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Downregulation of β-arrestin 1 suppresses glioblastoma cell malignant progression *vis* inhibition of Src signaling

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the most common brain malignancies worldwide and is typically associated with a dismal prognosis, yet the mechanisms underlying its aggressiveness remain unclear. Here, we revealed that β-arrestin 1 was overexpressed in GBM and contributed to poorer outcome. Knockdown of β-arrestin 1 suppressed the proliferation, invasiveness and glycolysis of GBM cells, and also enhanced temozolomide efficacy. Further, we discovered that knockdown of β-arrestin 1 decreased the activity of Src, and suppression of Src signaling was critically involved in β-arrestin 1 silencing-mediated suppression of GBM malignancies. Finally, we investigated the effect of β-arrestin 1 knockdown on the tumor growth and survival of xenograft models, and found that shβ-arrestin 1 apparently inhibited GBM growth *in vivo* and resulted in better survival of mice. Taken together, our findings suggest that knockdown of β-arrestin 1 can suppress GBM cell proliferation, invasion and glycolysis by inhibiting Src signaling. Thus, targeting β-arrestin 1 may be a potential therapeutic strategy for GBM treatment.

1. Introduction

Glioblastoma multiforme (GBM) is regarded as the most aggressive and lethal primary brain tumor in central nervous system [1,2]. Hallmarks of the GBM contain rapid growth, invasion to adjacent normal tissues, high rate of glycolysis, and chemoresistance [3–5]. Because of these features, GBMs are rarely curative and patients often have poorer outcome, with median survival < 15 months and median progression-free survival < 7 months [1,2]. This dismal situation motivates a search for molecules which are relevant to GBM malignant natures.

Beta-arrestin 1 belongs to the arrestin family and is ubiquitously distributed in mammalian tissues [6,7]. It is originally discovered as the major regulator of GPCRs (G protein-coupled receptors) internalization and desensitization [6,8–10]. With the knowledge of newly emerged researches, β-arrestin 1 is now recognized as it can scaffold many intracellular signaling molecules, and consequently modulate the strength and duration of numerous signalings including the Hedgehog, Wnt, Notch, Src and MAPK pathways [11–14]. Because of its universal expression and diversified functions, β-arrestin 1 plays critical roles in many physiological processes such as proliferation, differentiation and apoptosis [11,13]. Recently, it has been reported to be involved in tumorigenesis and progression in several types of cancer [15]. However, both the expression and biological function of β-arrestin 1 in GBM still remain poorly described.

Given the importance of β-arrestin 1 in human malignancies and its unclear role in GBM, in this study, we sought to determine the role of β-arrestin 1 in GBM progression. The results clearly showed that β-arrestin 1 contributes critically to proliferation, invasion and glycolysis of GBM. Importantly, we identified β-arrestin 1 as an upstream regulator of Src signaling, which mediated β-arrestin 1-induced malignancies of GBM.

2. Materials and methods

2.1. Patient samples

Thirty paired paraffin-embedded, archived GBM samples and their respective adjacent non-cancerous tissues as well as a survival cohort which contained 96 GBM specimens (for survival analysis only) were

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obtained from the Sanbo Brain Hospital, Capital Medical University (Beijing, China). All the samples were collected using protocols approved by the Ethics Committee of Sanbo Brain Hospital, and informed consents were obtained from all patients. The clinical and pathological classification and stage were determined according to the WHO classification of brain tumor criteria.

2.2. Immunohistochemistry (IHC) and scoring

Briefly, tissue sections were deparaffinized, soaked in Tris-EDTA buffer (pH 8.0) and boiled in the microwave, then incubated with the primary antibodies (described in immunoblotting) at 4 °C overnight. Next day, slides were washed and stained with the secondary antibody and DAB disclosure, counterstained with hematoxylin, dehydrated and mounted. The sections were reviewed and scored independently by two observers. IHC score was determined based on both the proportion of positively stained tumor (%) and the intensity of staining (weak: 1, moderate: 2, strong: 3), using the formula IHC score = percent of stained tumor (%)×intensity (1, 2, or 3). Cutoff value that defined low or high was 150 (low: score < 150; high: score≥150).

2.3. Cell culture and transfection

The human GBM cell lines H4, U87, U251 were purchased from the Cell Culture Center (Chinese Academy of Medical Sciences, Beijing, China); M059K were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All these cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and at 37 °C in 5% CO_2. Stable cell lines expressing shβ-arrestin 1was generated by transfection of pRS-β-arrestin 1 into U87 and U251 cells and cultured for 14 days with 400 μg/ml G418 or 0.5 μg/ml puromycin after infection. Positive clones were then selected and amplified for further analyses. For transient pCMV-β-arrestin 1 and pCMV-Src transfections (rescue experiments), pCMV-β-arrestin 1 and pCMV-Src, and their respective control vectors were transiently transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Both the pRS-β-arrestin 1 and pCMV-Src were obtained from OriGene.

2.4. Cell proliferation (MTS)/Colony formation

Briefly, a total of 3×10^3 cells in 100 μL of 10% FBS culture medium were seeded in 96-well plates. Once confluent, cells were cultured for 72 h before analysis. Then, the medium was aspirated and incubated with MTS solution (Promega, Madison, WI, USA) for 1 h. The viable cell number was reflected as the MTS absorbance which was measured spectrophotometrically at 490 nm. For evaluating the long-term proliferation of cells (colony formation assay), 1×10^3 tumor cells were plated into 60-mm dishes in 10% FBS culture medium. After 14 days, the cells were washed with PBS, fixed with methanol and 0.1% crystal violet. The colonies were counted and then photographed. All experiments were carried out in triplicate.

2.5. Transwell invasion assay

The Transwell invasion assay was performed using the Transwell chamber with Matrigel-coated. A total of 5×10^4 cells to be tested were starved in serum and growth factor-free medium overnight and then plated on the top chamber for 18–24 h, followed by removal of cells inside the upper chamber with cotton swabs, and the invasive cells on the lower side were fixed, stained with 0.1% crystal violet solution and counted using light microscope. The experiment was repeated three times.

2.6. AnnexinV/PI flow cytometry analysis

The Annexin V-FITC early apoptosis detection kit (Neobioscience, Shenzhen, China) was used to identify the apoptotic cells. Briefly, approximate 10^5 cells were harvested, washed with cold PBS twice and resuspended with 350 μL 1×Binding Buffer. Then, 5 μL of the Annexin V-FITC conjugate was added. After 20 min' light-prevented incubation at room temperature, cell suspension was diluted to a final volume of 500 μL /assay with ice cold 1×Binding Buffer. Next, 10 μL of the Propidium Iodide (PI) solution were added to each sample tube, and the samples were analyzed by FACS Canto™II cell analyzer (BD Biosciences, San Jose, CA, USA).

2.7. Measurements of glucose and lactate

A total of $6–8\times10^4$ cells per well were seeded in 12-well plates for 24 h, then medium was collected and the glucose and lactate levels were examined immediately. Glucose and lactate were measured using Glucose Assay Kit (Sigma-Aldrich) and Lactate Assay Kit (Sigma-Aldrich) respectively. The glucose consumption and lactate production were normalized to cell numbers (μmol per 4×10^4) and then determined relatively by the value of control.

2.8. Real-time PCR (qPCR)

Total RNA from cells was extracted with TRIzol (Invitrogen). First-strand cDNA was synthesized by using the Superscript II-reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR (qPCR) was conducted using SYBR Premix Ex Taq (Takara) on an ABI 7300 Real-Time PCR System (Applied Biosystems). All samples were normalized to GAPDH. Gene-specific qPCR primer pairs are provided as below.

ARRB1 (β-arrestin 1): Fw: 5′TTTGTGGCCAACGTACAGTG3′, Rev: 5′GTGAAAGGGTAAGCGTGCTC3′.

Slc2a1 (Glut1): Fw: 5′ CGGGCCAAGAGTGTGCTAAA 3′, Rev: 5′TGACGATACCGGAGCCAATG 3′.

Pkm2: Fw: 5′ CCA CTT GCA ATTATT TGA GGA A 3′, Rev: 5′ GTG AGC AGA CCT GCC AGA CT 3′.

Ldha: Fw: 5′ATCTTGACCTACGTGGCTTGGA3′, Rev: 5′CCATACAGGCACACTGGAATCTC 3′.

2.9. Immunoblotting

Total cell protein extracts were separated on 10% or 15% SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were subsequently probed with indicated primary antibodies and anti-mouse or anti-rabbit secondary antibodies, respectively. All of the first antibodies were diluted at 1:1000 except for Actin at 1:5000. The chemiluminescence signal was detected with Luminescent Image Analyzer LAS-4000 (Fujifilm). Blotting membranes were stripped and reprobed with anti-Actin as a loading control. Antibodies were described as followed: anti-β-arrestin 1 and anti-Glut1 antibodies were purchased from Abcam. Anti-Src, anti-p-Src (Tyr 416), anti-ERK (ERK1/2), anti-p-ERK (Thr202/Tyr204), anti-c-Myc, anti-MMP9, anti-Mcl-1, anti-Ki-67, anti-Actin, anti-mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technology.

2.10. Xenograft studies

Female, 5 weeks old, Nu/Nu mice were purchased from Vital River laboratories (Beijing, China). All animal care and experiments were carried out according to the Institutional Animal Welfare Guidelines of Chinese Academy of Medical Sciences. A total of 1×10^6 shβ-arrestin 1 U87, or shvector U87 were injected subcutaneously into mice. Measurement of tumor volume started from 2 weeks after injection and was operated every 6 days. At the end of each experiment, mice

were sacrificed, and tumors were calculated and paraffin-embedded. Sections of 5.0 μm were cut and subjected to IHC staining.

2.11. Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad software Inc., La Jolla, CA, USA). Paired GBM samples were analyzed by paired *t*-test. Survival curves were plotted using Kaplan–Meier estimates. One-way ANOVA test was employed for statistical analysis of remaining data. All tests were two-tailed Data are presented as means ± SEM. The $p < 0.05$ was considered statistically significant.

3. Results

3.1. Overexpressed β-arrestin 1 correlates with poor clinical outcomes in GBM patients

To explore the potential role of β-arrestin 1 in GBM, we first assessed its expression in 30 paired adjacent normal and GBM tissues using the immunohistochemistry (IHC) assay. Compared with the nontumorous tissues (median IHC score: 81), β-arrestin 1 was statistically overexpressed in GBM tissues (median IHC score: 182) (Fig. 1A and B). To investigate the relationship between β-arrestin 1 expression and patient outcome, we performed IHC staining of β-arrestin 1 on 96 GBM specimens with long-term follow-up records. High level of β-arrestin 1 was positively correlated with poorer overall survival of GBM patients (Fig. 1C). Collectively, these results suggest that β-arrestin 1 may play an oncogenic role in GBM.

3.2. Knockdown of β-arrestin 1 suppresses GBM malignancies

Next, we investigated the expression of β-arrestin 1 in human normal astrocyte (NHA) and a panel of GBM cells. Immunoblotting assay demonstrated that GBM cells exhibited a much higher level of β-arrestin 1 than the NHA (Fig. 2A). To examine whether β-arrestin 1 plays a role in the malignancies of GBM, U87 and U251 cell lines with stable arrestin 1 deficiency were established. The protein level of β-arrestin 1 was effectively downregulated by 70–80% in shRNA-transfected cells (Fig. 2B), leading to a significant decrease of proliferation and invasiveness in both shβ-arrestin 1 U87 and U251 compared with their vector control (Fig. 2C and D). To exclude the possibility that shβ-arrestin 1-inhibited GBM cell growth and invasion was induced by the off-target effect of our shRNA, we conducted rescue experiment, and found that transfection of β-arrestin 1 plasmid significantly reversed the downregulated β-arrestin 1 at both mRNA and protein levels (Supplementary Data 1A and 1B). We also discovered that the rescued β-arrestin 1 potentially increased the proliferation rate and invasiveness of the shβ-arrestin 1 cell (Supplementary Data 1C and 1D). Furthermore, colony formation assays showed that β-arrestin 1 ablation effectively suppressed the anchorage-dependent growth of U87 cells compared with its control vector (Fig. 2E). We next investigated whether knockdown of β-arrestin 1 could affect the anti-apoptotic ability of GBM cells. The flow cytometry (FCM) analysis demonstrated that downregulation of β-arrestin 1 markedly sensitized U87 cells to 100 μM temozolomide treatment (Fig. 2F). Similar results were also found in U251 cells (Fig. 2E and F). Altogether, these data reveal a critical role of β-arrestin 1 in GBM malignancies.

3.3. Knockdown of β-arrestin 1 inhibits GBM glycolysis

Because glycolysis provides energy for GBM proliferation and critically contributes to GBM malignant progression, we next investigated whether inhibition of β-arrestin 1 expression downregulated GBM glycolysis. As shown in Fig. 3A, β-arrestin 1- depleted cells showed significantly reduced levels of glycolytic indexes, such as glucose uptake and lactate production compared with control cells. We further evaluated the mRNA levels of key factors in glycolysis including *Slc2a1*, *Pkm2*, and *Ldha* in shβ-arrestin 1 and their vector cells. Results of qPCR assay demonstrated that expressions of these glycolytic factors were decreased when depleting β-arrestin 1 in U87 cells (Fig. 3B). Similar results were also obtained in U251 cells (Fig. 3B). Taken together, these data suggest that knockdown of β-arrestin 1 effectively inhibit GBM glycolysis.

3.4. Knockdown of β-arrestin 1 decreases the activity of Src

To explore the molecular mechanism of shβ-arrestin 1 in suppressing GBM malignancies, we assessed the alternation of Src and MAPKs activities, which had been reported to be regulated by β-arrestin 1 in panel researches [12–14]. Depletion of β-arrestin 1 significantly decreased the phosphorylated Src (Tyr416, p-Src) without affecting its total protein expression (Fig. 4A). However, depletion of β-arrestin 1 exerted no detectable effects on the phosphorylated status of MAPKs in GBM cells (Fig. 4A). Expressions of key effectors involved in cell growth, invasion, glycolysis and anti-apoptosis, such as c-Myc, MMP9, Glut1, and Mcl-1, were also decreased in shβ-arrestin 1 cells compared with their vector counterparts (Fig. 4B). We next investigated whether Src contributed to β-arrestin 1-mediated inhibition of GBM malignancies. We incubated control vector and β-arrestin 1-depleted U87 cells with 100 nM Src selective inhibitor dasatinib. As presented in Fig. 4C, 100 nM dasatinib effectively attenuated Src activation, and consequently decreased cell growth, invasion and glycolysis in the control vector cells (Fig. 4D-F). However, further inhibition of Src activity had no effect on those malignant phenotypes in β-arrestin 1-depleted U87 cells (Fig. 4D-F). In addition, we transiently transfected the Src plasmid into shβ-arrestin 1 cells (Fig. 4G), and observed that ectopic

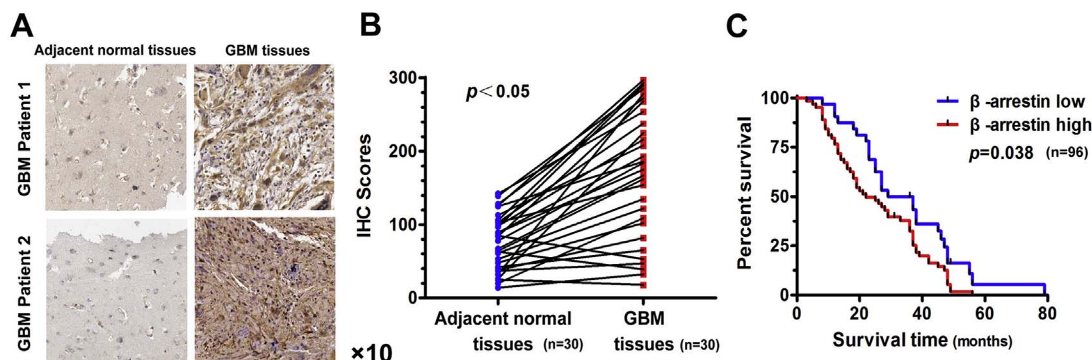

Fig. 1. Relationship between β-arrestin 1 expression and GBM progression. A. Representive IHC staining of β-arrestin 1 in adjacent normal and GBM tissues (Magnification, ×10 as indicated.). B. Analysis of β-arrestin 1 expression in adjacent normal and GBM tissues (n=30; ⋆, $p < 0.05$, paired *t*-test). C. Kaplan–Meier curves of GBM patients with low *versus* high expression of β-arrestin 1 (n=96; ⋆, $p < 0.05$, log-rank test).

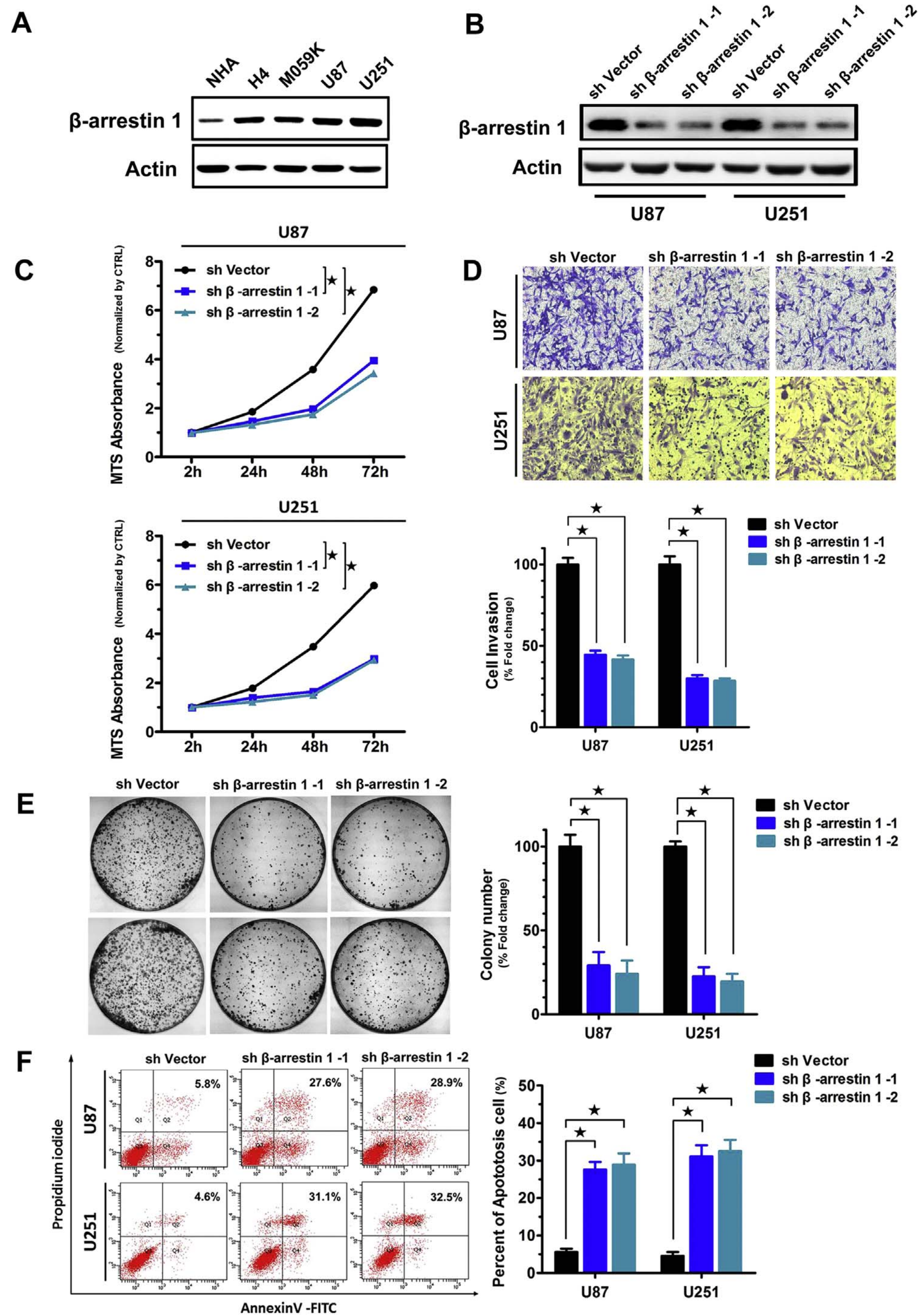

Fig. 2. Effects of β-arrestin 1 knockdown on the aggressiveness of GBM cells *in vitro*. A. Immunoblotting analysis of β-arrestin 1 protein levels in normal human astrocyte cells (NHA) and GBM cell lines (H4, M059K, U87, and U251). Expression levels were normalized to β-actin. B. Transfection efficacy of β-arrestin 1 shRNA in U87 or U251 cell lines was analyzed by immunoblotting, respectively. C-E. Evaluating the effects of β-arrestin 1 shRNA on the growth (C) invasion (D) and colony formation ability (E) of U87 and U251 cells. F. Evaluating the effects of β-arrestin 1 shRNA on the apoptosis of 100 μM temozolomide treated U87 and U251 cells by FCM assay. (⋆, $p < 0.05$; One-way ANOVA. Error bars, mean ± SEM of three independent experiments).

expression of Src restored the characteristics of proliferation, invasiveness and glycolysis in β-arrestin 1 knockdown U87 cells (Fig. 4H-J). Similar results were also obtained in U251 cells (Fig. 4H-J). These results reveal that inhibition of Src contributes to β-arrestin 1 knockdown- mediated GBM suppression.

3.5. Targeting β-arrestin 1 suppresses GBM progression in vivo

To evaluate if downregulation of β-arrestin 1 could inhibit GBM progression *in vivo*, we used U87 shβ-arrestin 1 and its vector cells, then subcutaneously injected them into nude mice. Obviously, the average tumor size of the β-arrestin 1 knockdown group was much smaller than the control group (Fig. 5A). Besides, tumors derived from the shβ-arrestin 1 cells demonstrated slower growth rate than their vector counterparts (Fig. 5B). More importantly, shβ-arrestin 1 U87–harbored mice had significantly longer survival time in contrast to the mice which were injected with control shRNA cells (Fig. 5C). Expression of proliferative marker Ki-67, c-Myc, tumor invasion factor

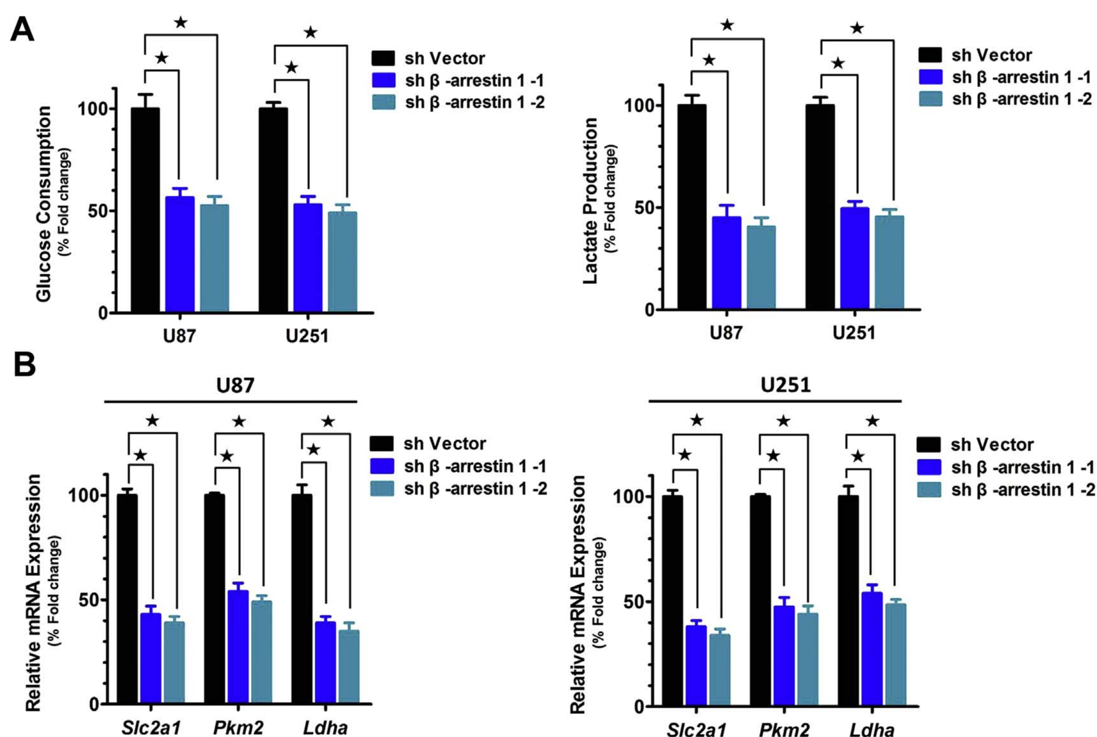

Fig. 3. Effects of β-arrestin 1 knockdown on the glycolysis of GBM cells *in vitro*. A. Alternation of glucose consumption and lactate production in vector control and shβ-arrestin 1 cells. B. Evaluating the effects of β-arrestin 1 shRNA on the glycolysis related gene expression in U87 and U251 cells using qPCR. (★, $p < 0.05$; One-way ANOVA. Error bars, mean ± SEM of three independent experiments).

MMP9, glycolysis marker Glut1 and p-Src, were further detected by immunohistochemistry. Consistent with the *in vitro* data, depletion of β-arrestin 1 potentially reduced the levels of Ki-67, p-Src, c-Myc, MMP9 and Glut 1 in subcutaneous tumors (Fig. 5D). Taken together, these results indicate that downregulation of β-arrestin 1 can inhibit GBM progression *in vivo*.

4. Discussion

Recently, growing evidences have indicated that nonvisual β-arrestins, which include β-arrestin 1 and β-arrestin 2, are involved in different pathological processes, especially promoting many oncogenic signaling in the progression of malignant tumors [16–18]. In the present study, we focused on the β-arrestin 1 in GBM based on our preliminary work, in which we did not find that β-arrestin 2 was differently (no statistical significance) expressed between the GBM tissues and adjacent normal tissues (data not shown). The results of β-arrestin 1 expression in GBM are consistent with a previous study [19]. Furthermore, in such study, using western blotting analysis, Mandell et al. also found a significant decrease in beta arrestin1 phosphorylation. So far, researches about β-arrestin 1 in different types of cancer mainly focused on the role of its total form [20–23]. However, phosphorylation of β-arrestin 1 mediates multiple biological functions [24,25]. Therefore, the role of β-arrestin 1 dephosphorylation in cancer progression should also be taken into consideration in the future work.

Although β-arrestin 1 has been implicated in certain cancers, such as non-small cell lung carcinoma, breast cancer, ovarian cancer and colorectal cancer [21,26–28], either the biological role or the underlying mechanism by which β-arrestin 1 functioned remains to be elucidated. In our study, we found that β-arrestin 1 was significantly elevated in GBM compared with adjacent normal tissues, and such a high level of β-arrestin 1 was tightly associated with malignancy of GBM, including the survival time of GBM patients, collectively demonstrating a pivotal role of β-arrestin 1 in the progression of GBM. Furthermore, we found that downregulating the expression of β-arrestins 1 in human GBM cell lines significantly inhibited GBM malignancies, typically cell proliferation, invasion and glycolysis. Resistance to temozolomide accounts for the dismal prognosis of GBM patients [29], and we also found that temozolomide-induced apoptosis in GBM cells was significantly enhanced by β-arrestin 1 silencing. Moreover, expression of anti-apoptotic factor Mcl-1 was decreased in shβ-arrestin 1 cells, suggesting that β-arrestin 1 might be essential for chemotherapy sensitization in GBM. These results strongly support that β-arrestin 1 may be a promising therapeutic target against GBM.

In this study, we found that inhibition of Src signaling, one of the most pivotal pathways which governs multitudes of malignant properties [30–32], might contribute to β-arrestin 1 knockdown-mediated GBM suppression. The original function of β-arrestin 1 is discovered to desensitize activated GPCRs. Nowadays, increasing evidences have shown that β-arrestin 1 is a well-established mediator of receptor endocytosis, ubiquitylation and G protein-independent signaling [33–35]. Recent global analyses of β-arrestin interactions and β-arrestin-dependent phosphorylation events have uncovered several previously unanticipated roles of β-arrestins in a range of cellular signaling events [11,13,14]. One of the most interesting findings is the observation that β-arrestin 1 can scaffold the tyrosine kinase Src to agonist-activating GPCRs, such as nAChR and ET_AR [28,36,37]. Previous studies have demonstrated that β-arrestin 1-mediated Src activation is majorly dependent on the recruiting function of GPCRs [12,14,38–40]. On the basis of the fact that GPCRs are critical in contributing to GBM progression, whether and how certain GPCRs participate in β-arrestin

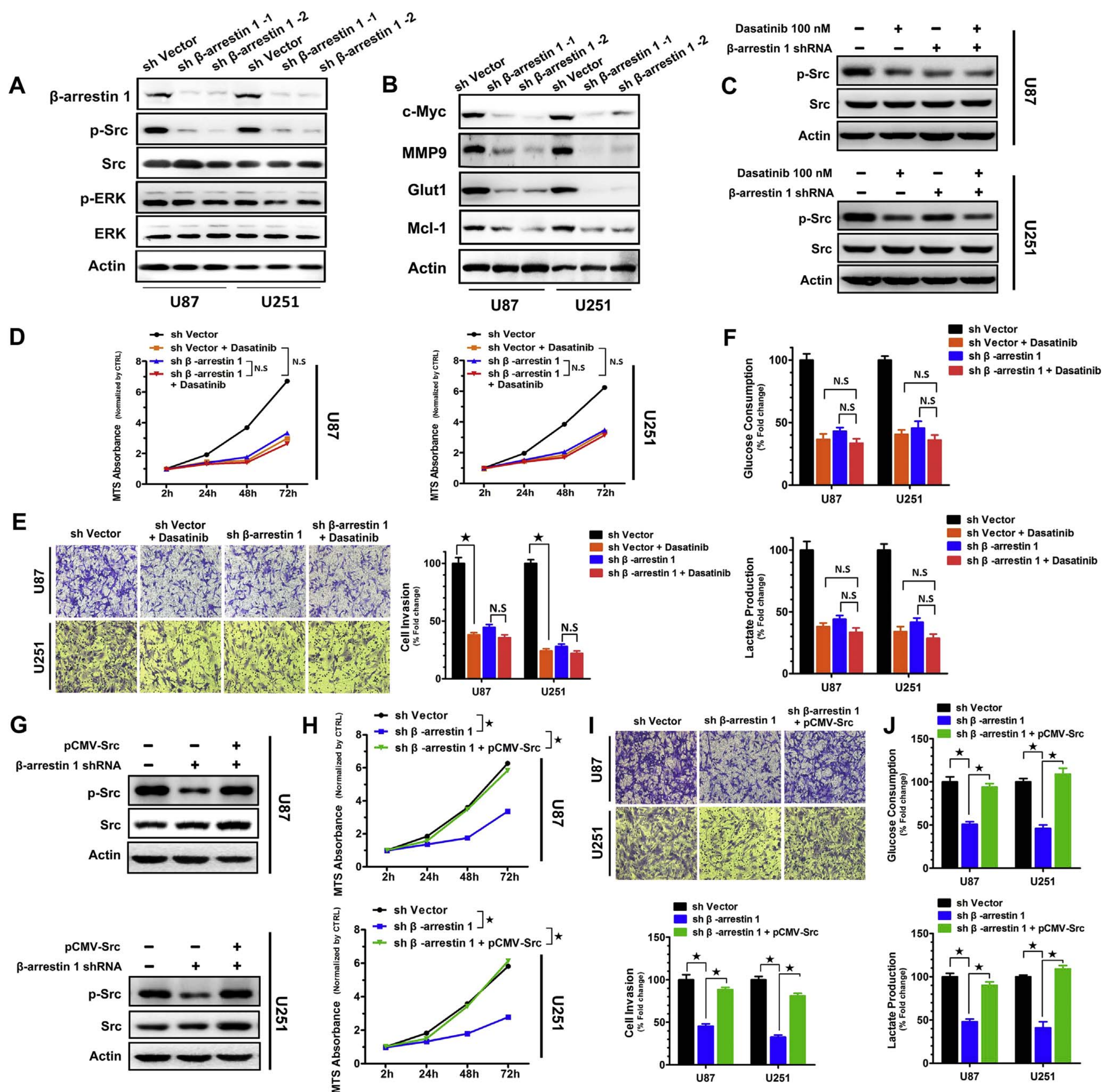

Fig. 4. Effects of β-arrestin 1 knockdown on the Src signaling in GBM cells. A. Changes of Src and MAPK signaling in shvector and shβ-arrestin 1 harboring GBM cells were analyzed by immunoblotting. B. Changes of downstream effectors in shvector and shβ-arrestin 1 harboring GBM cells were analyzed by immunoblotting. C. Detection of phosphorylated Src by immunoblotting in the shvector and shβ-arrestin 1 cells which were respectively treated with 100 nM Dasatinib. D-F. Evaluating the effects of β-arrestin 1 shRNA, 100 nM Dasatinib, or 100 nM Dasatinib in the presence of β-arrestin 1 shRNA on the growth (D), invasion (E) and glycolysis (F) of U87 and U251 cells. G. Phosphorylation status of Src in pCMV-Src transiently transfected shβ-arrestin 1 U87 and U251 cells were analyzed by immunoblotting. H-J. Evaluating the rescue effects of Src in β-arrestin 1 knockdown cells on the growth (H), invasion (I) and glycolysis (J) of U87 and U251. (⋆, $p < 0.05$, N.S, no significance; One-way ANOVA. Error bars, mean ± SEM of three independent experiments).

1-mediated Src activation in GBM remains a big question. These issues are needed to be elucidated in the future work.

A number of arrestin-regulating effectors are key players in cell proliferation, survival, and apoptotic death, which makes β-arrestin 1 the central node in GBM progression and ideal target for GBM therapy. However, since it is ubiquitously expressed and vital in both normal and tumorous cells, a serious problem, the side-effect, may arise from the targeting strategy towards β-arrestin 1. Consequently, further study is needed to determine whether GBM is more addicted to the over-expressed β-arrestin 1 than the normal human cells, which would make GBM cells relatively sensitive to the anti-β-arrestin 1 treatment.

Statement of author contributions

Tian Lan, Haoran Wang, Zhihua Zhang, Zitong Zhao and Xinyi Fan carried out experiments. Mingshan Zhang, Yanming Qu, and Tian Lan analyzed data. Qimin Zhan, Yongmei Song and Chunjiang Yu conceived experiments and wrote the paper. All authors had final approval of the submitted and published versions.

Conflict of interest statements

None.

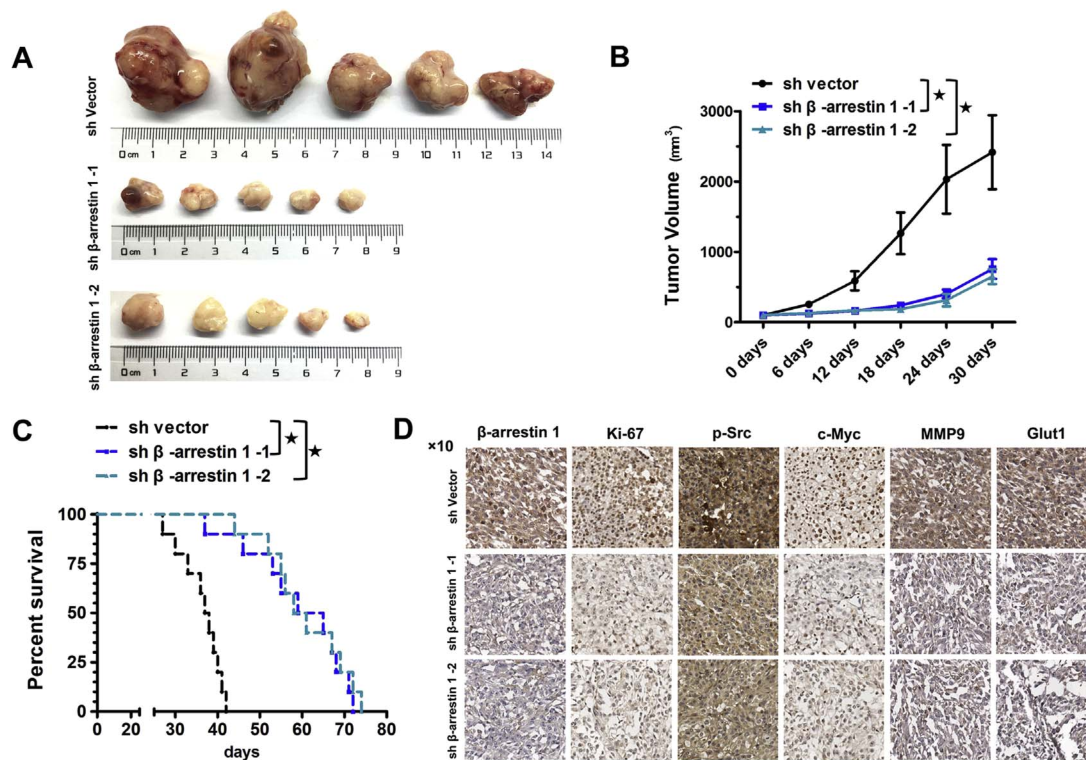

Fig. 5. Effects of β-arrestin 1 knockdown on the progression of GBM *in vivo*. A. Representive images of vector and β-arrestin 1 shRNA harboring tumors harvested from nude mice. B. Volume of β-arrestin 1 vector and shRNA derived subcutaneous tumor (calculated from 2 weeks after tumor cell injection). C. Kaplan–Meier curves of the survival periods in β-arrestin 1 shRNA tumor-bearing animal and their respective control group. D. IHC staining of β-arrestin 1, Ki-67, p-Src, c-Myc and Glut1 in β-arrestin 1 shRNA and vector derived tumor samples. (Magnification, ×10 as indicated.). (★, $p < 0.05$; One-way ANOVA for tumor volume, and log-rank test for survival. Error bars, mean ± SEM of three independent experiments).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2017.04.023.

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