

# Transforming growth factor- $\beta$ 1 induces fibrosis in rat meningeal mesothelial cells via the p38 signaling pathway

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**Abstract.** Leptomeningeal fibrosis is important in the pathogenesis of communicating hydrocephalus following subarachnoid hemorrhage; however, the underlying mechanisms of leptomeningeal fibrosis remain largely unclear. In the present study, primary meningeal mesothelial cells (MMCs) were used as a cell model to investigate the effect of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) on leptomeningeal fibrosis. Firstly, primary MMCs were isolated from rat brains and characterized by immunofluorescence, staining positive for keratin and vimentin, but negative for factor VIII. Upon TGF- $\beta$ 1 treatment, MMCs were induced to express connective tissue growth factor (CTGF), an indicator of fibrosis, in a dose-dependent manner. Furthermore, p38 mitogen-activated protein kinase (MAPK) signaling was significantly activated by TGF- $\beta$ 1. However, in the presence of a p38 MAPK inhibitor, TGF- $\beta$ 1-induced CTGF expression was markedly suppressed. Together, these data suggest that TGF- $\beta$ 1 could induce fibrosis of MMCs via the p38 MAPK signaling pathway, providing a novel potential target for intervention in TGF- $\beta$ 1-induced leptomeningeal fibrosis.

## Introduction

The meninges, the membranes located between the skull and brain, consist of three layers: Dura mater, arachnoid mater and pia mater. The latter two layers are often collectively

termed leptomeninges, as they have a similar embryonic origin and are closely associated (1). It has been demonstrated that leptomeningeal fibrosis is important in the pathogenesis of communicating hydrocephalus following subarachnoid hemorrhage (2,3). The predominant pathological features of leptomeningeal fibrosis include highly increased proliferation of leptomeningeal mesothelial cells and the accumulation of extracellular matrix (ECM), however the specific mechanisms are not clear (4-7). A previous study regarding meningeal fibrosis primarily focused on imaging and histology (7). However, investigations of its pathogenesis at the molecular level are lacking. Establishing an *in vitro* culture system of meningeal mesothelial cells (MMCs) could provide an improved experimental model for mechanistic studies of meningeal fibrosis.

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a critical fibrosis-inducing cytokine. It promotes cell proliferation, and stimulates overexpression and deposition of ECM proteins, such as fibronectin and collagen (8). The occurrence of many fibrotic diseases, including pulmonary fibrosis and renal fibrosis, has been demonstrated to be associated with the overexpression of TGF (9). Connective tissue growth factor (CTGF), an important downstream factor, could mediate the pro-fibrotic effects of TGF- $\beta$ 1, by promoting mitosis and fibroblast proliferation, inducing collagen synthesis, and mediating cell adhesion and chemotaxis (10). A previous study indicated that TGF- $\beta$ 1 is an important factor for the induction of meningeal fibrosis (11). TGF- $\beta$ 1 promotes fibrosis by inducing mRNA and protein expression of CTGF in MMCs, suggesting the possibility of blocking TGF- $\beta$ 1 signaling pathway to delay the progression of meningeal fibrosis. However, TGF- $\beta$ 1 exerts many important physiological effects, including immune modulation and the anti-inflammatory response, suggesting that blocking TGF- $\beta$ 1 function in a direct manner would be clinically unfeasible. Therefore, the identification of novel downstream targets of TGF- $\beta$ 1 is required to specifically inhibit the pro-fibrotic effect of TGF- $\beta$ 1. Previous studies have revealed that the p38 signaling pathway mediates TGF- $\beta$ 1-induced fibrosis in various types of cells, such as lung fibroblasts, mesangial cells and renal fibroblasts (12). However, whether the p38 signaling pathway is involved in TGF- $\beta$ 1-induced meningeal fibrosis remains unclear.

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The present study aimed to establish a cellular model of TGF- $\beta$ 1-induced meningeal fibrosis with primarily cultured MMCs. The specific role of the p38 mitogen-activated protein kinase (MAPK) signaling pathway in TGF- $\beta$ 1-induced meningeal fibrosis of mesothelial cells was investigated to identify novel therapeutic targets for the prevention and treatment of communicating hydrocephalus.

## Materials and methods

**Materials.** High-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). The following rabbit anti-mouse IgG antibodies were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China): Keratin (cat. no. BA2266-1), vimentin (cat. no. BS-0756R) and factor VIII (cat. no. BS-0434R). TGF- $\beta$ 1 was obtained from R&D Systems China Co., Ltd. (Shanghai, China). TRIzol reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Rabbit anti-mouse CTGF antibody was obtained from Abcam (Shanghai, China). Rabbit anti-mouse p38 MAPK antibody, rabbit anti-mouse phosphorylated-p38 MAPK antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were all purchased from Cell Signaling Technologies (Shanghai, China).

## Methods

**Cell culture and characterization.** Ten Sprague Dawley rats, aged 3-5 days, were obtained from the Laboratory Animal Center of Xinxiang Medical University (Xinxiang, China). The rats were immediately sacrificed by overdose of pentobarbital sodium (200 mg/kg i.p.; Sangon Biotech Co., Ltd., Shanghai, China) and whole brains were removed under sterile conditions, and washed with pre-cooled phosphate-buffered saline (PBS) (13). Leptomeninges were carefully peeled off in ice-cold DMEM (with 10% FCS) using ophthalmic tweezers and blood vessels were removed. Tissues were minced and triturated three times, and allowed to settle by gravity for 5 min. The supernatant was discarded and 5 ml complete medium (DMEM with 10% FCS, 100 U/ml penicillin and 100 U/ml streptomycin) was added. The tissues were pipetted up and down repeatedly before being transferred to 6-well plates and cultured in an incubator at 37°C with 95% humidity. After 24 h, one-half of the culture medium was replaced with fresh medium and impurities were removed by pipetting. Three days later, tissue fragments were aspirated and discarded. The culture medium was subsequently refreshed every three days. Cells were passaged routinely at 80% confluency. After three passages, the expression levels of markers of MMCs were investigated using immunofluorescence staining. Briefly, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with Triton X-100/PBS for 30 min. After two washes with PBS, cells were blocked with 4% bovine serum albumin (BSA; Sangon Biotech Co., Ltd.) for 1 h, followed by incubation with primary antibodies (keratin, vimentin and factor VIII) diluted 1:100 in Triton X-100/PBS/1% BSA at 4°C overnight. Following removal of the primary antibodies, cells were washed with PBS twice and the secondary

antibody, Invitrogen Alexa Fluor 488 goat anti-rabbit (cat. no. A11008; Thermo Fisher Scientific, Inc) with Triton X-100/PBS (dilution, 1:100) was added and incubated for 3 h at room temperature. For imaging, an Olympus IX71 (Nikon Corporation, Kanagawa, Japan) fluorescent microscope was used.

**Cell processing and testing.** MMCs of passage 3-8 were plated in a 60-ml flask at a density of  $1 \times 10^5$ /ml. At 70-80% confluency, the cells were treated with different concentrations of TGF- $\beta$ 1 (0, 1, 2 and 4 ng/ml), or pretreated with SB203580 (0, 1, 5 and 10  $\mu$ M) for 1 h followed by treatment with 2 ng/ml TGF- $\beta$ 1. Treated cells were cultured in an incubator at 37°C with 5% CO<sub>2</sub> and 95% humidity for a further 48 h before being harvested for total RNA and protein extractions.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions, and reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). mRNA levels were measured using an ABI Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Green qPCR Master mix (Kapa Biosystems, Inc., Wilmington, MA, USA). Gene-specific primers used for qPCR were as follows: Forward, 5'-TTG CCA AGC CTG TCA AGT TTG-3' and reverse, 5'-AAT GGC AGG CAC AGG TCT TG-3' for CTGF; forward, 5'-GGT CGG TGT GAA CGG ATT TG-3' and reverse, 5'-GCT TCC CAT TCT CAG CCT TGA-3' for GAPDH. The target mRNA level of control cells normalized to the level of GAPDH mRNA was set at 1 (14).

**Western blotting.** Cells were washed with PBS, and protein was extracted with lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and Roche's complete protease inhibitors (Roche Diagnostics, Shanghai, China)] and centrifuged at 15,000  $\times$  g for 15 min at 4°C (15). The protein concentration of the supernatants was determined using a Protein Assay kit II (Bio-Rad, Hercules, CA, USA). For western blotting, samples were separated by electrophoresis (150 V for 1.5 h), on a 12-15% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes. After blocking with 0.1% Tween-20 in PBS containing 5% skimmed milk, the membranes were incubated with the above-mentioned primary antibodies, keratin, vimentin and factor VIII. They were further incubated with a HRP-conjugated donkey anti-rabbit IgG (1:1,000; cat. no. D110056; Sangon Biotech Co., Ltd.) for 1 h. After three washes, the membranes were developed using chemiluminescence substrate. Immunoblot signals were quantified by measuring the immunoreactive protein band density with ImageJ 1.48 software (National Institutes of Health, Bethesda, MA, USA). For all immunoblot assays,  $\beta$ -actin served as a loading control.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard error of the mean. The group means were compared by two-way analysis of variance, and the significance of differences was determined by post hoc testing using Bonferroni's method.  $P < 0.05$  was considered to indicate a statistically significant difference.

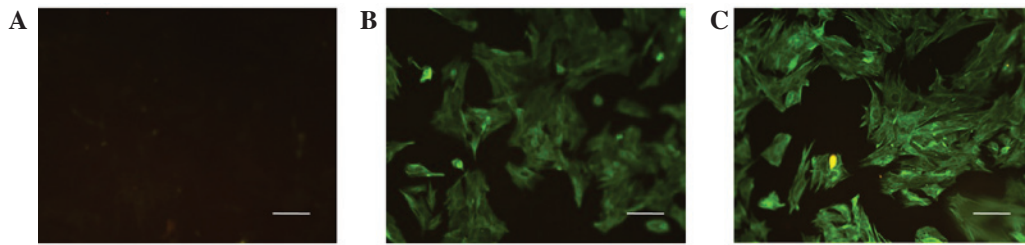


Figure 1. Identification of primary rat MMCs. Cells were gradually expanded and exponentially growing cells exhibited a reticular-like growth pattern, while cell cultures displayed a typical 'cobblestone' pattern upon reaching confluence. After three passages, primary rat MMCs were characterized by immunofluorescence with antibodies against (A) factor VIII, (B) vimentin and (C) keratin (scale bar, 20  $\mu$ m). Factor VIII served as a negative marker of MMCs, whereas keratin and vimentin staining was indicative of MMCs. MMCs, meningeal mesothelial cells.

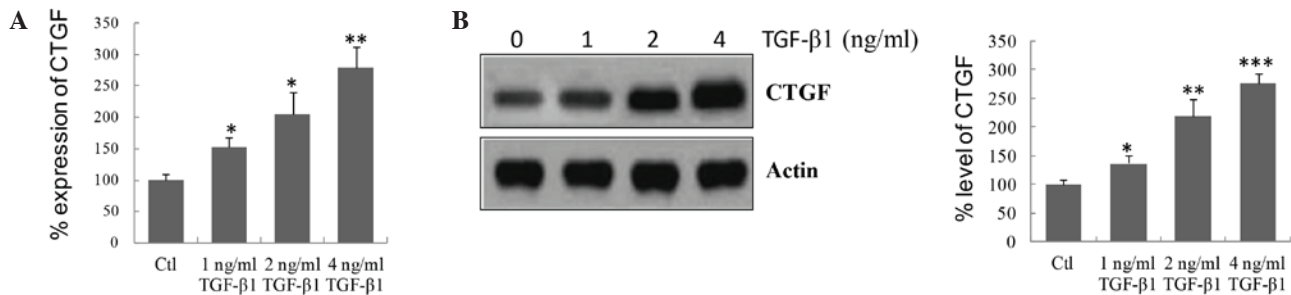


Figure 2. Fibrosis induction in MMCs by TGF- $\beta$ 1 *in vitro*. MMCs were treated with TGF- $\beta$ 1 at various concentrations for 2 days. (A) Quantitative polymerase chain reaction was conducted to determine the induction of CTGF mRNA. (B) Western blotting was performed to examine the induction of CTGF protein (upper panel), and the levels of CTGF protein are expressed as a percentage of the level measured in control cells (lower panel). \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. control. MMCs, meningeal mesothelial cells; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; CTGF, connective tissue growth factor; Ctl, control.

## Results

**Characterization of primary cultured rat MMCs.** After 24 h incubation of explant cultures, small quantities of spindle-shaped cells were observed. Cells then gradually expanded and exponentially growing cells exhibited a reticular-like growth pattern, while cell cultures displayed a typical 'cobblestone' pattern upon reaching confluence. After three passages, cells exhibited similar morphology to the primarily isolated cells. The cells were positive for keratin and vimentin, but negative for factor VIII following immunofluorescence staining (Fig. 1). Keratin and vimentin are commonly used markers for identifying mesothelial cells (16,17). Keratin and vimentin were predominantly localized in the cytoplasm, which is consistent with the characteristics of mesothelial cells. These results suggested that MMCs had been successfully isolated.

**TGF- $\beta$ 1 induces fibrosis in rat MMCs *in vitro*.** To determine the molecular mechanism of TGF- $\beta$ 1-induced fibrosis in MMCs, it was firstly examined whether TGF- $\beta$ 1 induced fibrosis in MMCs *in vitro*. The dissociated MMCs were treated with different concentrations of TGF- $\beta$ 1 and the effect on the induction of the fibrosis marker, CTGF was investigated. Results of qPCR revealed that a 48-h treatment with TGF- $\beta$ 1 induced the expression of CTGF in a dose-dependent manner ( $P$ =0.031,  $P$ =0.027, and  $P$ =0.042 for 1, 2 and 4 ng/ml TGF- $\beta$ 1, respectively vs. the control; Fig. 2A). Similarly, western blotting demonstrated the induction of CTGF protein by TGF- $\beta$ 1 ( $P$ =0.035,  $P$ =0.0077, and  $P$ =0.00058 for 1, 2 and 4 ng/ml TGF- $\beta$ 1, respectively vs. the control; Fig. 2B). Together, these data indicate that TGF- $\beta$ 1 may induce fibrosis in rat MMCs

*in vitro*, and that primary cultured MMCs may serve as an *in vitro* cellular model for the study of meningeal fibrosis.

**TGF- $\beta$ 1 induces activation of p38 MAPK signaling.** Previous studies have demonstrated that the p38 signaling pathway is closely associated with fibrosis, particularly TGF- $\beta$ 1-induced fibrosis, in many cell types (18,19). However, the association between the p38 signaling pathway and TGF- $\beta$ 1-induced fibrosis of MMCs is unclear. To elucidate this association, it was first investigated whether activation of the p38 signaling pathway was involved in TGF- $\beta$ 1-induced fibrosis. As displayed in Fig. 3, TGF- $\beta$ 1 at all concentrations significantly increased ( $P$ =0.0037,  $P$ =0.0085, and  $P$ =0.019 for 1, 2 and 4 ng/ml TGF- $\beta$ 1, respectively vs. the control) the phosphorylation of p38 in MMCs, indicating that TGF- $\beta$ 1 activates the p38 signaling pathway in MMCs.

**TGF- $\beta$ 1 induces fibrosis in rat MMCs via p38 MAPK signaling.** Although TGF- $\beta$ 1 may induce the activation of p38 signaling in MMCs as described above, whether p38 activation is associated with TGF- $\beta$ 1-induced fibrosis remains unknown. Therefore, the effect of the p38 inhibitor, SB203580 on the TGF- $\beta$ 1-induced expression of CTGF was examined. As displayed in Fig. 4A and B, SB203580 significantly inhibited the activation of p38 ( $P$ =0.0014,  $P$ =0.00039, and  $P$ =0.000086 for 1, 5 and 10  $\mu$ M SB203580, respectively vs. the control). In addition, SB203580 at various concentrations inhibited the induction of CTGF ( $P$ =0.029,  $P$ =0.014, and  $P$ =0.0012 for 1, 5 and 10  $\mu$ M SB203580, respectively vs. the control; Fig. 4C and D). As CTGF is an indicator of cell fibrosis, these data suggest that TGF- $\beta$ 1 induces fibrosis in rat MMCs via p38 MAPK signaling.

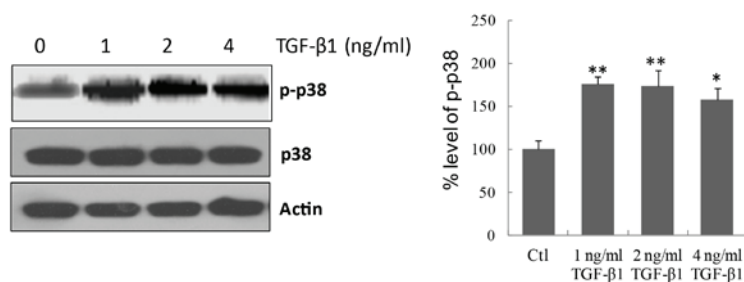


Figure 3. Activation of p38 mitogen-activated protein kinase signaling by TGF- $\beta$ 1. MMCs were treated with TGF- $\beta$ 1 at various concentrations for 30 min. Western blotting was performed to determine the levels of p-p38 (upper panel), and the levels of p-p38 were expressed as a percentage of the level measured in the control cells (lower panel). \* $P < 0.05$  and \*\* $P < 0.01$  vs. control. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; p, phosphorylated; Ctl, control; MMCs, meningeal mesothelial cells.

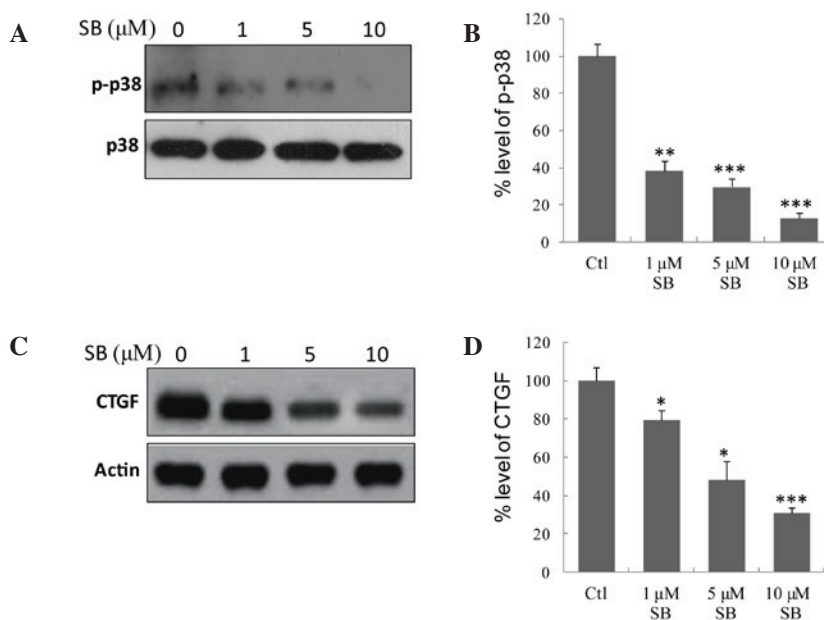


Figure 4. TGF- $\beta$ 1 induces fibrosis in MMCs via p38 mitogen-activated protein kinase signaling. MMCs were pre-treated with the p38 inhibitor SB at various concentrations, followed by incubation with TGF- $\beta$ 1 at 2 ng/ml. The levels of (A and B) p-p38 and (C and D) CTGF were determined by western blotting and expressed as a percentage of the levels measured in the control cells. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control. SB, SB203580; p, phosphorylated; CTGF, connective tissue growth factor; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; Ctl, control; MMCs, meningeal mesothelial cells.

## Discussion

Communicating hydrocephalus is usually secondary to subarachnoid hemorrhage, meningitis and traumatic brain injury, amongst other neurological disorders, as no effective therapeutic strategy for these conditions is available. Currently, it is proposed that the imbalance of cerebrospinal fluid secretion and absorption caused by fibrous adhesion of leptomeninges is the pathological basis of chronic hydrocephalus (7); however, the specific mechanisms remain unclear. Studies have indicated that tissue fibrosis is crucial in the imbalance between ECM protein synthesis and degradation, leading to the excessive deposition of ECM (20,21). This complex pathophysiological process involves various cytokines (22), among which TGF- $\beta$ 1 and its downstream effector, CTGF are possibly the most critical fibrosis-inducing factors. They have been demonstrated to be important in the induction of fibrosis in various organs and tissues, including the kidney, heart, liver, lung and skin (23,24). Previously,

the role of TGF- $\beta$ 1 in diseases of the central nervous system have begun to be investigated. It is reported that intrathecal injection of recombinant TGF- $\beta$ 1 in transgenic rats resulted in impaired cerebrospinal fluid flow and extensive meningeal adhesion, degeneration and thickening (22,25), indicating an important role of TGF- $\beta$ 1 in the occurrence of hydrocephalus and meningeal fibrosis. However, the underlying mechanisms of TGF- $\beta$ 1-induced meningeal fibrosis are not fully understood.

The present study demonstrated that TGF- $\beta$ 1 induces the expression of CTGF in MMCs in a dose-dependent manner, suggesting that cultured MMCs could be used as a reliable model with which to study meningeal fibrosis *in vitro*. To validate this cell model, the signaling pathway that is responsible for TGF- $\beta$ 1-induced fibrosis in MMCs was analyzed. As p38 signaling is known to be a key pathway mediating TGF- $\beta$ 1-induced fibrosis in cells of other types (12), the present study determined whether the p38 signaling pathway is involved in TGF- $\beta$ 1-induced fibrosis in MMCs. It was



demonstrated that TGF- $\beta$ 1 activates the p38 signaling pathway in MMCs. In addition, the p38-specific inhibitor, SB203580, significantly suppressed the induction of the fibrosis marker CTGF. This suggests that the p38 MAPK pathway is an important signaling pathway through which TGF- $\beta$ 1 induces the expression of CTGF. Thus, it may provide a novel strategy for therapeutic intervention, by blocking the p38 signaling pathway for the treatment of meningeal fibrosis induced by TGF- $\beta$ 1, rather than direct blocking of TGF- $\beta$ 1 function.

Studies have demonstrated that the mechanisms by which TGF- $\beta$ 1 induces the expression of CTGF are cell-specific. For example, Xie *et al* (26) observed that in airway smooth muscle cells, the extracellular signal-regulated kinase and c-Jun N-terminal kinase signaling pathways, but not the p38 signaling pathway, are involved in TGF- $\beta$ 1-induced CTGF expression. By contrast, Chang *et al* (27) demonstrated that p38 is involved in the TGF- $\beta$ 1-induced CTGF expression in buccal mucosa fibroblasts. In liver progenitor cells, p38 was demonstrated to be necessary for TGF- $\beta$ 1-induced CTGF expression and fibrosis (12). However, to the best of our knowledge, the mechanisms by which TGF- $\beta$ 1 act on MMCs have not yet been reported. In conclusion, the current study presents an important axis of the TGF- $\beta$ 1/p38 MAPK/CTGF signaling pathway in meningeal fibrosis, and provides a possible novel strategy for the clinical treatment of communicating hydrocephalus following subarachnoid hemorrhage.

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