Brain lipid-binding protein promotes proliferation and modulates cell cycle in C6 rat glioma cells

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Received April 11, 2017; Accepted September 18, 2017

DOI: 10.3892/ijo.2017.4132

Abstract. Gliomas are the most common primary brain tumors affecting adults. Four grades of gliomas have been identified, namely, grades I-IV. Brain lipid-binding protein (BLBP), which functions in the intracellular transport of fatty acids, is expressed in all grades of human gliomas. The glioma cells that are cultured *in vitro* are grouped into the BLBP-positive and BLBP-negative cell lines. In the present study, we found that C6 rat glioma cells was a distinct type of BLBP-negative cell line. Our results confirmed that in the C6 cells, the expression of exogenous BLBP increased the proliferation and percentage of cells in the S phase, in the culture medium containing 10 or 1% FBS. Moreover, exogenous BLBP was found to downregulate the tumor suppressors p21 and p16 in the 1% FBS culture medium, but only p21 in the 10% FBS culture medium. The results of the xenograft model assay showed that exogenous BLBP also stimulated tumor formation and downregulated p21 and p16. In conclusion, our study demonstrated that exogenous BLBP promoted proliferation of the C6 cells *in vitro* and facilitated tumor formation *in vivo*. Therefore, BLBP expression in glioma cells may promote cell growth by inhibiting the tumor suppressors.

Introduction

Gliomas are the most common brain tumors affecting adults and are known to originate from glial cells, which comprise astrocytes and oligodendrocytes (known as astrocytomas or oligodendrogliomas, respectively) (1). Malignant gliomas, such as glioblastoma (GBM), represent the most common lethal intracranial tumors, and the median survival for GBM patients is only 15 months (2). Brain lipid-binding protein (BLBP), also called fatty acid-binding protein 7 (FABP7), is a member of the fatty acid binding proteins (FABPs) family, which is involved in the intracellular transport of fatty acids. As a fatty acid transporter, the primary function of BLBP is to facilitate intracellular transport of the polyunsaturated fatty acids (PUFAs) (3), especially docosahexaenoic acid (DHA). In addition to DHA, BLBP also binds arachidonic acid (AA), although with a four-fold lower affinity than DHA (4).

BLBP expression is observed in all grades of human astrocytomas, but neoplastic cells with nuclear BLBP expression are only observed in the infiltrating type of tumors (5). Grade IV GBM displays a significantly higher number of BLBP/Sox2-positive tumor cells than diffuse astrocytoma (grade II) and anaplastic astrocytoma (grade III) (6). BLBP expression enhanced the motility of glioma cells (7), but human U87 glioma cell line was not observed to express BLBP *in vitro* (8). A recent study reported that glioblastoma stem-like cells (GSCs), which were similar to normal neural stem cells (NSCs) and could form neurospheres, showed higher expression of BLBP and that siRNA-mediated BLBP knockdown resulted in reduced proliferation and migration of GSCs *in vitro* (9).

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that act as transcription factors and regulate the expression of specific genes and are implicated in BLBP regulation. Three types of PPARs have been identified, namely, alpha (PPARα), beta/delta (PPARβ/δ) and gamma PPARγ (10). PPARs have been demonstrated to interact with FABPs, and PPAR antagonists are known to influence BLBP expression (9). PAX6 is another vital transcription factor expressed in the developing brain. BLBP is downregulated in PAX6 mutant rats, and overexpression of exogenous PAX6 was found to induce the ectopic expression of BLBP (11,12). Moreover, nuclear factor I (NFI) recognition sites have also been identified in BLBP and glial fibrillary acidic protein (GFAP) promoters, and all four members of the NFI family (NFIA, NFIB, NFIC, and NFIX) regulate the expression of BLBP and GFAP genes in the malignant glioma cells (13).

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Key words: BLBP, gliomas, C6 cell line, tumor suppressor

Tumor suppressor genes, also called anti-oncogenes, are a group of genes that function by inhibiting cancer cells. Loss or reduced expression of tumor suppressor genes usually leads to cancer progression (14). PPARs are crucial regulators of tumor suppressor genes, including p27 (15,16), p21 (15,17,18), and p16 (19-21). In addition, PAX6 has been demonstrated to influence cell proliferation by regulating p27, p21, and p16 expression (22-24). Moreover, p21 expression is known to be a primary factor influencing the roles of NFI family proteins in the cell cycle (25,26). Thus, we hypothesized that the effects of BLBP on glioma cell proliferation are primarily mediated by regulating the expression of these tumor suppressors.

In this study, we showed that BLBP could not be detected in the C6 cells. C6 is a rat glioma cell line that is histopathologically classified as an astrocytoma cell line and represents a widely used model for studying human GBMs (27). Our results revealed that the introduction of BLBP into the C6 cells stimulated cell proliferation *in vitro* and increased the percentage of cells in the S phase. Exogenous BLBP expression facilitated tumor formation in the C6 cells *in vivo*. Moreover, the observed increase in cell growth by exogenous BLBP may be mediated by downregulation of the tumor suppressors p21 and p16.

Materials and methods

Cell culture. The C6 cells were purchased from GeneChem (Shanghai, China). They were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS). Lentiviral vectors utilizing the GV287 lentiviral expression system (cDNA of rat BLBP driven by Ubi promoter, and enhanced green fluorescent protein (EGFP) driven by SV40 promoter) were also purchased from GeneChem. The titers of lentivirus overexpressing the rat BLBP cDNA (LV-BLBP group) and the negative control (NC) lentivirus (LV-NC group) were determined. For cell infection, in 6-well plates, the C6 cells were seeded at a density of $2x10^5$ cells/well in 10% FBS culture medium overnight; subsequently, lentivirus (MOI=20) and 5 μg/ml polybrene were added. After 24 h, the medium was replaced with fresh culture medium. After 3 days, cells at 80% confluence were harvested for the subsequent experiments.

PCR and gel electrophoresis. To detect BLBP gene expression in C6 cells, total RNA from LV-NC and LV-BLBP groups was extracted using UNIQ-10 Spin Column RNA Purification kit (Sangon Biotech, Shanghai, China). First strand cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). cDNA was subsequently analyzed on a Corbett RG-6000 PCR system (Qiagen, Dusseldorf, Germany) using QuantiNova™ SYBR Green PCR kit (Qiagen). Considering that no positive signals could be automatically detected in the LV-NC group at less than 40 cycles on a PCR instrument, so all PCR products were detected via 1.2% agarose gel electrophoresis directly. The following sense and antisense primers were synthesized and used for PCR: GAPDH 5'-GCAAGTTCAACGGCACAG-3', 5'-GCCAGTAGACTCC ACGACAT-3'; BLBP 5'-TGTGACCAAACCAACGGTGA-3', 5'-AGCTTGTCTCCATCCAACCG-3'.

CCK-8 assay. The C6 cells from the LV-NC and LV-BLBP groups were seeded at a density of $2x10^3$ cells/well into 96-well plates. All cells were cultured in 100 μl of 10% and 1% FBS culture medium for 24, 48, 72, 96 and 120 h. For the cell viability assay, the Cell Counting Kit-8 (CCK-8) reagent (Beyotime, Shanghai, China) was used. Briefly, the 10% or 1% FBS culture medium was discarded, after which 10 μl of CCK-8 reagent and 90 μl of serum-free DMEM/F12 medium were added to each well and incubated for 1 h. Optical density (OD) was measured using the Synergy 2 enzyme mark instrument (BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

Western blotting. The cells were seeded at a density of $2x10^5$ cell/well into 6-well plates. Fresh 10% or 1% FBS culture medium was added into the 6-well plates. After 24 h, all cells were washed with phosphate buffered saline (PBS) and the Tissue or Cell Total Protein Extraction kit (Sangon Biotech) was used for extracting total protein. Next, protein concentrations were determined using the Enhanced BCA Protein assay kit (Beyotime). Equal amounts of protein were resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE). Subsequently, gels were transferred onto polyvinylidene difluoride (PVDF) membranes using the Bio-Rad Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) at 25 V for 7 min, blocked with 5% milk in Tris-buffered saline Tween (TBST) buffer, and incubated with the respective primary antibody, namely, mouse anti-p27 (1:500, Beyotime, #AP027), mouse anti-p21 (1:500, Beyotime, #AP021), anti-p16 (1:500; Abgent, Suzhou, China; #AO1103b), and mouse anti-β-actin (1:5000; Cell Signaling Technology, Danvers, MA, USA; #3700), overnight at 4˚C. After incubation with the HRP-conjugated goat anti-mouse (1:5000; Beyotime; #A0216) or goat anti-rabbit (1:5000; Sangon; #D110058) secondary antibody at room temperature for 2 h, the blots were washed, and immunoreactive proteins were scanned using the Chemidoc XRS system (Bio-Rad). The OD values on the membrane were measured, and relative protein expression levels were quantified using the Image Lab software (Bio-Rad).

FACS assay for cell cycle. For the cell cycle assay, the C6 cells in the LV-NC and LV-BLBP groups were seeded into 25 ml culture flasks at a density of $2x10^5$ cells containing 5 ml of 10% or 1% FBS culture medium for 24 h. The cells were harvested and fixed in 70% ice-cold ethanol for at least 24 h at -20˚C. Next, they were stained with propidium iodide (PI) using the BD Cycletest™ Plus DNA Reagent kit (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at 37˚C, and all cells were performed in fluorescence-activated cell sorter (FACS) assay by using a FACSCalibur instrument (BD Biosciences).

EdU assay. For the EdU assay, the C6 cells from the LV-NC and LV-BLBP groups were seeded into 24-well plates and 25-ml culture flasks at densities of $2x10^4$ or $2x10^5$ cells/well, respectively, in 10 or 1% FBS culture medium. After 24 h, the cells were treated with the EdU (50 μM) reagent (Ribobio, Guangzhou, China) for 1 h and stained with the Apollo-567 reaction cocktail (Ribobio) for 30 min. After washing thrice with PBS, the cells in the 24-well plates were counterstained with Hoechst for nuclear staining and observed under a microscope, while the cells in the 25 ml culture flasks were assessed using FACSCalibur instrument (BD Biosciences) to determine

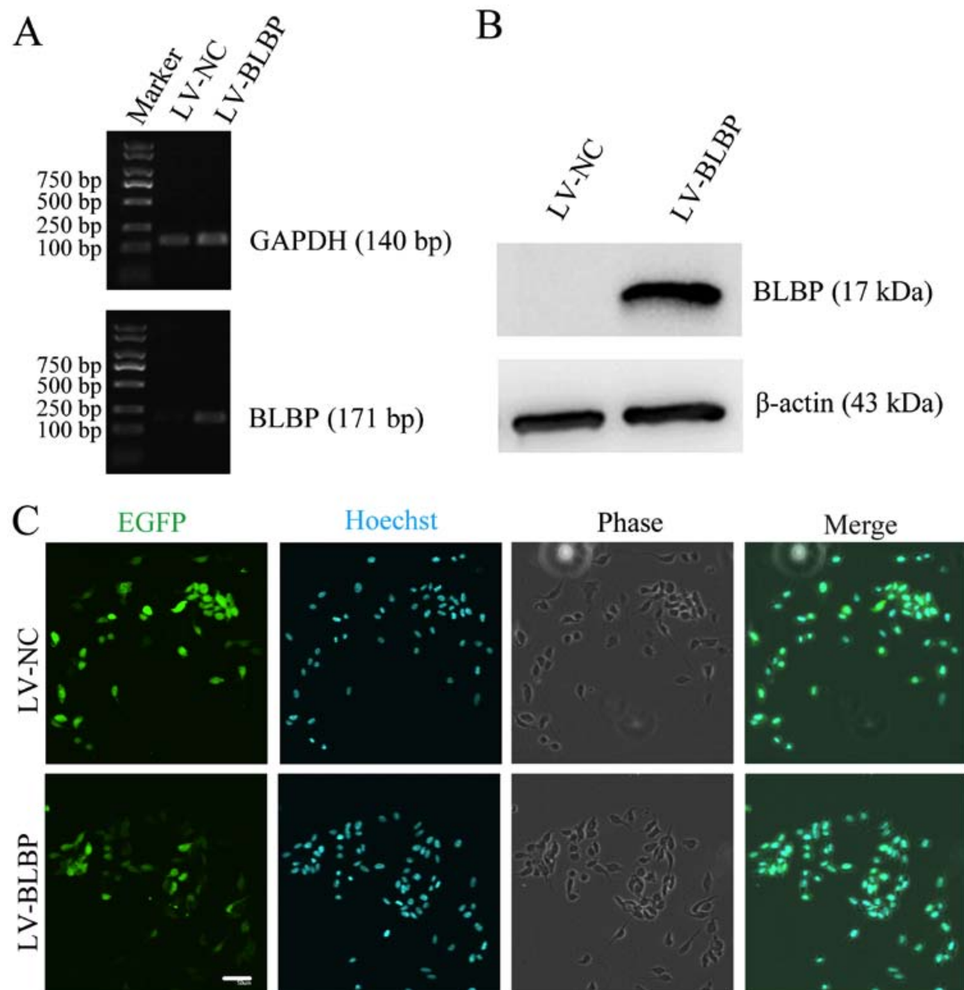

Figure 1. Following infection with lentivirus, the PCR results showed that the BLBP gene was positively amplified in the cells from the LV-BLBP group but not in those from the LV-NC group (A), and BLBP protein was detected only in the cells from the LV-BLBP group (B); all C6 cells both in LV-NC (C, upper panel) and LV-BLBP (C, lower panel) groups expressed EGFP, scale bar=50 μm.

the proportion of EdU-positive cells, all results were from three independent replicates.

Xenograft model. Twelve BALB/c nude mice were purchased from the Animal Experimental Center of Nantong University. All mice were treated under pathogen-free conditions in cages according to the Nantong University-approved protocols for the Care and Use of Laboratory Animals, and the ethical approval (no. 20161115-001) from the Lab Animal Ethics Committee of Nantong University was obtained for this study. Tumor models were established by subcutaneously implanting the C6 cells. Briefly, six mice were inoculated subcutaneously on the left flank with $5x10^6$ cells from the LV-BLBP group in 100 μl of serum free DMEM/F12 medium; the other six mice were inoculated with $5x10^6$ cells from the LV-NC group. After 21 days, all mice were euthanized by carbon dioxide (CO_2), and the flow rate did not displace more than 30% of the chamber volume/minute based on the American Veterinary Medical Association (AVMA) (28), which is recommended by the ethical guidelines of the University of Minnesota. Then all tumors were resected accordingly. Tumor volumes (V) were calculated using the following formula: $V = 1/2$ (length x width^2). The tumor tissues were cleaned with PBS, fixed with 4% paraformaldehyde for 2 h, and subsequently placed in 20% sucrose solution for 24 h. The tissues were frozen and cut into 10 μm sections for immunohistochemistry.

Microscope assay and immunohistochemistry. Cells infected with LV-NC or LV-BLBP were treated with 4% paraformaldehyde and then directly examined under the microscope using an EVOS FL Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) or examined using an Olympus laser confocal microscope (Olympus, Tokyo, Japan).

For Ki67 immunostaining, tumor tissue sections were incubated with rabbit anti-Ki67 antibody (1:400; Thermo Fisher Scientific; #PA5-19462) overnight at 4˚C and subsequently incubated with secondary antibodies (1:1000; Cell Signaling Technology; #8889S) for 2 h. After treatment with Hoechst for 15 min, all sections were examined using an Olympus laser confocal microscope.

Cell migration assay. Cell migration assay was performed on a 35-mm μ-dish with culture insert (Ibidi, Martinsried, Germany). Briefly, cells in the LV-NC and LV-BLBP groups were seeded at $2x10^4$ cells per well containing 70 μl of 10% FBS culture medium. After cell attachment for 24 h, culture

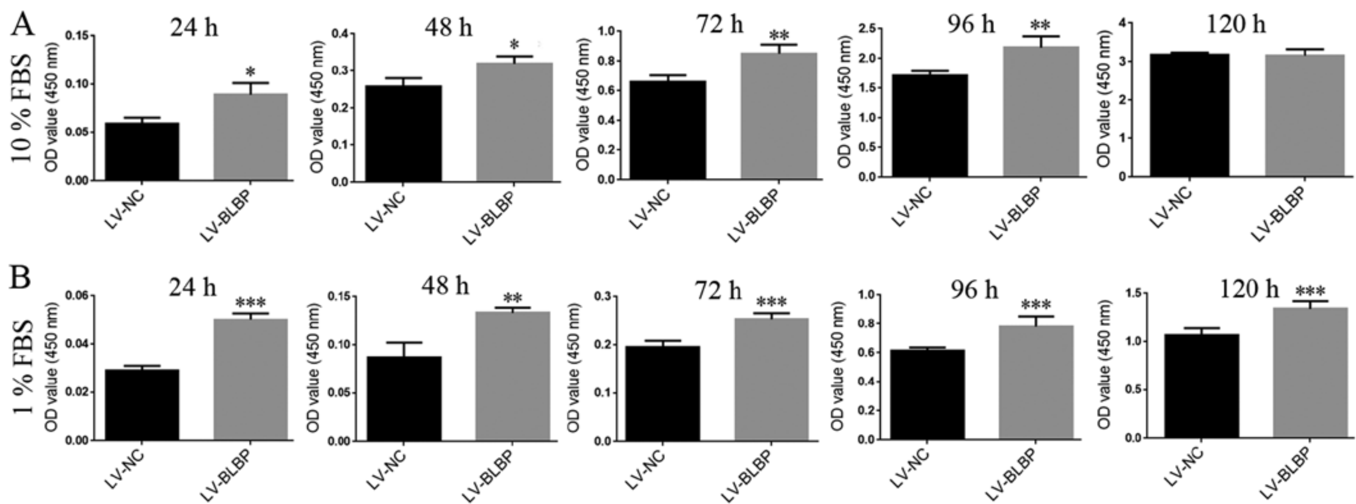

Figure 2. Cell viability of C6 cells was determined using the CCK-8 reagent by measuring the optical density (OD) values at 450 nm. When cultured in the 10% FBS medium, the OD values of cells from the LV-BLBP group increased from 24 to 96 h (*P<0.05; **P<0.01), but not at 120 h. After culturing in the 1% FBS medium, the OD values of cells from the LV-BLBP group were elevated from 24 to 120 h (**P<0.01; ***P<0.001).

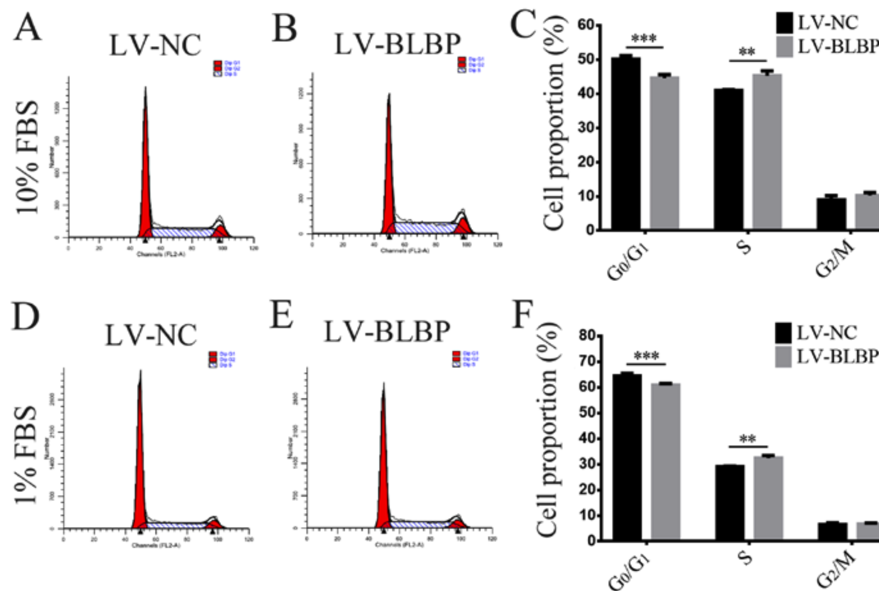

Figure 3. FACS analysis showed that the proportion of cells in the S phase increased (C and F) in the LV-BLBP group, both in the 10% (A and B) and 1% FBS (D and E) culture medium (**P<0.01); however, the proportion of cells in the G_0/G_1 phase decreased (***P<0.001).

inserts were gently removed using sterile tweezers to create a gap of ~500 μm. Next, wells were filled with 1 ml of fresh culture 10% FBS or 1% FBS culture medium. Photographs were captured immediately (0 h) or at 12, 24, 48 and 72 h.

Statistical analysis. Values were expressed as the mean ± SEM of at least three independent experiments. Unpaired Student's t-test was performed for statistical analyses using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The data were considered to be statistically significant at P<0.05.

Results

Exogenous BLBP was introduced into C6 cells. After infection with lentivirus, PCR (Fig. 1A) and western blotting (Fig. 1B) were performed to detect mRNA and protein expression levels of exogenous BLBP, respectively. The cells from the LV-NC group did not express endogenous BLBP; however, exogenous BLBP was clearly detected in the LV-BLBP group cells. The green fluorescence of the C6 cells from the LV-NC (Fig. 1C upper panel) and LV-BLBP groups (Fig. 1C lower panel) was observed under the microscope. BLBP expression has been previously observed in the human U251 glioma cells but not in the U87 glioma cells (8). In the current study, BLBP expression was not detected in the C6 cells, implying that C6 was another distinct type of BLBP-negative glioma cell line.

Exogenous BLBP stimulated growth in C6 cells in vitro. In the 10% FBS culture medium, results of the CCK-8 assay showed that the OD values increased from 24 to 96 h in the LV-BLBP group, but no significant differences were observed at 120 h (Fig. 2A), compared to the LV-NC group. The C6 cells grew

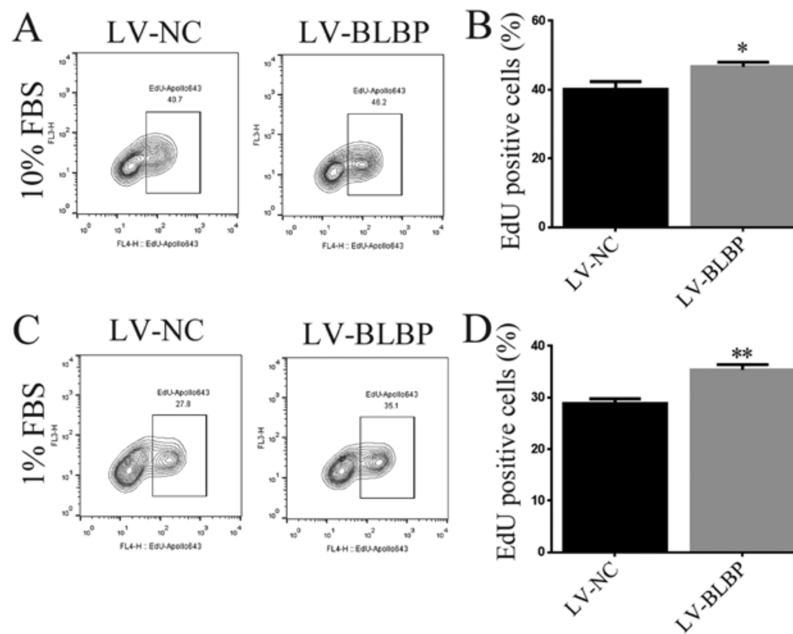

Figure 4. The EdU-labeled cells cultured in the 10% (A and B) or 1% FBS (C and D) medium were analyzed using FACS. Results showed that cells from the LV-BLBP group had a significantly higher number of EdU-labeled cells than those from the LV-NC group (*P<0.05, **P<0.01).

rapidly in the 10% FBS culture medium. We observed that all wells of the 96-well plate were potentially filled with cells after culturing for 120 h. Thus, the differences in OD values between the two groups were no longer apparent at this time point. FBS is derived from blood and is most widely used in *in vitro* cell cultures as a serum supplement. To minimize the effects of FBS on the culture medium, we reduced the concentration of FBS from 10 to 1%. The OD value were observed to increase from 24 to 120 h in the LV-BLBP group (Fig. 2B). These results suggested that exogenous BLBP expression increased cell viability in both the 10 and 1% FBS medium and that exogenous BLBP expression could serve as a potent factor for inducing cell proliferation.

Exogenous BLBP increases the percentage of C6 cells in the S phase. Next, we analyzed the cell cycle distribution of C6 cells after exogenous expression of BLBP. Compared to the LV-NC group, the LV-BLBP group showed higher cell population in the S phase, both in the 10% (Fig. 3A-C) and 1% (Fig. 3D-F) FBS culture media; however, cell population in the G_0/G_1 phase decreased (Fig. 3C and F). Considering the cell cycle is a continuous process, and the serum-contained medium is close to the internal environment in the organism, these results suggested most of the cells maintained their normal growth and the effect of BLBP in cell cycle could be reflected accurately in serum-contained medium.

The EdU assay was performed to confirm the increase in the proportion of cells in the S phase. EdU is a thymidine analogue that is incorporated into the DNA of dividing cells during the S phase. In the FACS assay, the proportion of EdU-positive cells in the LV-BLBP group was found to be higher than that in the LV-NC group in both the 10% (Fig. 4A and B) and 1% (Fig. 4C and D) FBS culture medium. Results also showed that the number of EdU-positive cells in the LV-BLBP group was considerably higher in both the 10% (Fig. 5A and B) and 1% (Fig. 5C and D) FBS culture medium than those in the LV-NC group. These findings implied that exogenous BLBP expression accelerated cell growth, evident by the higher percentage of cells in the S phase.

Exogenous BLBP decreases the expression of tumor suppressors p21 and p16. Tumor suppressor genes, such as p27 (15,16), p21 (15,17,18) and p16 (19-21) act as inhibitors of tumor growth. Inhibition or knockdown of these genes can lead to oncogenesis (29). We hypothesized that exogenous BLBP expression would potentially downregulate these tumor suppressors to stimulate cell proliferation. For the cells cultured in 10% FBS medium, only p21 expression levels were found to be lower in the LV-BLBP group compared to the LV-NC group. However, both p21 and p16 expression levels in cells from the LV-BLBP group cultured in 1% FBS medium were observed to be lower than those in the LV-NC group. Expression level of p27 showed no significant differences in the 10% or 1% FBS culture media (Fig. 6).

Exogenous BLBP facilitates tumor formation in the C6 cells in vivo. To further investigate the tumor-inducing effects of exogenous BLBP expression, we developed a xenograft model and calculated the tumor volumes after subcutaneous injection of the C6 cells in the LV-NC and LV-BLBP groups for 21 days. The average tumor volume (Fig. 7B) of samples in the LV-BLBP group (Fig. 7A, lower panel) were evidently larger than those in the LV-NC group (Fig. 7A, upper panel). Moreover, the number of Ki67-positive cells in the LV-BLBP group was also higher than that in the LV-NC group (Fig. 7C and D). Consistent with the *in vitro* results obtained using cells cultured in 1% FBS medium, western blotting results also showed that cells in the LV-BLBP group had lower p21 and p16 levels, but not p27 levels, when compared to cells in the LV-NC group. The above findings indicated that exogenous

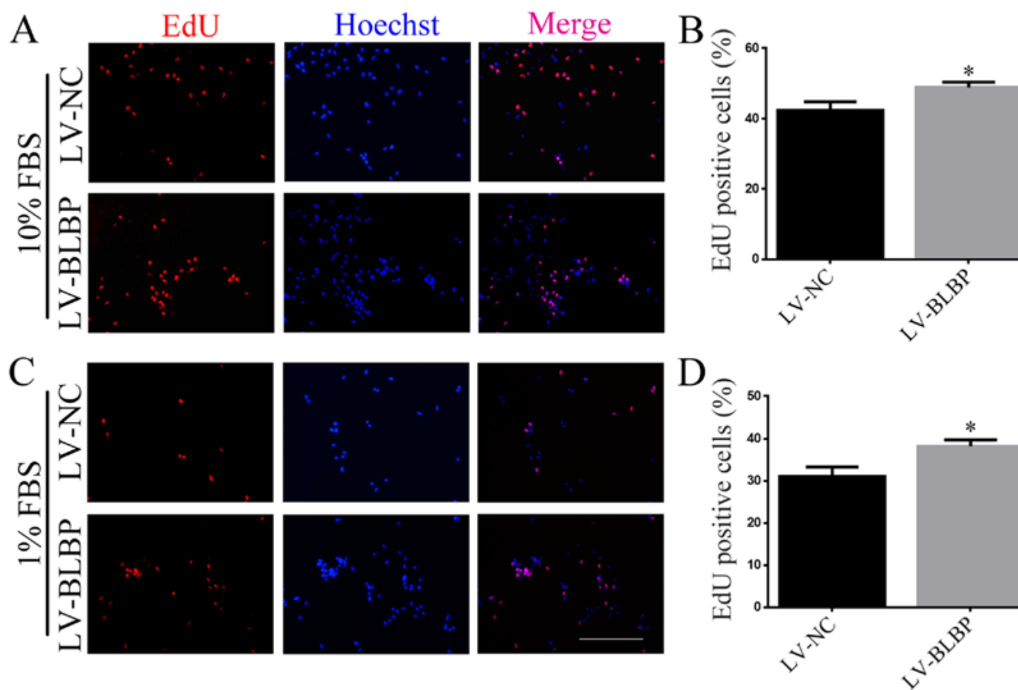

Figure 5. EdU-positive cells were examined under the microscope. The number of EdU-positive cells was higher in the LV-BLBP group in both the 10% FBS (A and B) and 1% FBS (C and D) culture medium (*P<0.05); scale bar, 200 μm.

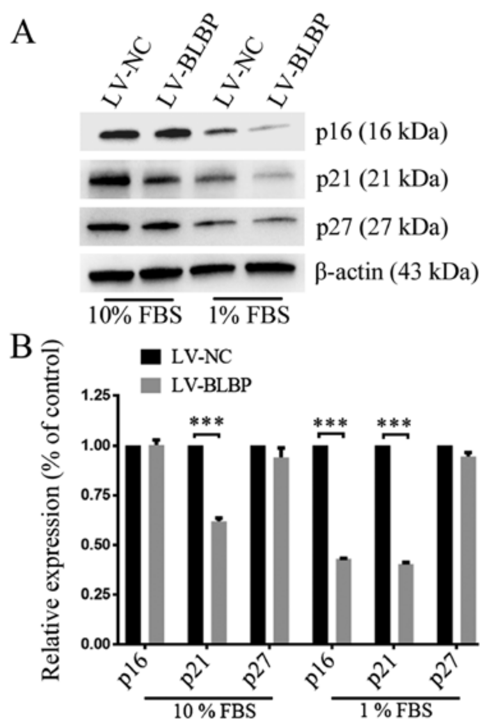

Figure 6. In western blot assay (A), both p21 and p16 expression levels decreased in cells in the LV-BLBP group when cultured in 1% FBS medium. However, only p21 levels decreased in cells in the LV-BLBP group when cultured in 10% FBS medium. No differences in p27 levels were observed between the two groups either in the 10% FBS or 1% FBS culture medium (B) (***P<0.001).

BLBP expression facilitated tumor growth *in vivo* and that the microenvironment of cells grown *in vivo* was more similar to the 1% FBS culture medium than to the 10% FBS medium *in vitro*.

Exogenous BLBP shows no effect on C6 cell migration. In the cell migration assay, the gap was filled with cells after treatment with 10% FBS at 24 h; on the other hand, for the 1% FBS medium, the gap was filled with cells at 72 h. The cells from the two groups did not show significant differences in terms of the cell migration distance both at 12 h in 10% FBS medium and at 12, 24, and 48 h in the 1% FBS medium (Fig. 8). These results suggested that exogenous BLBP does not promote cell migration in the C6 cells.

Discussion

BLBP promotes proliferation and modulates cell cycle in the C6 cells. BLBP, also called FABP7, is involved in the intracellular transport of fatty acids and is present in the proliferated astrocytes in the central nervous system (CNS), under physiological and pathological conditions (30-32). BLBP is also labeled in the radial glial cells (RGCs), which act as neural progenitor cells (NPCs) in the embryo's CNS and eventually differentiate into neurons and glia cells (33). Gliomas are the most common primary brain tumors in adults and can be classified into four grades (I-IV), based on their microscopic features. Grade IV astrocytoma is also known as GBM (3,34,35). Besides astrocytes and NPCs, BLBP expression has been also reported in all grades of glioma cells (5). Nuclear BLBP immunoreactivity is associated with poor prognosis in epidermal growth factor receptor (EGFR)-overexpressing GBM cells (5,36). As a marker for GBM (6), BLBP was considered to be involved in cell proliferation and invasion (9). Moreover, BLBP is expressed in the malignant glioma cell line U251, but not in U87. Additionally, exogenous BLBP enhances U87 cell motility and migration (37). However, little is known about the exact role of BLBP in the glioma cells. For instance, the specific cell cycle phases that

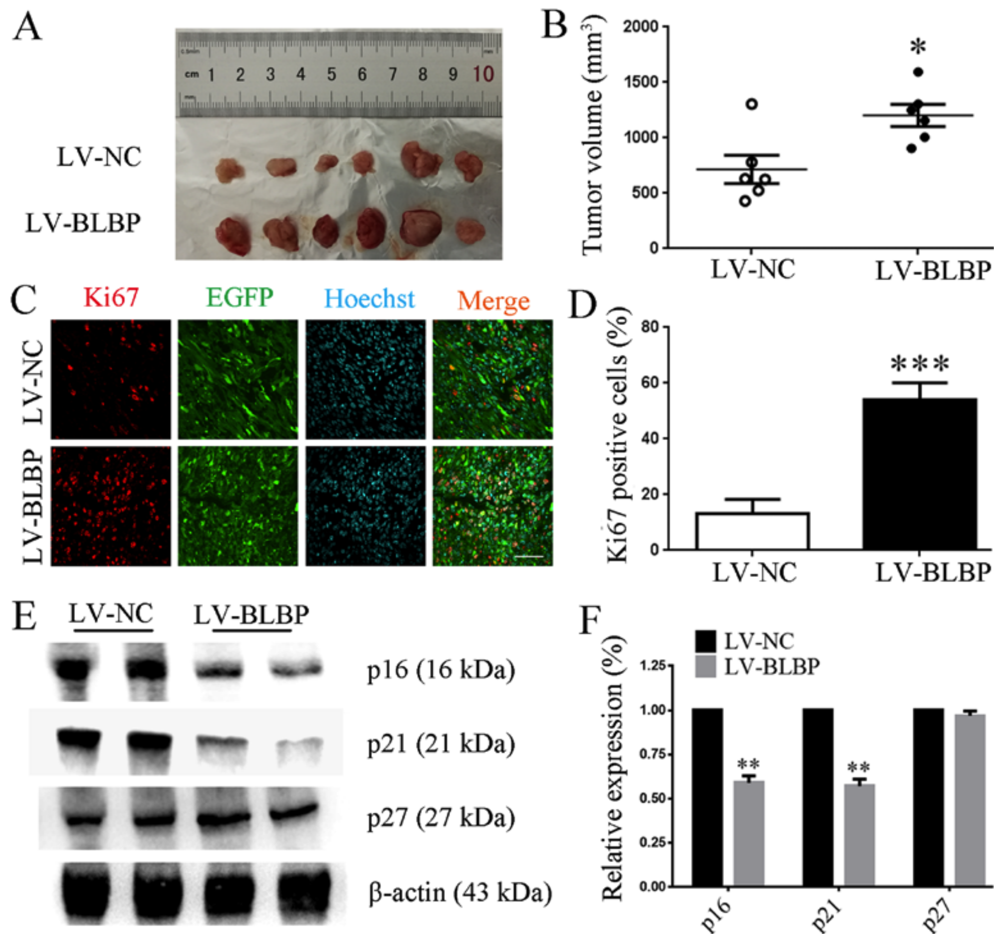

Figure 7. Tumor formation assay results showed that C6 cells from both the LV-NC group (A, upper panel) and LV-BLBP group (A, lower panel) formed tumors after subcutaneous injection for 21 days. Tumor volumes in mice from LV-BLBP were larger than those in the LV-NC group (B) (*P<0.05). The number of Ki67-positive cells (C) in tumor tissues of mice from the LV-BLBP group were considerably higher than those in the LV-NC group (D, ***P<0.001), scale bar, 50 μm. Expression levels of p21 and p16, but not p27, were markedly lower in the tumor tissues of mice in the LV-BLBP group (E and F) (**P<0.01).

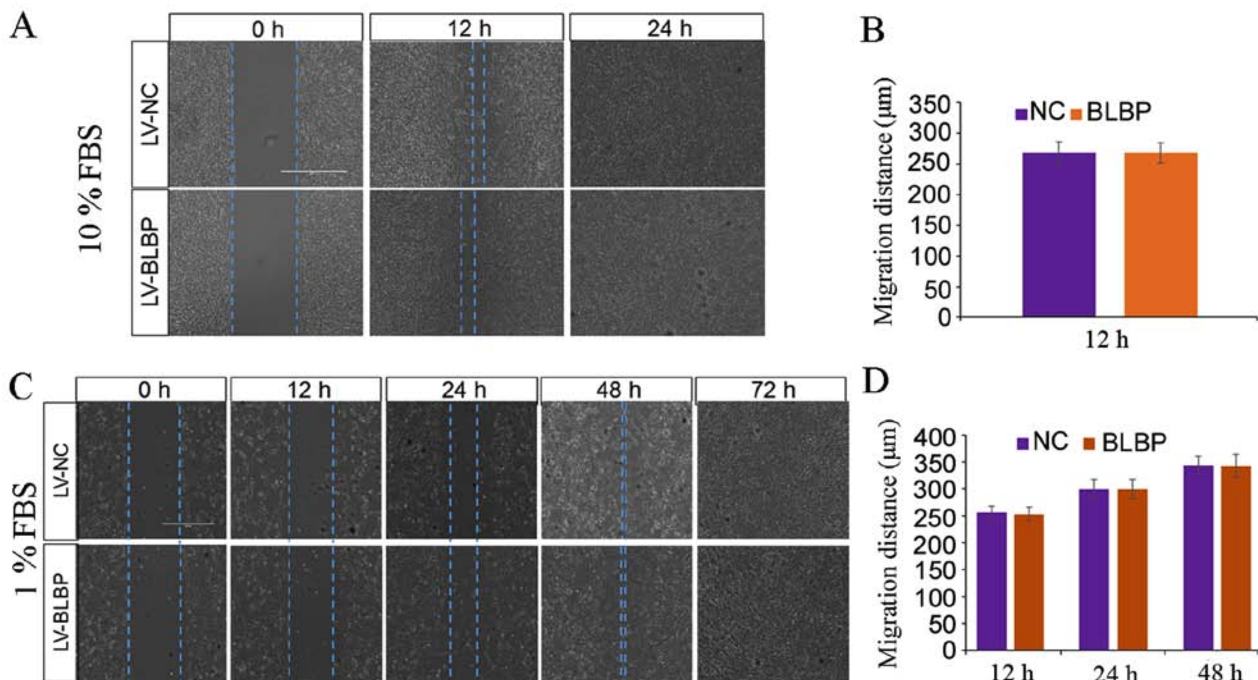

Figure 8. In the 10% FBS medium (A), cells from the LV-NC and LV-BLBP groups show no significant differences in cell migration distances at 12 h (B), scale bar, 400 μm. In the 1% FBS medium (C), cells from the LV-NC and LV-BLBP groups showed no significant differences in cell migration distances at 12, 24, and 48 h (D), scale bar, 400 μm.

are regulated by BLBP, as well as its downstream targets, are poorly understood.

The C6 cells showed upregulated expression of EGFR, human epidermal growth factor receptor 3 (Her3), platelet derived growth factor receptor β (PDGFβ), and insulin-like growth factor 1 (IGF1), which are often overexpressed in human gliomas. Thus, the C6 cell line is more similar to the human brain-derived tumors than to the astrocytes (38-41). Since the BLBP mRNA or protein expression was not detected in the C6 glioma cells (Fig. 1), we suggested that the C6 cells were also more likely to be similar to the BLBP-negative U87 cells but not to the BLBP-positive U251 cells. Considering that FBS is a mitogen that affects cell proliferation *in vitro*, we tried to reduce the concentration of FBS in the culture medium from 10 to 1%, which was the minimum concentration required for the survival of C6 cells.

In this study, exogenous BLBP expression was found to increase the viability of C6 cells in both the 10 and 1% culture medium (Fig. 2). FACS was performed to analyze the cell cycle distribution of cells and to verify the specific cell cycle phase that was regulated upon increased BLBP expression. The proportion of cells in the G_0/G_1 phase decreased; however, the proportion in the S phase increased in the LV-BLBP group both in the 10 and 1% FBS culture medium (Fig. 3). Moreover, results from the EdU assay using FACS or microscopy confirmed that the cell population in the S phase increased after exogenous BLBP expression both in the 10 and 1% FBS culture medium (Figs. 4 and 5). These results indicated that BLBP promoted proliferation and modulated cell cycle of cultured C6 cells *in vitro*. Although some studies showed the BLBP promoted the cell proliferation (9,42), we explored that the mechanism in this research and further verified its function. EdU is a thymidine analogue that is incorporated into the DNA of dividing cells during the S phase, our results showed that BLBP could stimulate the cell proliferation by increasing the population in S phase.

Exogenous BLBP downregulated tumor suppressors. The cell cycle is a continuous and dynamic process. S phase is a vital part of the cell cycle, DNA is replicated during this phase and precise DNA replication is necessary for facilitating cell proliferation. The cell proliferation is rapid after exposure of some growth-promoting signals (e.g. growth factors), and cells enter into S phase to duplicate DNA, so the S phase population increases in FACS assay. Moreover, if the cell growth is inhibited (e.g. DNA damage or stress), the cell cycle will be blocked at S phase. G_1/S transition is a stage between the G_1 and S phases (43,44). The cells decide whether to be quiescent, differentiate, or proliferate during the G_1/S transition. Thus, G_1/S transition is an important cell cycle check point which is regulated by cyclins, cyclin-dependent kinases (CDKs), oncogenes, and tumor suppressor genes (45,46).

The tumor suppressors that prevent cell cycle progression comprise genes from the CDK-interacting protein/kinase inhibitory protein (cip/kip) family, which includes p21, p27, and p57, and from the inhibitor of kinase 4/alternative reading frame (INK4a/ARF) family, which includes p14 and p16. Although several studies have reported that p27, p21 and p16 can induce cell cycle arrest at the G_2/M phase (47,48), these proteins primarily hinder progression of cells from the G_1 to S phase of the cell cycle (49-51).

Previous studies have demonstrated that BLBP expression is under the control of PPARs (8,9), PAX6 (11,12) and NFI (13), and these factors also control proliferation and cell cycle arrest by regulating p27 (15,16), p21 (15,17,18), and p16 (19-21). We observed that the expression of both p21 and p16 in the 1% FBS medium was decreased, while only p21 expression was downregulated in the 10% FBS medium (Fig. 6). Considering that proteins in the cip/kip and INK4a/ARF families primarily act by blocking the G_1/S transition, we suggested that the exogenous BLBP drove the G_1/S transition by downregulating p21 and/or p16. Thus, BLBP could regulate tumor suppressors to stimulate cell proliferation and modulate the cell cycle. However, besides tumor suppressors, G_1/S transition is also regulated by cyclins, and cyclin-dependent kinases (CDKs), we will detect these cyclins (e.g. cyclin D, cyclin E) or CDKs (e.g. CDK4/6 and CDK2) in our later experiments.

Exogenous BLBP facilitates tumor growth in xenograft models. Xenograft models are the most frequently used tools to evaluate the *in vivo* tumor formation ability. The C6 cells are subcutaneously implanted in the flanks of animals, after which the cells proliferated and developed into visible and measurable tumors after 21 days (Fig. 7A). Results showed that the C6 cells from both the LV-NC and LV-BLBP groups were capable of forming solid tumors *in vivo*. The volumes of tumors formed by the BLBP-expressing cells from the LV-BLBP group were significantly larger (Fig. 7B) and had a markedly higher number of Ki67-positive cells than those from the LV-NC groups (Fig. 7C and D). These findings demonstrated that exogenous BLBP expression promoted proliferation of the C6 cells *in vivo*. Considering the significant differences between the *in vivo* and *in vitro* microenvironments could serve as a major factor influencing tumor cell proliferation. Thus, we next measured the expression levels of the tumor suppressors p27, p21, and p16. Results showed that both p21 and p16 levels, but not p27 levels, decreased in the LV-BLBP group compared to those in the LV-NC group (Fig. 7E and F). The observed expression patterns of tumor suppressors *in vivo* were in accordance with the results obtained using cells cultured in the 1% FBS medium. Thus, our results implied that cell growth in the *in vivo* microenvironment was more similar to that in the *in vitro* culture conditions using the 1% FBS culture medium.

Exogenous BLBP was previously shown to enhance the migration ability of the U87 cells (37). Lastly, we detected the migration ability of C6 cells after exogenous expression of BLBP. However, we did not observe differences in terms of migration distance between the cells from the LV-NC and LV-BLBP groups (Fig. 8). Migration behavior induced by BLBP expression was suggested to be Cyclooxygenase-2 (Cox-2) expression-dependent (8); however, Cox-2 was not expressed in the C6 cells (52). Thus, we deduced that Cox-2 is a vital mediator of BLBP-dependent cell migration.

In summary, in this research, we used two groups of C6 cells (LV-NC group and LV-BLBP group), we compared certain data (e.g. viability, proteins expression, EdU positive cells) between these two groups in different serum concentration (10 and 1%) or at different time point (e.g. 24, 48, 72 h).

Our findings revealed that 1) exogenous expression of BLBP enhanced the *in vitro* proliferative ability of the BLBP-negative C6 glioma cell line; 2) exogenous BLBP expression prompted solid tumor growth *in vivo*; 3) the observed increase in cell proliferation upon treatment with exogenous BLBP is likely to be associated with the downregulation of tumor suppressors, such as p21 and p16, but not p27, both *in vivo* and *in vitro*. Furthermore, the involvement of BLBP in cell cycle regulation and cell migration requires further investigation.

Acknowledgements

This work was supported by grants of the Natural Science Foundation of Jiangsu Province (grant no. BK20140434), the National Natural Science Foundation of China (grant no. 81501133), the Innovative Training Program for College Students of Jiangsu Province (grant nos. 201613993001Y, 201510304089X).

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