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ORIGINAL ARTICLE

Periostin Is Expressed by Pericytes and Is Crucial for Angiogenesis in Glioma

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Abstract

The expression of the matricellular protein periostin has been associated with glioma progression. In previous work we found an association of periostin with glioma angiogenesis. Here, we screen gliomas for *POSTN* expression and identify the cells that express periostin in human gliomas. In addition, we study the role of periostin in an in vitro model for angiogenesis. The expression of periostin was investigated by RT-PCR and by immunohistochemistry. In addition, we used double labeling and in situ RNA techniques to identify the expressing cells. To investigate the function of periostin, we silenced *POSTN* in a 3D in vitro angiogenesis model. Periostin expression was elevated in pilocytic astrocytoma and glioblastoma, but not in grade II/III astrocytomas and oligodendrogliomas. The expression of periostin colocalized with PDGFRβ^+ cells, but not with OLIG2$^+$/SOX2$^+$ glioma stem cells. Silencing of periostin in pericytes in coculture experiments resulted in attenuation of the numbers and the length of the vessels formation and in a decrease in endothelial junction formation. We conclude that pericytes are the main source of periostin in human gliomas and that periostin plays an essential role in the growth and branching of blood vessels. Therefore, periostin should be explored as a novel target for developing anti-angiogenic therapy for glioma.

Key Words: Angiogenesis, Glioblastoma, Glioma, Matricellular protein, Periostin, Vasculogenesis.

INTRODUCTION

In order to find new targets for effective anti-angiogenic therapy for gliomas, the identification of molecules that play key roles in neovascularization is crucial. In spite of the fact that gliomas are among the tumors with highest degree of vascularization, anti-angiogenic therapies have not yielded major improvements in clinical outcome (1). It remains unclear why anti-angiogenic therapies largely fail, and whether the currently used drugs address all players in the complex process of angiogenesis. Levels of vascular endothelial growth factor (VEGF) are associated with tumor hypoxia that increases with tumor progression. VEGF inhibitors like bevacizumab are only used in patients with high-grade gliomas/glioblastomas (GBM) (2). The blood vessels in GBM show proliferation of endothelial cells, pericytes, and other mural cells, altogether designated as microvascular proliferation. However, notable changes in protein expression patterns of the vessel walls of gliomas that do not yet show microvascular proliferation have been recorded (3, 4). Given the notion that shifts in protein expression patterns have been recorded in the vasculature of low-grade gliomas, new targets for anti-angiogenic therapies in glioma should be explored.

In a previous study, we identified some proteins that are specifically upregulated in tumor angiogenesis (3). Among the proteins identified were αV-integrin and the matricelluar proteins tenascin-C and, most prominently, periostin. Matricellular proteins are expressed during development, tissue repair and cancer and contribute to angiogenesis by making the extracellular matrix permissive for new vascular sprouts (5–11). In various epithelial tumors increased levels periostin were found (5, 12–15) and a prominent role of periostin at sites of metastasis was reported (16). Periostin has been associated with glioma invasion and vasculature (3, 17–19), and recently its interference with anti-angiogenic therapies was highlighted (20). Most data were obtained in mouse models and data on the expression site of *POSTN* in human glioma are sketchy. In addition, the direct effects of periostin expression on glioma angiogenesis have not yet been investigated.

In this study, we explored the expression of periostin in human glioma samples by immunohistochemical detection of

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co-expression patterns and RNA in situ hybridization and found expression of periostin by PDGFRβ$^+$ pericytes without overlap with SOX2$^+$/OLIG2$^+$ glioma stem cells. Silencing of the *POSTN* gene in cultured pericytes resulted in a reduction of angiogenic capacity, proving the importance of periostin for glioma angiogenesis.

MATERIALS AND METHODS

Tissue Samples

Tissue samples of 21 GBM, 10 pilocytic astrocytomas (PA), 19 grade II and III astrocytoma (A II/III), and 9 oligodendrogliomas grade II/III (O II/III) were obtained from the Department of Pathology, Erasmus Medical Center, Rotterdam (Table 1). Pathology diagnoses were in accordance with the WHO criteria including the molecular criteria. IDH1 mutation was present in 2/21 GBMs, in 18/19A II/III, and in all O II/III. All oligodendrogliomas had 1p/19q codeletion (Table 1). In order to make comparisons with normal brain and other vascular lesions, we included 11 normal brain samples, 5 cavernous hemangiomas (CH), 10 arteriovenous malformations (AVM), and 10 hemangioblastomas (HB). The age and gender distributions of the patients are shown in Table 1. All patients had given consent for using their biomaterials and the study was approved by the medical ethical committee of the Erasmus Medical Center.

RNA Isolation and RT-PCR

Fresh-frozen samples (n = 67) and formalin-fixed paraffin-embedded (FFPE) samples (n = 28) were used for RNA isolation. For each fresh-frozen sample, 10–15 sections of 20-μm thickness were cut by a cryostat. Sections were collected in RNase free Eppendorf tubes, snap frozen on dry ice, and stored at –80°C until RNA isolation. To verify the presence of tumor in all the sections used for RNA isolation, 5-μm sections before and after sampling for RNA isolation were collected, H&E-stained and studied by a pathologist (J.M.K.). Total RNA was isolated using RNA-Bee (Campro, Veenendaal, The Netherlands) according to the instructions supplied by the manufacturer. For FFPE samples, RNA isolation and quality control was performed as described previously (3, 4).

Following isolation, RNA samples were diluted in nuclease-free water, snap frozen on dry ice and subsequently stored at –80°C. Total RNA quantity was determined by Nanodrop and RNA integrity was checked using gel electrophoresis. To generate cDNA, 1 μg of total RNA was reverse transcribed using the RevertAid cDNA synthesis kit (Fermentas, Waltham, MA). cDNA samples were stored at –20°C until they were measured by RT-PCR. siPOSTN sequences were purchased from Dharmacon (Cambridge, UK) (siPOSTN 1: catalogue #: J-020118-05-0005; siPOSTN 2: catalogue #: J-020118-06-0005). Exon-spanning TaqMan Gene Expression Assays of periostin (Hs00170815_m1, Applied Biosystems, Foster City, CA) was used to measure the expression of periostin. Expression of HPRT1 (Hs01003267_m1) and ACTB (Hs99999903_m1) were used as reference genes. RT-PCR to the endothelial marker CD31 was performed in order to correct for blood vessel density in a selection of samples (n = 67). PCR was performed in a 20 μL reaction volume in the Applied Biosystems 7500 Fast Real-Time PCR System. Negative controls using H_2O only samples were included and showed to be negative in all cases.

Mann-Whitney *U* test was used to perform statistical analysis. All glioma subgroups were compared with each other, and p value <0.01 was considered statistically significant.

Immunohistochemistry

FFPE samples corresponding to the same sample used for RNA isolation were used for immunohistochemistry. Antibodies for periostin, CD31, PDGFRβ, SOX2, and OLIG2 were used as previously described (2) (Table 2).

Confocal Imaging

A confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc., Thornwood, NY) was used. A diode laser was used for excitation of DAPI at 405 nm, an argon laser for FITC at 488 nm and a HeNe-laser for Cy5 at 633 nm. For DAPI an emission bandpass filter of 420–480 nm, for FITC the bandpass filter of 500–530 nm, and for Cy5 a bandpass filter of 650 nm were used.

In Situ Hybridization

The RNAscope 1 2.0 HD Brown Chromogenic Reagent Kit and Hs-*POSTN* probe (#409181) were used, according to

TABLE 1. Patient and Tumor Characteristics

	Mean Age(SD)	Sex(m/f)	IDH1wt/mut	1p/19q CodelYes/No	Total
Glioblastoma	47.4 (12.7)	15/6	19/2	0/21	21
Pilocytic astrocytoma	23.4 (18.6)	3/7			10
Astrocytoma (grade II/III)	43.2 (14.8)	7/12	1/18	0/19	19
Oligodendroglioma (grade II/III)	50.7 (7.8)	6/3	0/9	9/0	9
Normal brain	49.3 (14.8)	5/6			11
Cavernous hemangioma	19.4 (11.2)	1/4			5
Hemangioblastoma	1.3 (21.2)	6/4			10
Arterio-venous malformation	39.8 (18.3)	8/2			10

SD, standard deviation; m, male; f, female; IDH1, isodehydrogenase 1; wt, wild type; mut., mutation; codel, codeletion.

TABLE 2. *Z* Scores of Periostin Expression Tumors, Malformations and Normal Brain and p Values of Differences in Expression Between Tissues

			GBM	PA	AII/III	Oligo	n.b.	CH	HB	AVM
GBM	POSTN (–dCt)	Z		−1.10	**−3.50**	**−2.86**	**−2.98**	−0.81	−0.19	−0.82
		p		0.27	0.00	0.00	0.00	0.42	0.85	0.41
	POSTN/CD31 (–dCt)	Z		−0.39	**−2.22**		**−2.69**	−0.06	−1.80	−1.13
		p		0.70	0.03		0.01	0.95	0.07	0.26
PA	POSTN (–dCt)	Z	−1.10		**−3.30**	**−3.03**	**−3.39**	−0.25	**−1.66**	−1.74
		p	0.27		0.00	0.00	0.00	0.81	0.10	0.08
	POSTN/CD31 (–dCt)	Z	−0.39		**−2.72**		**−3.59**	−0.55	**−2.88**	−1.78
		p	0.70		0.01		0.00	0.58	0.00	0.08
AII/III	POSTN (–dCt)	Z	**−3.50**	**−3.30**		−0.49	−0.18	**−2.19**	**−3.60**	**−2.12**
		p	0.00	0.00		0.62	0.86	0.03	0.00	0.03
	POSTN/CD31 (–dCt)	Z	**−2.22**	**−2.72**			−0.32	−1.53	−1.29	−1.10
		p	0.03	0.01			0.75	0.13	0.20	0.27
Oligo	POSTN (–dCt)	Z	**−2.86**	**−3.03**	−0.49		−0.38	−1.91	**−3.56**	**−1.96**
		p	0.00	0.00	0.62		0.71	0.06	0.00	0.05
	POSTN/CD31 (–dCt)	Z								
		p								
n.b.	POSTN (–dCt)	Z	**−2.98**	**−3.39**	−0.18	−0.38		**−2.34**	**−3.43**	**−1.92**
		p	0.00	0.00	0.86	0.71		0.02	0.00	0.05
	POSTN/CD31 (–dCt)	Z	**−2.69**	**−3.59**	−0.32			**−2.15**	−1.76	−1.41
		p	0.01	0.00	0.75			0.03	0.08	0.16
CH	POSTN (–dCt)	Z	−0.81	−0.25	**−2.19**	−1.91	**−2.34**		−0.86	−1.47
		p	0.42	0.81	0.03	0.06	0.02		0.39	0.14
	POSTN/CD31 (–dCt)	Z	−0.06	−0.55	−1.53		**−2.15**		−1.47	−1.04
		p	0.95	0.58	0.13		0.03		0.14	0.30
HB	POSTN (–dCt)	Z	−0.19	−1.66	**−3.60**	**−3.56**	**−3.43**	−0.86		−1.10
		p	0.85	0.10	0.00	0.00	0.00	0.39		0.27
	POSTN/CD31 (–dCt)	Z	−1.80	**−2.88**	−1.29		−1.76	−1.47		−0.68
		p	0.07	0.00	0.20		0.08	0.14		0.50
AVM	POSTN (–dCt)	Z	−0.82	−1.74	**−2.12**	**−1.96**	**−1.92**	−1.47	−1.10	
		p	0.41	0.08	0.03	0.05	0.05	0.14	0.27	
	POSTN/CD31 (–dCt)	Z	−1.13	−1.78	−1.10		−1.41	−1.04	−0.68	
		p	0.26	0.08	0.27		0.16	0.30	0.50	

Abbreviations: GBM = glioblastoma; PA = pilocytic astrocytoma; A II/III = astrocytoma WHO grades II and III; OLIGO = oligodendroglioma WHO grades II and III; n.b. = normal brain; CH = cavernous hemangioma; HB = hemangioblastoma; AVM = arteriovenous malformation; POSTN = periostin. $P < 0.05$ highlighted.

the manufacturer's instruction (Advanced Cell Diagnostics, Hayward, CA). Briefly, prepared slides were baked for 1 hour at 60°C prior to use. After deparaffinization and hydration, tissue and cells were air-dried and treated with a peroxidase blocker before heating in a target retrieval solution (#320043) for 20 minutes at 95–100°C. Protease (#320045) was then applied for 30 minutes at 40°C. *POSTN* probe was hybridized for 2 hours at 40°C, followed by a series of signal amplification and washing steps. Hybridization signals were detected by chromogenic reaction using DAB chromogen followed by 1:1 (vol/vol)-diluted hematoxylin (Fisher Scientific, Pittsburg, PA) counterstaining.

Cell Culture Experiments

In order to investigate the main source of periostin, various cell lines were used. HUVEC (ScienCell-1800), human brain vascular pericytes (ScienCell-1200), and human astrocytes (ScienCell-8000) were cultured following the manual protocols. Periostin expression in cell lysates was measured by Western blotting using Periostin antibody (HPA012306, 1:50, Sigma Life Sciences, St. Louis, MO). In addition, GBM cell line U87 was cultured for 3 days. After that, U87-conditioned media was added to the cultures of HUVECs, pericytes and astrocytes for 3 days. The expression of periostin in the cell lysate and the media was measured in the 3 cells lines by Western blot.

Silencing of POSTN

Two different siRNA sequences of periostin were used (Dharmacon, GE Health Care, Eindhoven, The Netherlands). A mix of nontargeting siRNA (referred to as siSham) were also obtained from Dharmacon and used as a negative control for silencing. Silencing experiments were performed using transfection buffer 1, following the manufacturer's instructions. Human

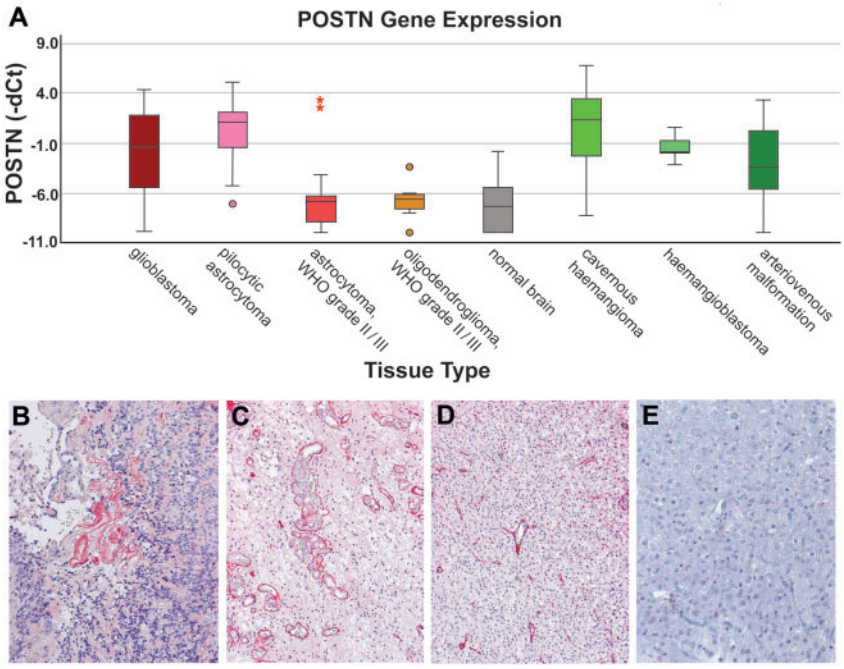

FIGURE 1. Results of RT-PCR to *POSTN* and immunohistochemistry to periostin. **(A)** Box plots of RT-PCR to *POSTN*. Highest expression levels of *POSTN* were found in GBM and PA. Significantly lower levels of expression are encountered in A II/III and O II/III. In HB and cerebral vascular malformations (CH and AVM), *POSTN* expression levels are high relative to A II/III and O II/III. In normal brain (n.b.) samples, lowest expression was recorded. **(B–E)** Periostin immunohistochemistry in GBM. Expression is concentrated around areas of MVP **(B)**. The expression is confined to the proliferated vessels in PA **(C)**. In A II/III **(D)** and O II/III **(E)**, periostin expression is found in around scattered blood vessels (×100).

brain vascular pericytes were transfected for 24 and 48 hours. RNA and proteins were isolated subsequently. The silencing efficiency was evaluated by RT-PCR and by Western blotting, following the protocols that were previously described.

3D In Vitro Angiogenesis Model

Pericytes were stained with DsRed and mixed with GFP labeled HUVECs at 1:5 ratio as described previously (21). To create a 3D in vitro angiogenesis model, bovine collagen type 1 was used. The parameters of angiogenesis, namely: number of tubule formation, length of tubule formation, and number of junctions were measured following 3 days of coculturing. These experiments were repeated 6 times.

RESULTS

POSTN Expression Is Associated With Angiogenesis in the Glial Tumors

The RNA expression of *POSTN* was corrected for vessel density by relating it to the expression of the endothelial marker CD31. *POSTN* expression was strongly elevated in PA and GBM as compared with that in normal brain (Fig. 1A). *POSTN* expression was also high in CH, HB, and AVM. The expression was low in A II/III and O II/III. The absolute expression and expression relative to vessel density (CD31 expression) are shown in Table 2. The results of immunohistochemistry were in line with those of the RT-PCR (Fig. 1B–E). In GBM, the expression of periostin was present in the perivascular area of hypertrophic and glomeruloid vessels, with dissemination in the neuropil (Fig. 1B). In the PAs expression was confined to the hypertrophied vasculature (Fig. 1C). The expression levels of periostin in A II/III and O II/III were comparable to those found in control brain samples (Fig. 1D, E). In AVM and CH, periostin was variably expressed in arteries and veins. In the HB, only a minority of capillaries was surrounded by perivascular periostin.

POSTN Is Expressed by Pericytes

In order to characterize the cells that express periostin, we performed double labeling fluorescent IHC. Periostin expression colocalized with PDGFRβ^+ pericytes (Fig. 2A). RNA in situ hybridization revealed that periostin protein expression localized with *POSTN* expression in scattered cells present just outside the cells expressing CD31 (Fig. 2B). GFAP-positive cells did not express *POSTN* (data not shown). IHC to the stem cell markers SOX2 and OLIG2 revealed that the expression of periostin does not colocalize with SOX2 and OLIG2-positive cells (Fig. 2C).

Pericytes Are the Main Source of POSTN

In order to study the function of *POSTN*, we first identified the periostin expressing cells in vitro. Western blotting using a periostin specific antibody confirmed that pericytes are the main source of expression. In addition, periostin expres-

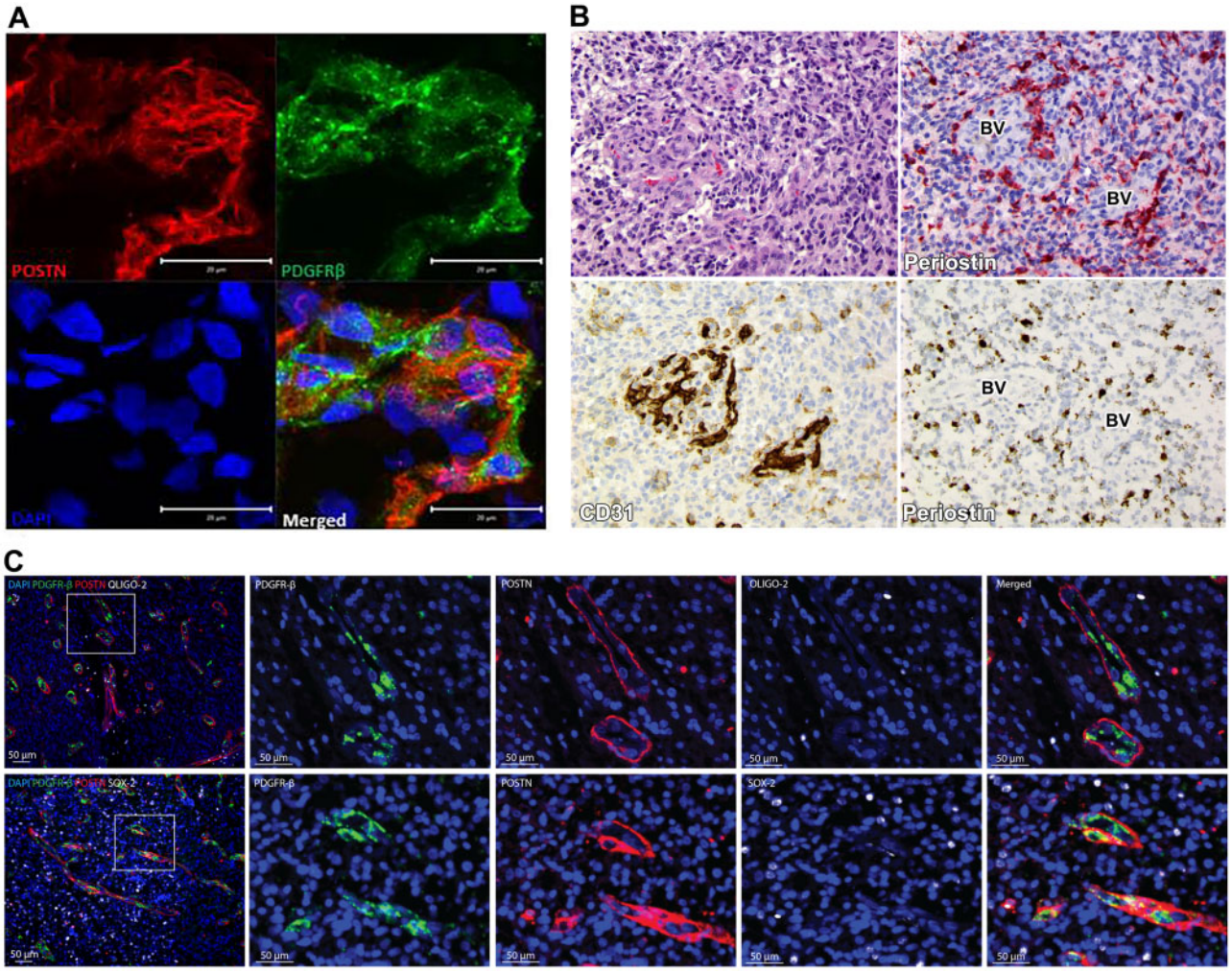

FIGURE 2. Periostin is expressed by pericytes. **(A)** Glioblastoma tissue immunostained for periostin and the pericyte marker PDGFRβ. There is overlapping expression of PDGFRβ and periostin (×400). **(B)** RNA in situ hybridization to *POSTN* in glioblastoma (lower right panel) reveals expression in scattered cells just outside of the endothelial layer. The CD31-positive endothelial cells (lower left panel) do not overlap with this RNA expression of *POSTN* (×40). **(C)** Cells expressing the stem cell transcription markers SOX2 and OLIG-2 do not overlap with the cells expressing periostin (IHC; ×40; DAPI counterstaining).

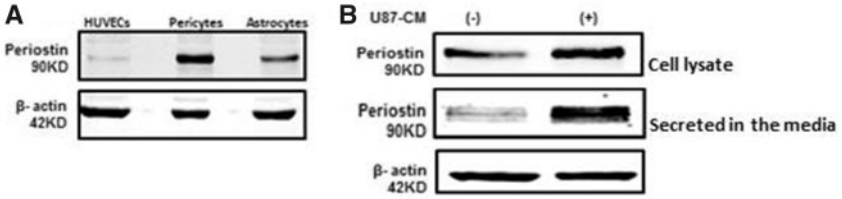

FIGURE 3. Western blotting to *POSTN* in cell cultures of various lineages. **(A)** Western blots for periostin in cell cultures of HUVEC (endothelial cells), pericytes, and normal astrocytes. *POSTN* expression is high in pericytes while expression is lower in astrocytes and absent from HUVEC. **(B)** Periostin protein expression by cultured pericytes w/wo cell lysates of the glioma cell line U87, or U87-conditioned media (U87-CM). Increased expression of periostin is observed following the addition of U87-CM, and after culturing the pericytes in the presence of U87 cell lysates.

sion was found in astrocytes also, but to a lesser extent than in pericytes. No expression was detected in endothelial cells (Fig. 3A). To investigate periostin expression in the presence of glial tumor cells, we added the conditioned media of U87 cells to the cultured pericytes and measured the expression of periostin after 24 hours. Glioma-conditioned media stimulated pericytes to express higher level of periostin (Fig. 3B). Increased periostin expression was also obtained following cul-

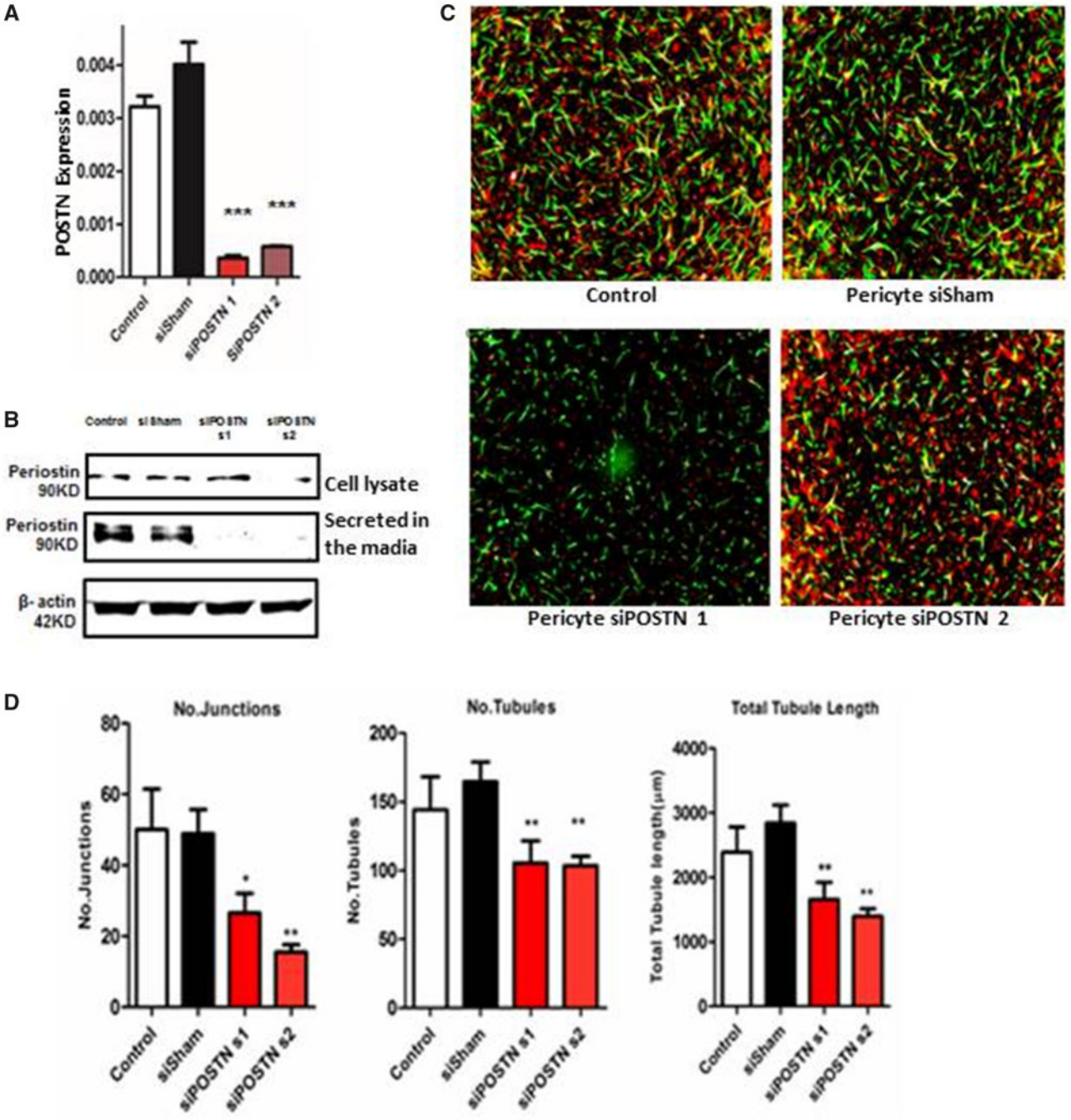

FIGURE 4. In vitro angiogenesis following silencing of *POSTN*. **(A)** Bar diagram of RT-PCR results showing the successful silencing of *POSTN* by 2 siRNAs. **(B)** Western blots showing levels of periostin in pericyte cell lysates and conditioned media, following silencing of *POSTN* in the pericytes. Silencing by sequence #2 resulted in significant reduction of periostin expression in the pericyte cell lysates and conditioned media. Silencing by sequence #1 resulted in reduced expression only in the conditioned media, not in the cell lysates. **(C)** Images of the 3D angiogenesis culture assay (pericytes stained with DsRed, HUVEC expressing GFP) using different conditions: Off-target silencing sequences (siSham) did not affect the formation of the blood vessels (upper panel right); effective silencing of *POSTN* resulted in significant reduction of formation of angiogenesis for both sequences (lower panels). **(D)** Bar diagrams showing the results of *POSTN* silencing in the pericytes on angiogenesis. For both silencing sequences, significant reductions in numbers of tubules (middle panel), tubular lengths (right panel), and number of vascular junctions (left panel) was achieved.

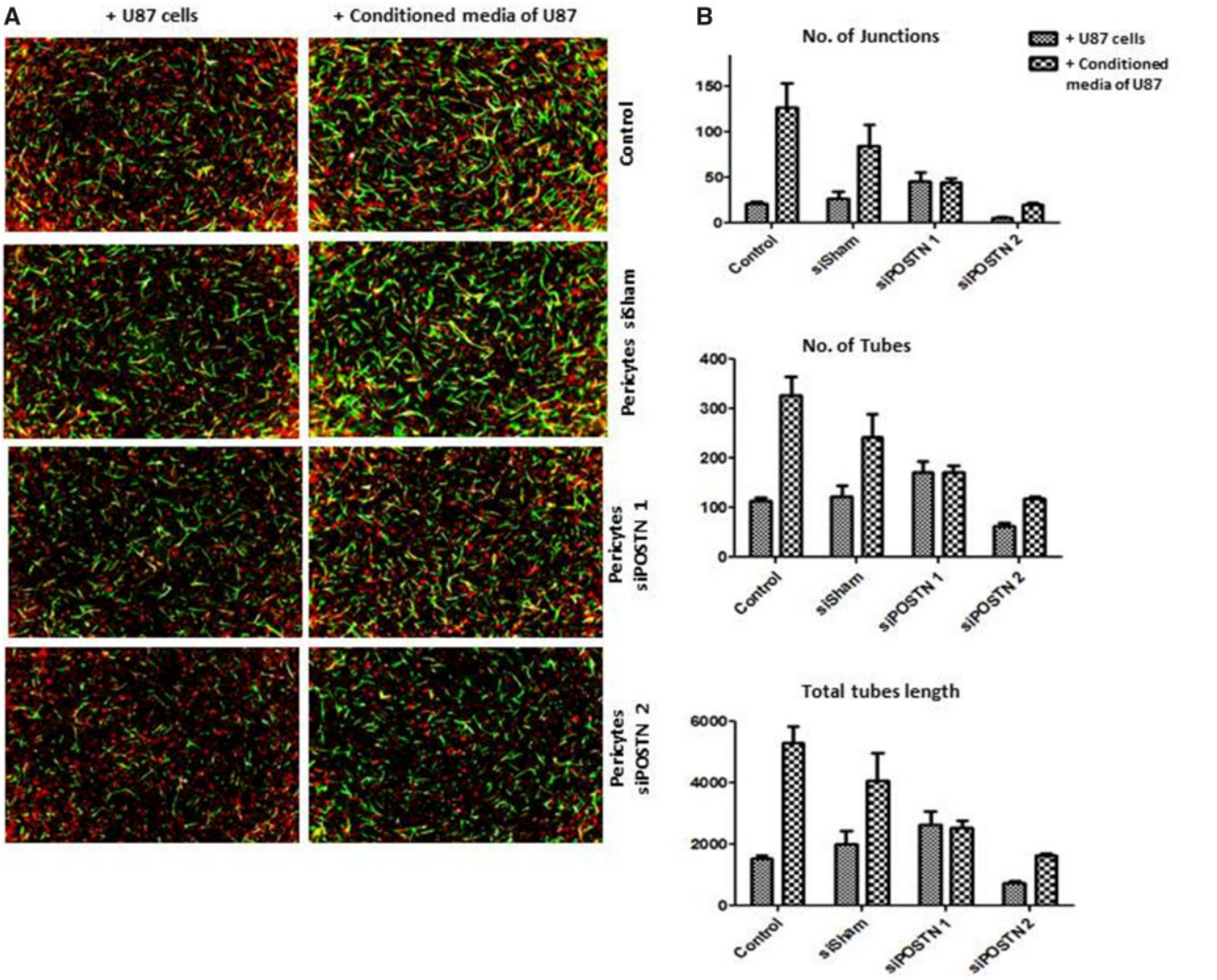

FIGURE 5. In vitro angiogenesis following silencing of *POSTN* in the presence of U87 cells or U87-conditioned medium. **(A)** Images of the angiogenesis culture assay following silencing of *POSTN* (using 2 different sequences) combined with U87 cells or U87 condition medium. **(B)** Quantification of the numbers and lengths of tubes and junctions. The effects of silencing *POSTN* on the number of vascular tubes, their length and the number of junctions were reduced in the presence of U87 cells or U87-conditioned medium.

turing the pericytes in the presence of U87 cell lysates (Fig. 3B).

POSTN Effect on In Vitro Angiogenesis

In functional assays, we silenced *POSTN* in pericytes and used a 3D in vitro angiogenesis assay. Silencing *POSTN* was achieved by 2 different siRNA sequences and *POSTN* was successfully downregulated using si*POSTN* for both sequences ($n = 3$; mean ± SEM; $p = 0.005$) (Fig. 4A). Effective downregulation of periostin protein in conditioned media and cell lysates of the cultured pericytes was achieved by using sequence #2. Following the use of sequence #1, downregulation of *POSTN* expression was only detected in the conditioned media, not in the cell lysates (Fig. 4B). The pericyte cultures silenced for *POSTN* were cocultured with endothelial cells in the 3D in vitro angiogenesis model. The number and length of the tubules and the number of junctions formed in the assay revealed significant differences for both silencing sequences (Fig. 4C, D).

In Vitro Angiogenesis Following Silencing of POSTN Partially Restored in the Presence of U87 Cells or U87 Conditioned Medium

The effects of silencing of periostin on angiogenesis was measured following the introduction of U87 (glioma) cells and following the addition of U87-conditioned media to the culture system. The angiogenesis-inhibiting effect of *POSTN* silencing in pericytes was partially saved by the addition of U87 cells or conditioned medium (Fig. 5A, B). The effects of silencing were stronger by using sequence #2.

DISCUSSION

In this study, we investigated the expression of periostin in gliomas of various malignancy grades and found the highest levels of expression in gliomas with proliferated microvasculature, that is, GBM and PA. Periostin expression appeared also to be high in other cerebral lesions with angiogenic activity, like HB and vascular malformations. In gliomas of lower malignancy grade, in which no visible changes of the vessel walls exist, the expression levels were comparable to those in normal brain. Both in the tissue samples of the patients and in the cell cultures, periostin was expressed by PDGFRβ$^+$ pericytes. However, in the cell cultures low-level expression by astrocytes was also observed. In the functional studies, we showed that periostin expression is necessary for proper formation of vasculature and that the presence of glioma cells (or their secretome) positively influences the angiogenesis-promoting effects of periostin.

Periostin is a matricellular protein and member of the tumor growth factor (TGF) family and its expression is induced by TGF-β and BMP-2 (22). Periostin promotes the incorporation of tenascin-C into the extracellular matrix (23) and interacts with bone morphogenic proteins 1/2 (BMP1/2) for the regulation of collagen cross-linking (24). Periostin interacts with various matricellular proteins in reparative processes and plays a role in the epithelial-mesenchymal transition in the context of neoplasia (2, 23–27). In recent years, it became clear that periostin plays roles in the proliferation, migration and the epithelial-mesenchymal transition of cancer (28–31). In breast cancer, periostin is expressed by tumor associated fibroblasts and promotes the proliferation and metastatic capacities of the tumor cells (32, 33). The N-terminal region of the molecule binds to integrins αVβ3, αVβ5, and α6β4 through its FAS domain (28), thereby promoting migration of tumor cells via the activation of Akt/PKB and focal adhesion kinase-mediated signaling pathways (14). In accordance, knock-down of *POSTN* in the ErbB2/Neu-driven murine breast tumor model results in reduced activity of the Notch signaling pathway and deceleration of tumor growth (34, 35). In breast and colonic cancer, it was shown that the expression of periostin by stromal cells is induced by tumor cells and is associated with cell proliferation, immune evasion, migration and genomic instability and decreases apoptosis of cancer cells (14, 36, 37). Periostin was associated with angiogenesis in wound healing and vascular heart disease, and also in neoplasia (38–44). In breast cancer-associated angiogenesis, the endothelial cells that navigate the branching of newly formed vessels, the vascular tip cells, also transiently express periostin (45).

To date, only few studies have focused on the expression of periostin in glial neoplasms and its expression was associated with tumor cell invasion (3, 17, 20, 46, 47). In contrast to periostin, the matricellular proteins tenascin-C and integrin-αV have been strongly associated with glioma angiogenesis (48–52). It is likely that endothelial cells and pericytes are responsible for the perivascular expression of tenascin-C while the proliferating glial tumor cells are the extravascular source of this protein (53, 54). The expression of tenascin-C is induced by several angiogenic factors, including VEGF, acidic and basic fibroblast growth factors (FGF), platelet-derived growth factor (PDGF), and tumor necrosis factor (TNF) (55). The perivascular presence of tenascin-C correlates with microvessel density and tumor cell proliferation (49, 50, 52, 53). Therefore, tenascin-C was selected as target for experimental tumor therapy with the use of radio-labeled anti-tenascin-C monoclonal antibodies (55). Integrin-αV is another molecule that interacts with periostin and not only plays a role in angiogenesis, but also in the proliferation, migration and invasion of the tumor cells (56). Integrins coordinate the interaction of the extracellular matrix with the cytoskeleton. Tenascin-C preferentially binds to integrin-αVβ3 (56). The expression of integrin-αV is increased during physiological angiogenesis (56, 57) and has been found upregulated in vascular malformations just as we found to be the case with respect to periostin (58). In the CNS, VEGF triggers the expression of integrin-αV by pericytes and endothelial and glial cells. The expression of integrin $\alpha_V\beta_3$ parallels the progression from low-grade to high-grade tumors (5, 59–61). Literature data point to upregulation of periostin expression by hypoxia and VEGF-driven angiogenesis (62–66). However, the expressional regulation seems more complex from the present findings. We found high expression of periostin in GBM as opposed to low expression in the lower-grade gliomas in which hypoxia is not yet dominant. However, hypoxia certainly drives angiogenesis in GBM, but the vascular proliferation in PA seems not essentially hypoxia-driven while the hypertrophied vasculature differs in architecture and protein expression patterns (67). We conclude that periostin expression contributes to aberrant angiogenesis, both in malformations and in gliomas, and that the formation and structure of the malformed blood vessels is a result of the cellular and environmental context of its expression.

Recently, it was suggested that periostin plays a role in the maintenance of stem cells in normal bone marrow and in the maintenance of leukemia-initiating cells (68). Among various extracellular matrix proteins, periostin was identified as important for the glioma stem cell niche (69). A similar effect of periostin on breast cancer stem cells has been described (32). In mice, it was shown that glioma stem cells defined by expression of SOX2 and OLIG2 produce periostin that stimulates the recruitment of tumor-associated macrophages through $\alpha_V\beta_3$ integrin signaling. In addition, periostin remodels the tumor micro-environment in concert with osteopontin and proteins of the CNN family by interaction with tumor-associated macrophages and other immune cells (69). Following their arrival in the brain, monocytes differentiate into M2-like macrophages that promote tumor progression and counteract the antitumor effects of T lymphocytes. In GBM the secretion of periostin was preferentially seen around cells marked by OLIG2 and SOX2 (69). In this study, we identified the PDGFRβ$^+$ pericytes as the source of periostin production. The perivascular RNA expression and the overlap with the protein by scattered cells corroborate this finding. In another recent paper, periostin secretion has been associated with glioma cell invasion, adhesion, migration and stem cell survival in gliomas, and correlates directly with glioma grade (70). Periostin expression was reportedly found in tumor cells but no double labeling for GFAP or other markers was provided.

The association between the expression levels of periostin on the one hand, and glioma grade and patient survival on the other, was explained by the increase in angiogenesis during glioma progression (70). Although we found expression of periostin in cultured astrocytes, we were unable to detect any expression in astrocytic tumor cells in the human glioma samples. We are, however, unable to confirm expression of periostin by any of the numerous cells expressing OLIG2 or SOX2, and we did not observe its overlap with the expression of SOX2 or OLIG2. Unfortunately, Zhou et al did not include immunohistochemistry to PDGFR in their study, which might have identified the true origin of periostin in their GBM samples. Therefore, we are unable to confirm expression of periostin by glioma tumor cells or glioma stem cells. The data indicate that the periostin expression in mice is only partly recapitulated in man.

In conclusion, periostin expression in gliomas serves a variety of functions that relate to neo-angiogenesis, an association that is also present in cerebral vascular malformations. The expression is significant in gliomas with microvascular proliferation (GBM and PA). We identified $PDGFR^{+}$ pericytes as the source of periostin, a finding that is relevant to new anti-angiogenic strategies in glioma.

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