# The Role of Matrix Metalloproteinase-3 in the Doxycycline Attenuation of Intracranial Venous Hypertension-Induced Angiogenesis

**Xiang Zou, MD, PhD**[‡*]
**Zehan Wu, MD, PhD**[‡*]
**Jun Huang, PhD**[§¶]
**Peixi Liu, MD, PhD**[‡]
**Xuanfeng Qin, MD, PhD**[‡]
**Liang Chen, MD, PhD**[‡]
**Wei Zhu, MD, PhD**[‡]
**Yao Zhao, MD, PhD**[‡]
**Peiliang Li, MD, PhD**[‡]
**Jianping Song, MD, PhD**[‡]
**Guo-Yuan Yang, MD, PhD**[§]
**Ying Mao, MD, PhD**[‡‖]

[‡]Department of Neurosurgery, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, China; [§]Med-X Research Institute, Shanghai Jiao Tong University, Shanghai, China; [¶]The Shanghai Institute of Hypertension, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [‖]State Key Laboratory of Medical Neurobiology, School of Basic Medical Sciences and Institutes of Brain Science, Fudan University, Shanghai, China

*These authors contributed equally to this work.

**Correspondence:**
Jianping Song, MD, PhD,
Department of Neurosurgery,
Huashan Hospital,
Fudan University,
No. 12 Wulumuqi Zhong Road,
Shanghai 200040, China.
E-mail: neurosurgerysong@foxmail.com

**Received,** September 9, 2017.
**Accepted,** December 22, 2017.
**Published Online,** February 16, 2018.



**BACKGROUND:** The molecular mechanism of brain arteriovenous malformation (BAVM) is largely unknown. Intracranial venous hypertension (VH) may enhance focal angiogenesis and promote BAVM development and progression. A rat VH model effectively simulates the hemodynamic microenvironment of this disease.

**OBJECTIVE:** To explore the effect of doxycycline in VH-related angiogenesis, as well as the role of matrix metalloproteinase-3 (MMP-3) and other molecular factors.

**METHODS:** A rat VH model was generated by common carotid artery and distal external jugular vein anastomosis. Microvessel density (MVD) in the perisinus area and expression of MMP-3/2/9, VEGF, TIMP-1, TGF-$\beta$, and HIF-1$\alpha$ were examined, with and without daily doxycycline treatment for 4 wk. The effects of doxycycline were verified in Vitro using human brain microvascular endothelial cells (HBMECs). MMP-3 overexpression or knockdown in HBMECs was used to confirm the role of MMP-3 in cell functions.

**RESULTS:** MVD in the perisinus cortex was greatly increased after VH. Doxycycline decreased MVD, suppressed MMP-3 overexpression, and reduced VEGF, TGF-$\beta$, and TIMP-1 levels compared with the controls ($P < .05$). In Vitro, doxycycline decreased HBMEC migration, tube formation, and the mRNA, protein, and enzymatic activity levels of MMP-3. MMP-3 overexpression in HBMECs promoted migration, while knockdown of MMP-3 significantly attenuated proliferation, migration, and tube formation ($P < .05$).

**CONCLUSION:** Our findings indicate that MMP-3 plays an important role in VH-related angiogenesis and the promotion of vascular remodeling. Suppression of MMP-3 overexpression by doxycycline may provide a potential strategy for inhibiting BAVM development.

**KEY WORDS:** Angiogenesis, Arteriovenous malformations, Doxycycline, Matrix metalloproteinases, Venous hypertension, Rats

*Neurosurgery 83:1317–1327, 2018* DOI:10.1093/neuros/nyx633 www.neurosurgery-online.com

Brain arteriovenous malformation (BAVM) is relatively infrequent, but is related with neurological impairment and death in young adults.[1,2] Treatment modalities for BAVM include lesion resection, endovascular intervention, and radiotherapy. However, a recent report from the unruptured brain arteriovenous malformations trial (ARUBA) indicated that invasive treatments for unruptured BAVM remained controversial.[3] For some high-risk BAVM with large volume, deep drainage vein, or invasion of eloquent areas, invasive or radiation therapies are ineffective.[3-6] On the other hand, evidence from ARUBA also showed that medical management alone could be superior to interventional treatment in patients with unruptured BAVM for the prevention of stroke and death.[3] Although this trial had shortcomings, it encouraged a search for alternative medical therapies to control existing BAVM progression without invasive treatments.

**ABBREVIATIONS: BAVM,** brain arteriovenous malformation; **DAPI,** 4′,6-diamidino-2-phenylindole; **DAVF,** dural arteriovenous fistula; **EJV,** external jugular vein; **HBMEC,** human brain microvascular endothelial cell; **MMP,** matrix metalloproteinase; **MVD,** microvessel density; **SD,** standard deviation; **VEGF,** vascular endothelial growth factor; **VH,** venous hypertension

Supplemental digital content is available for this article at www.neurosurgery-online.com.

The mechanism of BAVM formation, development, and progress is largely unknown. BAVM was long thought to be primarily a congenital disease, but may also be acquired after certain inciting events or subclinical injury, similar to dural arteriovenous fistula (DAVF).[7,8] The typical pathological feature of BAVM is the nidus, an abnormal vascular mass that directly shunts blood between the arterial and venous circulations. Intracranial venous hypertension (VH) caused by variable degrees of high flow through the feeding arteries, nidus, and draining veins is the most significant hemodynamic alternation in BAVM.[7,9] VH may enhance hypoxia-inducible factor 1$\alpha$ (HIF-1$\alpha$) and vascular endothelial growth factor (VEGF) expression, induce focal angiogenesis, and upregulate other angiogenic activities.[10] The continued excessive activation of angiogenesis and subsequent vascular remodeling is considered to be a potential mechanism of BAVM formation and development.[10-13]

The role of matrix metalloproteinases (MMPs) in VH is not clear. In this study, we explored MMP expression in response to VH using an in Vivo rat VH model. The effectiveness of doxycycline treatment was also examined. We further investigated the effect on vascular remodeling ability in Vitro of knocking down and overexpressing a key MMP identified from the in Vivo experiments.

## METHODS

### Surgical and Intervention Protocols

Animal experimental protocols were approved by the Institutional Review Board of our institution. Studies involving animals were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Twenty-seven rats were used in this study. To exclude potential hormonal effects on angiogenesis, male Sprague-Dawley rats weighing 350 to 400 g were used. All rats were bred and kept in a pathogen-free animal facility.

Rats underwent operation after intraperitoneal injection of 10% chloral hydrate at a dose of 0.2 mL/100 g and with local anesthesia. Standard sterile techniques were used during all procedures. Eighteen rats underwent surgery to induce intracranial VH. First, the proximal segment of the external jugular vein (EJV) and the right anterior facial vein were ligated with 10-0 nylon sutures. Next, the proximal common carotid artery was anastomosed to the distal EJV end-to-end using 11-0 nylon interrupted sutures. The initial portions of the right internal and external carotid arteries were ligated separately. The patency of the bypass and the subsequent morphological alteration of distal EJV were confirmed by 7.0-T magnetic resonance angiography (Bruker Biospec 70/20 USR; Ettlingen, Germany) 2 and 14 d after surgery, respectively. The other 9 rats, comprised by a sham group, underwent a procedure that involved exposure of cervical vessels but no occlusion or anastomosis.

VH rats were randomly assigned to 2 groups according to the intervention protocol. Rats in the doxycycline group (n = 9) and saline control group (n = 9) received an intraperitoneal injection of 1% doxycycline (Wellen Biological Pharmaceutical co LTD, Guangdong, China) at a dose of 2.5 mg/100 g or saline at a dose of 0.25 mL/100 g per day, respectively, starting after surgery. Rats in the sham group received intraperitoneal saline injection at the same dose as the control group. The intervention continued for 4 wk, and all rats were then euthanized. The brains were harvested from 3 rats in each group, fixed in 10% formalin, embedded in paraffin, and sectioned at 8 $\mu$m for immunohistochemical or immunofluorescent staining. For the other 6 rats in each group, the bilateral perisinus cortex was fractionated for western blot analysis. The perisinus cortex was defined as the area 2 mm lateral and 1 mm deep to the sagittal sinus in the coronal view, where a hypoxic microenvironment was mainly induced by the compromised drainage associated with VH.

Human brain microvascular endothelial cells (HBMECs) were used for in Vitro studies. Overexpression or knockdown of MMP-3 in HBMECs was induced using lentiviral vectors. Cell proliferation, migration, and tube formation assays were used to examine cell function. For detailed methods, please see the **Supplemental Methods**, **Supplemental Digital Content 1**.

### Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software Inc, La Jolla, California). Data are presented as means ± standard deviation (SD). Differences between 2 groups were analyzed by independent-sample *t*-tests. One-way analysis of variance was used for multiple comparisons, followed by posthoc Student–Newman–Keuls tests. A $P$ value $< .05$ was considered statistically significant.

## RESULTS

### Mortality in the Animal Model

Mortality among the VH rats was 14.3% (3/21). Three rats died secondary to operator error or surgical failure during VH induction. VH was successfully induced in 18 rats and a sham operation was performed on 9. The patency of the bypass and dilation of the distal EJV and transverse sinus were confirmed by magnetic resonance angiogram in all 18 VH rats (**Figure**, **Supplemental Digital Content 2**).

### Doxycycline Reduced Microvessel Density in the Perisinus Cortex

To verify the protective effect of doxycycline through inhibition of angiogenesis in VH rats, we analyzed microvessel density (MVD) in the perisinus cortex area (Figure 1A). Immunofluorescence staining for rats in the VH + Saline group showed $BrdU^+/CD31^+$ cells in the perisinus cortex, revealing that the microvessels were newly generated (Figure 1A). Furthermore, colabeling showed that MMP-3 was colocalized with CD31 (Figure 1A), indicating a role of MMP-3 in angiogenesis. We found that MVD was remarkably increased in VH rats (7.7 ± 1.4 vs 1.7 ± 0.8/field, $P < .001$) and was significantly decreased after doxycycline intervention compared with the control (4.0 ± 1.4 vs 7.7 ± 1.4/field, $P < .01$; Figure 1B).

### Doxycycline Suppressed MMP-3 Overexpression

MMP-3/2/9 levels in the perisinus cortex were assessed by immunostaining and western blot analysis. Compared with the control, MMP-3 was highly positive in the neurons, glial cells, endothelial cells, and tissue space of the VH rats (Figure 2A). Western blot analysis also demonstrated increased MMP-3 levels in VH rats relative to the controls (1.6 ± 0.2 vs 0.01 ± 0.00, $P < .001$; Figure 2B). After doxycycline treatment, MMP-3 levels

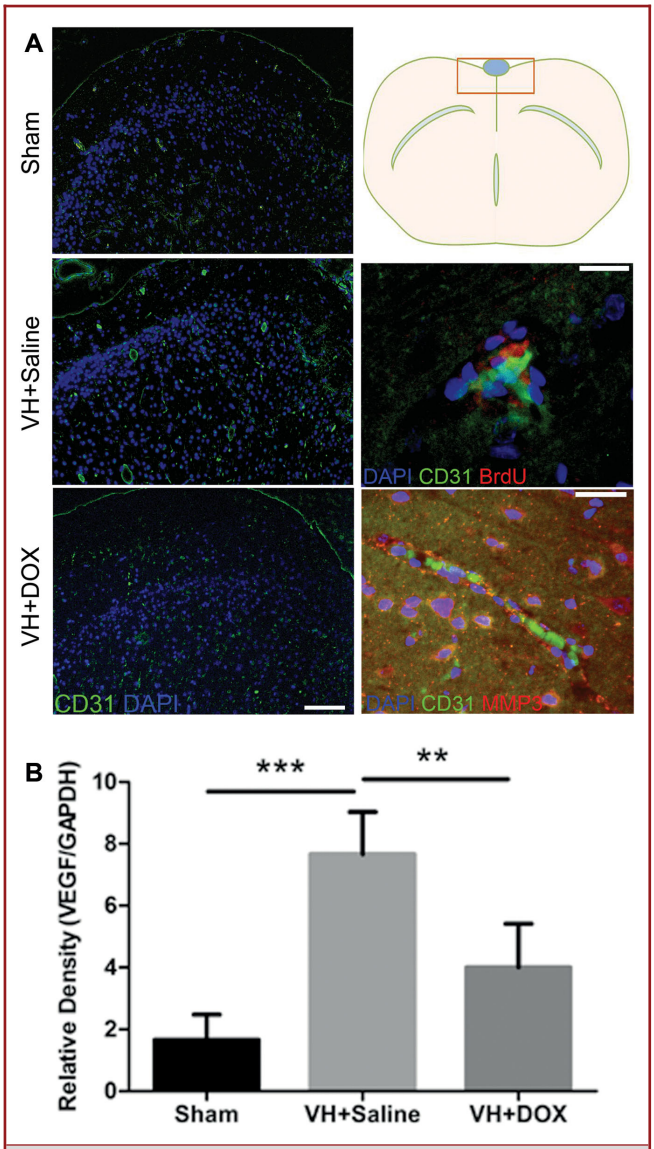


**FIGURE 1.** *MVD in a VH rat model.* **A**, *Left side: photomicrographs showing immunofluorescent staining in the sham, saline-treated VH, and doxycycline-treated VH rats. Green: CD-31, blue: 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China). Scale bar: 100 μm. Right side, top: schematic showing the observation area of the perisinus cortex (red frame) in the brain slice. Right side, middle: immunofluorescent staining in the VH + Saline group. Red: BrdU, green: CD-31, blue: DAPI. Scale bar: 20 μm. Right side, bottom: immunofluorescent staining in the VH + Saline group. Red: MMP-3, green: CD-31, blue: DAPI. Scale bar: 20 μm.* **B**, *MVD in the sham, VH + Saline and VH + Dox groups, n = 6 per group. Data are mean ± SD, **P < .01, VH + DOX vs VH + Saline, ***P < .001, VH + Saline vs Sham.*

were suppressed (0.01 ± 0.01 vs 1.6 ± 0.2, $P < .001$). By immunostaining, MMP-2/9-positive cells were mainly neurons and endothelial cells (Figure 2A). Interestingly, MMP-9 levels were not notably increased in VH rats, and showed no response to doxycycline. MMP-2 levels were elevated in VH rats, but also could not be suppressed by doxycycline.

### Decreased VEGF, TGF-β, and TIMP-1 Expression in the Perisinus Cortex After Doxycycline Treatment

Further immunostaining and western blot analysis were performed to investigate changes in other angiogenic (VEGF, TGF-β) and antiangiogenic factors (TIMP-1) in VH rats before and after doxycycline treatment. As expected, levels of VEGF and TGF-β were elevated in VH rats compared with the controls ($P < .001$ and $P = .003$; Figure 3A), together with levels of MMP-3. VEGF and TGF-β levels were also decreased after doxycycline administration ($P = .012$ and $P = .034$; Figure 3B). Interestingly, TIMP-1 expression was also decreased by doxycycline treatment ($P = .004$). However, doxycycline did not attenuate HIF-1α overexpression in the perisinus cortex (**Figure**, **Supplemental Digital Content 3**).

### Doxycycline Attenuated HBMEC Tube Formation and Migration Together with HIF-1α, MMP-2/3/9, and VEGF expression

A tube formation assay was performed to examine the effect of doxycycline on changes in the endothelial cell network formed by HBMECs. Branch points were significantly suppressed by doxycycline in a hypoxia group compared with vehicle-treated hypoxic HBMECs, after 12 h (34.50 ± 3.222 vs 52.83 ± 2.37, n = 6, $P = .001$; Figure 4A). However, for normoxic HBMECs, doxycycline did not suppress tube formation after 24 h. In a scratch wound-healing assay, hypoxia significantly increased HBMEC migration ability after 36 h compared with normoxia. This effect was suppressed by doxycycline. Moreover, doxycycline inhibited migration compared with vehicle-treated HBMECs, after 48 h culture under either normoxic (76.5 ± 2.36% vs 89.5 ± 2.26%, n = 6, $P = .003$) or hypoxic (67.00 ± 2.81% vs 88.17 ± 1.74%, n = 6, $P < .001$; Figure 4B) conditions. However, migration under normoxic and hypoxic conditions was already similar at that time point.

In addition, western blots showed that MMP-3 levels were decreased in hypoxic HBMECs by doxycycline after 24 h (1.00 ± 0.03 vs 1.32 ± 0.04, n = 6, $P < .001$) and 48 h (1.50 ± 0.05 vs 1.81 ± 0.05, n = 6, $P = .002$; Figure 4C). Similarly, MMP-3 enzymatic activity was decreased by doxycycline after 48 h (Figure 5A). Furthermore, real-time polymerase chain reaction analysis demonstrated that *MMP-3* mRNA levels increased at 48 h under hypoxic conditions, and were suppressed by doxycycline (Figure 5B). Conversely, protein levels of the MMP inhibitor, TIMP-1, were elevated after 24 h (1.15 ± 0.06 vs 0.49 ± 0.04, n = 6, $P < .001$) and 48 h (0.85 ± 0.04 vs 0.30 ± 0.034, n = 6, $P < .001$; Figure 4C). For HIF-1α, MMP-2/9, and VEGF, protein levels were all elevated by hypoxic culture, and doxycycline intervention decreased expression after 24 or 48 h (Figure 5C).

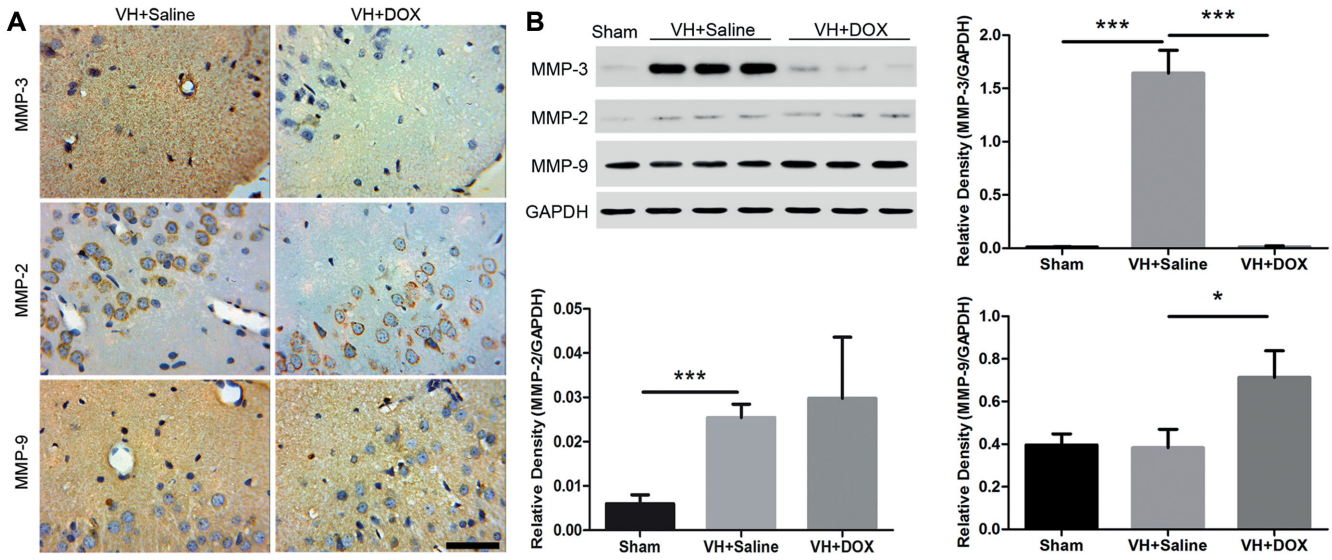


**FIGURE 2.** *Increased MMP-3 expression in VH rats.* **A**, *Photomicrographs showing immunostaining of MMP-3/2/9 in the saline-treated VH and doxycycline-treated VH rats. Scale bar: 50 μm.* **B**, *Western blots and histograms showing relative levels of MMP-3/2/9 in the sham, saline-treated VH and doxycycline-treated VH rats, n = 6 per group. Data are mean ± SD, *P < .05, VH + DOX vs VH + Saline for MMP-9 relative expression, ***P < .001, VH + DOX vs VH + Saline and VH + Saline vs Sham for MMP-3 relative expression, VH + Saline vs Sham for MMP-2 relative expression.*

### MMP-3 Knockdown Suppressed HBMEC Proliferation

A CCK-8 (Cell Counting Kit-8, Sigma-Aldrich Chemical Company, St Louis MO, USA) assay was used to examine the effect of MMP-3 expression level on HBMEC proliferation (Figure 6A). There was no difference in proliferation rate between MMP-3-overexpressing and control HBMECs (Figure 6B). However, proliferation was significantly inhibited in MMP-3 shRNA-treated HBMECs compared with their controls, on days 3 (2.00 ± 0.16 vs 3.64 ± 0.11, $P < .001$) and 4 (2.34 ± 0.11 vs 4.40 ± 0.24, $P < .001$; Figure 6B). A tube formation assay in matrigel was performed to examine the effect of MMP-3 expression on changes in endothelial cell network formation. We found no difference between MMP-3-overexpressing and control HBMECs. However, tube formation was significantly suppressed in MMP-3 shRNA-treated HBMECs after 12 h compared with their controls (6.00 ± 5.50 vs 51.00 ± 19.80, $P < .001$; Figures 6C and 6D).

### MMP-3 Promoted HBMEC Migration and Invasion

We performed transwell assays to investigate whether MMP-3 affects HBMEC migration and invasion (Figure 7A). MMP-3 cDNA-transduced HBMECs exhibited significantly increased migration relative to controls (217.3 ± 42.5 vs 110.0 ± 57.0, $P = .011$; Figure 7B). On the other hand, when MMP-3 was knocked down, migration ability was suppressed ($P = .031$; Figure 7B). MMP-3 overexpression also significantly increased HBMEC invasion compared with the control ($P < .001$; Figure 7B). MMP-3-overexpressing HBMECs actively migrated from both edges and showed a significantly higher healing rate compared with the control, at 24 and 48 h postwounding. In addition, HBMEC wound-healing rates were significantly suppressed in MMP-3 shRNA-treated HBMECs ($P < .001$; Figures 7C and 7D).

## DISCUSSION

Many researchers have demonstrated that MMP-mediated vascular remodeling is involved in BAVM formation and development.[11-15] MMP-3 and MMP-9 have been of particular interest because of their extensive involvement in vascular diseases and key roles in vascular remodeling.[14,16-23] Hashimoto et al[11] showed that MMP-9 is overexpressed in BAVM patients. An imbalance of TIMPs and MMP-9 is a potential mechanism of BAVM rupture.[11,13,24] MMP-3 is a crucial pro-MMP activator that is also involved in remodeling and turnover of the extracellular matrix[21,22,25] and is overexpressed in human BAVM tissues.[16] Previous studies found that single nucleotide polymorphisms of MMPs, specifically MMP-3 and MMP-9, are respectively associated with the risk of BAVM formation and rupture.[16,18,26] In contrast, although MMP-2 is involved in arterial lesion progression, such as atherosclerosis, it seems to be inactivated or minimally activated in BAVM.[11,20,21]

However, in this study, only MMP-3/2, and not MMP-9, were overexpressed in response to VH accompanied with elevated MVD. In Hashimoto's report, BAVM tissues were mostly collected from ruptured or embolized cases[11,20]; therefore, we assume that MMP-9 is the prevailing MMP in those tissues.

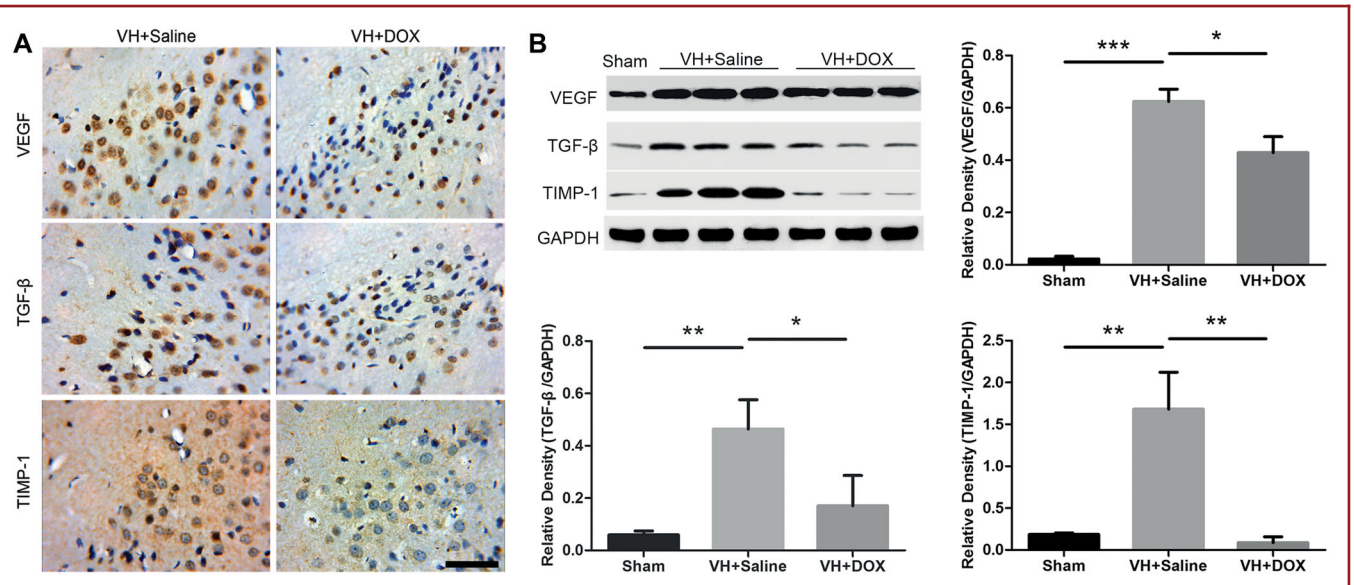


**FIGURE 3.** *VEGF, TGF-β, and TIMP-1 expression in VH rats.* **A**, *Photomicrographs showing VEGF, TGF-βm, and TIMP-1 immunostaining in saline-treated VH and doxycycline-treated VH rats. Scale bar: 50 μm.* **B**, *Western blots and histograms showing relative levels of VEGF, TGF-β, and TIMP-1 expression in the sham, saline-treated VH, and doxycycline-treated VH rats, n = 6 per group. Data are mean ± SD, *P < .05, VH + DOX vs VH + Saline for VEGF and TGF-β relative expression, **P < .01, VH + Saline vs Sham for TGF-β and TIMP-1 relative expression, and VH + DOX vs VH + Saline for TIMP-1 relative expression, ***P < .001, VH + Saline vs Sham for VEGF relative expression.*

We suggest that MMP-3 overexpression might be related to the formation and development of BAVM (Table), whereas elevated MMP-9 could be responsible for rupture.[17,18,26] The VH rat model is more likely to mimic the initial or early stages of BAVM formation or development under a VH hemodynamic environment, and subsequently stimulate very active angiogenesis or vascular dysplasia.[10,27] Similar to previous reports, we found that the expression of MMP-2 was very limited.[20] Therefore, the role of MMP-3 overexpression in angiogenesis might be more important at this early stage.

To investigate the effects of altered MMP-3 expression in vascular endothelial cells, we overexpressed or knocked down MMP-3 in HBMECs in Vitro. Previous researches showed a functional role of MMP-3 in cell proliferation, migration, and invasion in other diseases, but not in HBMECs.[28-30] Similar to previous studies, we found that, although MMP-3 upregulation did not increase HBMEC proliferation and tube formation, it notably enhanced HBMEC migration, suggesting that MMP-3 overexpression might enhance vascular structure remodeling via angiogenesis. Interestingly, we also found that MMP-3 downregulation led to a strong antiangiogenesis effect by attenuating HBMEC proliferation, tube formation, migration, and invasion. The dose effect of doxycycline in HUVECs has previously been reported, suggesting that 80 μM doxycycline inhibits cell proliferation most effectively,[31] a dose confirmed in this study. These findings suggest that MMP-3 plays an important role in promoting angiogenesis and that the suppression of MMP-3 alone offers a potential treatment strategy for vascular inhibition (Table).

Although there is a lack of specific MMP inhibitors for use in clinical practice, doxycycline, a clinically applicable antibiotic and also a nonspecific MMP inhibitor, has been considered as a potential safe and effective medication to stabilize BAVM.[20,32] Doxycycline binds and blocks access to the active zinc ion of MMPs to alter their 3-dimensional protein structure, thus deactivating the enzyme.[33] Furthermore, doxycycline may also activate the TIMP-1 pathway to enhance the inhibition of MMPs.[33] The unique small molecular and liposoluble features of doxycycline allow it to pass the blood–brain barrier.[33] Several studies showed that anti-MMP treatment using doxycycline might be safe and promising for the stabilization of BAVM.[20,32]

We tried to determine whether doxycycline could be used for angiogenic inhibition in response to VH. In the present study, the decrease in perisinus MVD after doxycycline treatment suggested its efficacy in angiogenic inhibition. However, interestingly, further analysis showed that overexpression of MMP-3, but not MMP-2/9, was significantly suppressed in the VH rats after doxycycline treatment. This novel finding indicated that doxycycline not only deactivates MMPs, but might also act on the underlying pathway to suppress MMP-3 expression.

An accompanying antiangiogenesis mechanism of MMP-3 suppression by doxycycline could be the concomitant decrease of VEGF and TGF-β. VEGF and TGF-β are known angiogenic factors in the BAVM formation pathway[7,15,34-36]; VEGF

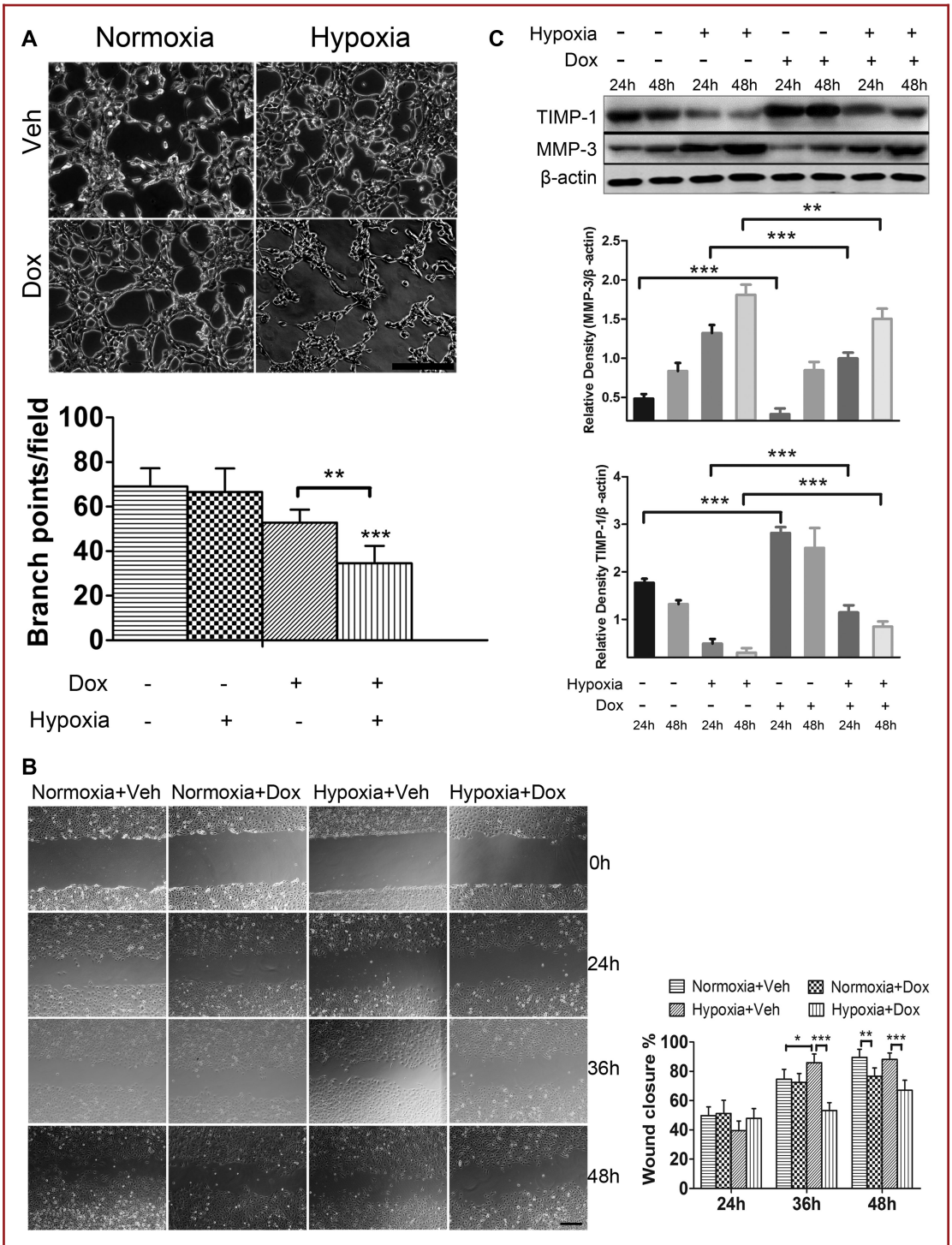


**FIGURE 4.** *Doxycycline attenuated HBMEC tube formation and migration together with MMP-3 upregulation and TIMP-1 downregulation in Vitro.* **A**, *Tube formation of different groups of HBMECs.* $^{**}P < .01$, *Hypoxia + Dox vs Hypoxia + Veh. Scale bar: 100 μm. Veh, Vehicle; Dox, doxycycline.* **B**, *Wound closure rate of HBMECs following 24, 36, and 48 h of doxycycline treatment.* $^{*}P < .05$, *Normoxia + Veh vs Hypoxia + Veh;* $^{**}P < .01$, *Normoxia + Dox vs Normoxia + Veh;* $^{***}P < .01$, *Hypoxia + Dox vs Hypoxia + Veh. Scale bar: 100 μm. Veh, Vehicle; Dox, doxycycline.* **C**, *Western blots and histograms showing relative amounts of MMP-3 and TIMP-1 expression.* $^{**}P < .01$, $^{***}P < .001$. *Dox, doxycycline. Data are mean ± SD, n = 6 per group.*

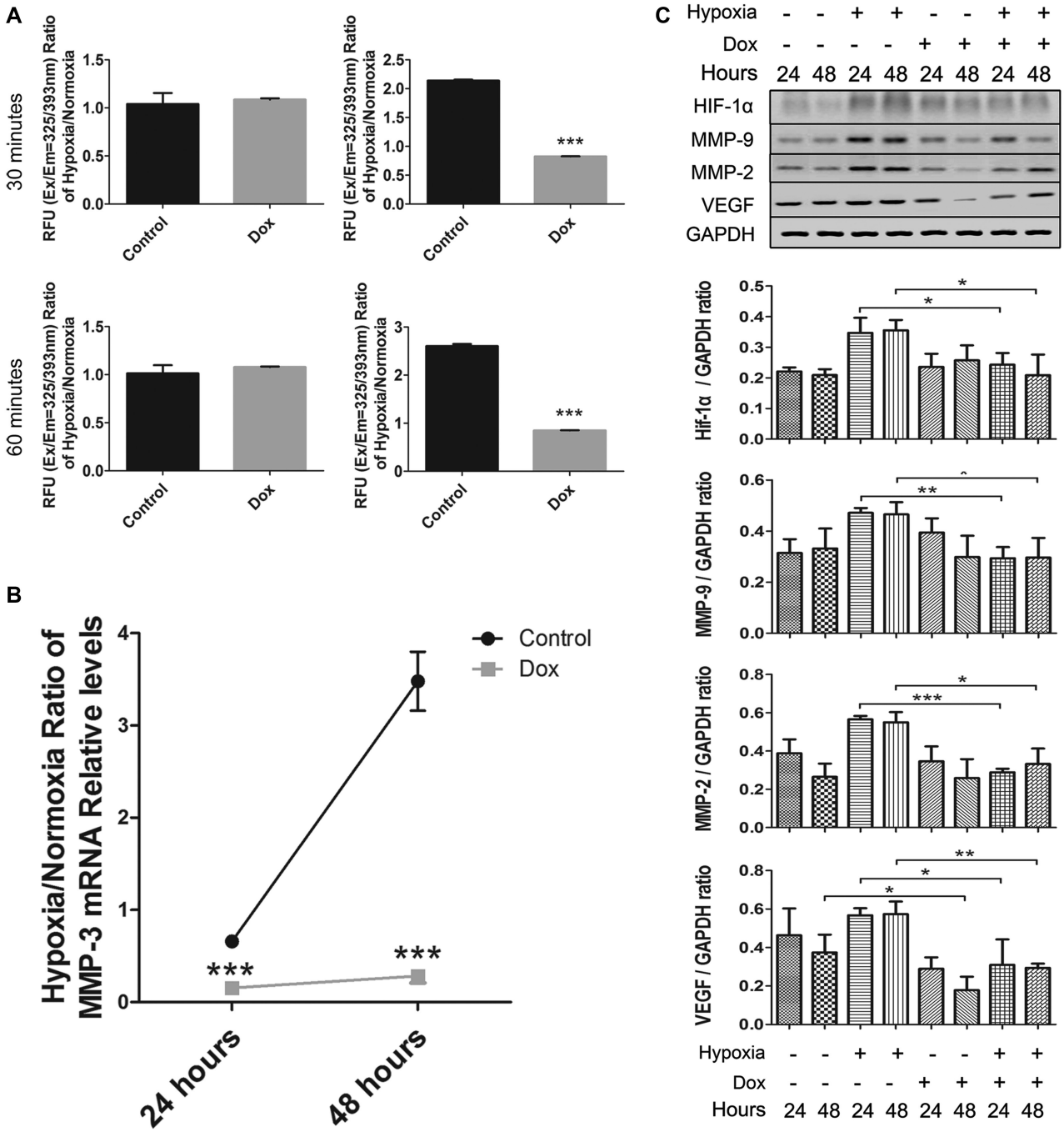


**FIGURE 5.** *Doxycycline attenuated MMP-3 mRNA together with MMP-3 enzymatic activity and HIF-1α, MMP-2/9 and VEGF expression in Vitro.* **A**, *Changes in MMP-3 enzymatic activity (over 30 and 60 min) after 24 h (left) and 48 h (right) of doxycycline (Dox) treatment, presented as Hypoxia/Normoxia ratios.* ***$P < .001$ *compared with control.* **B**, *MMP-3 mRNA levels after 24 and 48 h doxycycline treatment, presented as Hypoxia/Normoxia ratios.* ***$P < .001$ *compared with control.* **C**, *Western blots and histograms showing relative amounts of HIF-1α, MMP-2/9, and VEGF expression.* *$P < .05$, **$P < .01$. *Dox, doxycycline. Data are mean ± SD, $n = 6$ per group.*

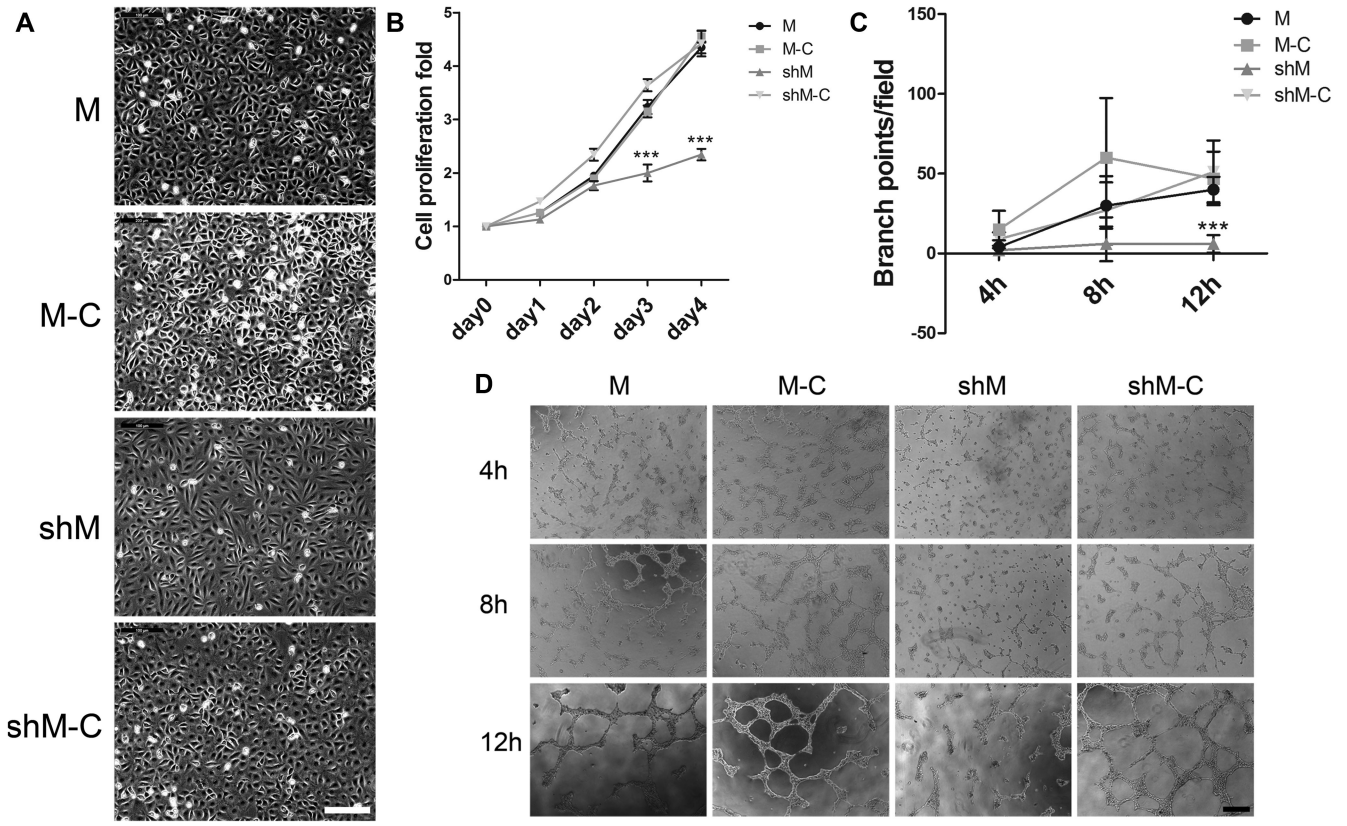


**FIGURE 6.** *MMP-3 knockdown decreased HBMEC proliferation and tube formation in Vitro.* **A**, *Photomicrographs showing the proliferation of 4 HBMEC treatment groups on the fourth day after transduction. Scale bar: 100 μm.* **B**, *Quantified HBMEC proliferation over 4 days in the 4 treatment groups.* ***$P < .001$, *shM vs shM-C.* **C**, *Quantified tube formation over 12 h for the 4 treatment groups.* ***$P < .001$, *shM vs shM-C.* **D**, *Photomicrographs showing tube formation over 12 h in the 4 treatment groups. Scale bar: 100 μm. M, MMP-3 overexpression group; M-C, control for the M group; shM, MMP-3 knockdown with shRNA; shM-C, control for the shM group. Data are mean ± SD, n = 6 per group.*

is upstream of MMP activation, and the TGF-$\beta$ pathway is independently related to endothelial cell proliferation.[7,15,36-38] Expression of both proteins significantly decreased after doxycycline administration in VH rats. Because these proteins do not appear to be direct targets of doxycycline, we believe that their downregulation corresponded to the attenuation of angiogenesis by MMP-3 suppression. Surprisingly, activation of the antiangiogenic factor, TIMP-1, was also reduced after doxycycline intervention. Hashimoto et al[11] previously found that TIMP-1 activation was strongly negatively correlated with MMP-9 activation. Therefore, we assume that, in the VH environment, the antiangiogenesis effect of doxycycline mainly depends on suppression of MMP-3, but not MMP-9, and therefore the TIMP-1 pathway is not activated (Table).

The therapeutic value of doxycycline for VH appears to be through antiangiogenesis and vascular stabilization, but not recovery of the abnormal hemodynamic microenvironment. In this study, VH was induced by carotid artery to EJV anastomosis. Doxycycline administration can only improve the pathological angiogenesis, but not the VH. For further strict investigation of the relationship between MMP-3 and VH angiogenesis, transgenic animals are needed. HIF-1$\alpha$ is related to anoxia and is considered an early messenger in angiogenesis activity triggered by the VH environment.[10] There was no difference in HIF-1$\alpha$ expression between VH- and doxycycline-treated VH rats, but an in Vitro experiment demonstrated that HIF-1$\alpha$ levels were suppressed after 24 and 48 h by doxycycline in hypoxic conditions, similar to other tetracyclines such as minocycline,[39] suggesting that a higher dose of doxycycline may be effective in Vivo.

## Limitations

There were several limitations in this study. First, the VH model is more likely to be a DAVF model, rather than a BAVM model. Until now, only one animal model has been found to induce severe vascular dysplasia such as a nidus-like lesion in the brain. However, this model is based on stimulation with VEGF in combination with conditional deletion of *Alk1*, which is a

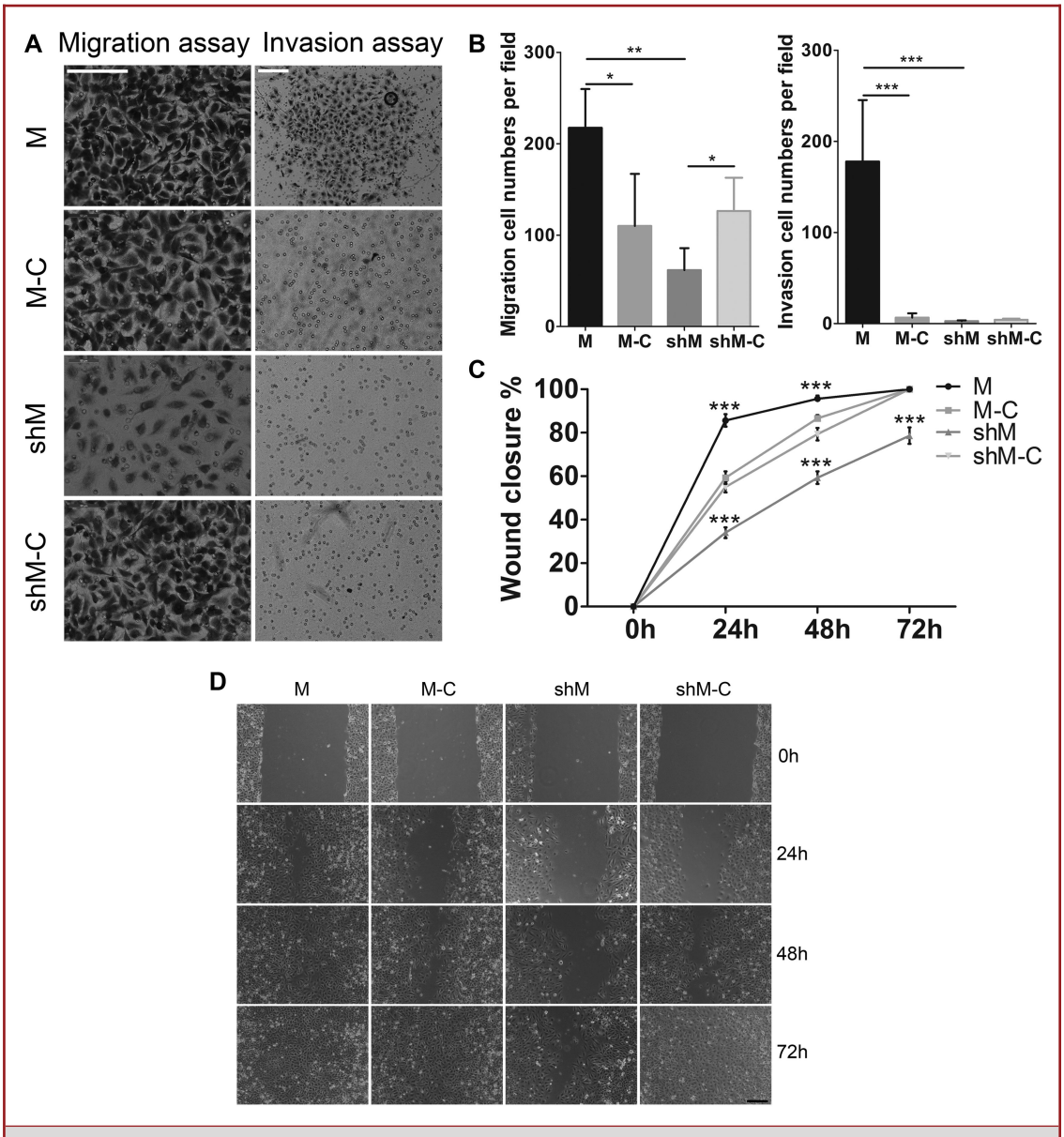


**FIGURE 7.** *MMP-3 contributed to HBMEC migration and invasion.* **A**, *Photomicrographs showing the migration and invasion of 4 HBMEC treatment groups. Scale bar: 100 μm.* **B**, *Semi-quantification of HBMEC migration and invasion in the 4 treatment groups. *P < .05, M vs M-C and shM vs shM-C, **P < .01, M vs shM, ***P < .001, M vs M-C and shM.* **C**, *Wound closure rates in the four groups following 72 h of treatment. ***P < .001, M vs M-C and shM vs shM-C.* **D**, *Photomicrographs showing wound healing over 72 h in the 4 treatment groups. Scale bar: 100 μm. M, MMP-3 overexpression group; M-C, control for the M group; shM, MMP-3 knockdown with shRNA; shM-C, control for the shM group.*

known gene deficit in familial hemorrhagic telangiectasia, not sporadic BAVM.[40] Therefore, this model is not strictly BAVM, but has implications for it. Compared with transgenic methods, the VH model is still a valuable and important surrogate model of DAVF or BAVM.[10] A macroscopic nidus or fistula-mimicking lesion, more than angiogenesis, can be seen in the rat brain in this VH model.[41] Although a true "nidus" does not develop in the VH model, it still mimics important features of existing or early BAVM that trigger abnormal angiogenesis in the nearby cortex, such as a direct arteriovenous shunt, and secondary VH causing a high-velocity and low-resistance shunt environment. Moreover, it is more commonly used and is easily replicable. Our aim is to find a way to inhibit further abnormal angiogenesis under an existing BAVM with VH. Therefore, we believe that this model is the

**TABLE. Summary of the Study's Findings and Their Implications.**

| Findings | Implications |
|---|---|
| Doxycycline reduces the increase in MVD seen in VH rats. | MMP inhibition is a potential therapeutic strategy for BAVM. |
| Doxycycline reduces the increase in MMP-3, VEGF, TGF-$\beta$, and TIMP-1 expression seen in VH rats. | These gene products are associated with angiogenesis in VH rats. |
| Doxycycline reduces migration, tube formation and MMP-3 expression in HBMEC. | The effects of doxycycline are mediated by effects on endothelial cell angiogenesis. |
| MMP-3 knockdown reduces proliferation, migration and tube formation in HBMEC. MMP-3 overexpression increases HBMEC migration. | MMP-3 mediates the effects of doxycycline and is a potential target for novel BAVM therapies. |

BAVM, brain arteriovenous malformation; HBMEC, human brain microvascular endothelial cells; MMP, matrix metalloproteinase; MVD, microvessel density; VH, venous hypertension.

most suitable for our experiments. Second, although we initially found that the effect of doxycycline on suppressing MMP-3 was remarkable, both in Vivo and in Vitro, after comparison with other important angiogenesis factors, it was not clear that MMP-3 was the main causal factor underlying changes in MVD. We could reach this conclusion definitively only if MMP-3 was specifically knocked down in an in Vivo model.

## CONCLUSION

Our study used VH models to provide novel insights into the potential angiogenic function of MMP-3 in the development and progression of BAVM. The proliferation, migration, and tube formation of vascular endothelial cells were inhibited in Vitro by downregulation of MMP-3 compared with overexpression of MMP-3. Doxycycline attenuated angiogenesis in response to a VH and hypoxia environment in Vivo and in Vitro, perhaps predominantly by suppressing MMP-3 expression.

### Disclosures

Funding was from the National Natural Science Foundation of China (Grant numbers 81400962, 81601706, 8157110, 30500524, 30973103, 81070936, 81571117, 81200889) and the National Key Technology R&D Program of the Ministry of Science and Technology of China (Grant number 2011BAI08B06); National Basic Research Program of China 973 Program (Grant No. 2015CB755500) and Shanghai Sailing Program (Grant No. 15441904500). The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.

## REFERENCES

1. Group Tams. Arteriovenous malformations of the brain in adults. *N Engl J Med*. 1999;340(23):1812-1818.
2. Halim AX, Johnston SC, Singh V, et al. Longitudinal risk of intracranial hemorrhage in patients with arteriovenous malformation of the brain within a defined population. *Stroke*. 2004;35(7):1697-1702.
3. Mohr JP, Parides MK, Stapf C, et al. Medical management with or without interventional therapy for unruptured brain arteriovenous malformations (ARUBA): a multicentre, non-blinded, randomised trial. *Lancet*. 2014;383(9917):614-621.
4. Crowley RW, Ducruet AF, McDougall CG, Albuquerque FC. Endovascular advances for brain arteriovenous malformations. *Neurosurgery*. 2014;74(suppl 1):S74-S82.
5. Bradac O, Charvat F, Benes V. Treatment for brain arteriovenous malformation in the 1998–2011 period and review of the literature. *Acta Neurochir*. 2013;155(2):199-209.
6. Hamilton MG, Spetzler RF. The prospective application of a grading system for arteriovenous malformations. *Neurosurgery*. 1994;34(1):2-6; discussion 6-7.
7. Lawton MT, Rutledge WC, Kim H, et al. Brain arteriovenous malformations. *Nat Rev Dis Primers*. 2015;1:15008.
8. Lawton MT, Jacobowitz R, Spetzler RF. Redefined role of angiogenesis in the pathogenesis of dural arteriovenous malformations. *J Neurosurg*. 1997;87(2):267-274.
9. Hacein-Bey L, Young WL. Hemodynamic perturbations in cerebral arteriovenous malformations and management implications. *Interv Neuroradiol*. 1999;5(1suppl):177-182.
10. Zhu Y, Lawton MT, Du R, et al. Expression of hypoxia-inducible factor-1 and vascular endothelial growth factor in response to venous hypertension. *Neurosurgery*. 2006;59(3):687-696; discussion 687-696.
11. Hashimoto T, Wen G, Lawton MT, et al. Abnormal expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in brain arteriovenous malformations. growth and bleeding in BAVM: another role for MMPs. *Stroke*. 2003;34(4):925-931.
12. Lee CZ, Xue Z, Zhu Y, Yang GY, Young WL. Matrix metalloproteinase-9 inhibition attenuates vascular endothelial growth factor-induced intracerebral hemorrhage. *Stroke*. 2007;38(9):2563-2568.
13. Hashimoto T, Lawton MT, Wen G, et al. Gene microarray analysis of human brain arteriovenous malformations. *Neurosurgery*. 2004;54(2):410-425; discussion 423-425.
14. Kim H, Pawlikowska L, Chen Y, Su H, Yang GY, Young WL. Brain arteriovenous malformation biology relevant to hemorrhage and implication for therapeutic development. *Stroke*. 2009;40(3 suppl):S95-S97.
15. Xu B, Wu YQ, Huey M, et al. Vascular endothelial growth factor induces abnormal microvasculature in the endoglin heterozygous mouse brain. *J Cereb Blood Flow Metab*. 2004;24(2):237-244.
16. Huai C, Song J, Ma Z, et al. Allelic variation of the MMP3 promoter affects transcription activity through the transcription factor C-MYB in human brain arteriovenous malformations. *PLoS One*. 2013;8(3):e57958.
17. Sun B, Qiu H, Zhao F, et al. The rs9509 polymorphism of MMP-9 is associated with risk of hemorrhage in brain arteriovenous malformations. *J Clin Neurosci*. 2012;19(9):1287-1290.
18. Zhao Y, Li P, Fan W, et al. The rs522616 polymorphism in the matrix metalloproteinase-3 (MMP-3) gene is associated with sporadic brain arteriovenous malformation in a Chinese population. *J Clin Neurosci*. 2010;17(12):1568-1572.
19. Chen Y, Fan Y, Poon KY, et al. MMP-9 expression is associated with leukocytic but not endothelial markers in brain arteriovenous malformations. *Front Biosci*. 2006;11(1):3121-3128.
20. Hashimoto T, Matsumoto MM, JF Li, Lawton MT, Young WL, University of California SFBSG . Suppression of MMP-9 by doxycycline in brain arteriovenous malformations. *BMC Neurol*. 2005;5(1):1.
21. Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochem Pharmacol*. 2008;75(2):346-359.
22. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res*. 2003;92(8):827-839.
23. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. 2002;90(3):251-262.

24. Dasu MR, Barrow RE, Spies M, Herndon DN. Matrix metalloproteinase expression in cytokine stimulated human dermal fibroblasts. *Burns*. 2003;29(6):527-531.
25. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem*. 1992;267(6):3581-3584.
26. Rangel-Castilla L, Russin JJ, Martinez-Del-Campo E, Soriano-Baron H, Spetzler RF, Nakaji P. Molecular and cellular biology of cerebral arteriovenous malformations: a review of current concepts and future trends in treatment. *Neurosurgical Focus*. 2014;37(3):E1.
27. Yang ST, Rodriguez-Hernandez A, Walker EJ, Young WL, Su H, Lawton MT. Adult mouse venous hypertension model: common carotid artery to external jugular vein anastomosis. *J Vis Exp*. 2015;(95):50472. doi: 10.3791/50472.
28. Laurent M, Martinerie C, Thibout H, et al. NOVH increases MMP3 expression and cell migration in glioblastoma cells via a PDGFR-alpha-dependent mechanism. *FASEB J*. 2003;17(13):1919-1921.
29. Shih WL, Liao MH, Yu FL, Lin PY, Hsu HY, Chiu SJ. AMF/PGI transactivates the MMP-3 gene through the activation of Src-RhoA-phosphatidylinositol 3-kinase signaling to induce hepatoma cell migration. *Cancer Lett*. 2008;270(2):202-217.
30. Lan YY, Yeh TH, Lin WH, et al. Epstein-Barr virus Zta upregulates matrix metalloproteinases 3 and 9 that synergistically promote cell invasion in vitro. *PLoS One*. 2013;8(2):e56121.
31. Su W, Li Z, Li F, Chen X, Wan Q, Liang D. Doxycycline-mediated inhibition of corneal angiogenesis: an MMP-independent mechanism. *Invest Ophthalmol Vis Sci.* 2013;54(1):783-788.
32. Frenzel T, Lee CZ, Kim H, et al. Feasibility of minocycline and doxycycline use as potential vasculostatic therapy for brain vascular malformations: pilot study of adverse events and tolerance. *Cerebrovasc Dis*. 2008;25(1-2):157-163.
33. Yao JS, Shen F, Young WL, Yang GY. Comparison of doxycycline and minocycline in the inhibition of VEGF-induced smooth muscle cell migration. *Neurochem Int*. 2007;50(3):524-530.
34. Walcott BP. BMP signaling modulation attenuates cerebral arteriovenous malformation formation in a vertebrate model. *J Cereb Blood Flow Metab*. 2014;34(10):1688-1694.
35. Jonker L. TGF- beta & BMP receptors endoglin and ALK1: overview of their functional role and status as antiangiogenic targets. *Microcirculation*. 2014;21(2):93-103.
36. Pawlikowska L, Tran MN, Achrol AS, et al. Polymorphisms in transforming growth factor-beta-related genes ALK1 and ENG are associated with sporadic brain arteriovenous malformations. *Stroke*. 2005;36(10):2278-2280.
37. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7(5):359-371.
38. Pufe T, Harde V, Petersen W, Goldring MB, Tillmann B, Mentlein R. Vascular endothelial growth factor(VEGF) induces matrix metalloproteinase expression in immortalized chondrocytes. *J Pathol.* 2004;202(3):367-374.
39. Li CH, Liao PL, Yang YT, et al. Minocycline accelerates hypoxia-inducible factor-1 alpha degradation and inhibits hypoxia-induced neovasculogenesis through prolyl hydroxylase, von Hippel-Lindau-dependent pathway. *Arch Toxicol*. 2014;88(3):659-671.
40. Walker EJ, Su H, Shen F, et al. Arteriovenous malformation in the adult mouse brain resembling the human disease. *Ann Neurol.* 2011;69(6):954-962.
41. Chen L, Mao Y, Zhou LF. Local chronic hypoperfusion secondary to sinus high pressure seems to be mainly responsible for the formation of intracranial dural arteriovenous fistula. *Neurosurgery*. 2009;64(5):973-983; discussion 983.


***Supplemental digital content*** *is available for this article at www.neurosurgery-online.com.*

**Supplemental Digital Content 1. Supplemental Methods.**
**Supplemental Digital Content 2**. **Figure**. Magnetic resonance angiography of a venous hypertension model.
**Supplemental Digital Content 3**. **Figure**. HIF-1$\alpha$ expression in venous hypertension rats.

## Acknowledgments

We sincerely thank Xiaodi Qiu and Xiao Song for experimental assistance. We thank Nicholas Rufaut, PhD, for editing the English text of a draft of this manuscript.