Steroid receptor coactivator-1 enhances the stemness of glioblastoma by activating long noncoding RNA XIST/miR-152/KLF4 pathway

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Abstract
Glioblastoma (GBM) recurrence is attributed to the presence of therapy-resistant glioblastoma stem cells. Steroid receptor coactivator-1 (SRC-1) acts as an oncogenic regulator in many human tumors. The relationship between SRC-1 and GBM has not yet been studied. Herein, we investigate the role of SRC-1 in GBM. In this study, we found that SRC-1 expression is positively correlated with grades of glioma and inversely correlated with glioma patient's prognosis. Steroid receptor coactivator-1 promotes the proliferation, migration, and tumor growth of GBM cells. Notably, SRC-1 knockdown suppresses the stemness of GBM cells. Mechanistically, long non-coding RNA X-inactive specific transcript (XIST) is regulated by SRC-1 at the post-transcriptional level and mediates the function of SRC-1 in promoting stemness-like properties of GBM. Steroid receptor coactivator-1 can promote the expression of Kruppel-like factor 4 (KLF4) through the XIST/microRNA (miR)-152 axis. Additionally, areobufagin and bufalin, SRC small molecule inhibitors, can reduce the proliferation and stemness of GBM cells. This study reveals SRC-1 promotes the stemness of GBM by activating the long noncoding RNA XIST/miR-152/KLF4 pathway and provides novel markers for diagnosis and therapy of GBM.

KEYWORDS
cancer stem cell, glioblastoma, lncRNA XIST, posttranscriptional regulation, steroid receptor coactivator-1
1 | INTRODUCTION

Glioblastoma (GBM) has an extremely grim prognosis and is the most common aggressive brain tumor.\textsuperscript{1,2} It was classified as grade IV glioma of the astrocytic lineage by the WHO.\textsuperscript{1,3} Standard therapy, consisting of surgery and the combination of temozolomide (TMZ) chemotherapy and radiation, offers median survival of only 15-18 months after initial diagnosis.\textsuperscript{4-8} The poor prognosis of GBM patients is related to the oncogenic nature of the GBM cell and the existence of a small subpopulation of glioblastoma stem cells (GSCs). Glioblastoma stem cells have the capability of self-renewal and multiple differentiation and are resistant to chemotherapy and radiotherapy. Consequently, they are likely responsible for failure of treatment and high recurrence rates in GBM.\textsuperscript{9,11}

Steroid receptor coactivator-1 (SRC-1, also called NCOA1) is a transcriptional coactivator in the SRC family that also contains SRC-2 (TIF2 or GRIP1) and SRC-3 (AIB1 or ACTR). These coactivators promote transcription by interacting with nuclear receptors such as estrogen and progesterone receptors, as well as other transcription factors such as activator protein-1, nuclear receptors such as estrogen and progesterone receptors, as well as other transcription factors such as activator protein-1, nuclear factor-κB, β-catenin, Ets-2, PEA3, and HOXC11.\textsuperscript{12,13} A previous study showed that SRC-1 promotes metastasis through mediating Ets-2-mediated human epidermal growth factor receptor-2 (HER2) expression and by activating colony stimulating factor-1 expression for macrophage recruitment.\textsuperscript{14} Loss-of-function deletion of the SRC-1 gene in mice reduces estrogen effect on the vascular injury response.\textsuperscript{15} Additionally, recent studies reported that SRC-1 is overexpressed in breast cancer and hepatocellular carcinoma, and regulates the proliferation of breast cancer, prostate cancer, and hepatocellular carcinoma.\textsuperscript{12,14,16-22} Furthermore, SRC-1 is important for cancer stem-like cells in lung and breast cancer.\textsuperscript{23} However, the relationship between SRC-1 and GBM has not yet been studied in detail.

In this study, we found that SRC-1 is positively correlated with grades of glioma and inversely correlated with glioma patient’s prognosis. Steroid receptor coactivator-1 promoted the proliferation, migration, and tumor growth. Additionally, SRC-1 enhanced stem-like characteristics of GBM cells. Mechanistic studies showed that SRC-1 enhances the stemness by activating the long noncoding RNA (lncRNA) X-inactive specific transcript (XIST)/microRNA (miR)-152/Kruppel-like factor 4 (KLF4) pathway in GBM cells. Our findings show that SRC-1 could be used as a diagnostic marker and therapeutic target for GBM.

2 | MATERIALS AND METHODS

2.1 | Tissue microarray

Paraffin-embedded tumor tissue microarrays were purchased from Shanghai Outdo Biotech. Brain tumor classification was conducted according to WHO criteria.\textsuperscript{3} There were 59 specimens on the tissue microarray, including three normal brain tissues, 20 astrocytoma, 34 glioblastoma, and two between astrocytoma and glioblastoma. The clinical characteristics of the cohort are described in Table S1. The immunohistochemical staining was scored according to the four-point system (score 0-3) as previously reported.\textsuperscript{24-26} A score of 2 or 3 was considered high expression, and the score of 0 or 1 was low expression. The mean immunoreactive score (IRS) was considered as the final IRS (Table S1).

2.2 | Cell culture

LN229, U251, U87-MG, T98G, U118, C6, SVG-p12 (human fetal glial cell), HEK293T, and HeLa cell lines were obtained from ATCC. All cells were incubated in DMEM (Gibco) supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin at 37°C under 5% CO\textsubscript{2}.

2.3 | Lentivirus production and virus infection

The shRNA knockdown plasmids (GV248-shSRC1-1 and GV248-shSRC1-2) targeting SRC-1 as well as a nontargeting shRNA plasmid were purchased from Genechem. Full-length cDNA encoding human SRC-1 was generated by PCR from the SRC-1 recombinant plasmid from Genechem and its sequence was confirmed (NM_003743) by DNA sequencing. The fragment was subcloned into pCDH-CMV-MCS-EF1-Puro plasmid (System Biosciences). To generate recombinant lentivirus, these constructs were cotransfected with lentiviral packaging vectors (pSPAX2 and pMD2.G) into 293T cells by using Lipofectamine 2000 (Invitrogen). Then media supernatant was collected. For transduction of GBM cells, lentiviral supernatant was added into the culture medium for 24 hours. Puromycin (1 μg/mL) was used to remove noninfected GBM cells. Surviving clones were isolated and examined by real-time PCR and western blot to determine SRC-1 knockdown or overexpression efficiency. Primers used for PCR are shown in Table S2.

2.4 | Cell proliferation assay and colony formation assay

The MTT and colony formation assays were carried out as we described previously.\textsuperscript{27-29}

2.5 | Cell cycle analysis

Cell cycle analysis was carried out as we described previously.\textsuperscript{29,30}

2.6 | Wound healing assay

Wound healing assay was carried out as we described previously.\textsuperscript{29,31-33}
2.7 | Western blot

The western blot assay was carried out according to our previous studies. Antibodies information is summarized in Table S3.

2.8 | RNA extraction and RT-quantitative PCR

The RNA extraction and RT-quantitative PCR (qPCR) were carried out according to our previous studies. Primers used for qPCR are shown in Table S4.

2.9 | Immunofluorescence

Immunofluorescence (IF) was carried out as we described previously. Antibody information is listed in Table S3.

2.10 | Sphere formation assay

The sphere formation assay was carried out according to our previous studies.

2.11 | CD133 marker detection by flow cytometry

Glioblastoma stem cells were cultured and enriched by sphere formation, and then GSCs were harvested, dissociated, washed with PBS, and incubated with 2 μL CD133-PE Ab (1:50) (Miltenyi Biotec) for 30 minutes. The GSCs were then washed, suspended in PBS, and subjected to flow cytometry (BD AccuriC6) to determine the percentages of viable CD133+ cells.

2.12 | RNA sequencing

The RNA sequencing (RNA-seq) experiments were undertaken by Novogene.

2.13 | Animal and tumor models

2.13.1 | Tumor xenografts in nude mice

Five-week-old nude mice (BALB/c) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. To initiate tumors, 1 × 107 cells in 100 μL PBS + Matrigel (2:1, v/v; BD Biosciences) were injected s.c. into the dorsal flank of each nude mouse by two blinded technicians. After the tumor volume reached approximately 100 mm³, the tumor volumes (A × B²/2; A being the greatest diameter and B being the diameter perpendicular to A) were measured twice a week by digital calipers. At the end of the studies, mice were sacrificed, and tumors were dissected, weighed, stored, and fixed.

2.13.2 | Rat brain orthotopic implantation model

Male Wistar rats (200-300 g) were purchased from Liaoning Changsheng Technology Co., Ltd. The C6 GBM cell suspension (1 × 10⁶ cells in 10 μL PBS) was injected stereotactically over a 10-minute period using a Hamilton syringe at a depth of 5 mm. At the end of the studies, mice were sacrificed, and brains were excised and fixed. Tumor volumes were calculated using the formula A × B²/2, where A was the maximum long-dimension tumor diameter and B was the maximum short-dimension tumor diameter.

All animal procedures and animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals under the approval of the SPF Laboratory Animal Center at Dalian Medical University.

2.14 | Immunohistochemistry and H&E staining

Tumors and brains were fixed in 4% paraformaldehyde (Sigma-Aldrich) and prepared for histological analysis. All H&E and immunohistochemical (IHC) staining was carried out as previously described. Antibody information is listed in Table S3.

2.15 | Promoter reporter and dual luciferase assays

Human XIST promoter was amplified from the human genomic DNA template and inserted into pGL3-basic vector (Promega). For the luciferase reporter assays, cells were seeded in 24-well plates and transfected with the indicated plasmids. For normalization of transfection efficiency, pRL-TK (Renilla luciferase) reporter plasmid was added to each transfection. Forty-eight hours after transfection, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega).

2.16 | Statistical analysis

All statistical analyses were carried out using SPSS 19.0 software. The results are represented as means ± SD. Statistical differences between two groups were analyzed by two-tailed Student’s t test. Levene’s test was used to test variance equality. The relationship between SRC-1 expression and clinicopathologic parameters was analyzed by the χ² test. Statistically significant differences were considered significant when P < .05 (*P < .05, **P < .01, ***P < .001) and not significant when P > .05.
3 | RESULTS

3.1 | Steroid receptor coactivator-1 expression is positively correlated with grades of glioma and inversely correlated with glioma patient’s prognosis

Western blot and IF were used to detect the expression and distribution of SRC-1 in glioma tumor tissue and GBM cell lines. The data from western blot revealed that SRC-1 expression was upregulated in several GBM cells compared with SVG p12 (Figure 1A). Immunofluorescence revealed that SRC-1 was a dominantly nuclear-expressing protein of GBM tissue and glial fibrillary acidic protein expressed in cytoplasm of GBM cells (Figure 1B).

To further confirm the potential clinical implications of SRC-1 in gliomas, IHC was carried out using a glioma tissue microarray (Table S1). No statistically significant correlations were identified between SRC-1 expression and gender or age (Figure 1C). In low-grade tumors (grades I and II), the percentage of high expression of SRC-1 was 25.00% (3/12), whereas in high-grade tumors (grades III and IV), the percentage of high expression of SRC-1 was 86.36% (38/44). According to our analysis, we found that the expression of SRC-1 was strongly associated with the grade of the tumors (Figure 1C,D). Representative images of SRC-1 by IHC staining in different WHO grades of glioma tissues and normal brain tissue are shown in Figure 1E. Moreover, patients with high expression of SRC-1 showed poorer overall survival than those with low expression (GSE44412, GSE4271) (Figure 1F). Together, our findings indicate that SRC-1 expression is positively correlated with clinical glioma malignant grade and inversely correlated with patient’s prognosis.

3.2 | Steroid receptor coactivator-1 promotes cell proliferation and migration in GBM cells

To explore the possible functions of SRC-1 in GBM, SRC-1 knockdown and overexpression cells were developed and named as...
Steroid receptor coactivator-1 (SRC-1) promotes the cell proliferation of glioblastoma cells. A-D, Cell viability was examined by MTT assay for U251, U87, C6, and LN229 cells in 48 h (n = 3). E, F, Cell colony formation ability was examined in U251 and LN229 cells (n = 3). G, I, Flow cytometry analysis was used to examine cell cycles in U251 and LN229 cells (n = 3). PI, propidium iodide. H, J, Western blot analysis for SRC-1, proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin-dependent kinase 4 (CDK4) in U251, U87, C6, and LN229 cells. K, L, Cell viability was detected in U251 cells, which were treated with temozolomide (TMZ; 500 and 1000 μmol/L) and sorafenib (SOR; 50 and 80 μmol/L). *P < .05, **P < .01
Steroid receptor coactivator-1 (SRC-1) promotes the cell migration of glioblastoma cells. A-D, Migration ability was detected in U251, U87, C6, and LN229 cells by wound healing assay (n = 3). Scale bar, 500 and 200 μm. E, F, Western blot analysis for SRC-1, N-cadherin, E-cadherin, Vimentin, and TWIST1 in U251, U87, C6, and LN229 cells. G, H, Representative immunofluorescence images of U251 and LN229 cells stained for Vimentin (red) and DAPI (blue). Scale bar, 50 μm. **P < .01, ***P < .001.
FIGURE 4  Steroid receptor coactivator-1 (SRC-1) enhances the self-renewal ability of glioblastoma stem cells. A-D, Self-renewal ability was detected in U251, U87, C6, and LN229 cells by sphere formation assay. Scale bar, 100 μm. E, Representative images and quantitative assessment of CD133 were examined in LN229-SRC1 spheres compared with LN229-EV spheres using flow cytometry. F, G, Expression of SRC-1, CD133, and Kruppel-like factor 4 (KLF4) were detected by western blot analysis in U251, U87, C6, and LN229 cells. *P < .05, **P < .01, ***P < .001
U251-shSRC1, U87-shSRC1, C6-shSRC1, and LN229-SRC1. These cells were used to investigate the effects of SRC-1 on cell proliferation and migration in GBM cells. First, the results of MTT and colony formation revealed that SRC-1 knockdown significantly suppressed U251, U87, and C6 cell proliferation, whereas SRC-1 overexpression increased cell proliferation in LN229 cells (Figure 2A-F). SRC-1 knockdown increased the percentage of U251 cells in the G0/G1 phase with a concomitant decrease in S and G2/M phase (Figure 2G). Furthermore, SRC-1 knockdown suppressed the expression of proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin-dependent kinase 4 (CDK4) (Figure 2H). In contrast, SRC-1 overexpression significantly reduced the percentage of LN229 cells in the G0/G1 phase and upregulated the expression of PCNA, cyclin D1, and CDK4 (Figure 2I,J).

To further evaluate the functional significance of SRC-1 in the chemoresistance of GBM, cell viability was examined in the U251-EV and U251-shSRC1 cells under treatment with increasing concentrations of TMZ and sorafenib (SOR). Silencing SRC-1 remarkably reduced the proliferation of U251 cells treated with TMZ or SOR (Figure 2K,L).

Next, migration rate was examined in U251, U87, C6, and LN229 cells by wound healing assay. As shown in Figure 3A-D, SRC-1 knockdown significantly reduced U251, U87, and C6 cell migration rates, and the reverse was shown in LN229-SRC1 and LN229-EV cells. To verify the roles of SRC-1 on the epithelial-mesenchymal transition (EMT) process, the protein expression of N-cadherin, E-cadherin, Vimentin, and TWIST1 was detected by western blot and IF. Our data confirmed that SRC-1 plays an important role in promoting EMT of GBM cells (Figure 3E-H). Together, these data suggest that SRC-1 promotes GBM cell proliferation and migration.

### 3.3 Steroid receptor coactivator-1 enhances stem-like characteristics in GBM cells

To determine the roles of SRC-1 in GSCs, the self-renewal capacities of U251, U87, C6, and LN229 cells was detected by sphere formation assay. The results showed that SRC-1 knockdown significantly decreased sphere initiation and growth, and opposite results were shown in LN229-SRC1 cells compared with LN229-EV cells (Figure 4A-D). To further clarify the roles of SRC-1 on the GSCs at the molecular level, the expression levels of CD133 and KLF4 were examined by flow cytometry and western blot. The results of flow cytometry showed that the percentage of CD133+ cell counts were significantly increased in LN229-SRC1 cell spheres when compared with LN229-EV cell spheres (Figure 4E). The expression of CD133 and KLF4 was decreased in SRC-1 knockdown cells compared to control cells and was increased in LN229-SRC1 cells compared to LN229-EV cells (Figure 4F,G). These results indicate that SRC-1 can enhance the self-renewal capacity and stem-like characteristics in GBM cells.

### 3.4 Steroid receptor coactivator-1 knockdown inhibits GBM growth in vivo

To further verify the in vivo tumorigenesis-promoting effect of SRC-1, a xenograft tumor growth assay was carried out on nude mice. LN229-EV and LN229-SRC1 cells were injected s.c. into the dorsal flanks of nude mice. Tumor volume, weight, and protein expression were evaluated in these mice. Tumors derived from LN229-SRC1 cells grew faster than those derived from the control cells (Figure 5A,B). The results of western blot and IHC showed that the expression of SRC-1, PCNA, KLF4, and Vimentin of the SRC-1 overexpression group was distinctly higher than in the control group (Figure 5C,D). These findings indicate that overexpression of SRC-1 promotes tumor growth and development of GBM.

The rat brain orthotopic implantation model of C6 cells was established to further investigate the effect on glioma growth of SRC-1. The results shown by pathological pictures, glioma volume, and H&E indicated that SRC-1 knockdown efficiently suppressed GBM growth (Figure 5E-G). Furthermore, SRC-1 knockdown inhibited the expression of PCNA, CD133, KLF4, and Vimentin of GBM (Figure 5H,I). Together, these data suggest that SRC-1 promotes tumorigenesis of GBM and the downregulation of SRC-1 reduced intracranial GBM growth in vivo.

### 3.5 Steroid receptor coactivator-1 promotes stem-like characteristics through the IncRNA XIST/miR-152/KLF4 pathway

In further efforts to investigate the downstream target genes for SRC-1 in GBM, we extracted total RNA from both U251-EV and U251-shSRC1 cells and generated the global gene expression profile by RNA-seq (Figure 6A). The RNA expression level of IncRNA XIST was less than eightfold in U251-shSRC1 cells compared with U251-EV cells (Figure 6B and Table S5). Furthermore, XIST was recently determined to be a downstream target gene for SRC-1 mediated glioma stemness. By analyzing GEO databases (GSE2223 and GSE4058), we found that XIST was notably elevated in human glioma and GBM tissues (Figure 6C,D). Furthermore, XIST expression was significantly higher in high-grade glioma and low-grade glioma tissues than in normal tissues, but no significant difference in XIST expression levels was observed between high- and low-grade glioma tissues (Figure 6E). We validated the RNA-seq data by using RT-qPCR analysis. The results confirmed that XIST expression was robustly inhibited following SRC-1 knockdown and that XIST expression was increased following SRC-1 overexpression (Figure 6F,G). MicroRNA-152 acts as a competing endogenous RNA of XIST and KLF4 is a downstream target of miR-152. By analyzing a GEO database (GSE2223), we found that KLF4 was upregulated in human GBM tissues, but KLF4 expression was not significantly higher in glioma tissues than in normal tissues (Figure 6H-J). The expression of KLF4 and miR-152 was detected by RT-qPCR and
Figure 5  Overexpression of steroid receptor coactivator-1 (SRC-1) increases glioblastoma (GBM) tumorigenesis and SRC-1 knockdown inhibits orthotopic GBM growth in vivo. A, Tumor volume was measured and calculated. n = 6. B, Tumor weight was measured in 8 weeks. n = 6. C, Western blot analysis for SRC-1, Kruppel-like factor 4 (KLF4), Vimentin, and proliferating cell nuclear antigen (PCNA) in LN229-EV and LN229-SRC1 tumors. D, H&E staining showing the tissue morphology and immunohistochemical (IHC) staining showing the SRC-1 and PCNA expression pattern in xenograft tumors of nude mice. Scale bar, 100 μm. E, Pathological picture of orthotopic gliomas after 28 days. F, Glioma volume was measured and calculated after 28 days. n = 6. G, Representative H&E staining showing the tissue morphology. Scale bar, 1 mm and 20 μm. H, IHC staining showing the SRC-1, PCNA, and CD133 expression patterns in rat brain orthotopic glioma. I, Western blot analysis for SRC-1, KLF4, Vimentin, PCNA, and CD133 in normal brain and glioma tissues. *P < .05, **P < .01
Steroid receptor coactivator-1 (SRC-1) promotes the expression of X-inactive specific transcript (XIST) and Kruppel-like factor 4 (KLF4) and suppresses microRNA (miR)-152 expression. A, An unbiased transcriptome expression profiling heat map between U251-EV and U251-shSRC1 cells. B, All differentially expressed genes are listed in the column graph according to their fold change. Right panel, enlarged section showing log2 fold change <−6. C–E, XIST levels were analyzed in glioma tissues of GSE2223 and GSE4058 databases. F, G, RT-quantitative PCR (qPCR) analysis for SRC-1 and XIST in U251 and LN229 cells. H–J, KLF4 levels were analyzed in glioma tissues of GSE2223 databases. K, L, RT-qPCR analysis for miR-152 and KLF4 in U251 and LN229 cells. *P < .05, **P < .01, ***P < .001. GBM, glioblastoma; NS, not significant.
western blot. The results revealed that KLF4 expression was inhibited and miR-152 expression was upregulated by SRC-1 knockdown, whereas overexpression of SRC-1 increased KLF4 expression and decreased miR-152 expression (Figure 6K,L). Then XIST expression was reverse rescued by transient siRNA transfection in LN229-SRC1 cells. The RT-qPCR results revealed that XIST reversed the expression of miR-152 but not KLF4 at RNA level, and western blot analysis showed that KLF4 protein expression was significantly decreased (Figure 7A,B). Furthermore, knockdown of XIST by siRNA abolished the induced sphere-forming ability of SRC-1 in LN229-SRC1 cells (Figure 7C). Next, to further confirm the role of miR-152 for SRC-1-regulated KLF4 and SRC-1-mediated stem-like characteristics in GBM cells, miR-152 expression was rescued by transient transfection of miR-152 mimic in LN229-SRC1 cell. The results revealed that miR-152 reversed the expression of KLF4 and XIST, and that miR-152 mimic abolished the induced sphere-forming ability of SRC-1/XIST in LN229-SRC1 cells (Figure 7D-F).

To further investigate how SRC-1 affects XIST expression, we first examined whether SRC-1 regulates the XIST RNA transcription process. To this end, we cloned the sequence of XIST promoter (−1982, +219) by PCR and inserted the sequence into luciferase reporter pGL3-basic plasmid. Dual-luciferase reporter results showed that firefly luciferase did not significantly change after SRC-1 overexpression in 293T cells, which suggested that SRC-1 does not influence the proximal XIST promoter activity (Figure 7G). These results implied that SRC-1 might posttranscriptionally regulate XIST. U251 and LN229 cells were treated with actinomycin D (Act D), which blocks de novo RNA synthesis, and total RNA was isolated at indicated time points after Act D application. Relative XIST RNA levels were measured by RT-qPCR.
normalized to that at 0 hour. Knockdown of SRC-1 resulted in a decrease of the half-life of XIST from 7 to 5 hours in U251 cells, whereas overexpression of SRC-1 increased its half-life from 0.5 to 4.5 hours in LN229 cells, indicating that SRC-1 promotes the stability of XIST RNA after transcription (Figure 7H,I). Taken together, these data confirm that SRC-1 stabilizes XIST RNA and enhances stem-like characteristics, at least partly, through upregulating the XIST/miR-152/KLF4 pathway.

3.6 | Arenobufagin and bufalin inhibit proliferation and stemness of GBM cells

Our previous study reported that arenobufagin (ARE) and bufalin (BFU; SRC inhibitor), homogeneous bufadienolides, could inhibit SRC-1 expression in HER2-overexpressing breast cancer cells. Thus, in this study, ARE and BFU were used to treat LN229-EV, LN229-SRC1, and U251 cells at 50 nmol/L for 48 hours. The results showed that the expression of SRC-1, XIST, and KLF4 had all obviously declined in control and SRC-1 overexpression cells (Figure 8A-C). We then tested the efficacy of ARE and BFU on proliferation of GBM cell lines. After treatment with different concentrations (0.01-10 μmol/L) of ARE and BFU for 48 hours, the proliferation of cells was significantly suppressed in a dose-dependent manner (Figure 8D,E). Furthermore, ARE and BFU decreased the sphere-forming ability of SRC-1 overexpressed cells (Figure 8F). Collectively, these data indicate that bufadienolides could inhibit proliferation and stemness through suppressing SRC-1 expression in GBM cells.

4 | DISCUSSION

It has been reported that SRC-1 is overexpressed and plays a crucial promoting role in several human cancers, including breast cancer, liver cancer, lung cancer, and prostate cancer. Accumulated evidence has revealed the pivotal roles that sex steroid hormones and steroid receptor coactivators play in the regulation of brain...
Steroid receptor coactivator-1 also participates in the regulation of vascular endothelial growth factor expression and its expression was upregulated by progesterone in D54 cells. However, there were no in-depth or detailed studies about the expression and roles of SRC-1 in GBM.

Accumulated studies have revealed that SRC-1 is the predominant p160 family member in the brain and mostly localized in the neurons, but some astrocytes are also positive for SRC-1. Our data of brain tissue microarray and analysis of GEO databases indicated that SRC-1 expression is positively correlated with clinical glioma malignant grade and inversely correlated with glioma patient’s prognosis, which implied that SRC-1 might be involved in tumorigenesis of GBM. We also determined the possible function of SRC-1 in the procession of GBM. The results showed that SRC-1 knockdown induces G2/M arrest and suppresses proliferation of GBM cells. Our data also revealed that SRC-1 promotes migration of GBM cells by upregulating the expression of EMT process; this is consistent with previous reports that SRC-1 promotes breast cancer metastasis by upregulating the expression of transcription factor TWIST1, which could increase the expression of N-cadherin and Vimentin. Furthermore, in our study, SRC-1 also promoted tumorigenesis in a xenograft mouse model and SRC-1 knockdown inhibited GBM growth in a rat intracranial brain tumor model.

Our data primarily indicate that SRC-1 promotes stem-like characteristics and the self-renewal ability of GBM cells, consistent with a recent report that SRC-1 is important for the proliferation of breast and lung cancer stem-like cells. A number of other reports have indicated possible associated roles for GSCs in GBM initiation, propagation, and maintenance, as well as for recurrence and chemoresistance. There are many stemness factors, ALDH1, CD44, CD133, c-Kit, KLF4, Nanog, Nestin, Oct4, and Sox2, that promote self-renewal and survival pathways in GBM. Therefore, to combat the growth of glioblastoma, identifying and targeting a key stemness factor that drives the most aggressive cell proliferation is crucial. The data from Figure 4 reveal that SRC-1 promotes stem-like characteristics and the self-renewal ability of GBM cells. Furthermore, SRC-1 upregulates the expression of KLF4, which is a master regulator of GSCs. Collectively, our data strongly suggest SRC-1 as a potential target to GBM stemness, thus overcoming GSCs mediated drug resistance. U251 cells were treated with SRC-1 knockdown or GBM chemotherapy drug (TMZ and SOR), and the results revealed that knockdown of SRC-1 enhanced the sensitivity of GBM cells to chemotherapy drugs, which could be of great significance in the clinical treatment of GBM.

Next, we investigated the mechanism of SRC-1 promoting GBM stemness. From the results of RNA-seq, we obtained a series of differentially expressed genes, including the XIST gene (−8.3378-fold), which was recently revealed to be a key factor associated with GSCs and transcriptional silencing of the X chromosome in mammals. Previous studies have also confirmed the function of IncRNA XIST as a tumor regulator in various human cancers, including breast cancer, ovarian cancer, and hepatocellular carcinoma. A recent study showed that the IncRNA XIST is essential for long-term survival of hematopoietic stem cells. In addition, knockdown of IncRNA XIST exerts tumor-suppressive functions by upregulating miR-152, which functions as a tumor suppressor by targeting KLF4 in human GSCs.

Furthermore, our data from GEO database analyses indicated that XIST and KLF4 are upregulated in human GBM samples. Our results showed that SRC-1 promotes the expression of XIST and KLF4 and suppresses miR-152 expression. Knockdown of XIST abolishes the induced sphere-forming ability of SRC-1 and rescues the expression of miR-152 and KLF4. Similarly, miR-152 mimic restores the stemness of SRC-1. Overall, these data confirm that SRC-1 increases KLF4 expression and stem-like characteristics, at least in part, through upregulating the XIST/miR-152 pathway. Further studies assessed how SRC-1 regulates IncRNA XIST. We found that SRC-1 enhances XIST RNA expression at the posttranscriptional level rather than through promoting its transcriptional activity. A previous study showed that JPX and TSIX could activate transcription of XIST by evicting CTCF protein or in a sex-specific manner; however, two earlier studies used nuclear run-on analysis and measurements of steady-state levels to argue that XIST is posttranscriptionally regulated by RNA stabilization rather than by increased transcriptional rate.

Recent studies have shown that altering coactivator function is a new entrée into the treatment of cancer. For instance, our previous study showed that ARE and BFU can inhibit SRC-1 and SRC-3 expression in HER2-positive breast cancer cells. Additionally, ARE and BFU can block breast cancer, glioma, and lung cancer cell proliferation. In our study, ARE and BFU suppressed the expression of SRC-1 in GBM cells. Even though SRC-1 was overexpressed, ARE and BFU restrained the cell proliferation and sphere-forming ability of GBM cells. These data confirm that inhibiting SRC-1 expression with ARE and BFU can block proliferation and stemness of GBM cells. From these data, we can also conclude that ARE is more effective in inhibiting proliferation and stemness of GBM cells than BFU.

In summary, our study indicates that SRC-1 is overexpressed in both GBM cells and tissue as well as being positively correlated with clinical glioma malignant grade. The SRC-1 expression level is inversely correlated with glioma patient’s prognosis. In addition, SRC-1 is a key regulator of the cell cycle, EMT, stemness, and tumorigenesis of GBM. Furthermore, our results show that SRC-1 enhances stemness of GBM through the XIST/miR-152/KLF4 regulatory pathway (Figure 8G). Knockdown of SRC-1 could enhance the sensitivity of clinical chemotherapy drugs. In conclusion, our findings suggest that SRC-1 could be a therapeutic target as well as a diagnostic marker in GBM.

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DISCLOSURE
The authors have no conflict of interest to declare.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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