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ORIGINAL PAPER

Malignant gliomas induce and exploit astrocytic mesenchymal-like transition by activating canonical Wnt/β-catenin signaling

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Abstract The complex microenvironment of malignant gliomas plays a dynamic and usually cancer-promoting role in glioma progression. Astrocytes, the major stromal cells in the brain, can be activated by glioma microenvironment, resulting in a layer of reactive astrocytes surrounding the gliomas. Reactive astrocytes are universally characterized with the upregulation of glial fibrillary protein and glycoprotein podoplanin. In this work, we investigated the role of reactive astrocytes on malignant glioma microenvironment and the potential mechanism by which glioma cells activated the tumor-associated astrocytes (TAAs). The reactive astrocytes were observed around gliomas in the intracranial syngeneic implantation of rat C6 and mouse GL261 glioma cells in vivo, as well as primary astrocytes cultured with glioma cells condition medium in vitro. Besides, reactive astrocytes exhibited distinct epithelial-to-mesenchymal (-like) transition and enhanced migration and invasion activity, with the decrease of E-cadherin and concomitant increase of vimentin and matrix metalloproteinases. Furthermore, canonical Wnt/β-catenin signaling was activated in TAAs. The Wnt/β-catenin pathway inhibitor XAV939 and β-catenin plasmid were used to verify the regulation of Wnt/β-catenin signaling on TAAs and their invasion ability. Taken together, our findings established that glioma cells remarkably activated astrocytes via upregulating Wnt/β-catenin signaling, with obviously mesenchymal-like transition and increased migration and invasion ability, indicating that glioma cells may stimulate adjacent astrocytes to degrade extracellular matrix and thereby promoting tumor invasiveness.

Keywords Tumor-associated astrocytes (TAAs) · Malignant glioma · GFAP · PDPN · Wnt/β-catenin signaling · Tumor microenvironment

Introduction

Malignant gliomas are histologically heterogeneous malignant tumors of the central nervous system, with inexpressive response to current therapeutic approaches including surgical resection followed by chemotherapy or radiation therapy [1, 2]. A major obstacle for successful therapy is the topographically diffuse nature of the malignant gliomas [3]. Thus, developing a new strategy that targets the aggressive cells or inhibits their migratory capacity is probably to promote the advance of glioma therapy.

Numerous studies have demonstrated that the tumor microenvironment actively contributes to tumor initiation, progression and metastasis [4, 5]. The microenvironment of malignant gliomas includes various cellular components including neurons, astrocytes, microglias, pericytes, fibroblasts and endothelial cells [6]. Astrocytes, comprised approximately 50 % of the cells in the brain, have traditionally been considered as supportive glial cell components in neural tissue, with additional roles in homeostatic

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and communicative functions. In fact, astrocytes are also capable of responding to many kinds of insults to the central nervous system (CNS) [7, 8]. In these conditions, astrocytes become activated with distinctive morphological and biochemical features such as cell hypertrophy, upregulation of intermediate filaments (glial fibrillary acidic protein, GFAP) and increased cell proliferation [9]. The GFAP staining of both organotypic brain slice cultures and human brain metastasis samples reveals that activated astrocytes around tumor cells form a barrier at the brain–tumor interface as well [10, 11]. Podoplanin (PDPN), a mucin-like transmembrane glycoprotein, plays a major role in various cellular processes, including tumor migration and invasion. It has been reported to be a novel cell surface marker for reactive astrocytes in the vicinity of gliomas and nonneoplastic brain lesions [12]. Meanwhile, overexpression of PDPN is correlated with malignant progression of astrocytic tumors [13]. All these implicate that the intricate cross talk between reactive astrocytes and tumor cells may influence tumor invasiveness and promote tumor malignancy.

In fact, glioma-associated reactive astrocytes have been shown to contribute to the altered brain microenvironment and facilitate the survival and invasion of malignant gliomas [6, 14]. Astrocytes attracted to the glioma tumors have been shown to promote brain invasion by secretion of glial-derived neurotrophic factor (GDNF) [15] and IL-23 [16]. Besides for paracrine regulation, a gap junction channel protein connexin43 which is enhanced significantly in glioma-associated astrocytes could facilitate the formation of tumor invasive niche and increase the ability of glioma cells invasion [17]. Furthermore, it is well known that glioma invasion can be promoted by matrix metalloproteinase (MMPs)-mediated degradation of the extracellular matrix in the glioma environment, while tumor-associated astrocytes have also been found to be able to support glioma invasion by increased expression and activation of extracellular matrix degrading proteases such as MMP2, MMP9 and membrane-type 1 matrix metalloproteinase (MT1-MMP) [18]. Although the role of astrocytes on the development of malignant gliomas is gradually characterized, the impact of gliomas on their neighboring astrocytes still remains unclear.

In this work, we established intracranial glioma animal model and identified numbers of activated astrocytes adjacent to gliomas with high expressions of GFAP and PDPN. The primary astrocytes were cultured with CM, and it was found that reactive astrocytes exhibited distinct mesenchymal-like transition and enhanced invasion ability, with the decrease of E-cadherin and concomitant increase of vimentin and MMPs. Wnt/β-catenin signaling was activated during this process, which was verified by the reduction of GSK3β-mediated β-catenin degradation and the increase of β-catenin nuclear translocation using a specific inhibitor XAV939 and β-catenin overexpression plasmid. Our study showed that glioma cells exploited astrocytes to increase breakdown of extracellular matrix and thereby promoting tumor invasiveness.

Materials and methods

Animals

Sprague–Dawley rats were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The TCF/Lef:H2B-GFP transgenic mice (C57) were purchased from the Jackson laboratory (Farmington, CA, USA). Rats and Mice were housed in a temperature controlled room with a standard 12-h light/12-h dark cycle. All procedures were approved and performed in accordance with guidelines of the ethics approval from the Experimentation Ethics Review Committee of China Pharmaceutical University.

Cell lines and tumor-conditioned medium (CM)

Rat C6 and mouse GL261 glioma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), routinely cultured in DMEM containing 10 % fetal bovine serum (Wisent, Nanjing, China), 1 % penicillin and streptomycin, and maintained in a humidified atmosphere of 95 % air, 5 % CO_2 at 37 °C. Glioma cells derived conditioned medium (CM) was produced by the incubation of C6 cells in serum-free medium for 48 h.

Reagents and antibodies

Anti-GFAP, anti-E-cadherin, anti-vimentin, anti-GSK3β and anti-p-β-catenin (Ser552) were obtained from CST Technology Inc. (Danvers, MA, USA). Anti-β-catenin and anti-MMP9 were purchased from Abcam (Cambridge, MA, USA). Anti-cyclin D1, anti-p-GSK3β (S9) and anti-laminA were acquired from Bioworld Technology Inc. (St. Louis, MN, USA). Anti-podoplanin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and anti-GAPDH was from Proteintech (Chicago, IL, US). Secondary antibodies (goat anti-rabbit HRP or goat anti-mouse HRP) were purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). Alexa fluor 546 donkey anti-rabbit lgG and Alexa fluor 488 donkey anti-mouse lgG were purchased from Life Sciences (Farmingdale, NY, USA). DAPI was acquired from Life Sciences, and Phallodin-FITC was from Sigma Aldrich (St. Louis, MO, USA).

XAV939 was from Selleck Chemicals (Houston, TX, USA). β-catenin plasmid was obtained from Dr. Haiwei

Zhang (Chongqing Cancer Hospital, Chongqing, China), pGL-3 control plasmid was from Promega (Madison, WI, USA), and transfection reagent was from Vazyme Biotech Co., Ltd. (Nanjing, China).

Primary culture of rat astrocytes

Primary brain astrocytes were isolated from the cerebral cortices of 1-day-old SD rats [19]. In brief, animals were killed, meninges were removed, and cortices were minced and gently dissociated and then resuspend with DMEM/F 12 (Gibco) medium containing 100 IU/mL of penicillin, 100 mg/mL of streptomycin, 10 % fetal bovine serum (Gibco), transferred into 75 cm^2 culture flasks (5×10^{10} cells/flask), and incubated at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2. After 40 min, the adherent cells were removed by transferring the supernatant soliquoid to a new culture flask. The medium was changed after 2 days, and thereafter twice a week. After 9 days of cell culture, floating microglias were removed by shaking the flask vigorously. The culture was subcultured twice before the experiments were performed. This protocol produced cultures with >95 % glial fibrillary acidic protein (GFAP)-positive cells.

Western blot assay

Cell lysates were separated by 10 % SDS–polyacrylamide gel electrophoresis and electrobloted onto PVDF membranes by standard procedures. After blocking with 3 % BSA in PBS for 1.5 h at 37 °C, the membranes were incubated for another 1.5 h with the primary antibody at 37 °C, followed by peroxidase-conjugated secondary antibody for 1 h at 37 °C. Immunoreactive protein bands were detected with Tanon 5200 chemiluminescence imaging system (Shanghai, China).

Real-time quantitative PCR

Total RNA was extracted from astrocytes using TRIzol®-Reagent (Invitrogen, Carlsbad, CA, USA) and then the quality of total RNA was assessed by the ratio of A_{260}/A_{280} using a BioPhotometer (Eppendorf, Hamburg, Germany). 1 μg of total RNA was reversely transcribed using First-strand cDNA synthesis superMix (TransGen Biotech, Beijing, China). Quantitative real-time PCR (q-PCR) was conducted following the protocol supplied by Vazyme SYBR Green Master Mix kit. Sequences of the primers for target genes tested in this study were designed as in Table 1. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those for GAPDH (ΔCt).

Intracranial glioma models

Animals were anesthetized with 3 % pentobarbital sodium, and a 1.0-mm-diameter hole was drilled through the skull. C6 cells (5×10^5 cells) in 10 μL PBS were injected intracerebrally into the striatum of SD rats (position: 3.0 mm lateral to the midline, 1.0 mm anterior to the bregma and 6.0 mm ventral to the dura) and GL261 cells (1×10^5 cells) in 2 μL PBS were injected intracerebrally into the striatum of C57 mice (position: 2.5 mm lateral to the midline, 1.0 mm anterior to the bregma and 3.0 mm ventral to the dura). At about 14 days after injection, animals were killed and brains were fixed with 4 % paraformaldehyde for histology and immunohistochemistry.

Immunofluorescent staining

Cells were washed with PBS and fixed with methyl alcohol for 20 min at −20 °C. TritonX-100 for 20 min at 4 °C was used for permeabilization. Cells were blocked with PBS containing 3 % BSA for 1 h at 37 °C. After that, cells were incubated with the primary antibody for 2 h at 37 °C followed by the secondary antibody for 1 h at 37 °C. Nuclei were stained with DAPI. Immunofluorescence photomicrographs were captured using a fluorescent microscope (Carl Zeiss, Germany).

Immunohistochemistry

Tissues were embedded and cut into 5-μm brain paraffin section. After blocked by goat serum, sections were incubated with primary antibodies for 2 h at 37 °C. Slides were washed three times in PBS and exposed to HRP-conjugated secondary antibodies for 2 h at room temperature. And then immunohistochemistry kit of KeyGen was used. The immunoreactivity was finally examined under a microscope of Jiangnan XD-202 (Nanjing, China).

In vitro invasion assay

In vitro invasiveness of astrocytes was examined using the BD Matrigel invasion assay system (BD Biosciences, San Jose, CA, USA). 5×10^4 Cells resuspended by DMEM or CM were plated onto the matrigel-coated upper chamber. Serum-containing media were placed in the bottom chamber. After incubation at 37 °C for 24 or 48 h, non-migrated cells from the upper chamber were removed, and migrated cells on the lower side of the membrane were fixed, stained with crystal violet, and counted.

Wound healing assay

About 1×10^5 cells were seeded in six-well plates, and an incision was made in the central area of the confluent

culture to create an artificial wound. In turn, cells were incubated with conditioned media of C6 for 24 h or 48 h. Images of the wound area were captured by microscope.

Statistical analysis

All experiments reported here were performed with at least triplicate independent replications. All data were represented as means ± SDs and compared by one-way analysis of variance (ANOVA) and Student's *t* test using SPSS software (SPSS Inc., Chicago, IL, USA). *P < 0.05 and **P < 0.01 were considered to be statistically significant.

Results

Tumor-associated astrocytes were activated with the overexpression of GFAP and PDPN

The presence of reactive astrocytes in brain tumor is usually characterized by their enhanced GFAP and PDPN immunoreactivity [12]. To corroborate that reactive astrocytes appeared in the peri-tumoral area with overexpressed GFAP and PDPN, we established an in vivo rat model consisting of intracranial syngeneic implantation of rat C6 glioma cells. Implanted C6 glioma cells were observed to infiltrate into surrounding brain tissues (Fig. 1a). There was a noticeable increase of PDPN and GFAP protein levels in the tumor border cells compared to the tumor core and distal astrocytes, indicating that the astrocytes around gliomas were activated by their neighboring glioma cells (Fig. 1b).

To further investigate the potential mechanism underlying reactive astrogliosis induced by glioma cells in vitro, primary astrocytes were cultured with C6 CM to imitate the activation of TAAs. As shown in Fig. 1d–h, the protein levels of GFAP and PDPN in primary astrocytes were significantly upregulated by the stimulation of C6 CM for 24 h, accompanied by the increased mRNA levels of GFAP. This demonstrated that primary astrocytes were activated by C6 CM treatment (Fig. 1e, f). Importantly, after cultured with C6 CM for 24 h, primary astrocytes displayed abnormal mesenchymal-like cell shape and a loss of well-organized cell–cell contact (Fig. 1c, d, h). Taken together, tumor-associated astrocytes can be activated by glioma cells with upregulated GFAP and PDPN.

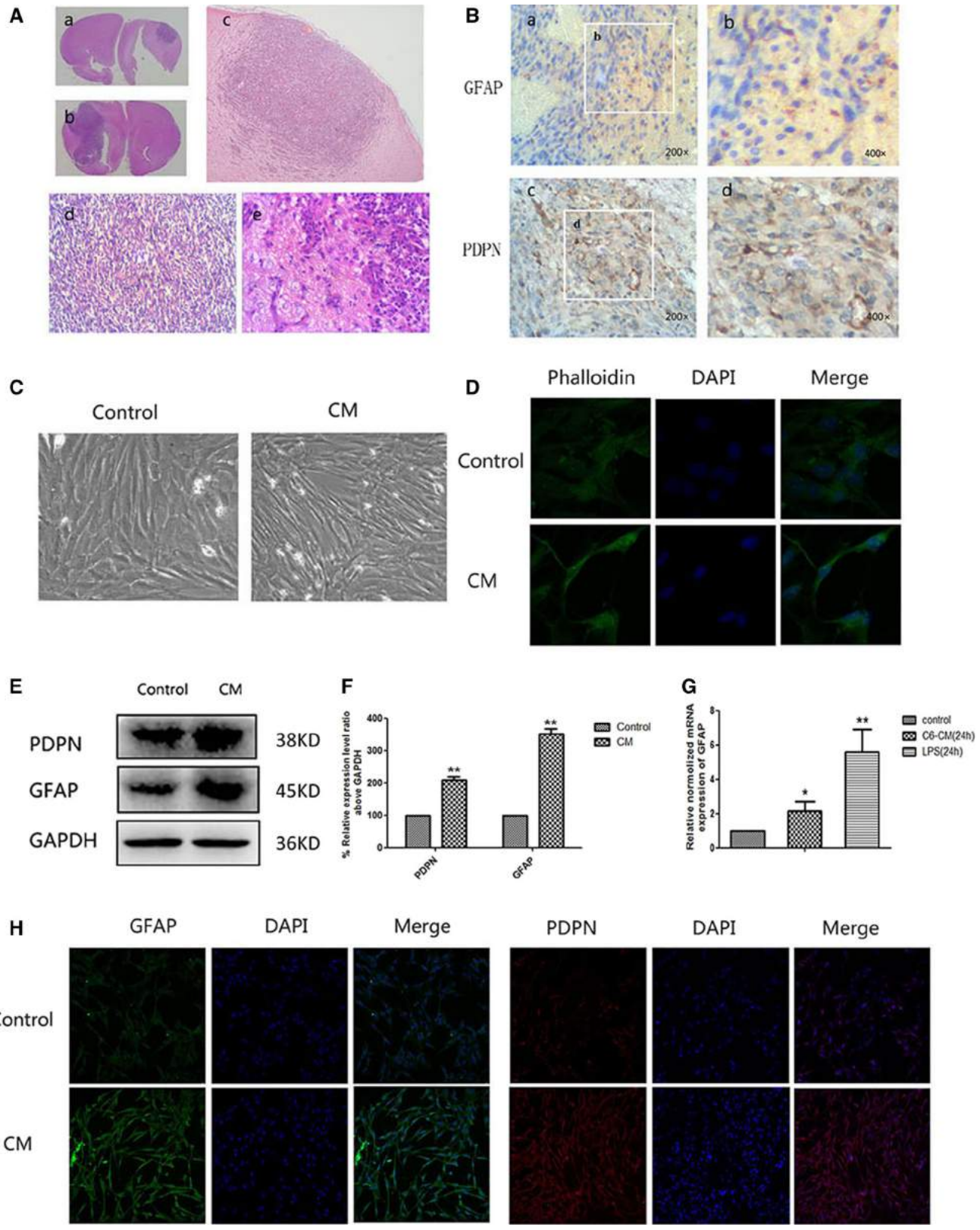
Fig. 1 Expressions of GFAP and PDPN in tumor-associated astrocytes were upregulated both in vivo and in vitro. **a** Representative images of hematoxylin and eosin (H&E) stains in intracranial implantation of C6 cells were showed. **b** GFAP and PDPN immunoreactivity were visualized by brown staining. **b** and **d** Magnified images of GFAP and PDPN in TAAs. **c** Primary astrocytes cultured with normal medium or with glioma CM showed different morphology. **d** The fluorescence of TAAs was detected by phalloidin staining. **e** and **f** The protein expressions of GFAP and PDPN were measured by Western blot. **g** The mRNA expression of GFAP was determined by real-time PCR. LPS was used as a positive control. **h** Immunofluorescence stainings with GFAP antibody (*green*) and PDPN antibody (*red*) were showed. DAPI was for visualization of the nuclei (*blue*). Data (means ± SDs) were representative of at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with control ▶

Glioma cells caused mesenchymal-like transition of tumor-associated astrocytes

Based on the above results, we further detected the expression levels of epithelial-to-mesenchymal-related markers as well as the migration and invasion ability of TAAs. After stimulated with C6 CM for 24 h, the increased protein expressions of vimentin and MMP9 and decreased expression of E-cadherin were observed in TAAs (Fig. 2a). Real-time PCR showed similar results, with upregulation of MMP2, MMP7 and downregulation of E-cadherin gene (cdh1) (Fig. 2b). Subsequently, cell migration and invasion assays showed that C6 CM-treated

Table 1 List of primers used for quantitative real-time PCR

Gene	Primer sequence	
	Forward	Reverse
GAPDH	5′-TGGTATCGTGGAAGGACTCA-3′	5′-CAGTAGAGGCAGGGATGATG-3′
GFAP	5′-GCTCCAAGATGAAACCAACC-3′	5′-CCAGCGACTCAACCTTCCT-3′
c-myc	5′-ATCACCAGCAACAGCAGAGC-3′	5′-GCAACATAGGACGGAGAGCA-3′
c-jun	5′-TGACTGCAAAGATGGAAACG-3′	5′-CCAGGTTCAAGGTCATGCTC-3′
ccnd1	5′-ATTTGCACACCTCTGGCTCT-3′	5′-TGTGGTCCCTACCCTCCATA-3′
MMP2	5′-CCCGTTATGAGACCCTGAGC-3′	5′-AGACCAATCGTGCCTCCATC-3′
MMP7	5′-CTGGGTCTGGGTCACTCTTC-3′	5′-AACTTCTGGATGCCTGCAAT-3′
cdh1	5′-ATTACAAGTTCCCGCCATCC-3′	5′-GGCTCTTTGACCACCGTTCT-3′


A
a
b
c
d
e
B
GFAP
PDPN
200×
400×
C
Control
CM
D
Phalloidin
DAPI
Merge
Control
CM
E
Control
CM
PDPN
GFAP
GAPDH
38KD
45KD
36KD
F
% Relative expression level ratio above GAPDH
Control
CM
PDPN
GFAP
G
Relative normolized mRNA expression of GFAP
control
C6-CM(24h)
LPS(24h)
H
GFAP
DAPI
Merge
PDPN
DAPI
Merge
Control
CM

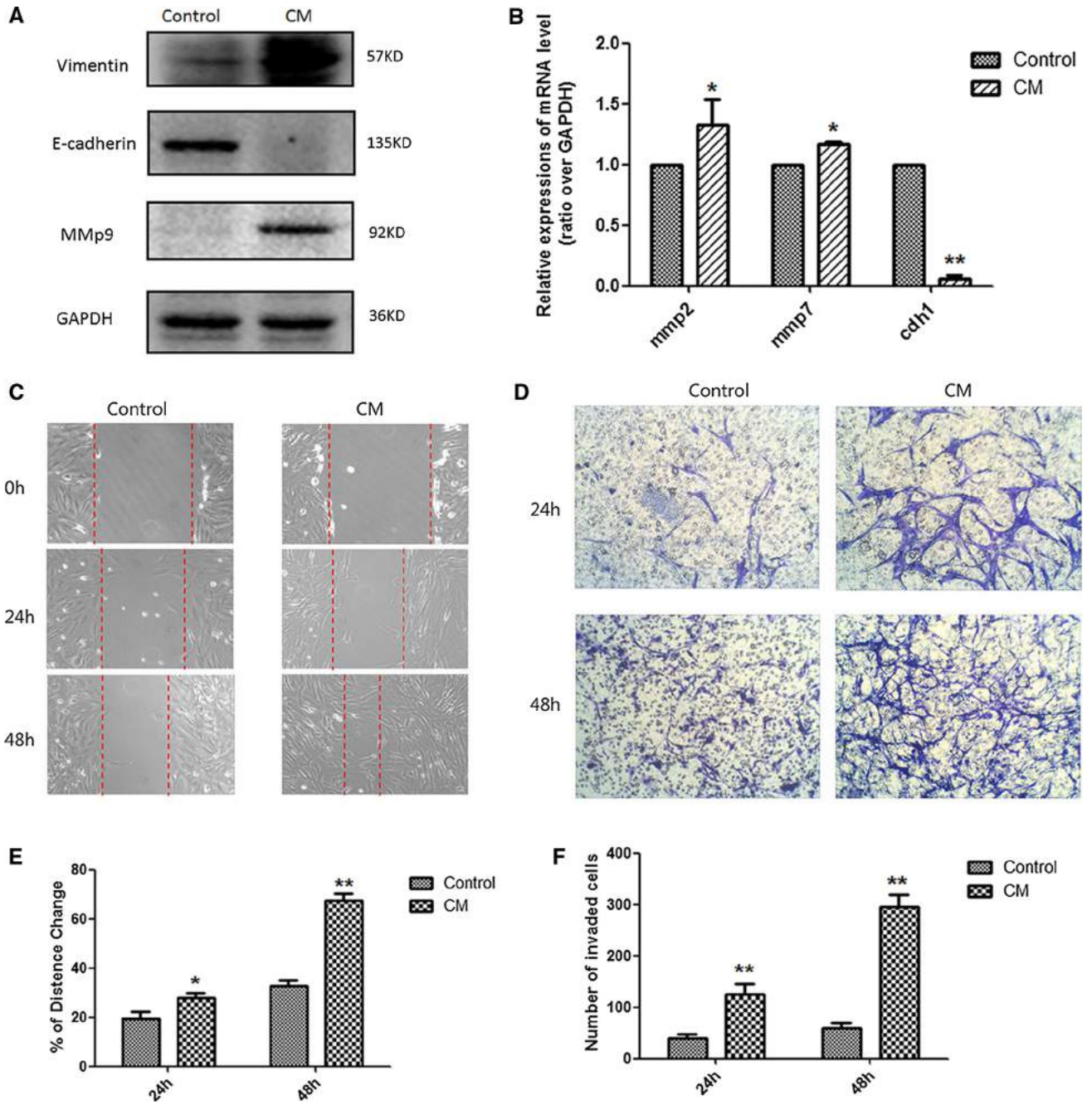


Fig. 2 Glioma CM promoted the invasiveness of astrocytes. **a**, **b** The migration of astrocytes was measured after 24 and 48 h in a 6-well format with or without CM. **c**, **d** The invasion of TAAs was detected by transwell assay. The upper chamber with matrigel contained 100 μL cell suspension with serum-free medium, and lower chamber was loaded with 500 μL DMEM or glioma CM with 10 % FBS. The migrated cells were detected after 24 and 48 h. Cells permeating the membrane filters were stained by crystal violet and counted using a light microscope. **e** Western blot of vimentin, E-cadherin and MMP9. **f** The expressions of MMP2, MMP7 and cdh1 mRNA were measured using real-time PCR. GAPDH was used as an endogenous housekeeping gene. Data (means ± SDs) were representative of at least three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with control

astrocytes exhibited significantly accelerated wound closure (Fig. 2c, e) and increased cell invasion activity both at 24 and 48 h (Fig. 2d, f). Collectively, our results suggested that glioma cells activated astrocytes, resulting in mesenchymal-like transition and enhanced expression of MMPs as well as increased invasion and migration potency.

Tumor-associated astrocytes were activated by glioma cells via canonical Wnt/β-catenin signaling pathway

β-catenin is a crucial factor in Wnt/β-catenin signaling that regulates the tumor cell proliferation and invasion in malignant glioma. Various evidences show that Wnt/β-catenin signaling plays an important role in EMT [20]. We then looked at whether canonical β-catenin signaling was involved in the glioma cell-induced activation of astrocytes. Compared with control astrocytes, total β-catenin, p-β-catenin (Ser552) and p-GSK3β (S9) expressions were significantly increased in TAAs (Fig. 3a, b). The phosphorylation at Ser552 of β-catenin is associated with its nuclear accumulation and transcriptional activation of its target genes. Next, the increased nuclear translocation of β-catenin in TAAs was found as shown in Fig. 3d, e, and the transcriptions of three downstream genes of β-catenin signaling including ccnd1, c-myc and c-jun were also significantly enhanced (Fig. 3c). These findings suggested that canonical Wnt/β-catenin signaling was involved in this glioma cell-induced activation of astrocytes.

We then established a GL261 intracranial animal model in Tcf/Lef C57 mice. The TCF/Lef:H2B-GFP transgenic line is a faithful readout of canonical Wnt signaling activity at the single-cell-level resolution. It represents a unique tool for live imaging the in vivo processes triggered by Wnt/β-catenin signaling [21]. At the periphery of glioma, cells with green fluorescence suggested the transcriptional activation of β-catenin (Fig. 2f). By costaining with anti-PDPN, we showed that the red fluorescence staining PDPN merged with green fluorescence, which demonstrated that Wnt/β-catenin signaling was activated in TAAs around implanted glioma cells. All these evidences proved that glioma cells induced the activation of astrocytes via activating canonical Wnt/β-catenin signaling.

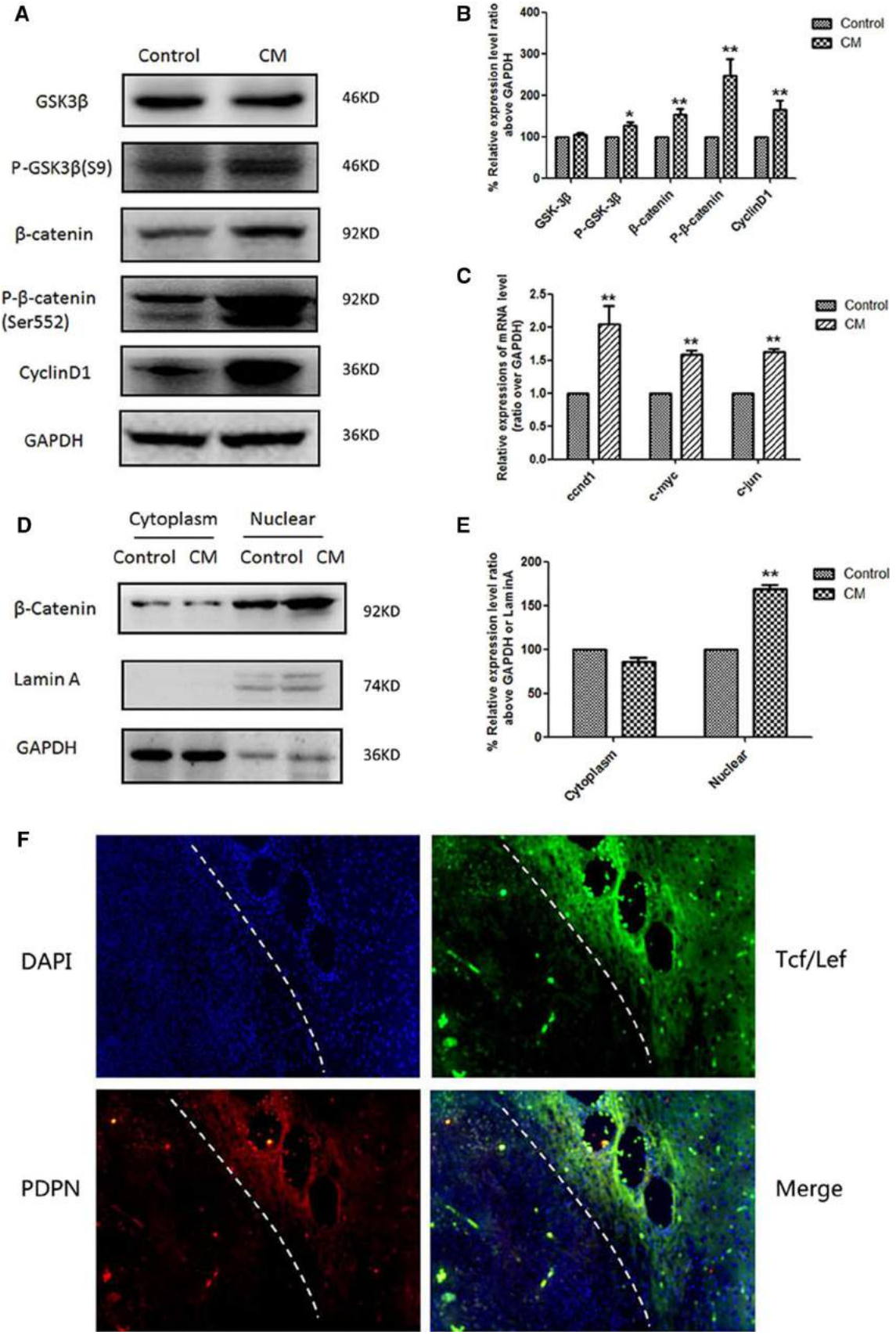
The enhanced invasion of TAAs was mediated by canonical Wnt/β-catenin signaling

To explore whether increased invasion of TAAs was due to the activation of β-catenin signaling, a selective and potent Wnt/β-catenin inhibitor XAV939 was used in TAAs. XAV939 stimulates β-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex, resulting in the reduction of β-catenin nuclear accumulation. As shown in Fig. 4a, c, XAV939 significantly downregulated β-catenin expression and the transcription of its downstream genes such as ccnd1, c-myc and c-jun (Fig. 4c). XAV939 was able to reverse the activation of astrocytes induced by glioma CM, as verified by the downregulation of GFAP and PDPN (Fig. 4a). Invasiveness of TAAs with the treatment of XAV939 was consistently decreased (Fig. 4g), and the alterations of MMP2 and MMP7 mRNA levels presented similar tendency (Fig. 4e).

On the contrary, astrocytes transfected with β-catenin overexpression plasmid presented the opposite effect. Overexpressed β-catenin in astrocytes upregulated GFAP and PDPN protein levels as shown in Fig. 4b, d. At the same time, these astrocytes showed enhanced invasion potency (Fig. 4g) and coinstantaneous upregulation of MMP2 and MMP7 mRNA levels (Fig. 4f). Taken together, these results showed that the activation and invasion ability of TAAs was regulated by canonical Wnt/β-catenin signaling.

Discussion

Recently, there are growing evidences suggesting that astrocytes also play a critical role in the evolution of many primary brain tumors, gliomas, and a less prominent, though significant, role in the progression of metastases [22, 23]. Using the intracerebral transplantation model of malignant glioma cells in GFAP-luciferase mice, Lee et al. showed a significantly positive correlation between tumor size and astrogliosis [24]. Indeed, astrocytes attracted to the tumors are proven to promote tumor cell growth by secretion of IL-1, IL-6, FGF, TGF-β1 and TNFα [25]. Astrocytes are also able to support glioma invasion by increasing the expression of GDNF, IL-23 and promoting the activation of MMP2, MMP9 and MT1-MMP expressed by gliomas.


A
Control
CM
GSK3β
46KD
P-GSK3β(S9)
46KD
β-catenin
92KD
P-β-catenin (Ser552)
92KD
CyclinD1
36KD
GAPDH
36KD
B
% Relative expression level ratio above GAPDH
Control
CM
GSK-3β
P-GSK-3β
β-catenin
P-β-catenin
CyclinD1
C
Relative expressions of mRNA level (ratio over GAPDH)
Control
CM
ccnd1
c-myc
c-jun
D
Cytoplasm
Nuclear
Control CM Control CM
β-Catenin
92KD
Lamin A
74KD
GAPDH
36KD
E
% Relative expression level ratio above GAPDH or LaminA
Control
CM
Cytoplasm
Nuclear
F
DAPI
Tcf/Lef
PDPN
Merge

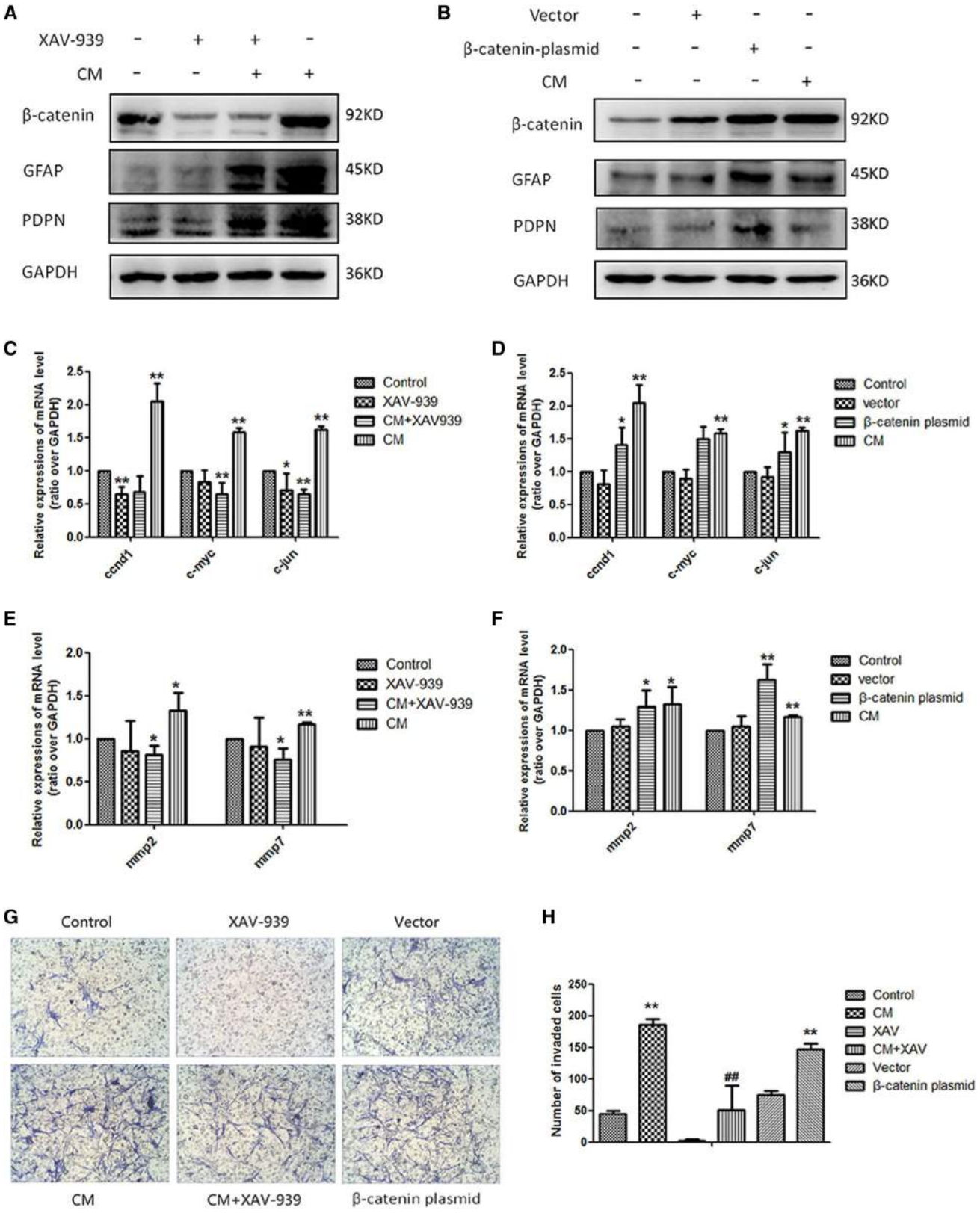
◀**Fig. 3** Canonical Wnt/β-catenin signaling was activated in tumor-associated astrocytes. **a** The expressions of GSK3β, p-GSK3β (S9), β-catenin, p-β-catenin (ser552) and cyclin D1 were measured by Western blot, and the quantifications of their expressions were normalized to GAPDH (**b**). **c** The mRNA expressions of ccnd1, c-myc and c-jun in astrocytes and TAAs were measured using real-time PCR. GAPDH was used as an endogenous housekeeping gene. **d** The nuclear translocation of β-catenin and quantification of its expression normalized to GAPDH (**e**). **f** Intracranial implantation of GL261 cells in Tcf/Lef C57 mouse and the immunofluorescence stainings of β-catenin (*green*), PDPN (*red*) and DAPI (*blue*) were detected. Results were representative of those obtained from three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with control

Besides for GFAP, PDPN has been recently reported to be a novel cell surface marker for reactive astrocytes in the vicinity of gliomas as well as nonneoplastic brain lesions [12]. Using a syngeneic intracranial glioma rat model, we showed that both PDPN and GFAP were highly expressed in a subset of astrocytes within and adjacent to gliomas, indicating that the astrocytes around gliomas were activated by their neighboring glioma cells. However, different from the classical reactive astrocytes in nonneoplastic brain lesions, glioma reactive astrocytes did not exhibit cell hypertrophy changes. As many factors secreted by reactive astrocytes are released by other cell types as well, it is still extremely difficult to assess the effects of astrocytes on cancer progression [2]. Therefore, we attempted to establish an in vitro model for further mechanism study of glioma reactive astrocytes. The primary astrocytes cultured with C6 CM showed overexpressed GFAP and PDPN demonstrating that the activation of TAAs was successfully imitated.

Several studies prove that the overexpression of PDPN in cancer-associated fibroblasts is able to enhance peripheral tumor progression [26]. Besides, tumor cells often upregulate PDPN as they undergo epithelial mesenchymal transition, and this upregulation is correlated with increased tumor motility and metastasis [27]. Our study showed that TAAs with highly expressed PDPN had similarly EMT-like transition and enhanced invasion ability. Upregulation of mesenchymal markers vimentin and downregulation of epithelial markers E-cadherin, as well as the upregulation of MMPs, were also observed in TAAs. Collectively, it was demonstrated that glioma cell CM could activate astrocytes in vitro, resulting in its EMT-like transition. The loss of well-organized cell–cell contact in TAAs induced by EMT-like transition could help to weaken the barrier around glioma and thereby developing a loose microenvironment for tumor cell migration and invasion.

It is well known that EMT plays a critical role in epithelial cancer invasion and progression. Recently, Kahlert et al. linked EMT to glioblastoma progression and invasion [28]. It has been demonstrated that activation of the canonical Wnt/β-catenin pathway promotes the migratory and invasive capacity of glioblastoma. Modulation of Wnt signaling alters the expression of epithelial-to-mesenchymal transition activators, for example ZEB1, suggesting a role of this process in the regulation of glioma motility [20, 29]. β-catenin, the major mediator of the canonical Wnt signaling, is degraded through interactions with Axin, APC, and the protein kinase GSK-3 in cells not exposed to a Wnt signal [30]. When the canonical Wnt signal is activated, the inactivation of GSK3β via phosphorylation at Ser9 increases the cytoplasmic pool of β-catenin protein, accompanied by its translocation to the nucleus and interaction with TCF/LEF, resulting in transcriptional activation of the Wnt-responsive genes including cyclin D1, MMPs and others [31]. Both in vitro and in vivo study proved the activation of β-catenin signaling in TAAs responded to gliomas. Furthermore, the expression of p-GSK3β (S9) was upregulated in TAAs, which demonstrated that the increased β-catenin nuclear translocation was regulated by the reduction of GSK3β-mediated β-catenin degradation. Besides, the regulation of β-catenin on TAAs' activation and invasion was further verified by the specific inhibitor XAV939 and β-catenin overexpression plasmid.

The cross talk between the cancer cells and their non-cellular surroundings, the extracellular matrix (ECM), is one of the important steps in cancer invasion and metastases. Targeting ECM can disrupt cancer progression effectively [32]. MMPs are a family of zinc-dependent endopeptidases that play a crucial role in regulation of ECM in the tumor microenvironment [33]. The upregulated expression of MMPs in tumor-activated astrocytes may help to break the ECM barrier around glioma and facilitate the invasion of glioma cells.


A
XAV-939 - + + -
CM - - + +
β-catenin
GFAP
PDPN
GAPDH
92KD
45KD
38KD
36KD
B
Vector - + - -
β-catenin-plasmid - - + -
CM - - - +
β-catenin
GFAP
PDPN
GAPDH
92KD
45KD
38KD
36KD
C
Relative expressions of mRNA level (ratio over GAPDH)
Control
XAV-939
CM+XAV939
CM
ccnd1
c-myc
c-jun
D
Relative expressions of mRNA level (ratio over GAPDH)
Control
vector
β-catenin plasmid
CM
ccnd1
c-myc
c-jun
E
Relative expressions of mRNA level (ratio over GAPDH)
Control
XAV-939
CM+XAV-939
CM
mmp2
mmp7
F
Relative expressions of mRNA level (ratio over GAPDH)
Control
vector
β-catenin plasmid
CM
mmp2
mmp7
G
Control
XAV-939
Vector
CM
CM+XAV-939
β-catenin plasmid
H
Number of invaded cells
Control
CM
XAV
CM+XAV
Vector
β-catenin plasmid

◀**Fig. 4** Invasion of TAAs was regulated by β-catenin. β-catenin overexpression plasmid (**a**) and the β-catenin signaling inhibitor XAV939 (**b**) were used in astrocytes, and the proteins levels of β-catenin, GFAP and PDPN were measured. The mRNA levels of ccnd1, c-myc and c-jun (**c**, **d**), as well as MMP2 and MMP7 (**e, f**), were measured using real-time PCR. **g**, **h** The invasive ability was tested by transwell. Data (means ± SDs) were representative of at least three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with control

In conclusion, we first verified that glioma cells CM could induce the activation of astrocytes in vitro. The glioma-activated astrocytes presented EMT-like changes with increased MMPs expressions and invasion ability. This process was regulated by canonical Wnt/β-catenin signaling. Our study provides evidences for understanding the effects of astrocytes in tumor microenvironment and raises new approaches to control glioma invasion and progression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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