

Activation of the Notch signaling pathway promotes neurovascular repair after traumatic brain injury

Qi-shan Ran^{*}, Yun-hu Yu, Xiao-hong Fu, Yuan-chao Wen

Department of Neurosurgery, the First People's Hospital of ZunYi/the Third Affiliated Hospital of ZunYi Medical College, Zunyi, Guizhou Province, China

***Correspondence to:**

Qi-shan Ran, ranqishan1203@126.com.

orcid:

0000-0002-4949-8327 (Qi-shan Ran)

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Abstract

The Notch signaling pathway plays a key role in angiogenesis and endothelial cell formation, but it remains unclear whether it is involved in vascular repair by endothelial progenitor cells after traumatic brain injury. Therefore, in the present study, we controlled the Notch signaling pathway using overexpression and knockdown constructs. Activation of the Notch signaling pathway by Notch1 or Jagged1 overexpression enhanced the migration, invasiveness and angiogenic ability of endothelial progenitor cells. Suppression of the Notch signaling pathway with Notch1 or Jagged1 siRNAs reduced the migratory capacity, invasiveness and angiogenic ability of endothelial progenitor cells. Activation of the Notch signaling pathway *in vivo* in a rat model of mild traumatic brain injury promoted neurovascular repair. These findings suggest that the activation of the Notch signaling pathway promotes blood vessel formation and tissue repair after brain trauma.

Key Words: nerve regeneration; endothelial progenitor cells; traumatic brain injury; Notch signaling pathway; cell migration; invasion; angiogenesis; jetPEI™ system; neural regeneration

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Introduction

Endothelial progenitor cells (EPCs) differentiated from hematopoietic stem cells play important roles in angiogenesis and regeneration. These cells can differentiate into vascular endothelial cells and repair blood vessels (Burger and Touyz, 2012). EPCs have been shown to help repair secondary nerve injury after traumatic brain injury (TBI) (Li et al., 2013; Park et al., 2014; Wang et al., 2015a).

Vascular repair plays a major role in recovery after TBI (Franzblau et al., 2013; Brew et al., 2014; Badaut et al., 2015). Wei et al. (2010) showed that the number of circulating EPCs is low during the acute phase, and then increases gradually but dramatically after TBI in patients. Elevating EPC levels in circulating blood with atorvastatin improves memory and spatial cognitive function in a rat model of TBI (Liu et al., 2007). Although these findings suggest that EPCs play a critical role in recovery after TBI, the molecular mechanisms regulating EPC number and function remain poorly understood.

The Notch signaling pathway has been shown to regulate angiogenesis and endothelial cell formation, and is found in numerous cell types (Artavanis-Tsakonas et al., 1999). Li et al. (2010) showed that the Notch signaling pathway regulates proliferation and differentiation as well as angiogenesis. Furthermore, Liu et al. (2014) reported that in transgenic mice, persistent activation of the Notch pathway inhibits basic

fibroblast growth factor-induced angiogenesis, and prevents the development of ovarian follicles. Lee et al. (2014) showed that the Notch pathway targets the proangiogenic regulator Sox17 to inhibit angiogenesis. Our group found that Notch1 protein and mRNA levels are reduced in injured tissue, but then gradually increase to normal levels in the early stage of TBI. We also found that mRNA levels of the Notch1 ligand, Jagged1, are reduced after injury (Xu et al., 2009). In the present study, we investigated the role of the Notch signaling pathway in vascular repair after TBI.

Materials and Methods

In vitro experiments

EPC culture

Ten healthy specific-pathogen-free male Wistar rats, 20 weeks old, were purchased from Vital River Laboratories, Beijing, China (animal license No. SCXK (Jing) 2012-0001). All rats were housed at 24°C in a room with a 12-hour light/dark cycle, and allowed free access to food and water. The protocols were approved by the Laboratory Animal Care and Use Committee, the Third Affiliated Hospital of ZunYi Medical College, China.

Approximately 10 mL of blood was taken from each rat *via* cardiac puncture. Plasma was isolated following gradient centrifugation of whole blood, and monocytes were obtained and seeded in 10-cm-diameter culture dishes with

stem cell medium for 6 days. Suspended cells were removed, and adherent cells were placed in fibronectin-containing medium (Shanghai Qianchen Biotechnology Co., Ltd., Shanghai, China) until the number of cells reached 1×10^6 /mL (Ahrens et al., 2011). Cells were digested with trypsin. CD34 and CD133, the markers for angiogenesis, were identified by flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA) (Shim et al., 2015).

Cell transfection

Cells were transfected at 70% confluence. A 1- μ g aliquot of plasmid (Notch1 OE, Jagged1 OE, si-Notch1 or si-Jagged1; Genechem, Shanghai, China) was dissolved in serum-free medium. Vegofect transfection reagent (1:1,000; Vigorous Biotechnology, Beijing, China) was dissolved in serum-free medium in accordance with the manufacturer's instructions. The plasmid and transfection solution were mixed and incubated without stirring for 15 minutes. The mixture was added dropwise to the serum-free medium. The culture dish was shaken slightly to evenly distribute the transfection mix. Cells were cultured for 48 hours before the experiments.

Western blot assay

Cells on the culture dish were collected and lysed using pre-cooled RIPA lysis buffer. Loading buffer was added, the sample was boiled, and SDS-PAGE was performed. Samples were transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour, and then incubated with goat anti-Notch1 polyclonal antibody, rabbit anti-Jagged1 polyclonal antibody and goat anti-rat GAPDH monoclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The membrane was washed with TBS-Tween three times and incubated with rabbit anti-goat IgG or goat anti-rabbit IgG (1:2,000; Beijing Zhongshan Golden Bridge, Beijing, China). Reactive bands were detected with a chemiluminescence analyzer (Bio-Rad, Hercules, CA, USA).

Luciferase reporter assay

Promoter activity of Hes1, a major downstream target of the Notch signaling pathway, was detected as previously reported (von Grabowiecki et al., 2015). A luciferase reporter construct containing the Hes1 promoter was transfected into cells and cultured for 24 hours. Luciferase activity was measured with a dual luciferase reporter assay kit (Vigorous Biotechnology, Beijing, China). All steps were strictly carried out in accordance with the instructions in the kit.

Scratch test

A marker pen was used to draw a horizontal line on the back of the six-well plates, and 1×10^6 EPCs were seeded and incubated until 90% confluence. A 200 μ L pipet tip was oriented vertical to the surface of the plate and used to make a scratch through the cell layer. Detached cells were removed by gently washing with PBS. The dish was incubated for a further 24 hours and then fixed with paraformaldehyde. The distance of cell migration was analyzed (Giordano et al., 2014).

Transwell assay

The Transwell assay was performed strictly in accordance with the instructions in the Transwell assay kit (Corning, Corning, NY, USA). The Transwell chamber insert was coated with matrigel (GE Healthcare, Bethesda, MD, USA) and hydrated before use. Cells were digested, resuspended with serum-free medium containing bovine serum albumin, and cell density was adjusted to 5×10^5 /mL. Cells were cultured in the Transwell insert, which was placed in 24-well plates, and 600 μ L medium was added and incubated for 24 hours. Cells were quantified with the indirect counting method. Matrigel and cells in the apical part of the Transwell insert were removed with a cotton swab. A 500- μ L aliquot of complete medium containing MTT was added into the 24-well plates, and then the Transwell chamber was placed in the 24-well plates for 4 hours at 37°C. After removal of the medium, 500 μ L of dimethyl sulfoxide was added to the Transwell chamber and incubated for 10 minutes with shaking. The Transwell insert was taken out, the optical density was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA), and the invasive ability of the cells was determined (Sun et al., 2014).

Immunofluorescence assay

EPCs were incubated in a culture dish containing a pretreated coverslip for 24 hours after transfection. The coverslip was washed with PBS twice, fixed with 4% paraformaldehyde, and treated with 2% PBS-Tween for 10 minutes. After three washes with PBS (each for 5 minutes), cells were blocked with bovine serum albumin for 30 minutes, incubated with goat anti-CD34 polyclonal antibody (1:200; Santa Cruz Biotechnology) at 4°C overnight, and then with rabbit anti-goat IgG (1:2,000; Beijing Zhongshan Golden Bridge) at 37°C for 2 hours. Nuclei were counterstained with Hoechst 33342 dye. Samples were mounted with mounting medium, and photographed under a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan).

In vivo experiments

Rat models of mild TBI

A total of 15 adult specific-pathogen-free male Wistar rats, 6–8 months of age and weighing approximately 250 g, were housed at 24°C under a 12-hour light/dark cycle, and allowed free access to food and water. The rats were acclimated for 1 week, and then anesthetized with 5% chloral hydrate. The head was fixed in a stereotaxic apparatus (Beijing Zongshi Dichuang Technology, Beijing, China). After shaving, a 3-cm sagittal incision was made 0.5 cm posterior to the midpoint between the eyes along the midline of the head to expose the skull and to identify the bregma. A hole was drilled in the skull to expose the dura mater. Using a PinPoint™ precision brain injury impactor (Wuhan Yihong Technology Co., Ltd., Wuhan, Hubei Province, China), the dura mater was impacted at 3 m/s to induce craniocerebral injury. The scalp was then sutured.

Injection using the jetPEI™ system

All procedures were in strict accordance with instructions

in the *in vivo*-jetPEI™ kit (Polyplus, Visalia, CA, USA). Rats with mild TBI were randomly divided into five groups ($n = 3$): empty plasmid, Notch1 overexpression, Jagged1 overexpression, Notch1 knockdown, and Jagged1 knockdown. Using the *in vivo*-jetPEI™ delivery system, the Notch1 and Jagged1 plasmids and the Notch1 and Jagged1 siRNAs were injected *via* puncture 0.3 cm lateral to the midline and 0.5 cm anterior to the coronal suture (Stefini and Rasulo, 2008). pcDNA-Notch1 and pcDNA-Jagged1 were purchased from GeneChem, Shanghai, China. The Notch1 and Jagged1 siRNAs were purchased from Sigma, St. Louis, MO, USA. A 5- μ g aliquot of empty plasmid encapsulated using *in vivo*-jetPEI™ was injected in the empty plasmid injection group. The shRNA sequences used were as follows: Notch1 knockdown, 5'-UUC UCC GAA CGU GUC ACG U-3'; Jagged1 knockdown, 5'-GAA UGU GAG GCC AAA CCU U-3'.

Western blot assay and immunofluorescence analysis

Fourteen days after injection, rats in each group were sacrificed and brain tissues were collected. One sample of brain tissue was cut into 1-mm³ blocks with a tissue homogenizer, treated with pre-cooled lysis buffer, and boiled with loading buffer. Notch1 and Jagged1 expression levels were evaluated using western blot assay (Koob et al., 2012). Antibodies were identical to those used for the *in vitro* western blot assay.

The other brain tissue sample was immersed in optimal cutting temperature medium, frozen using liquid nitrogen, and sectioned with a cryostat. The sections were placed at room temperature for 30 minutes, fixed with acetone, treated with 2% PBS-Tween, and then used for immunofluorescence staining (Kunishio et al., 1989). Antibodies were identical to those used for the *in vitro* western blot assay.

Statistical analysis

Data are expressed as the mean \pm SD, and were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences between groups were compared using one-way analysis of variance and the nonparametric Mann-Whitney *U* test. A value of $P < 0.05$ was considered statistically significant.

Results

Specific activation and inhibition of the Notch signaling pathway in EPCs

Notch1 and Jagged1 plasmids were transfected into EPCs *in vitro*. Western blot assay demonstrated that expression levels of Notch1 and Jagged1 were significantly increased in the transfected EPCs ($P < 0.05$). Luciferase reporter assay revealed that the Notch signaling pathway was activated after overexpression of Notch1 and Jagged1 (Figure 1A). Notch1 and Jagged1 protein levels were knocked down after Notch1 and Jagged1 siRNAs were transfected into cells ($P < 0.05$). Luciferase reporter assay showed that Hes1 promoter activity was significantly inhibited when the Notch signaling pathway was suppressed by transfecting the siRNAs (Figure 1B). These data suggest that the Notch signaling pathway was specifically activated or suppressed.

Activation of the Notch signaling pathway enhanced the migration of EPCs

The scratch test showed that compared with the empty plasmid control group (vector group), the distance migrated by the cells was greater after overexpression of Notch1 and Jagged1 ($P < 0.05$; Figure 2A). Compared with the scrambled siRNA group, migratory ability diminished after the Notch signaling pathway was inhibited by Notch1 and Jagged1 knockdown with siRNA ($P < 0.05$). These results are similar to those obtained using DAPT to block the Notch signaling pathway ($P > 0.05$; Figure 2B). These data indicate that the Notch signaling pathway promotes EPC migration.

Activation of the Notch signaling pathway enhanced EPC invasiveness

A Transwell chamber insert coated with matrigel was used to assess EPC invasiveness. Compared with the empty plasmid control group (vector group), cell invasiveness was significantly enhanced after the Notch signaling pathway was activated by the overexpression of Notch1 and Jagged1 ($P < 0.05$). Moreover, this effect was similar to that achieved with the Notch agonist Jagged1 ($P > 0.05$; Figure 3A). After knockdown of Notch1 and Jagged1, the Notch signaling pathway was inhibited. Compared with the scrambled siRNA group, knockdown of Notch1 and Jagged1 significantly reduced EPC invasiveness ($P < 0.05$), and this effect was similar to that obtained using DAPT to block the Notch signaling pathway ($P > 0.05$; Figure 3B). These findings show that the Notch signaling pathway enhances EPC invasiveness.

Activation of the Notch signaling pathway enhanced the angiogenic ability of EPCs

CD31, also known as platelet endothelial cell adhesion molecule, is a marker of endothelial cells and can be used to examine the formation of blood vessels (Shim et al., 2015). Compared with the control group (vector group), the fluorescence intensity of CD31 was significantly enhanced in EPCs transfected with Notch1 or Jagged1 overexpression constructs ($P < 0.05$). This effect was similar to that produced by Jagged1 ($P > 0.05$; Figure 4A). Compared with the scrambled siRNA group, Notch1 or Jagged1 knockdown significantly diminished the fluorescence intensity of CD31 in EPCs ($P < 0.05$). This effect is similar to that produced by DAPT, which blocks the Notch signaling pathway ($P > 0.05$; Figure 4B). These data show that the Notch signaling pathway enhances the angiogenic ability of EPCs.

In vivo activation of the Notch signaling pathway promoted angiogenesis and tissue repair in the injured brain

Western blot assay revealed that when Notch1 or Jagged1 overexpression constructs were injected into the brain, the corresponding protein levels were noticeably increased in brain tissue (Figure 5A). If siRNA for Notch1 or Jagged1 was injected into the brain, expression of the respective protein was knocked down (Figure 5B). Immunofluorescence staining revealed that when the Notch signaling pathway was activated in rats with mild TBI, CD31 labeling was robust compared with the control group. Conversely, when the

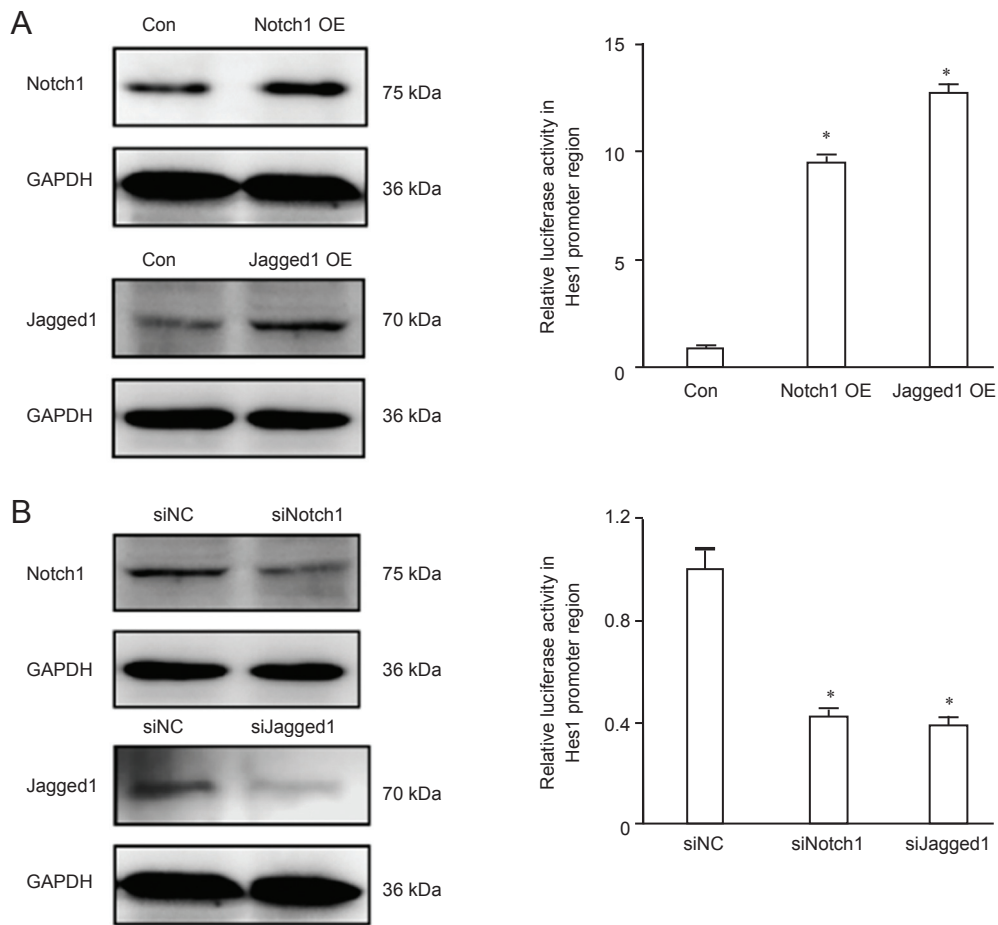


Figure 1 Specific activation or inhibition of the Notch signaling pathway in endothelial progenitor cells.

(A) Left: Western blot assay showing the overexpression of Notch1 and Jagged1. Con: Control group; Notch1 OE: Notch1 overexpression group; Jagged1 OE: Jagged1 overexpression group. Right: Hes1 promoter activity measured with luciferase reporter assay. (B) Left: Western blot assay showing knockdown of Notch1 and Jagged1. siNC: Control group; siNotch1: Notch1 knockdown group; siJagged1: Jagged1 knockdown group. Right: Hes1 promoter activity measured with luciferase reporter assay. Hes1 promoter activity is presented as a ratio to the control group. Data are expressed as the mean \pm SD. Differences between groups were compared using one-way analysis of variance and nonparametric Mann-Whitney *U* test. Experiments were performed in triplicate. **P* < 0.05, vs. control group.

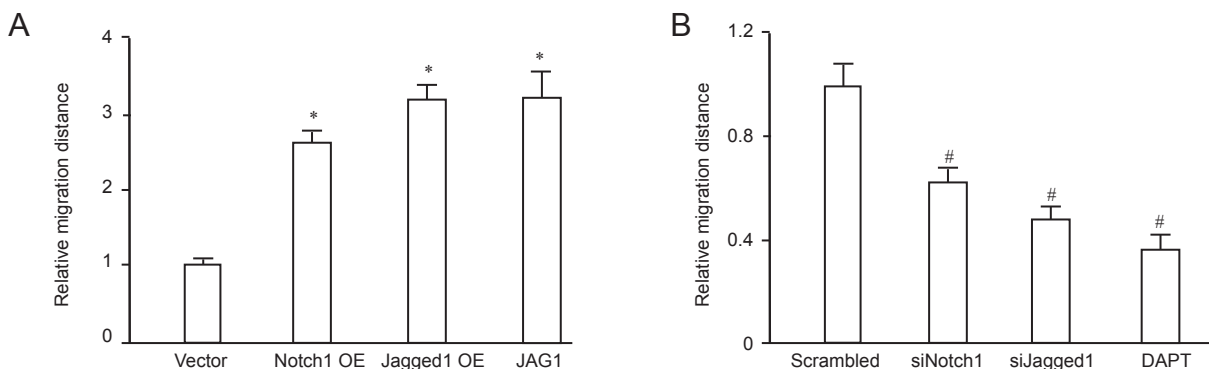


Figure 2 Activation of the Notch signaling pathway enhanced the migration of endothelial progenitor cells.

(A) Scratch test for evaluating the distance of cell migration in the Notch signaling pathway activation groups. The distance of cell migration is presented as a ratio to the vector group. (B) Scratch test for evaluating the distance of cell migration in the Notch signaling pathway inhibition groups. The distance of cell migration is presented as a ratio to the scrambled siRNA group. Data are expressed as the mean \pm SD. The difference between groups was compared using one-way analysis of variance and nonparametric Mann-Whitney *U* test. Experiments were performed in triplicate. **P* < 0.05, vs. vector group; #*P* < 0.05, vs. scrambled siRNA group. Notch1 OE: Notch1 overexpression group; Jagged1 OE: Jagged1 overexpression group; JAG1: Ligand for multiple Notch receptors and involved in the mediation of Notch signaling; siNotch1: Notch1 knockdown group; siJagged1: Jagged1 knockdown group; DAPT: γ -secretase inhibitor IX.

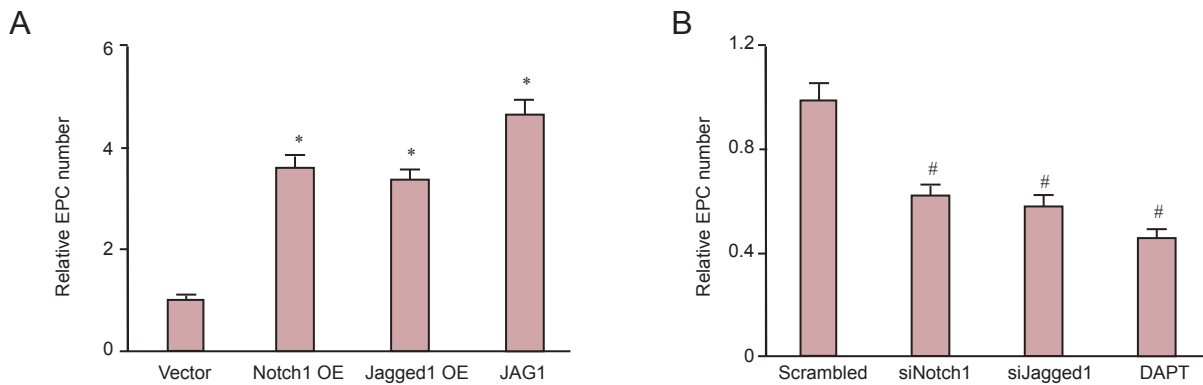


Figure 3 Activation of the Notch signaling pathway enhanced the invasiveness of endothelial progenitor cells (EPCs).

(A) Transwell assay showing the number of invasive EPCs in the Notch signaling pathway activation groups. The number of EPCs is presented as a ratio to the vector group. (B) Transwell assay showing the number of invasive EPCs in the Notch signaling pathway inhibition groups. The number of EPCs is presented as a ratio to the scrambled siRNA group. Data are expressed as the mean \pm SD. Differences between groups were compared using one-way analysis of variance and nonparametric Mann-Whitney *U* test. Experiments were performed in triplicate. * $P < 0.05$, vs. vector group; # $P < 0.05$, vs. scrambled siRNA group. Notch1 OE: Notch1 overexpression group; Jagged1 OE: Jagged1 overexpression group; JAG1: Ligand for multiple Notch receptors and involved in the mediation of Notch signaling; siNotch1: Notch1 knockdown group; siJagged1: Jagged1 knockdown group; DAPT: γ -secretase inhibitor IX.

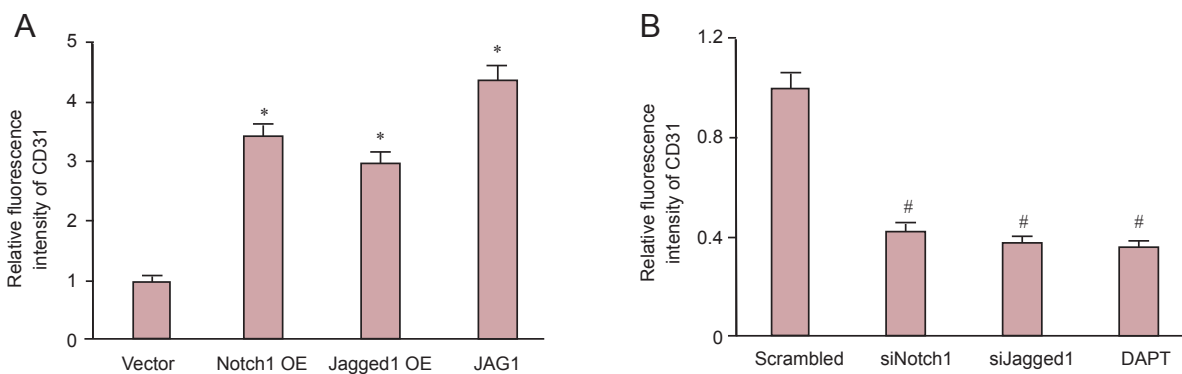


Figure 4 Activation of the Notch signaling pathway enhanced the angiogenic ability of endothelial progenitor cells.

(A) Immunofluorescence assay showing the fluorescence intensity of CD31 labeling in the Notch signaling pathway activation groups. The fluorescence intensity of CD31 is presented as a ratio to the vector (control) group. (B) Immunofluorescence assay showing the fluorescence intensity of CD31 labeling in the Notch signaling pathway inhibition groups. The fluorescence intensity of CD31 is presented as a ratio to the scrambled siRNA group. Data are expressed as the mean \pm SD. Differences between groups were compared using one-way analysis of variance and nonparametric Mann-Whitney *U* test. Experiments were performed in triplicate. * $P < 0.05$, vs. vector group; # $P < 0.05$, vs. scrambled siRNA group. Notch1 OE: Notch1 overexpression group; Jagged1 OE: Jagged1 overexpression group; JAG1: Ligand for multiple Notch receptors and involved in the mediation of Notch signaling; siNotch1: Notch1 knockdown group; siJagged1: Jagged1 knockdown group; DAPT: γ -secretase inhibitor IX.

Notch signaling pathway was suppressed, CD31 immunofluorescence was reduced ($P < 0.05$; **Figure 5C, D**).

Discussion

EPCs differ from other progenitor cells; they have an abundant source, are easily extracted, and can be used autologously. Consequently, EPCs are likely to become a new source for cell transplantation. Wang et al. (2015b) showed that the tropic and paracrine capacity of EPCs can be enhanced by modification, permitting these cells to be used to treat TBI. Recombinant human erythropoietin can increase the number of circulating EPCs in rats and improve neurologic recovery after TBI (Wang et al., 2015a). Zhang et al. (2013) showed that the transplantation of vascular endothelial colony-forming

cells could effectively treat TBI in a mouse model.

Jagged1 has been shown to regulate the Notch signaling pathway and promote angiogenesis (Hellström et al., 2007; Benedito et al., 2009; Suchting and Eichmann, 2009). Kamei et al. (2012) found that EPCs promote astrocyte proliferation by activating Jagged1-dependent Notch signaling after spinal cord injury. Kwon et al. (2008) showed that EPCs derived from Jagged1^{-/-} knockout mice have reduced neovascularization capacity and lower therapeutic potential for ischemic injury. Xie et al. (2015) demonstrated that simvastatin enhanced regeneration at the site of injury by activating Notch signaling, thereby contributing to the recovery of neurological function after TBI. Despite these recent advances, the functional roles of the Notch signaling pathway

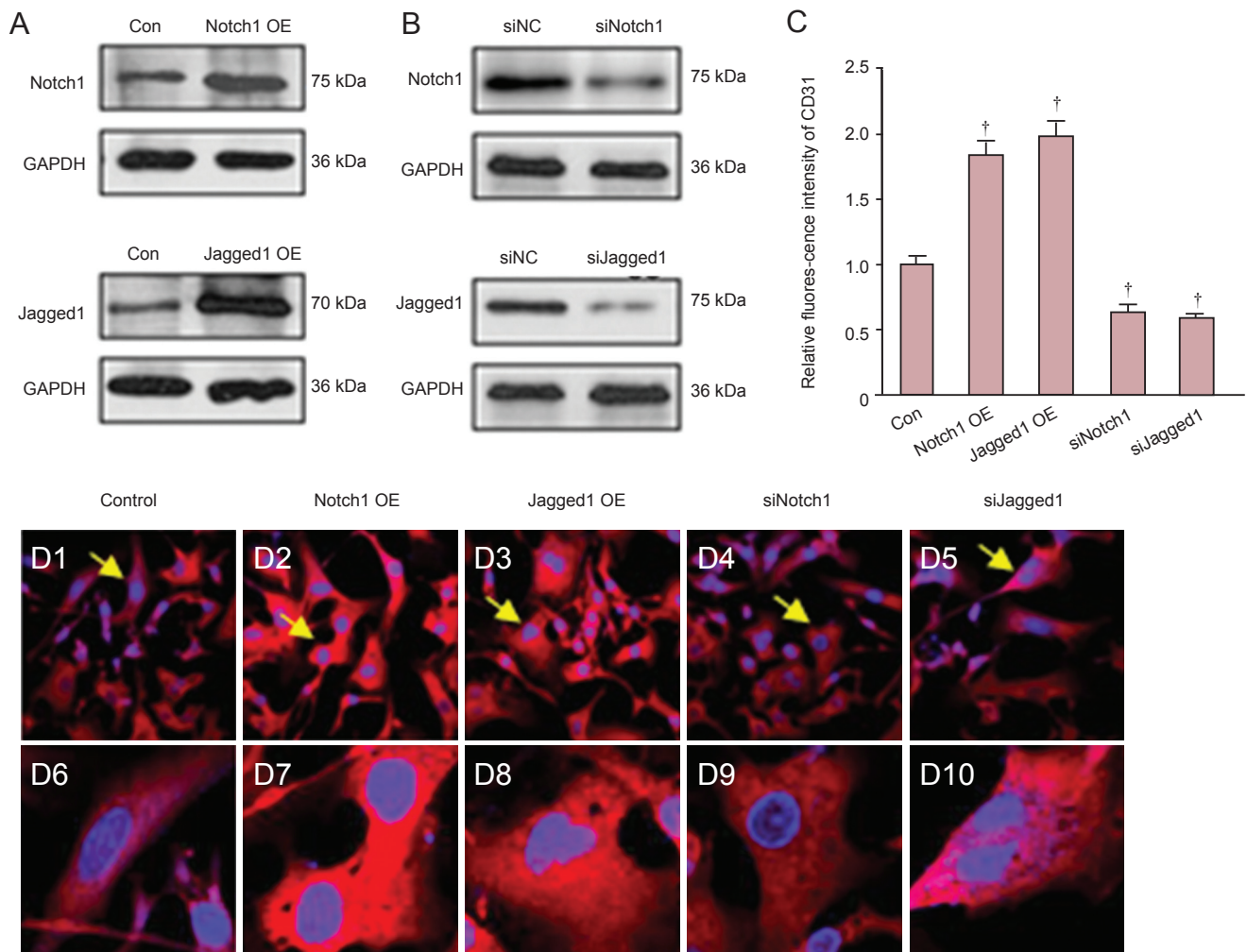


Figure 5 *In vivo* activation of the Notch signaling pathway promoted neurovascular repair in the brain after traumatic brain injury.

(A) Western blot assay showing the effects of overexpression of Notch1 and Jagged1 *in vivo*. (B) Western blot assay showing the effects of knock-down of Notch1 and Jagged1 expression *in vivo*. (C) Immunofluorescence assay showing the fluorescence intensity of CD31 labeling in the Notch signaling pathway activation and inhibition groups. The fluorescence intensity of CD31 is presented as a ratio to the control group. Data are expressed as the mean \pm SD ($n = 5$ in each group). Differences between groups were compared using one-way analysis of variance and nonparametric Mann-Whitney U test. $\dagger P < 0.05$, vs. vector group. (D) Effects of Notch signaling pathway activation and inhibition in rats with traumatic brain injury (D1–D5, $\times 40$; D6–D10, $\times 100$). D1–D5: Arrows indicate CD31 expression. Notch1 OE: Notch1 overexpression group; Jagged1 OE: Jagged1 overexpression group; JAG1: Ligand for multiple Notch receptors and involved in the mediation of Notch signaling; siNotch1: Notch1 knockdown group; siJagged1: Jagged1 knockdown group; DAPT: γ -secretase inhibitor IX.

in EPCs remain unclear. Further investigation is required to clarify whether activation of the Notch signaling pathway can improve neurovascular repair and functional recovery after brain injury.

In summary, in this study, we found that overexpression of Notch1 or Jagged1 activates the Notch signaling pathway, enhances EPC migration and invasiveness, and promotes angiogenesis. Further studies are required to elucidate the molecular mechanisms and effectors mediating these effects of Notch1/Jagged1.

Author contributions: YHY provided the data and ensured the integrity of the data. QSR conceived and designed the study, wrote the paper, was in charge of manuscript authorization, and obtained the funding. XHF participated in statistical analysis and data analysis. YCW provided technical and data

support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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