown.

To address the possible roles of Arhgef1 in NPCs, we first confirmed that Arhgef1 is highly expressed in NPCs. Next, we used shRNA-mediated knockdown inhibition of the Arhgef1 expression in mouse NPCs. Interestingly, we observed that the changes of Arhgef1 levels strongly influenced the neurite outgrowth in NPC-derived neurons. In addition, we analyzed the role of RhoA pathway, which transduces Arhgef1 function to the regulation of neurite outgrowth. Our results provide a new understanding of molecular mechanisms that govern neurite outgrowth of NPC-derived neurons.

2. Materials and methods

2.1. DNA constructs, antibodies and reagents

Plasmid DNA was prepared using a Plasmid Maxi Kit (Qiagen). pSUPER shRNA-producing plasmids (pSUPER-Arhgef1-shRNA) was constructed based on the sequence published [22], and its scrambled control sequence is 5′-GGTGAGGCGACGACATTCT-3′. Efficiency and specificity of knockdown have verified previously [21,22].

We used the following primary antibodies: Arhgef1 and β-tubulin III were purchased from Sigma (St. Louis, MO, USA); GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Nestin and GFAP from Millipore (Darmstadt, Germany); RhoA and β-actin from Cell Signaling Technology (Beverly, MA, USA). BrdU from Abcam (Cambridge, MA, USA).

Epidermal growth factor (EGF) and Fibroblast growth factor-2 (FGF2) were purchased from Peprotech (New Jersey, USA); Rho Inhibitor I from Cytoskeleton Inc (Denver, Colorado, USA); poly-D-lysine and laminin from Sigma.

2.2. Cell culture

Primary NPCs were isolated and cultured as previously described with some modifications [23]. Briefly, 13.5-day-old (E13.5) ICR mouse embryos were sacrificed and the embryos were removed from the uterus. Then, their brains were removed and freed from meninges. The cortices were isolated, minced and incubated for 15 min at 37 °C in a 0.05% trypsin (Gibco, USA) and 0.15% DNase I solution (Roche Diagnostics) and triturated to obtain single cell suspension. Subsequently, cells were plated in cell culture flasks and incubated at 37 °C, 5% CO_2 at a cell density of 1×10^5/mL in DMEM/F12 (Gibco, USA) supplemented 2% (v/v) B27 (Gibco, USA), 1% (v/v) N2 supplement, 20 ng/mL bFGF, 20 ng/mL EGF, penicillin-streptomycin (100 U/mL). After 3–5 days, the cells formed neurospheres. At 3-days intervals, the neurospheres were passaged by dissociation into single cells.

For neuronal differentiation, neurospheres were dissociated into single-cell suspensions and plated on poly-D-lysine/laminin-coated coverslips at a density of 2×10^4 cells per coverslip. At 1 h after plating, the medium was replaced with neuron differentiation medium [24] (Neurobasal medium, 2% (v/v) B27, 2 mM L-Glutamine, 0.06% D-Glucose and 100U/mL penicillin-streptomycin) for another 5 days.

For undirectional differentiation assays, dissociated NPCs were plated on 12 mm glass coverslips coated with poly-D-lysine/laminin in 24 well plates at 2×10^4 cells per well. The cells were cultured in Neurobasal medium, supplemented with 2% (v/v) B27, 5 (v/v) % FBS and 100 U/mL penicillin-streptomycin. After 5 days, differentiated cells are stained with β-tubulin III and GFAP for lineage analysis.

Mouse neuroblastoma Neuro-2a cells (ATCC, Manassas, VA, USA) were grown in MEM (Life Technologies) supplemented with 10% FBS and 100U/ml penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Primary cortical neurons were prepared and cultured from E14.5 ICR embryos as previously described [25].

2.3. Transient transfection

Neurospheres were dissociated into single cells with trypsin. Transfection in dissociated cortical progenitor cells was performed using Lipofectamine 2000 (Life Technologies). Briefly, 3 μg Scr-shRNA or Arhgef-shRNA and 1 μg GFP were co-transfected into 5×10^5 progenitor cells using 4 μl Lipofectamine 2000, then cells were plated on 13 mm glass coverslips coated with poly-D-lysine/laminin and fed with differentiation medium. Neuronal morphology was analyzed after 5 days in vitro (DIV).

2.4. Immunohistochemistry and immunocytochemistry

For immunohistochemistry, brain of embryos were dissected, fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, and impregnated with 10–30% sucrose overnight at 4 °C. Brains were then embedded in O.C.T compound and frozen in −80 °C. Frozen brains were sliced in 10 μm thick coronal sections using a cryostat (Leica, Germany). Cryosections were immunostained with primary antibodies in blocking solution (3% BSA, 10% Goat serum, 0.3% Triton X-100) overnight at 4 °C and with secondary antibodies in PBS for 2 h at room temperature (RT). The concentrations of antibodies and dye used were: rabbit anti-Arhgef1 (1:200), mouse anti-Nestin (1:200), mouse anti-β-tubulin III (1:1000), and DAPI (1:2000). The sections were mounted with Hydromount (National Diagnostics, Atlanta, USA). Images were obtained with Zeiss 700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

For immunocytochemistry, cultured cells on coverslips were fixed with 4% PFA in PBS for 20 min and washed three times with PBS for 5 min each, blocked for 30 min with a blocking solution (1% BSA, 4% Goat serum, 0.4% Triton X-100), and incubated with primary antibody overnight at 4 °C. Coverslips were incubated with appropriate secondary antibodies for 2 h at room temperature (RT). Coverslips were mounted and photographed with the Zeiss Axio Imager A2 microscope (Carl Zeiss AG, Oberkochen, Germany). Cells with neurites were defined as cells that possessed at least one neurite more than one cell body diameter in length. Quantifications of the neurites were performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

2.5. GTPase activation assay

Briefly, Neuro-2a cells transfected with Arhgef1 knockdown plasmid. After 24 h in culture, the cells were washed and lysed in the dish in 50 mM Tris (pH 7.5), 500 mM NaCl, 1% Triton X-100,

10% Glycerol, 0.5% sodium deoxycholate, 20 mM $MgCl_2$, 10 μg/mL leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Active RhoA was investigated using the GST-RBD binding assay, Bound active RhoA were detected by western blot using antibodies to RhoA.

2.6. BrdU incorporation assay

NPCs proliferation was determined using 5-bromodeoxyuridine (BrdU) incorporation. Briefly, BrdU was added to the neurosphere culture medium 4 h before cell harvest at a final concentration of 10 μm. In order to count the number of cells that incorporated BrdU, neurospheres were dissociated, the single-cell suspension was plated and individual cells were fixed 1 h later. For BrdU detection, cells were treated with 2 M HCL at room temperature for 20 min to denature the nucleotides and then neutralized with 0.1 M sodium borate at room temperature for another 20 min. Cells were extensively washed in PBS and blocked with 5% bovine serum albumin, then cells were incubated with anti-BrdU antibody and secondary antibodies as described in immunocytochemistry section.

2.7. Statistical analysis

Data were presented as means ± standard error of the mean (s.e.m.). The two-sided unpaired Student's *t*-test was used to evaluate the statistical significance of differences in two groups and multiple groups, respectively. Probabilities <5% ($P < 0.05$) was considered as significant. All experiments were independently repeated at least three times.

3. Results

3.1. The expression of Arhgef1 in the developing cerebral cortex

We have previously shown that Arhgef1 is predominantly expressed in early stages of neural development [21]. To determine the precise distribution of Arhgef1 protein in developing cortex, we performed immunostaining for Arhgef1 on cryostat sections of developing mouse brain, focusing on the cerebral cortex. As shown in Fig. 1A, Arhgef1 is expressed throughout in the E12.5 cerebral cortex, with strongest expression near the ventricular surface. These regions contained highly proliferative cells, as revealed by positive staining of Nestin, a neural progenitor cell-specific intermediate filament protein (Fig. 1B). However, at E16.5, Arhgef1 expression was highly concentrated at cortical plate (CP), where most differentiated neuronal cells reside, and weaker expression was also detected in the ventricular zone (VZ; Fig. 1A, C). These results suggest that Arhgef1 is expressed in NPCs and newly-generated neurons, and it may be a regulator of neuronal differentiation of NPCs during development of the cerebral cortex.

3.2. Arhgef1 is highly expressed in NPCs

The expression profile of Arhgef1 in cerebral cortex suggests that it might play a role in NPC development. To investigate this, NPCs were isolated from E13.5 mouse neocortex and plated at a clonal density to allow neurosphere formation (Fig. 2A, B). After five days in culture, the neurospheres (Fig. 2C) and dissociated cells (Fig. 2D) were positive for Nestin. These NPCs are multipotent and can be induced to differentiate toward neurons (β-tubulin III-positive) and astrocytes (GFAP-positive) in vitro (Fig. 2E). High expression of Arhgef1 in the NPCs was confirmed by western blot analysis (Fig. 2F) and immunofluorescent staining (Fig. 2G). Furthermore, we found that endogenous Arhgef1 is expressed in the NPCs at the perinuclear region, and it was partly colocalized with the Golgi marker GM130 (Fig. 2H). To further determine whether Arhgef1 is expressed in proliferating NPCs, we have combined the techniques of BrdU pulse labeling and immunostaining. We found that all proliferating NPCs (BrdU positive) expressed Arhgef1 (Fig. 2I). Taken together, these results confirmed that Arhgef1 is highly expressed in NPCs.

3.3. Selective silencing of Arhgef1 promotes neurite outgrowth of neurons derived from NPCs

The expression profile of Arhgef1 in developing cortex suggests that it might be involved in neuronal differentiation of NPCs. To further investigate this possibility, we used unidirectional protocol to allow the NPCs to differentiate. Interestingly, we observed that Arhgef1 is found in elevated levels in a subset of the β-tubulin III-positive neuronal population (Fig. 3A), but not in GFAP-positive astrocytes examined (Fig. 3B). These observations are consistent with our previous observations in vivo that Arhgef1 is expressed in NPCs and newly-generated neurons. In addition, our previous studies showed that Arhgef1 inhibits neurite outgrowth in cultured cortical neurons [21]. Thus, our observations led us to investigate whether Arhgef1 might also negatively regulate neurite outgrowth during neuronal differentiation of NPCs. Considering that Arhgef1 may be mainly involved in neuronal development, NPCs were cultured in Neurobasal medium supplemented with 2% (v/v) B27, 2 mM L-Glutamine and 0.06% D-Glucose for 5 days (Fig. 3C). Using this protocol, the majority of the differentiated cells were neurons (~80% β-tubulin III positive cells) (Fig. 3D).

To verify whether Arhgef1 indeed plays an inhibitory role in neurite outgrowth of these newly-generated neurons, we first examined the expression of Arhgef1 along neuronal differentiation of NPCs. Consistent with our previous studies in cultured cortical neurons [21], Arhgef1 was strongly expressed in NPCs, and gradually decreased along differentiation (Fig. 3E). Next, we transfected an Arhgef1 shRNA plasmid into NPCs, and endogenous Arhgef1 expression was clearly downregulated 24 h after transfection (Fig. 3F). Interestingly, reducing Arhgef1 expression strongly influenced the neuronal morphology (Fig. 3G), indicated by the remarkable increase of both primary neurite number (Fig. 3H) and the longest neurite length (Fig. 3I). These findings suggest that Arhgef1 act as a negative regulator in neurite outgrowth of neurons derived from NPCs.

3.4. Arhgef1 modulates neurite outgrowth of NPC-derived neurons, which may involve RhoA activation

We next investigate the mechanisms underlying the effect of Arhgef1 knockdown on neurite outgrowth in NPCs. As a specific GEF for RhoA, Arhgef1 is involved in diverse biological processes through the regulation of RhoA activation [19,26,27]. Our previous studies confirmed that Arhgef1 regulates neurite outgrowth through activation of RhoA signaling pathway [21]. Therefore, we examined the possibility that the involvement of RhoA activation in Arhgef1-regulated neurite outgrowth of NPC-derived neurons. Indeed, transfection of Arhgef1 shRNA strongly suppressed the activation of RhoA in Neuro-2a cells (Fig. 4A, B). To address whether the inhibition of RhoA activity is actually promote NPCs differentiation, we analyzed the effects of RhoA inhibition on NPCs differentiation (Fig. 4C). We found that RhoA inactivation by its inhibitor significantly increased the number of primary neurite (Fig. 4D) and the longest neurite length (Fig. 4E), which is similar to the differentiation phenotype induced by Arhgef1-knockdown, indicating that RhoA may act downstream of Arhgef1.

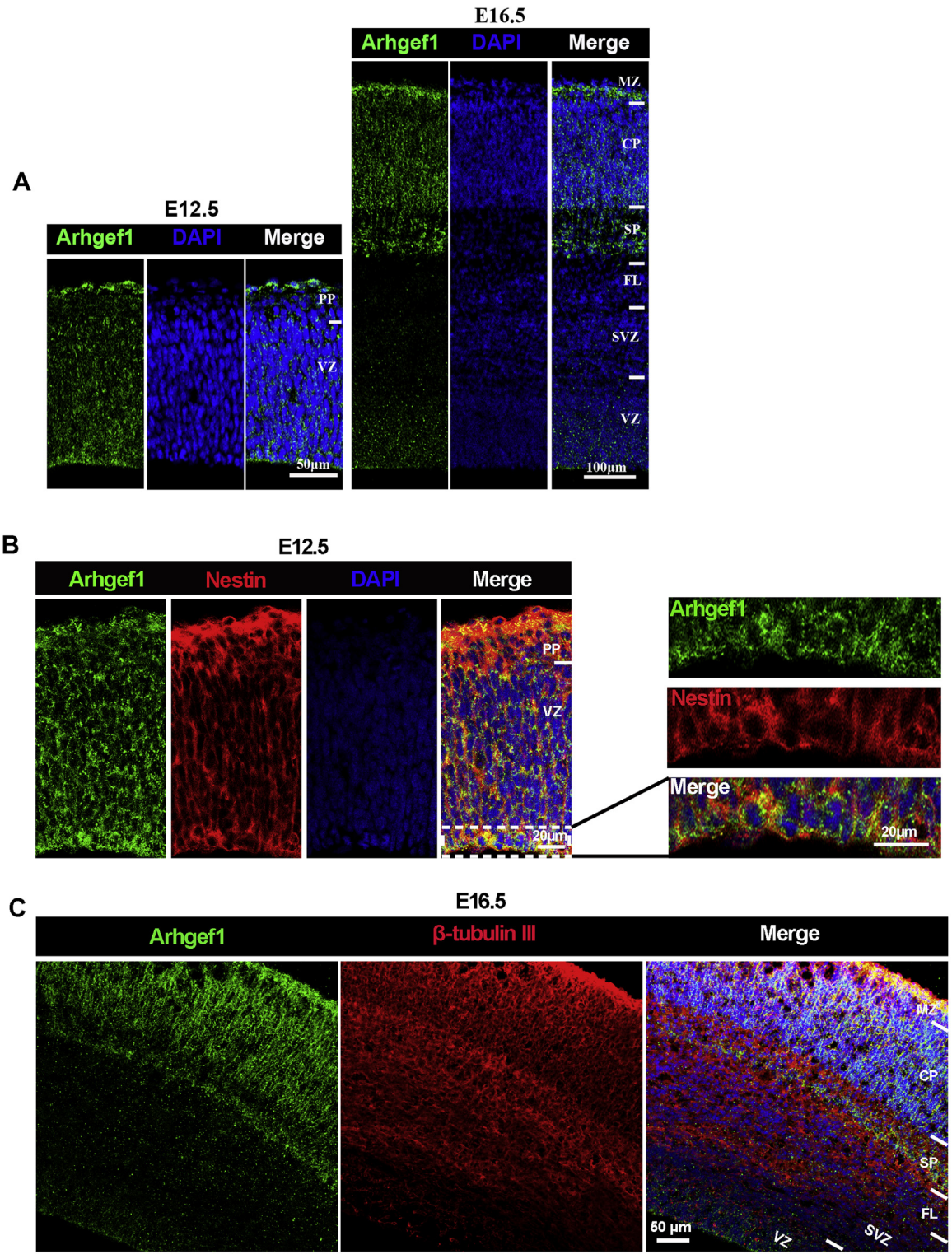

Fig. 1. Arhgef1 is expressed in the developing cerebral cortex. (A) Arhgef1 protein (green) distribution was determined by immunofluorescence in coronal sections of telencephalon at E12.5 and E16.5. Nuclei are visualized with DAPI (blue). Scale bars, 50 μm in E12.5; 100 μm in E16.5. (B) Arhgef1 (green) is expressed in Nestin (red)-positive cells at embryonic Day 12.5 (E12.5). Nuclei are visualized with DAPI (blue). Scale bars, 20 μm. (C) Arhgef1 (green) is expressed at E16.5 in β-tubulin III (red)-positive neuronal cells. Scale bars, 50 μm. The preplate (PP), marginal zone (MZ), cortical plate (CP), subplate (SP), intermediate zone (IZ), subventricular zone (SVZ) and ventricular zone (VZ) in the cortical region are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

During cortical development, neural stem cells (NSCs) and progenitor cells (NPCs) that reside in the VZ and Subventricular zone (SVZ) undergo a delicate process of migration and differentiation to produce accurate number of neurons and glial cells needed for building the nervous system. The processes of neurogenesis, neurite initiation, extension, axon/dendrite specification, and integration into neural circuits are central questions in the field of brain development. Any new understanding of these pro-

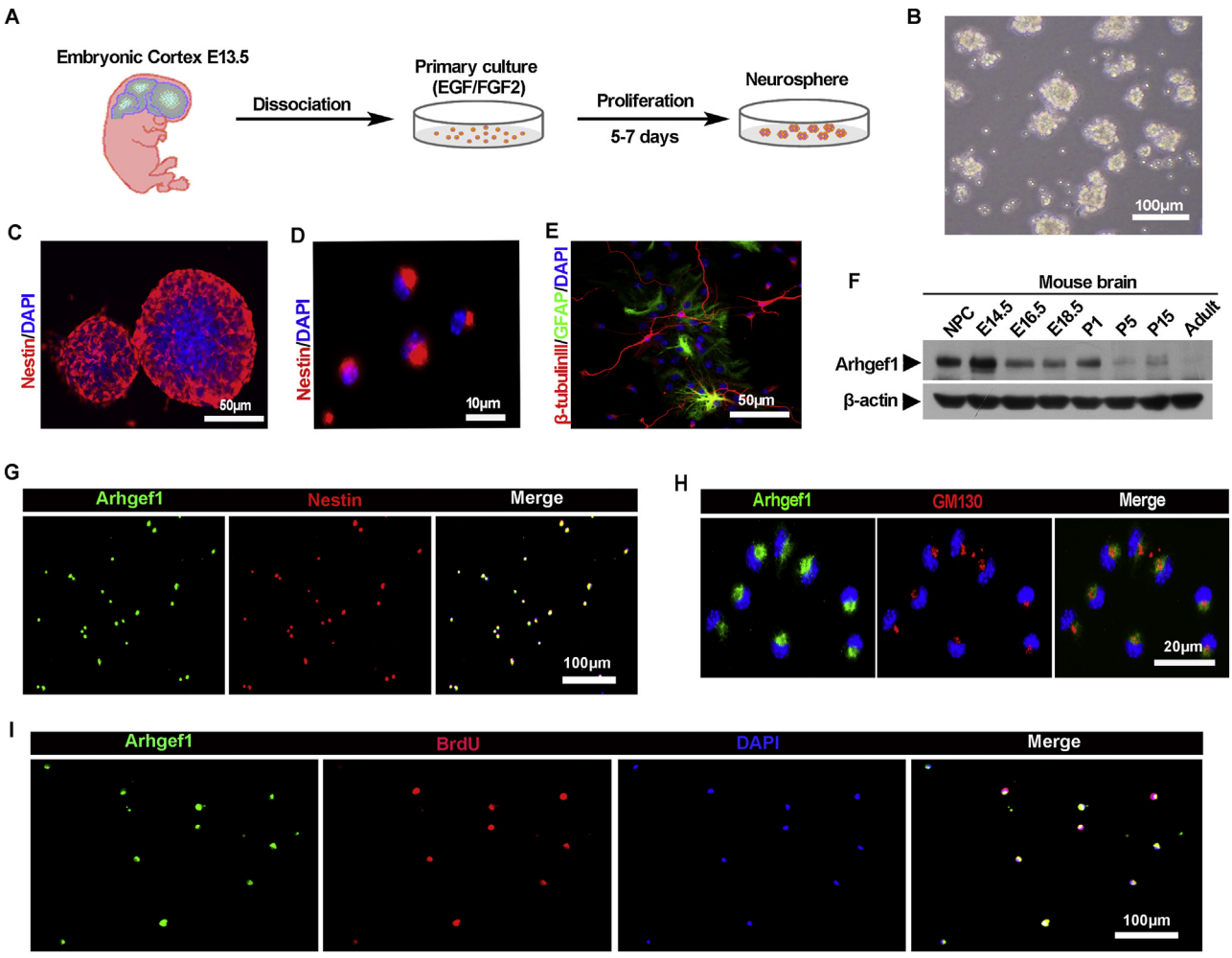

Fig. 2. Arhgef1 is expressed in NPCs. (A) A schematic depiction of NPC isolation and cell culture. NPCs were dissected from embryonic mouse neocortex at 13.5 days old, followed by culturing with both EGF and FGF2 for 5–7 days. (B) Neurospheres were normally formed after 5 d in culture. Scale bar, 100 μm. Neurospheres (C) and dissociated cells (D) were neural progenitor marker Nestin (red) positive. DAPI (blue) staining shows all cells. Scale bar, 50 μm in (C), 10 μm in (D). (E) β-tubulin III (red) and glial fibrillary acidic protein (GFAP) (green) labeling of NPC-derived neurons and astrocytes, respectively. Scale bar, 50 μm. (F) Western blot analyses of Arhgef1 in mouse brain at different developmental stages and in cortical progenitor cells. β-actin was used as a loading control. (G) Arhgef1 (green) and Nestin (red) labeling of NPCs. Scale bar, 100 μm. (H) Undifferentiated NPCs were plated, and probed for Arhgef1 (green) and GM130 (red). Nuclei are visualized with DAPI (blue). Scale bar, 20 μm. (I) Co-immunostaining of Arhgef1 (green) and BrdU (red). NPCs were pulse-labeled with BrdU for 4 h prior to immunostaining analysis.Cell nuclei are stained by DAPI (blue). Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cesses would advance knowledge of normal neurodevelopment and provide valuable knowledge how pathological conditions may be caused when alterations of these processes occur. In the present study, our findings provide evidence, for the first time, that Arhgef1 is expressed strongly in NPCs, and its expression is rapidly down-regulated upon neuronal differentiation. This suggestes that Arhgef1 may plays a critical role during cortical neurogenesis. Indeed, using Arhgef1 shRNA to knockdown the expression of Arhgef1 has a significant effect on NPC differentiation, resulting in enhanced neurite outgrowth of the newly differentiated neurons.

Rho small GTPase, such as RhoA, Rac1, and Cdc42 are key regulators of neurite extension or retraction induced by extracellular stimuli and biological responses [28]. Rho GTPases are activated by a large number of RhoGEFs [12]. Several RhoGEFs are known to regulate neuronal morphogenesis in response to a variety of extracellular cues [14,29–31]. Although previous findings showed that inhibition of the RhoA signaling pathway promotes neurite outgrowth and neuronal differentiation of mouse NSCs [32], information on the spatio-temporal regulation of this signaling pathway in neuronal differentiation and neurite outgrowth of NSC-derived neurons is limited. Our previous study has shown abundant expression of Arhgef1 in the early stages of neural development [21]. Here, we examined the expression and location of Arhgef1 in the developing cerebral cortex. Interestingly, we observed that Arhgef1 is expressed at E12.5 in Nestin^{+} NPCs and the expression was subsequently reduced in differentiating neurons. The temporal expression of Arhgef1 prompted us to investigate whether Arhgef1 regulates neuronal development. As expected, Arhgef1 was also expressed in cultured NPCs during self-renewal stages. Upon differentiation, Arhgef1 was decreased but remained expressing in neurons, similar to the pattern seen in vivo. To further analyze the effect of Arhgef1 in neuronal development, we used shRNA to selectively reduce Arhgef1 expression in cortical progenitor cells. Interestingly, we showed that inhibition of Arhgef1 strongly promoted neurite outgrowth of neurons. Furthermore, the involvement of RhoA activity is further confirmed by the promotion of neurite outgrowth when RhoA activity was blocked by Rho inhibitor I. Thus, the findings in the present study may uncover Arhgef1 as a mediator of RhoA signaling during the development of newly differentiated neurons.

In conclusion, the present study suggestes that Arhgef1 is a functionally essential modulator of the RhoA pathway and neu-

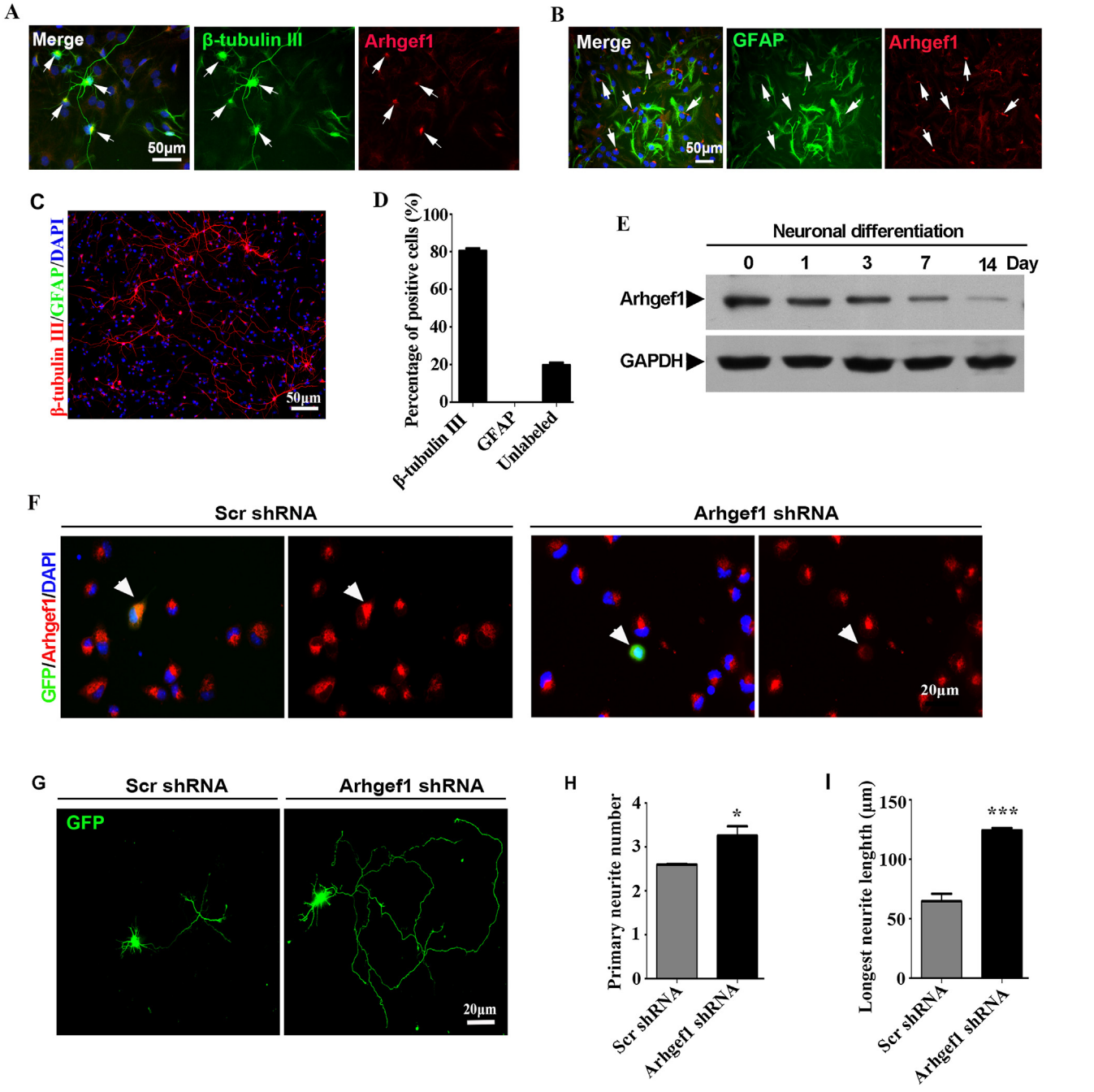

Fig. 3. Loss of Arhgef1 promotes neurite outgrowth in NPC-derived neurons. NPCs were induced to differentiate undirectionaly in Neurobasal medium, supplemented with 2% (v/v) B27 and 5 (v/v) % FBS. After 5 days, cells were fixed and probed for Arhgef1 (red) and β-tubulin III (green) (A) or GFAP (green) (B). Arhgef1-positive cells are indicated by arrows. Scale bar, 50 μm. (C) NPC differentiated with neuronal differentiation medium and stained with anti-β-tubulin III (red), anti-GFAP (green) and DAPI (blue) after 5 day culture. Scale bar, 50 μm. (D) The percentage of cells of each population was calculated, and the total cells were visualized by DAPI staining of nuclei. (E) Detection of Arhgef1 protein expression during NPCs differentiation by western blot. GAPDH was used as a loading control. (F) NPCs were co-transfected with GFP together with Arhgef1 shRNA or Scr shRNA construct and cultured for 24 h. Cells were immunostained using Arhgef1 antibody (red). Arrowheads indicate transfected cells. Scale bar, 20 μm. (G) NPCs co-transfected with the same set of plasmids described in (F) were differentiated for 5 days. The GFP expression allowed visualization of neurites. Scale bar, 20 μm. Primary neurite number (H) and average length of the longest neurite (I) were measured. *$p < 0.05$, ***$p < 0.001$, Student's *t*-test. At least 30 cells/group were analyzed in each experiment, n = 3. Error bars, s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rite outgrowth in NPC-derived neurons (Fig. 4F). Understanding the function and regulation of Arhgef1 in this context may contribute to a better understanding the spatio-temporal RhoGTPase signaling in regulation of neuronal differentiation. However, there are still several questions that remain to be resolved, including how Arhgef1-RhoA signaling regulates neurite outgrowth and cortical development, and whether Arhgef1-RhoA signaling participate in the maintenance and differentiation of NPCs.

Conflict of interest

The authors declare no conflicts of interest.

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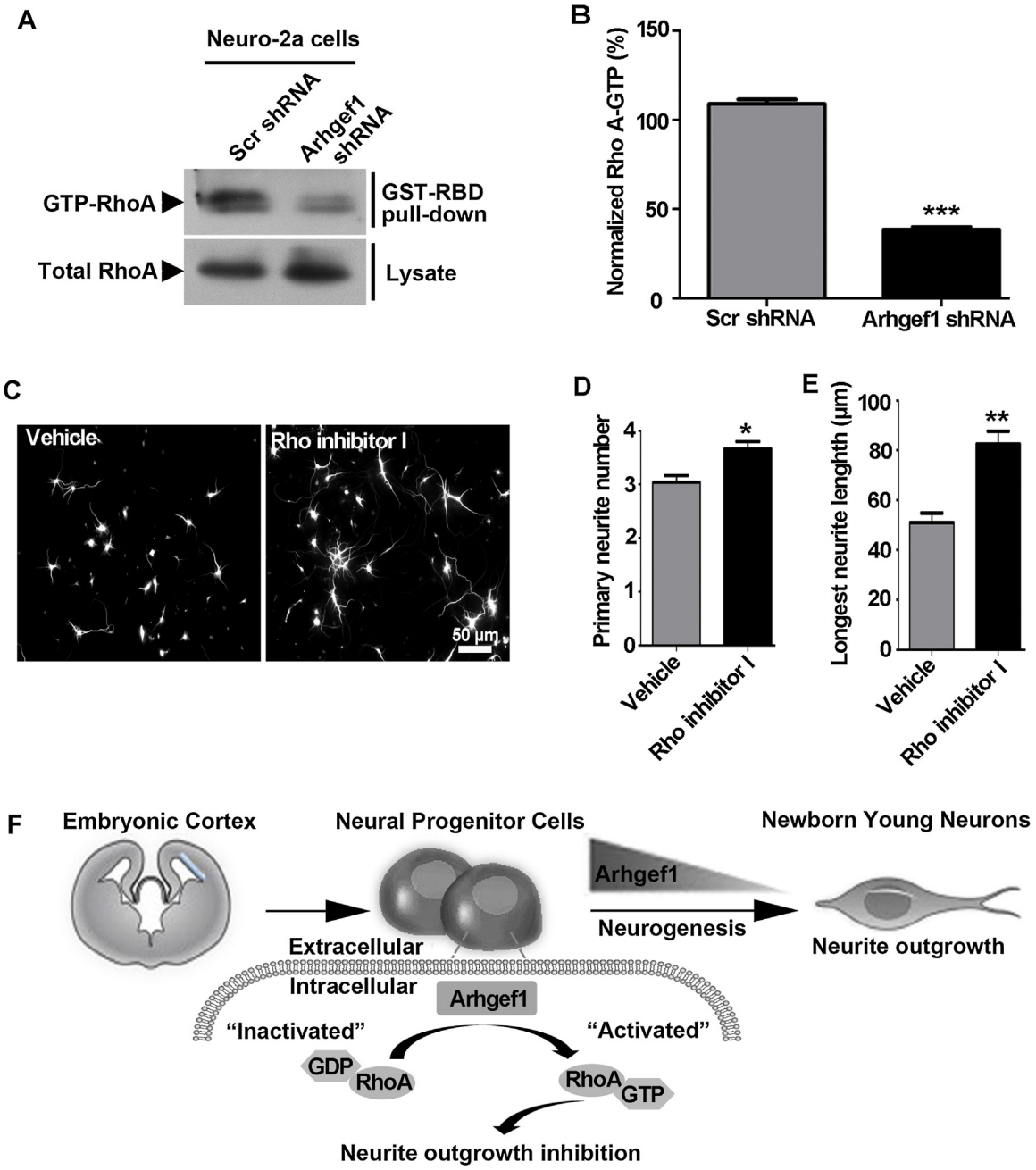

Fig. 4. Arhgef1-mediated RhoA activation is essential for neurite outgrowth in NPC-derived neurons. (A) Neuro-2a cells were transfected with Arhgef1-shRNA construct, followed by culturing for 24 h. Cell lysates were then subjected to RhoA activity assay for detection of GTP-bound RhoA (RhoA-GTP). (B) Rho A-GTP levels were quantified and normalized. ***$p < 0.001$, Student's *t*-test from three independent experiments. (C) NPCs were treated with Rho inhibitor I 0.2 μM, a RhoA inhibitor, or its vehicle PBS, and differentiation for 5 days. Cells were immunostained using β-tubulin III antibody for visualization of neurites. Scale bar, 50 μm. Primary neurite number (D) and the longest neurite length (E) were measured. *$p < 0.05$, **$p < 0.01$, Student's *t*-test. At least 100 cells/group were analyzed in each experiment, n = 3. Error bars, s.e.m. (F) A schematic of proposed role of Arhgef1 in regulating neurite outgrowth of newly differentiated neurons from NPCs.

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Authors' contributions

Xiang and L.Shi designed the experiments; X.Xiang, X.Zhuang and S.Li performed the experiments; X.Xiang and L.Shi did the data analysis; X.Xiang and L.Shi wrote the paper.

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