Downregulation of Pygopus 2 inhibits vascular mimicry in glioma U251 cells by suppressing the canonical Wnt signaling pathway

HAIDONG WANG[1], JIANHUA FU[2], DIANSHUANG XU[1], WEIWEI XU[1], SHIYONG WANG[1], LIU ZHANG[1] and YONGSHENG XIANG[1]

[1]Department of Neurosurgery, Affiliated First Hospital of Jinan University, Guangzhou, Guangdong 510630; [2]Department of Neurosurgery, The Fifth Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 580001, P.R. China

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Abstract. Gliomas are the most common type of malignant primary brain tumor, and the Wnt signaling pathway is associated with glioma malignancy. Pygopus protein plays an important role in developmental brain patterning, and has been identified to be a component of the Wnt signaling pathway. In the present study, the Pygopus 2 (Pygo2) protein was examined in 80 glioma tissue samples. Short hairpin (sh) RNA-Pygo2 was transfected into glioma U251 cells, and the cell proliferation, colony formation and bromodeoxyuridine (BrdU) incorporation were analyzed. Western blot analysis and reverse transcription-polymerase chain reaction were used to detect the expression of Pygo2. A vascular mimicry assay was performed to examine the vascular mimicry of U251 cells. A luciferase reporter assay was used to detect the β-catenin/Wnt system. The cyclin D1 protein was also detected using western blot analysis. The results demonstrated that inhibition of the expression of Pygo2 significantly triggered the decrease of cell proliferation, colony formation and BrdU incorporation compared with the cells treated with scramble control shRNA (shRNA-Scr). shRNA-Pygo2 transfection was found to inhibit vascular-mimicry and block the Wnt signaling pathway compared to the cells transfected with shRNA-Scr. The transfection of shRNA-Pygo2 also decreased the expression of the Wnt target gene cyclin D1. In conclusion, shRNA-Pygo2 suppressed glioma cell proliferation effectively and inhibited vascular mimicry by inhibiting the expression of cyclin D1 in the canonical Wnt/β-catenin pathway in brain glioma cells.

Correspondence to: Professor Yongsheng Xiang, Department of Neurosurgery, Affiliated First Hospital of Jinan University, 613 Huangpu West Road, Guangzhou, Guangdong 510630, P.R. China
E-mail: 19289637@qq.com; 13751836608@139.com

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Introduction

Brain tumors account for 90% of all central nervous system cancers in adults (1,2), and gliomas are the most common type of malignant primary brain tumor. Glioma is a diffuse and highly-invasive tumor, resulting in a dismal prognosis, and long-term survivors are rare (3). Gliomas are also characterized by high migratory and proliferative abilities (4). However, the molecular mechanism that triggers the development of gliomas is elusive and requires additional investigation. Therefore, the treatment strategies for glioma therapy and drugs for the treatment of the tumors are also limited (5,6). Exploration of the specific mechanism of the invasion and inhibition of glioma is required.

Previous studies have concluded that deregulation of developmentally important genes contributes to malignant disease in adults (7). For example, the canonical Wnt/β-catenin pathway is required for normal embryonic development, as well as controlled proliferation of adult stem cells (8,9). However, mutations in the components of the Wnt/β-catenin pathway have been associated with a number of human cancers derived from breast, colorectal, ovarian and neuroectodermal tissues (10,11), which is an important method for the identification of novel therapeutic targets.

Sareddy *et al* (12) reported that the Wnt/β-catenin pathway plays a major role in tumorigenesis and tumor progression in N-ethyl-N-nitrosourea-induced rat gliomas. Pu *et al* (13) demonstrated that the downregulated expression of Wnt2 and β-catenin by small interfering RNA (siRNA) suppressed malignant glioma cell growth. According to the two aforementioned studies, it was hypothesized that the Wnt/β-catenin pathway may participate in glioma progression and development.

The Pygopus genes were initially identified as a mechanism that controls Wnt/β-catenin signaling in *Drosophila melanogaster*. There are two Pygopus genes that exist in mammals, which code for the Pygopus 1 (Pygo1) and Pygopus 2 (Pygo2) proteins (14,15). Previous studies have reported that Pygo2 is

essential for the growth of epithelial ovarian (16) and breast cancers (17). Therefore, the present study investigated the role of Pygopus in glioma U251 cells, and may aid in the identification of anti-glioma drugs for glioma therapy.

Materials and methods

Tissue samples. In the present study, 80 recently resected glioma samples were obtained from patients that underwent primary surgery at the Department of Neurosurgery, Pearl River Hospital, Nanfang Medical University (Guangzhou, China). Additionally, five normal brain tissue specimens, which were obtained from internal decompression performed on patients with cerebral injury, or from temporal lobe resection for the treatment of epilepsy, were also included. All glioma samples were diagnosed by two independent neuropathologists, and were paraffin-embedded and archived. The patient protocol in the present study has been approved, and all patients provided informed consent. The present study was approved by the ethics committee of Jinan University (Jinan, China). All specimens were collected according to approved protocols at Jilin No. 1 Hospital. The paraffin-embedded tissue samples were cut into 4-μm thick sections for analysis.

Cell culture and transfection with short hairpin (sh)RNA for Pygo2. Human glioblastoma U251 cells were purchased from the Cell Center of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The U251 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies) and 100 μg/ml streptomycin. The cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2. The cells of the experimental group were treated with Pygo2 shRNA (shRNA-Pygo2) , the negative control group was treated with scramble control shRNA (shRNA-Scr) , and the control group was treated with Lipofectamine alone (Con). All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions.

Immunohistochemical (IHC) analysis. IHC analysis was performed according to the manufacturer's instructions for streptavidin-peroxidase (SP; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), and immunochemistry was performed using a rabbit anti-human Pygo2 polyclonal antibody (sc-74878; Santa Cruz Biotechnology Co., Ltd., Dallas, TX, USA) at a dilution of 1:75. Pygo2-positive tissues were defined by the presence of intracellular staining. Pygo2-negative tissues were defined by the absence of specific intracellular staining, as observed in the negative control. The expression levels of Pygo2 and cyclin D1 in each specimen were scored between 0 and 4. The absence of staining or presence of weak staining was scored as 0 and 1, respectively. Intensive staining of 25% of the tumor cells or moderately intensive staining of <80% of the cells was scored as 2. Intensive staining of 25-50% of cells or moderately intensive staining of >80% of the cells was scored as 3, and intensive staining of >50% of the tumor cells was scored as 4. The samples were independently evaluated under light microscopy (CX31 microscope; Olympus Corporation, Tokyo, Japan) by two pathologists without prior knowledge of the clinical data of the patients.

Synthesis of shRNA. Two shRNA sequences were designed in the present study. One shRNA sequence was designed using a specific pair of oligonucleotides to yield shRNA-Pygo2, the Pygo2-targeting shRNA, and the shRNA-Scr sequence was designed using the scramble control sequences. shRNA-Pygo2 was designed and synthesized based on the human Pygo2 cDNA sequences (GenBank accession no., NW_925683), followed by insertion into the pSUPER vector (Oligoengine, Seattle, WA, USA). The primers used to design the oligonucleotides are listed in Table I. The sequence was specific and no sequences were homologous with any relevant human genes.

Reverse transcription-polymerase chain reaction (RT-PCR). The total RNA of the cultured U251 glioma cells was isolated using the TRIzol reagent (Invitrogen, Groningen, Netherlands). RT-PCR was performed according to the manufacturer's instructions, and as previously described by Xu *et al* (18). The primers for Pygo2 were as follows: Forward, 5'-CCATTCTGT GTGAGGCCTCCT-3'; and reverse, 5'-CCATGCCCTCAC GGATGT-3'. The primers produced a 165-bp fragment. The primers for the internal control GAPDH were as follows: Forward, 5'-ACCACAGTCCATGCCATCA-3'; and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. The GAPDH primers produced a 450-bp fragment. The PCR reaction procedure was performed as previously described by Chen *et al* (19).

Western blot analysis. The U251 cells were harvested at 48 h post-transfection, and the total proteins were extracted with cell lysis buffer containing protease inhibitor. The cell lysis buffer was fixed as previously described by Xu *et al* (18). The western blot gels were then incubated overnight at 4˚C with mouse anti-human Pygo2 monoclonal antibody (sc-98744; dilution, 1:1,000) or mouse anti-human cyclin D1 (A12; dilution, 1:1,000) antibodies (Santa Cruz Biotechnology Co., Ltd.). The specific western blot assay method was performed as described by Wang *et al* (20). β-actin (dilution, 1:2,000; Santa Cruz Biotechnology Co., Ltd.) was employed as the internal control to quantify the protein expression of the aforementioned proteins. MTT assays were performed by adding MTT (Sigma-Aldrich, St. Louis, MO, USA) to the culture medium at a final concentration of 5 mg/ml at 37˚C. The reaction was terminated by removal of the supernatant and addition of 200 μl DMSO to dissolve the formazan product. The plates were read at 540 nm on a micro-ELISA plate reader (Thermo MK3; Thermo Labsystems, Santa Rosa, CA, USA). Each assay was performed in at least four wells. The procedure used in this assay was performed as previously described by Wang *et al* (21).

Luciferase reporter assay. The U251 cells were transiently transfected using the basic nucleofector kit (Amaxa Biosystems GmbH, Cologne, Germany), according to the manufacturer's instructions. Briefly, the cells were trypsinized, counted using a hemocytometer (15170-172, VWR International LLC, Radnor, USA) and centrifuged at 4,00 x *g*. Subsequently, ~5x10^6 cells were resuspended in 100 μl of Basic Nucleofector

Table I. Primers for designing the oligonucleotides.

Primers	Sequence
shRNA-Pygo2	
Forward	5'-GATCCCCTGTGAGGCCTCTTGTCAGAAATTCAAGAGATTTCTGACAAGAGGCCTCACATTTTTA-3'
Reverse	5'-AGCTTAAAAATGTGAGGCCTCTTGTCAGAAATCTCTTGAATTTCTGACAAGAGGCCTCACAGGG-3'
shRNA-Scr	
Forward	5'-GATCCCCGTGGTTTCATCGCATCTGCTTCAAGAGAGCAGATGCGATGAAACCACTTTTTA-3'
Reverse	5'-AGCTTAAAAAGTGGTTTCATCGCATCTGCTCTCTTGAAGCAGATGCGATGAAACCACGGG-3'

Solution (Amaxa Biosystems GmbH). The cells were transfected with 1 μg of reporter plasmid, for both the transcription factor reporter plasmid (pTOPflash) and the mutant control plasmid (pFOPflash), and 1 μg of the internal control pRL-TK, with or without 4 μg of dominant negative (DN)-TCF plasmids (program A33 onthe Nucleofector). Subsequent to transfection, ~1x10^4 cells were transferred to each well of a 24-well plate containing fresh, pre-warmed DMEM and maintained for 48 h at 37˚C in a 5% CO_2 atmosphere. The cells were then lysed in lysis buffer, and 20 μl of each lysate was monitored for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Light units were measured using Lmax II 384 (Molecular Devices LLC, Sunnyvale, CA, USA). The control reporter pRL-TK contains a herpes simplex virus thymidine kinase promoter driving a *Renilla* luciferase gene, and *Renilla* luciferase activity was used to normalize the results for transfection efficiency. The reporter activities were expressed as the ratio of the luciferase activity of TOPflash to the luciferase activity of FOPflash from experiments performed in triplicate.

Vascular mimicry assay. The formation of vascular mimicry in U251 cells was tested using a reduced growth factor Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA). Eight-chamber slides were pre-coated with 0.1 ml Matrigel per well and were incubated at 37˚C for 30 min. Floating spheres of glioma stem-like cells (GSLCs) were resuspended in the endothelial cell basal medium-2 (Lonza, Basal, Switzerland) containing 2% FCS, with or without 10 ng/ml VEGF (PeproTech, Rocky Hill, NJ, USA). After 4 days, the tubules formed by GSLCs were counted using light microscopy. The formation of vascular mimicry was analyzed using Image Image Lab software, version 4.1 (Bio-Rad, CA, USA).

The specific definition for vascular mimicry was as follows: One closed and network-like lumen under the microscope represented one typical vascular mimicry; one opened ring structure represented an atypical vascular mimicry; and an unordered structure demonstrated no vascular mimicry. The number of vascular mimicries on 100 random fields was calculated.

Analysis of bromodeoxyuridine (BrdU) incorporation. BrdU was added to the cells 48 h post-transfection for 30 min, and the U251 cells were stained for BrdU using a BrdU flow kit (BD Biosciences), according to the manufacturer's instructions. The detailed processes were performed as described by Wang *et al* (3).

Statistical analysis. Statistical analyses were performed using SPSS software, version 19.0 (IBM, Armonk, NY, USA). The association between the expression of the Pygo2 protein and the clinicopathological characteristics of patients with glioma was analyzed using Fisher's exact test. All data were expressed as the mean ± standard error. Differences between groups were analyzed for statistical significance using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Pygo2 protein expression was enhanced in human primary glioma samples. Immunochemistry was utilized to examine the Pygo2 protein in human brain glioma tissue. The results indicated that the Pygo2 protein was not expressed in the normal brain tissue samples (Fig. 1A). However, low expression of the Pygo2 protein was observed in the grade II glioma sections (Fig. 1B), and high expression of the Pygo2 protein was observed in the grade IV glioma tissues (Fig. 1C).

Expression of the Pygo2 protein was detected in 52 out of 80 glioma samples, and no Pygo2 expression was detected in the normal brain tissues (Table II). There were no significant differences between the incidence of Pygo2 protein expression in males and females, and no significant age association was observed (Table II).

Specific shRNA inhibits Pygo2 protein expression. In the present study, shRNA was revealed to target the Pygo2 gene and downregulate the expression of the Pygo2 protein. The RT-PCR results revealed that shRNA-Scr did not affect the transcription of Pygo2 mRNA (Fig. 2A). However, shRNA-Pygo2 significantly downregulated Pygo2 mRNA expression compared with the shRNA-Scr group (Fig. 2A; $P<0.01$). In addition, the expression of the Pygo2 protein was also detected in the present study. According to the results of the western blot analysis, the Pygo2 protein levels in the cells treated with shRNA-Pygo2 were significantly reduced compared with the shRNA-Scr treated cells (Fig. 2B; $P<0.01$). The RT-PCR and western blot analysis each illustrated that shRNA-Pygo2 was able to efficiently and specifically inhibit the expression of Pygo2 in U251 cells (Fig. 3B).

Transfection with shRNA-Pygo2 affects the proliferation of U251 cells. In order to identify whether shRNA-Pygo2 affected the growth of glioma cells, MTT and plate colony formation

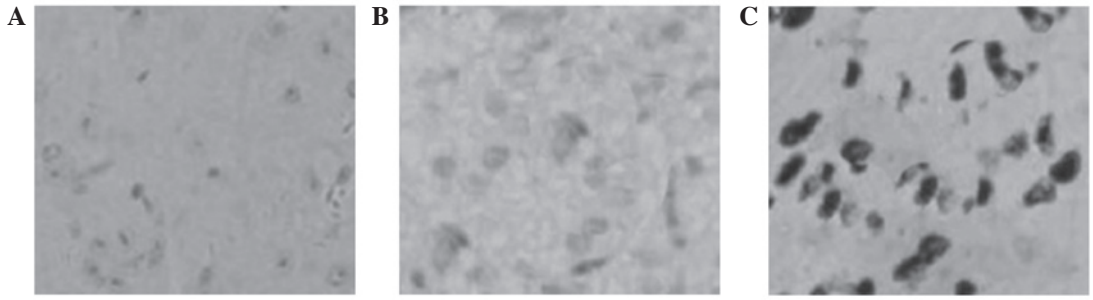

Figure 1. Pygo2 protein expression in normal and glioma tissues was identified by immunohistochemical assay. (A) No Pygo2 expression was detected in normal tissues. Weak and strong Pygo2 expression was identified in (B) grade II and (C) grade IV glioma tissues, respectively. Magnification, x400. Pygo2, Pygopus 2 protein.

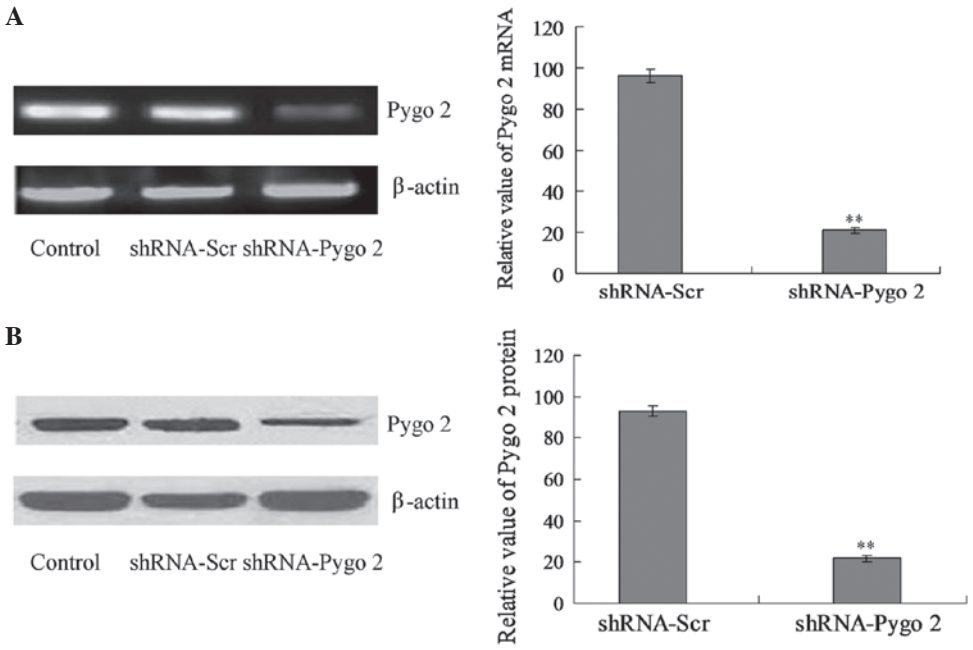

Figure 2. Pygo2 expression in shRNA-silenced tissues, assessed by western blot analysis and PCR. (A) Reverse transcription-PCR was performed to assess Pygo2 mRNA expression in the control, shRNA-Scr and shRNA-Pygo2 treated cells, with β-actin mRNA expression in U251 cells acting as the loading control. (B) Western blotting of Pygo2 expression following treatment with shRNA-Scr or shRNA-Pygo2. **P<0.01 vs. shRNA-Scr group. Pygo2, Pygopus 2 protein; PCR, polymerase chain reaction; shRNA, short hairpin RNA; shRNA-Scr, scramble control shRNA; shRNA-Pygo2, shRNA targeting Pygo2.

assays were employed to detect the proliferation and activity of the U251 cells. Transfection of cells with shRNA-Pygo2 significantly reduced the cell proliferation compared with the shRNA-Scr treated cells (Fig. 3A; P<0.05). In addition, there were no significant differences between the control cells and the cells treated with shRNA-Scr.

To determine the anti-proliferative effects of shRNA-Pygo2, the ability of shRNA-Pygo2 to suppress colony formation was examined. The results indicated that shRNA-Pygo2 induced significant inhibition of colony formation in U251 cells.

In addition, the BrdU method was also used to confirm the effects of shRNA-Pygo2 on the U251 cell proliferation. As indicated in Fig. 3C, treatment of shRNA-Pygo2 may significantly decrease levels of BrdU incorporation, which indicated that shRNA-Pygo2 may inhibit DNA synthesis of the tumor cells.

shRNA-Pygo2 transfection inhibits vascular mimicry. The findings of the vascular mimicry assay indicated that there was no significant difference in vascular mimicry between the control group and group transfected with shRNA-Scr (Fig. 4A and B). However, shRNA-Pygo2 successfully disturbed the expression of Pygo2 and significantly decreased the vascular-mimicry compared with the U251 cells transfected with shRNA-Scr (P<0.01; Fig. 4C).

shRNA-Pygo2 downregulates the Wnt signaling pathway. To obtain further evidence that the Wnt pathway is involved in the vascular mimicry of U251 cells, a transcription factor (TCF) reporter gene containing five optimal TCF-binding sites, TOPflash, or the mutant control plasmid FOPflash was transfected into the U251 cells. The results indicated that the reporter activity of the Wnt pathway in the group treated with shRNA-Pygo2 was significantly reduced compared with the group treated with shRNA-Scr and the control group (P<0.05; Fig. 5).

shRNA-Pygo2 triggers the downregulation of cyclin D1 expression. To identify the specific pathway through which the

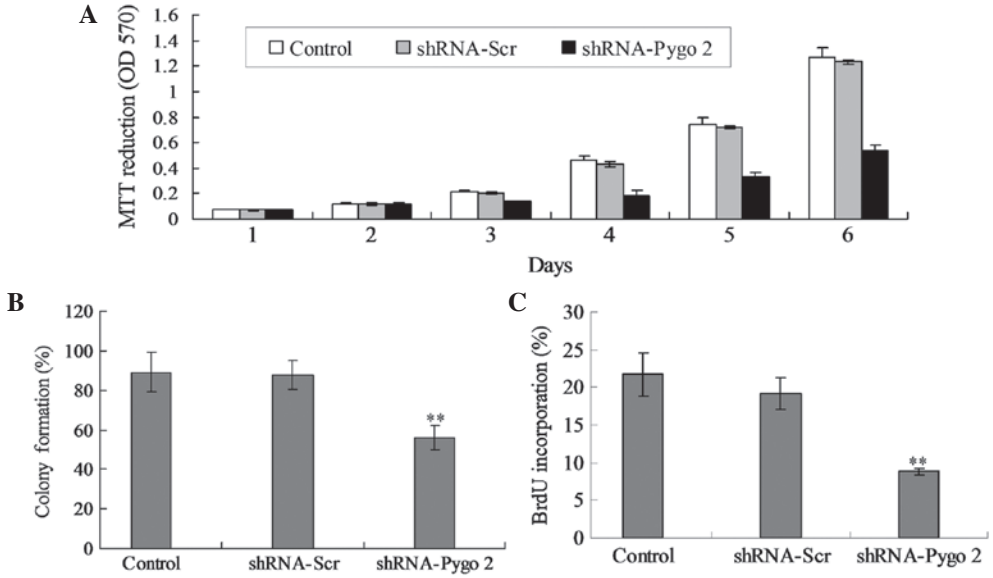

Figure 3. Pygo2 suppression inhibits the proliferation of U251 cells. (A) An MTT assay was used to examine the activity of the U251 cells, and it was found that suppression of Pygo2 inhibits the activity of U251 cells. (B) A colony formation assay was used to examine anchorage-independent growth in U251 cells, and it was found that Pygo2 suppression inhibits the anchorage-independent growth of U251 cells. (C) BrdU incorportation was used to assess the DNA synthesis of cells, and it was found that Pygo2 suppression inhibits the DNA synthesis of U251 cells. **P<0.01 vs. shRNA-Scr group. Pygo 2, Pygopus 2 protein; BrdU, bromodeoxyuridine; shRNA, short hairpin RNA; shRNA-Scr, scramble control shRNA; shRNA-Pygo2, shRNA targetin Pygo2; OD570, optical density at 570 nm.

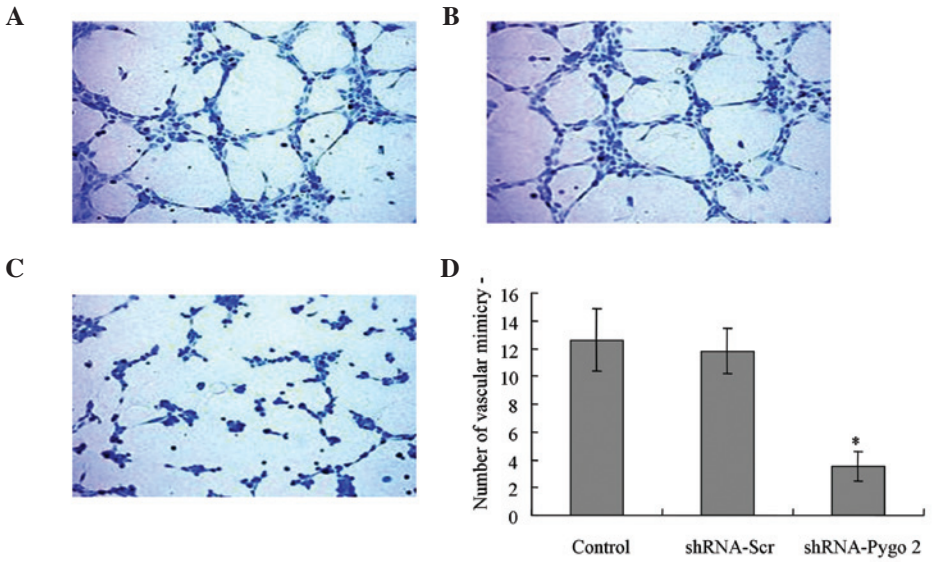

Figure 4. shRNA-Pygo2 transfection affects vascular mimicry in U251 cells. (A) Vascular mimicry in control cells. (B) Vascular mimicry in shRNA-Scr treated cells. (C) Vascular mimicry in shRNA-Pygo2 treated cells. (D) Statistical analysis of the vascular-mimicry in the three groups. shRNA, short hairpin RNA; shRNA-Pygo2, shRNA targeting Pygopus 2 protein; shRNA-Scr, scramble control shRNA. *P<0.05 vs. shRNA-Scr group.

shRNA-Pygo2 inhibited cell proliferation, the Wnt/β-catenin pathway component cyclin D1 was detected by western blot analysis. The cyclin D1 protein levels were significantly decreased in the cells treated with shRNA-Pygo2 compared with the cells treated with shRNA-Scr and the control group cells (Fig. 6; P<0.01).

Discussion

Malignant glioma is known to be a highly invasive tumor of the central nervous system that is challenging to treat. Although therapeutic methods have been investigated for numerous years (22-24), only a small amount of progress has been achieved in patients with malignant gliomas. Therefore, the identification of novel therapeutic targets or methods of treatment is an extremely urgent and promising undertaking for the clinical treatment of gliomas.

Numerous studies have reported that the Pygopus protein plays an important role in developmental brain patterning (25,26). In the present study, the association between the expression of the Pygo2 protein and the clinical grade of brain glioma was investigated. The present results

Table II. Association between the expression of Pygo2 and the clinicopathological characteristics of brain glioma patients.

Clinicopathological characteristic	Total, n	Pygo2-positive, n (%)	P-value
Gender			
Male	52	67.3	0.626
Female	28	60.7	
Age, years			0.121
<44	37	19 (51.4)	
≥44	43	33 (76.7)	
Brain tissue type			
Glioma	80	52 (65.0)	<0.001
Normal	5	0 (0.0)	

Pygo2, Pygopus 2 protein.

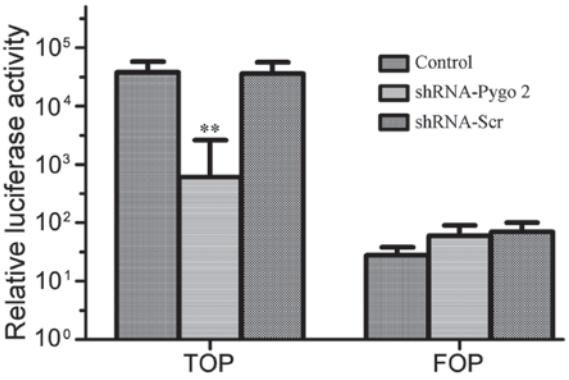

Figure 5. Downregulation of the Wnt signaling pathway in cells treated with shRNA-Pygo2. **P<0.01 represents the luciferase activity of TOP in shRNA-Pygo 2 group compared to control group. shRNA, short hairpin RNA; shRNA-Pygo2, shRNA targeting Pygopus 2 protein; shRNA-Scr, scramble control shRNA; FOP, mutant control plasmid; TOP, transcription factor reporter plasmid.

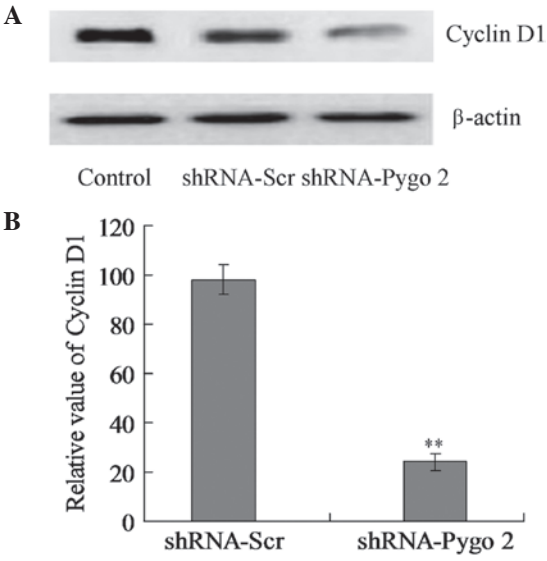

Figure 6. Effect of Pygo2 inhibition on the cyclin D1 expression of U251 cells. (A) Western blot analysis of cyclin D1 following treatment with shRNA-Scr or shRNA-Pygo2 in U251 cells. (B) Statistical analysis of the western blot analysis. **P<0.01 vs. shRNA-Scr. shRNA, short hairpin RNA; shRNA-Pygo2, shRNA targeting Pygopus 2 protein; shRNA-Scr, scramble control shRNA.

indicated that the expression level of Pygo2 increased with the clinical grade. Therefore, it was confirmed that the tumor grade exhibited a positive association with Pygo2 expression in brain glioma. The present results were consistent with previous studies that indicated that Pygo2 protein expression was enhanced in breast (17) and epithelial ovarian cancers (16). However, the two aforementioned studies indicated that Pygo2 is necessary for the proliferation of breast and epithelial ovarian cancer cells, and the specific function of Pygo2 in the progress of gliomas also remains elusive. Prior to the present study, it was hypothesized that the role of Pygo2 in the development of gliomas may be associated with certain signaling pathways.

Tumors may result from the interruption of the developmental signaling pathways that regulate embryonic development in form and structure (27). The Wnt/β-catenin signaling pathway is a major developmental pathway that regulates central nervous system development during embryogenesis, as well as during adulthood (28). The Wnt/β-catenin signaling pathway also plays an important role in carcinogenesis (29). However, the Wnt/β-catenin signaling pathway-associated cascade genes in gliomas has not been investigated in previous studies. Furthermore, the association between the downstream genes in the Wnt/β-catenin signaling pathway and the malignant progression of glioma is also poorly studied. Thus, in the present study, specific shRNA against Pygo2 mRNA was established and termed shRNA-Pygo2. The shRNA-Pygo2 was then transfected into the U251 cells. The results indicated that Pygo2 knockdown inhibited cell proliferation, colony formation and BrdU incorporation.

In the present study, the vascular mimicry assay revealed that shRNA-Pygo2 significantly decreased the vascular mimicry in U251 cells compared to treatment with shRNA-Scr. The formation of vascular mimicry may be due to the blocking expression of Pygo2. The decreased vascular mimicry also triggers the inhibition of the differentiation and proliferation of U251 cells in a later step.

The present study hypothesized that the vascular mimicry induced by shRNA-Pygo2 may involve the Wnt signaling pathway, and therefore the vascular mimicry assay was performed. The results indicated that the Wnt pathway

participated in the development of vascular mimicry and cell proliferation induced by shRNA-Pygo2.

Previous studies have also transfected the siRNA sequences specific to Pygo protein mRNA, and inhibited the TCF/LEF-mediated transcriptional activation of reporter genes (30,31), which suggested that Pygo2 members may be involved in TCF/β-catenin-driven transcription. In the present study, the expression of cyclin D1 protein in the U251 cells treated with shRNA-Pygo2 was investigated. The results revealed that the expression of cyclin D1 was significantly reduced by Pygo2 shRNA, which was consistent with the decreased expression of cyclin D1 in breast cancer cells following Pygo2 antisense RNA treatment (17).

In conclusion, Pygo2 expression is associated with the clinical grade of glioma malignancy. shRNA-Pygo2 effectively suppresses the proliferation of glioma cells by inhibiting the cyclin D1 expression of the canonical Wnt/β-catenin pathway for brain glioma.

Acknowledgements

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