Sonic hedgehog secreted by neurons regulates angiopoietin expression in neighboring fibroblasts

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Abstract. Lately, the importance of the communication between different cell types and the understanding of the cell communication pathway has been emphasized, as it may provide a novel therapeutic strategy for the regeneration of damaged tissue. In the present study, we suggest that sonic hedgehog (Shh) is a mediator of cell communication between neurons and fibroblasts. Recombinant Shh (rShh) affected the expression of the angiogenic factors, angiopoietin (Ang)-1 and Ang-2, in fibroblasts, but not in neurons or neural progenitor cells (NPCs). The expression of the Shh downstream transcription factor, Gli1, was markedly increased in neurons and NPCs, indicating that neurons and NPCs responded to rShh. However, rShh did not affect Ang-1 and Ang-2 expression in neurons and NPCs. It should be noted that Shh was strongly expressed in neurons, but that Shh expression was undetectable in fibroblasts. We performed a co-culture assay using neurons and fibroblasts to investigate whether the expression of Ang-1 and Ang-2 is regulated by cell communication, without rShh treatment. Ang-1 expression in fibroblasts was markedly upregulated by co-culture with neurons, whereas Ang-2 expression was decreased by co-culture with neurons. Moreover, when an Shh-neutralizing antibody was added, this effect was diminished. Collectively, our data suggest that Shh-expressing neurons regulate angiopoietin expression in neighboring fibroblasts in a paracrine manner.

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Introduction

Sonic hedgehog (Shh) is a secretory glycoprotein and acts as an autocrine and paracrine factor (1). Shh binds to its receptor, patched 1 (Ptch1). In conditions in which Shh is inactivated, Ptch1 inhibits the action of the receptor, Smoothened. When Shh binds to Ptch1, this suppression of Smoothened is reversed, which in turn activates the transcription factor, Gli1 (1,2). Gli1 upregulates several downstream signaling genes, including Ptch1 and Gli1 (3,4). Shh plays an important role in the developmental process, which includes the regulation of axonal guidance, cell differentiation and the proliferation of neural progenitor cells (NPCs) (5-7). In adults, Shh, is involved in hypoxia-induced neural progenitor proliferation in the brain injured by stroke (8), controls stem cell maintenance in postnatal and adult brain neurogenesis (9), regulates angiogenesis and vasculogenesis (10,11) and strengthens vascular tightness (12).

Angiopoietin (Ang)-1 and Ang-2 belong to the angiopoietin family, and play an important role in blood vessel formation both in normal development and pathological conditions (13,14). The balance between Ang-1 and Ang-2 is important for the maintenance of the vasculature network (15). Ang-1 upregulates vascular integrity by modulating tight junction proteins (16,17), enhances endothelial regeneration in diabetic mice (18) and recovers ischemic limb injury through the recruitment of bone marrow-derived progenitor cells (19). In addition to the role of Ang-1 and Ang-2 in angiogenesis, previous studies have shown that they have a broad target cell spectrum. Ang-1 blocks cell death and improves the survival of fibroblasts, skeletal myocytes and cardiomyocytes (20-22). It also induces neurite outgrowth (23), as well as the proliferation and differentiation of NPCs (24). Ang-2 also regulates NPC differentiation and migration (25). These reports indicate the importance of angiopoietin as a broad spectrum regulatory factor.

We have previously reported that Shh affects Ang-1 and Ang-2 mRNA expression only in fibroblasts (26). In the present study, we demonstrate that Shh is produced by neurons, and that the mRNA expression of Ang-1 and Ang-2 in fibroblasts is

modulated by co-culture with neurons, without exogenous Shh treatment. Moreover, Shh-neutralizing antibody significantly blocked the regulation of Ang-1 and Ang-2 in fibroblasts which was induced by co-culture with neurons. Our data suggest that fibroblasts and neurons communicate with each other through Shh signaling. Thus, we propose the concept of fibroblast/ neuron cross-talk.

Materials and methods

Animals, cell isolation and culture of neural progenitors and neurons. All the animal experiments were performed under the approval of the Institutional Animal Care and Use Subcommittee of Inha University Hospital (Incheon, Korea). For the culture of fresh NPCs, the cortices of fetuses obtained from CD1 mice [embryonic day (E)14-16] were isolated, minced and incubated in a PBS solution with 0.25% trypsin and 0.01% DNase I for 20 min as previously described (26). The cells were resuspended in DMEM/F12 medium (Gibco/ Invitrogen, Carlsbad, CA, USA) containing 1% FBS, N2 supplement (Gibco/Invitrogen), 0.6% glucose, HEPES (50 mmol/l), bFGF (20 ng/ml), EGF (20 ng/ml), heparin (5 μg/ml) and penicillin/streptomycin/amphotericin B (1:100 dilution; Gibco/ Invitrogen). The cells were plated at $2x10^5$ cells/well into 24-well plates coated with poly-D-lysine and laminin (PDL/L plates; Biocoat Inc., Horsham, PA, USA; BD Biosciences, Franklin Lakes, NJ, USA). After 24 h, 1% FBS was removed. For the neuron cultures, mouse cortices (E15-17) were harvested as described in our previous study (26). The cells were seeded at $3x10^5$ cells/well into PDL/L plates (Biocoat Inc.) and cultured in defined neuron culture medium; neurobasal medium (Gibco, Invitrogen) containing 2% B27, glutamine (2 mmol/l) and 1% penicillin/streptomycin. On days 1-3, glutamate (25 μg/ml) and β-mercaptoethanol (10 μmol/l) were added. On day 3, Ara-C (10 μmol/l) was added for 24 h, and the medium was then exchanged with fresh defined neuron culture medium.

Cell line and reagents. NIH3T3 mouse embryonic fibroblasts were purchased [American Type Culture Collection (ATCC), Manassas, VA, USA] and cultured in DMEM containing 10% FBS (Gibco/Invitrogen) and 1% penicillin/streptomycin. Mouse recombinant Shh (mrShh) was purchased from GenScript (Piscataway, NJ, USA; Cat. no. Z03050) and the cells were incubated with 10 or 50 nM rShh for 16 h. MAB4641, a neutralizing antibody against Shh [10 μg/m; R&D Systems (Minneapolis, MN, USA)] was used for the specific blocking of Shh in the culture medium.

Quantitative (real-time) reverse transcription PCR (RT-qPCR). Total RNA was extracted using the QIAshredder and the RNeasy Mini kit (Qiagen, Hilden, Germany). Total RNA (1-2 μg) was converted into cDNA using the PrimeScript™ 1st strand cDNA Synthesis kit (Takara Bio, Inc., Shiga, Japan). For real-time PCR assay, mouse Ang-1, Ang-2, Ptch1, Shh and Gli1 were analyzed with an ABI TaqMan Gene Expression Assay primer and FAM probe sets (Applied Biosystems, Foster City, CA, USA). Transcript levels were normalized to the 18S rRNA. Real-time samples were run on an ABI PRISM-7500 sequence detection system (Applied Biosystems). In addition, end-point PCR for Ang-1 and Ang-2 was performed using following primers and GAPDH was used

for normalization: Ang-1 forward, 5'-aaacagcaaatgggaacagg-3' and reverse, 5'-gggcaggtgaactccactaa-3' (melting temperature, 60˚C; 35 cycles); Ang-2 forward, 5'-caaggcactgagagacacca-3' and reverse, 5'-ctgaactcccacggaacatt-3' (melting temperature, 60˚C; 35 cycles); and GAPDH forward, 5'-ccactggcgtcttcaccac-3' and reverse, 5'-cctgcttcaccaccttcttg-3' (melting temperature, 60˚C; 27 cycles).

Western blot analysis. The cells were harvested and lysed in lysis buffer (40 mM Tris pH 7.4, 10 mM EDTA, 120 mM NaCl, 0.1% NP-40) containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Total protein (10-30 μg) was fractionated in SDS-PAGE and immunoblotted with specific antibodies against Ang-1 (Novus Biologicals, Littleton, CO, USA) and Ang-2 (Novus Biologicals). Actin (Calbiochem, La Jolla, CA, USA) was used as an internal control. We used the Kodak Image Station 400R to detect the bands and Kodak Molecular Imaging Software version 4.0. The quantification of band intensity was analysed using TINA 2.0 (Raytest, Straubenhardt, Germany) and normalized to the intensity of actin.

Co-culture assay. Neurons and NIH3T3 fibroblasts were co-cultured as previously described with certain modifications (16). Fibroblasts were seeded at the bottom of a 12-well plate. Neurons were plated into a Transwell chamber at $2x10^5$ cells/well (pore size, 0.4 μm; Corning Life Science, Tewksbury, MA, USA). The Transwell chamber with the confluent neurons was then placed in the 12-well plate and was further incubated for 16 h in the defined neuron culture medium. During co-culture, a neutralizing antibody against Shh (MAB4641; 10 μg/ml; R&D Systems) was added to the bottom well. Total RNA was extracted from the fibroblasts to evaluate the effects of Shh secreted from the neurons.

Statistical analysis. All the results are expressed as the means ± standard deviation. The differences between the groups were compared by an unpaired t-test or one-way analysis of variance (ANOVA). P-values ≤0.05 were considered to indicate statistically significant differences. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA).

Results

rShh regulates the expression of Ang-1 and Ang-2 in fibroblasts, but not in neurons and NPCs. We determined the expression of Ang-1 and Ang-2 following treatment with recombinant sonic hedgehog (rShh) (Fig. 1A). Ang-1 mRNA expression was increased in fibroblasts only, whereas Ang-2 mRNA expression was decreased by rShh treatment. Ang-1 and Ang-2 mRNA expression showed no change in either the neurons or the NPCs. Gli1 mRNA levels significantly increased following treatment with rShh in all cell types, indicating that neurons and NPCs responded to rShh normally; however, rShh did not affect Ang-1 and Ang-2 mRNA expression in neurons and NPCs (Fig. 1A), which is in accordance with the results of our previous study (26). To confirm the effects of rShh on angiopoietin at the protein level we performed western blot anlaysis (Fig. 1B). Ang-1 protein expression showed a marked
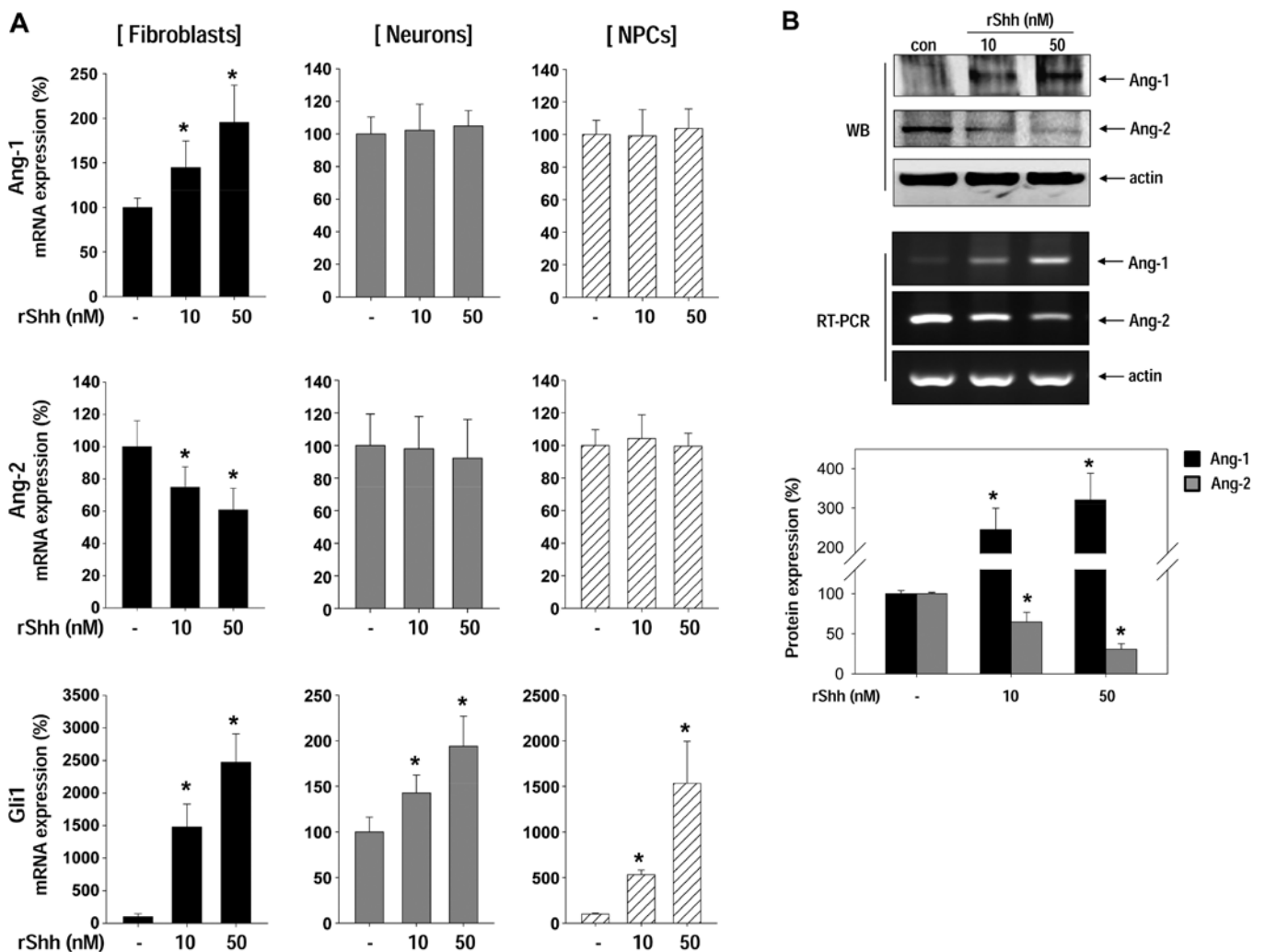
Figure 1. The expression of angiopoietin (Ang)-1 and Ang-2 is regulated by recombinant Shh (rShh) only in fibroblasts. (A) Fibroblasts, neurons and neural progenitor cells (NPCs) were incubated with 10 or 50 nM rShh for 16 h, and RT-qPCR was performed. Ang-1 mRNA expression was increased, whereas Ang-2 mRNA expression was decreased following treatment with rShh only in fibroblasts (n=5). Ang-1 and Ang-2 mRNA expression showed no change in either the neurons (n=4) or the NPCs (n=3), even though Gli1 mRNA expression was upregulated following treatment with rShh (*p<0.001). (B) The expression of Ang-1 and Ang-2 in fibroblasts was confirmed by western blot analysis (WB; top panel, n=3) and RT-qPCR (middle panel, n=3). Quantitative graphs for western blot analysis (bottom panel, n=3; *p<0.05). Con, control (untreated cells).

increase in a dose-dependent manner, while Ang-2 expression decreased in a dose-dependent manner.

Shh-expressing cells and Shh-responsive cells differ. We then investigated Shh mRNA expression in 3 types of cells, fibroblasts, neurons and NPCs, in order to determine Shh basal levels (Fig. 2A). Shh was not expressed in fibroblasts, but it was expressed in both neurons and NPCs. Of note, Shh expression levels in the neurons were much higher than those in the NPCs (Fig. 2A). It is known that Shh acts in an autocrine and paracrine manner (1). Shh binds to its receptor, Ptch1, and activates its signaling with the upregulation of Ptch1 and Gli1 expression (3). Thus, we examined the mRNA expression of Ptch1 and Gli1 to clarify which cells are influenced by rShh (Fig. 2B and C). We hypothesized that rShh mainly affects neurons even though all 3 types of cells were affected by rShh, as endogenous Shh was highly synthesized in neurons. It should be noted however, that Shh downstream signaling, Ptch1 and Gli1, was upregulated in fibroblasts, but not in neurons (Fig. 2B

and C). These data indicate that although neurons are one of the major cell sources for Shh expression, fibroblasts are the most responsive cells to Shh. Therefore, we suggest that Shh-expressing cells and Shh-responsive cells differ, and we suggest the existence of a cell cross-talk in Shh-mediated angiopoietin regulation.

Shh secreted by neurons regulates Ang-1 and Ang-2 gene expression in fibroblasts in a co-culture system. As shown in Fig. 2, we found that Shh expression was markedly increased in neurons, whereas Shh mainly affects fibroblasts as opposed to neurons. We suggested the existence of a cross-talk between neurons and fibroblasts. To confirm our hypothesis, we performed co-culture assay (Fig. 3). Fibroblasts were seeded at the bottom well of 12-well plate, and then a Transwell chamber with confluent neurons was placed in the 12-well plate (Fig. 3A). Following further incubation, total RNA was extracted from the fibroblasts and the Ang-1 and Ang-2 mRNA expression was determined (Fig. 3B-D). It is of interest to note that Ang-1
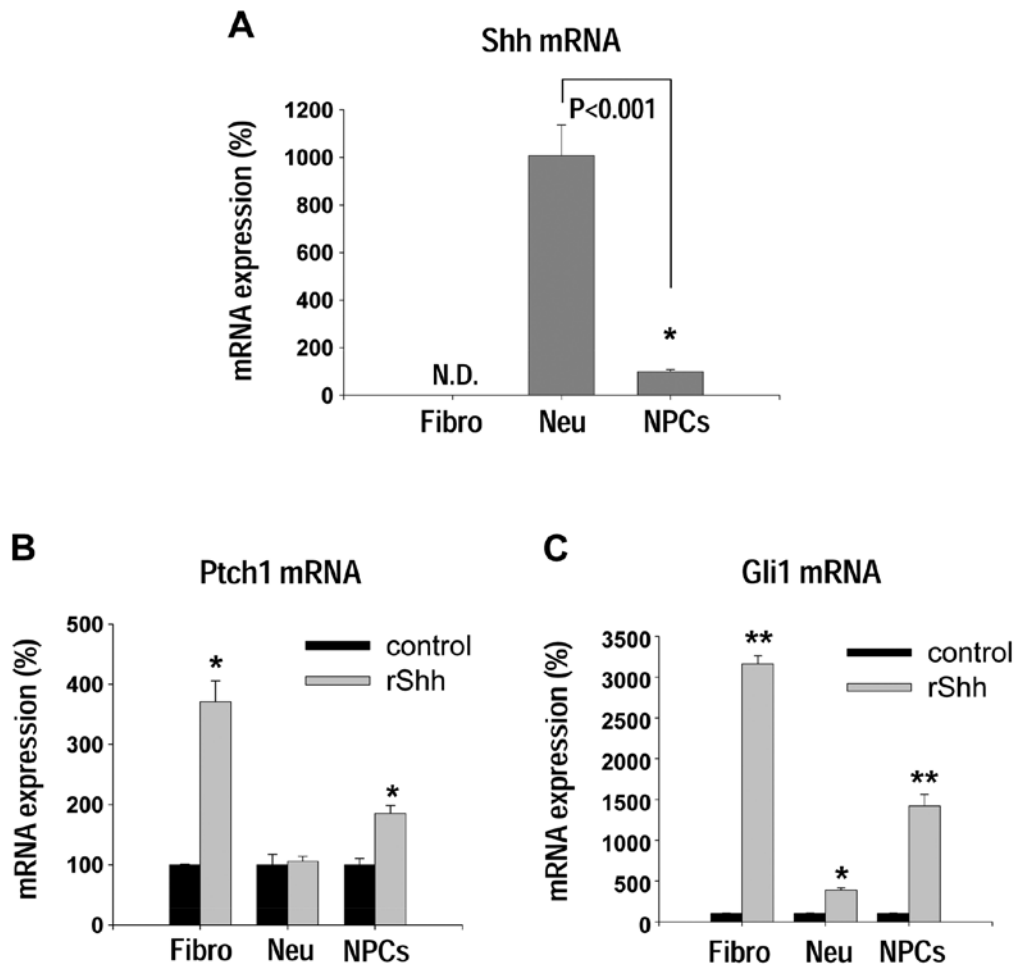
Figure 2. Sonic hedgehog (Shh) expression and the regulation of the Shh downstream targets, patched 1 (Ptch1) and Gli1, in 3 types of cells. (A) RT-qPCR for the determination of Shh mRNA expression revealed a high expression in neurons (Neu) (n=7). (B and C) Fibroblasts (Fibro), neurons and neural progenitor cells (NPCs) were treated with 50 nM recombinant Shh (rShh) for 16 h. The expresson of Ptch1 and Gli1, the downstream targets of Shh, was markedly increased following treatment with rShh in fibroblasts (n=3 each) (*p<0.05, **p<0.005).

expression was markedly increased only when the neuron co-culture system was used (Fig. 3B). Moreover, the co-culture-induced Ang-1 upregulation was markedly inhibited by the addition of a Shh-neutralizing antibody (MAB4641; Fig. 3B). On the other hand, Ang-2 mRNA expression in the fibroblasts decreased in the co-culture group; however, this reduction in Ang-2 expression following co-culture was reversed by the addition of Shh-neutralizing antibody (Fig. 3C). Our results suggest that Shh released from neurons, which may influence neighboring fibroblasts, thus regulating Ang-1 and Ang-2 expression (Fig. 3E).

Discussion

In this study, we found that neurons and fibroblasts communicate with each other and that the main source of Shh expression is from neurons, not fibroblasts or NPCs. In addition, the regulation of angiopoietin expression in fibroblasts was affected by neurons in the co-culture system, suggesting the existence of a cross-talk between neurons and fibroblasts. Lately, the importance of gaining an understanding of the communication pathway between different cell types has been emphasized in the research field of drug development. In the research area

of neurovascular and oligovascular units, there is increasing evidence that there is a communication between blood vessels and brain cells (27,28). Cerebral endothelial cells may secrete trophic factors that affect neighboring cells (27,28). Likewise, astrocytes/endothelial coupling regulate the development of the cerebral vasculature, i.e., the blood-brain barrier, through Ang-1 (16). Nerves serve as a template for arteries and determine the arterial differentiation through the vascular endothelial growth factor (29,30). Osteoblast-secreted Ang-1 promotes the survival and maintenance of hematopoietic stem cells (31). Endothelial cells stimulate fibroblast differentiation following ischemic injury and therefore increase tissue fibrosis following myocardial infarction (32). These studies have shown that the complex interplay between types of cells is extensively regulated in normal physiology.

As this cell cross-talk between cells may involve common growth factors and may be regulated in an interconnected manner, molecules, such as Shh hold great therapeutic potential. Shh upregulates vascularization and osteoblastic differentiation (33), and can also regulate both angiogenesis and myogenesis (34). Ang-1 decreases both vascular damage and cardiomyocyte death, thus improving cardiac function following myocardial infarction (17). Ang-1 also stimulates
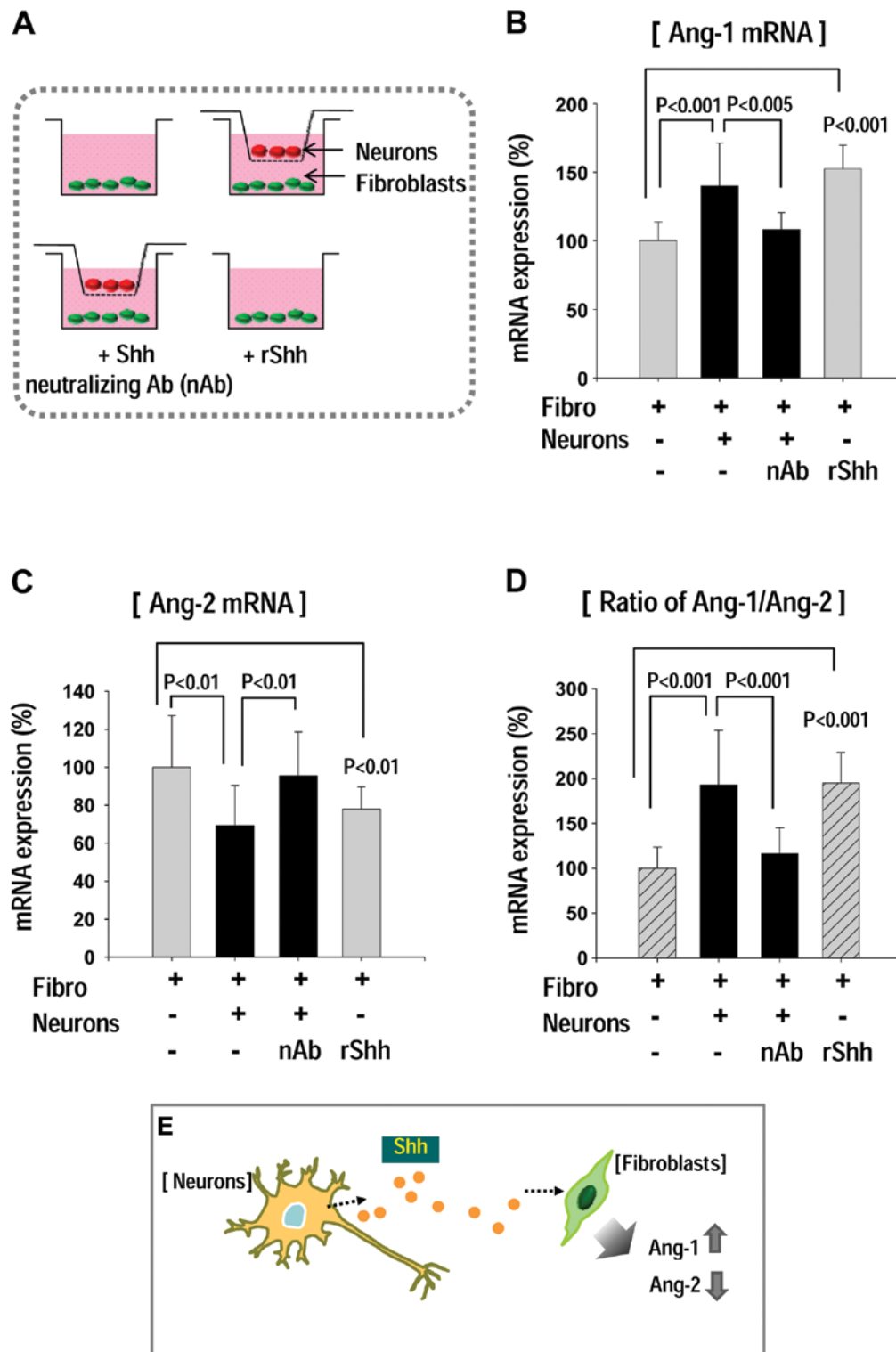
Figure 3. Neurons regulate angiopoietin (Ang)-1 and Ang-2 gene expression in fibroblasts through sonic hedgehog (Shh) secretion in a co-culture system. (A) Schematic illustration of co-culture between fibroblasts and neurons. Fibroblasts were seeded at the bottom of a 12-well plate. A Transwell chamber with confluent neurons was then placed in the 12-well plate and was further incubated. Total RNA was extracted from the fibroblasts and performed RT-qPCR was performed. (B and C) Ang-1 mRNA expression was increased by co-culture with neurons, whereas Ang-2 mRNA expression was decreased (n=4). Neutralizing antibody (nAb) against Shh (MAB4641, 10 μg/ml) was added to the bottom well medium during co-culture, which blocked the effect of neurons on the expression of Ang-1 and Ang-2 mRNA in fibroblasts. Treatment of fibroblasts with recombinant Shh (rShh) protein (50 nM) was performed as a positive control. (D) The ratio of Ang-1 to Ang-2 expression was calculated (n=4). (E) Schematic diagram of cell cross-talk between fibroblasts and neurons in angiopoietin gene regulation. Shh was markedly in neurons. Shh was expressed and released from neurons, thus influencing neighboring fibroblasts to inversely regulate Ang-1 and Ang-2 expression through paracrine mechanisms. Fibro, fibroblasts.

muscle regeneration after muscle injury in mice by promoting muscle satellite cell self-renewal (35).

Moreover, previous studies have suggested that the combined transplantation of different cell types improves

therapeutic potential. Vascular regeneration in hindlimb ischemia was shown to be enhanced by the transplantion of a combination of embryonic stem cell-derived endothelial and mural cells, compared with single-cell transplantation (36). The combination of Shh gene transfer and bone marrow-derived progenitor cells has also been shown to improve angiogenesis and muscle regeneration in limb ischemia to a greater degree than with each treatment individually (37). Therefore, an improved understanding of cell communication and the functions of regulatory cytokines, such as Shh may provide a novel strategy for the regeneration of damaged tissue.

Acknowledgements

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