

Exosomal circRNA 0001445 promotes glioma progression through miRNA-127-5p/SNX5 pathway

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ABSTRACT

Background: Glioma is one of the most wide-spreading brain cancers worldwide. Exosomes have emerged as essential regulators in intercellular communication, and exosomal circular RNAs (circRNAs) are critical for cancer progression. In this study, we aimed to investigate the role of exosomal circRNAs in glioma progression and associated mechanisms.

Methods: Exosomes derived from glioma cells were isolated and identified by transmission electron microscopy and nanoparticle tracking analysis (NTA). CCK-8, wound healing assays, transwell invasion assays, and flow cytometry assays were performed to assess glioma progression. RNA sequencing, RT-qPCR, western blotting, fluorescence *in situ* hybridization assay, luciferase assays, and cell transfection assay were performed to investigate related molecular mechanisms.

Results: The results demonstrated that exosomes derived from glioma cells promoted glioma progression. Also, exosomal circRNA 0001445 was taken up and upregulated in glioma cells treated with exosomes. In addition, exosomal circRNA 0001445 acted as a sponge for miRNA-127-5p to upregulate the expression of sorting nexin 5 (SNX5). Lastly, the effect of exosomal circRNA 0001445 was mediated by miRNA-127-5p/ SNX5 signaling pathway.

Conclusion: These results demonstrated that exosomal circRNA 0001445 promoted glioma progression through miRNA-127-5p/SNX5 signaling pathway. This study provides a novel understanding of the molecular mechanism of glioma progression.

INTRODUCTION

Malignant glioma is one of the most wide-spreading brain cancers worldwide and has a poor prognosis with an overall 5-year survival rate of 10% [1]. Clinicopathologically, glioma is categorized into several grades, from I to IV, based on the criteria published by the World Health Organization (WHO) [2]. It has been reported that the majority of diagnosed glioma cases are grade IV, namely, glioblastoma (GBM) or high-grade astrocytoma [3, 4]. Despite the advances in diagnostic strategy and aggressive therapies, more than fifty percent of patients die within one year after diagnosis [5, 6]. In China, brain

tumors have emerged as the seventh leading cause of death among malignant tumors, with an increase in the mortality of 194% relative to the 1970s [7]. Currently, the standard therapies for patients with glioma are surgical resection followed by adjuvant chemotherapy and/or radiotherapy. However, the therapeutic effect is often limited by the complexity of neuronal anatomy and the developed resistance to chemotherapeutic agents and radiation [8], leading to poor prognosis and recurrence. Thus, it is urgent to thoroughly investigate the molecular mechanisms underlying tumorigenesis and the progression of glioma and develop more effective therapeutic strategies for glioma patients.

Exosomes are one group of nano-sized bilayer-bound vesicles, with 30-150 nm in diameter [9]. Exosomes can be generated and secreted from almost all cell types and are readily accessible in various body fluids, such as saliva, urine, blood, and ascites [9]. It has been demonstrated that exosomes present in intercellular spaces or circulate in the body fluids, in which exosomes can be taken up by neighboring cells or remote cells, thereby influencing the biological processes of recipient cells [10]. Notably, exosomes also act as bioactive molecules shuttles to transport various types of bioactive cargos, including mRNAs, DNAs, circular RNAs (circRNAs), and microRNAs (miRNAs), from donating cells to recipient cells [11]. To date, growing evidence demonstrates that exosomes derived from cancer cells are essential for tumor initiation, progression, and metastasis [12]. For example, exosomes derived from cisplatin-resistant lung cancer cells are associated with enhanced cisplatin resistance through transferring miRNA-100-5p [13]. Also, exosomes derived from hepatocellular carcinoma cells can shuttle circRNA 0051443 to inhibit hepatocellular carcinoma progression [14]. Furthermore, exosomal circRNA CircNFIX facilitates temozolomide resistance in glioma through targeting miRNA-132 [15]. As such, it is suggestive that exosomes derived from tumor cells may be a promising avenue to uncover glioma progression and development.

In this study, we sought to investigate the effect of exosomes in glioma progression and related mechanisms. We demonstrated that exosomes derived from glioma cells promoted proliferation, migration, and invasion of glioma cells. Also, we uncovered that circRNA 0001445 was taken up and significantly upregulated in glioma cells treated with exosomes, as well as promoted glioma cell progression. Moreover, our results indicated that the effect of exosomal circRNA 0001445 in glioma was mediated by miRNA-127-5p/sorting nexin 5 (SNX5) signaling pathway. Thus, this study provides a novel understanding of the role of exosomal circRNA in glioma progression and insight into developing exosome-based treatments for patients with glioma.

MATERIALS AND METHODS

Cell culture

Human glioma cell lines A172 and U373 and human embryonic kidney cell line HEK293T were purchased from Shanghai Institute of Biochemistry and Cell Biology (SIBCB). Cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% exosome-free fetal bovine serum (FBS) (Gibco, USA), 100 ng/mL streptomycin, and 100 U/mL penicillin (Gibco, USA). Cells were cultured in an incubator at

37° C in a humidified atmosphere containing 5% CO₂. For isolating exosomes, exosome-free FBS was generated by centrifuging at 100,000 g at 4° C overnight to remove the bovine-derived exosomes.

Extracellular vesicles (EVs) isolation and identification

EVs isolation was carried out by using differential ultracentrifugation as previously described [16]. Briefly, cell culture supernatant was obtained by centrifuging 50 mL cell culture at 10,000×g for 20 min at 4° C. The supernatant was then transferred to a centrifuge tube and centrifuged at 100,000×g for 60 min at 4° C. After removing the supernatant, the sediments were resuspended with PBS and filtered with a 0.22 μm cell strainer. The filtered liquid was centrifuged at 100,000×g for 60 min. After repeating the previous step, the EVs were collected for subsequent experiments. The level of exosomal protein was determined using BCA Protein Assay Kit (Abcam, China) according to the manufacturer's instructions. In each experiment, 10 μg of EVs were resuspended in 100 μL PBS and added to the respective cell culture for 24 h. For exosome identification, transmission electron microscopy was applied to determine the morphology of EVs as previously described [17]. The size distribution and concentration of EVs were determined by Nanoparticle tracking analysis (NTA) via using NanoSight NS300 (Malvern Instruments, UK) according to the manufacturer's instruction.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from EVs by using the exoRNeasy Midi Kit (Qiagen, USA) or from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. The quality of total RNAs was examined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNAs were synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN, USA) according to the manufacturer's instruction. PCR reactions were performed using the qPCR SYBR Green Mix (Bio-Rad, USA) on the ABI 7500 real-time PCR system (Applied Biosystems, USA) under recommended reaction conditions. PCR reactions were performed in triplicate, and PCR data were analyzed by using the 2^{-ΔΔC_t} approach [18]. GAPDH was used as the internal control for mRNA and circRNA, and U6 was used as the internal control for miRNA. The primers were as following: GAPDH: 5'-AGAAGGCTGGGGCT CATTG-3' (forward) and 5'-AGGGGCCATCCACAG TCTTC-3' (reverse); U6: 5'-CTCGCTTCGGCAGCAC A-3' (forward) and 5'-AACGCTTCACGAATTTGCG

T-3' (reverse); circRNA 0001445: 5'- TTAATTAAGG AGGCTTGTGGATCAGAAT-3' (forward) and 5'-TCC CCGCGGCTTTTGTCTCTATAGT-3' (reverse); miRNA-127-5p: 5'- CTCTTCAAGCTCCAAACCAA C-3' (forward) and 5'-GTATCCACCAGAACCACCAG G-3' (reverse); and SNX5: 5'-ACGTTTCAGAGCCCA GAGTT-3' (forward) and 5'-TCGAGGACCATCAAAG TCG-3' (reverse).

Western blotting

Total protein was isolated from cells (1×10^6) or EVs (10 μ g) using cell lysis buffer (Thermo Scientific, USA). The total protein was quantified by using BCA Protein Assay Kit (Abcam, China). The same amount of total protein of cells or EVs was denatured in sodium dodecyl sulfate (SDS). Total protein was then separated by SDS-PAGE gel and then transferred onto PVDF (polyvinylidene difluoride) membranes (Invitrogen, USA). The membranes were blocked in 5% non-fat milk for 1 h and incubated with primary antibodies overnight at 4° C, followed by incubation with secondary antibodies at room temperature for 1 h. The primary antibodies were as follows: CD9 (1:1000), SNX5 (1:500), GAPDH (1:2000), CD54 (1:1000), Annexin (1:1000), Bcl-2 (1:1000), cleaved-caspase3 (1:1000), cleaved-caspase9 (1:2000), E-cadherin (1:1000), N-cadherin (1:1000), Vimentin (1:1000), Calnexin (1:500), and MMP9 (1:1000), and secondary antibody goat anti-mouse IgG-HRP (1:2000) were purchased from Santa Cruz Biotechnology (Shanghai, China). The protein bands were visualized by the ECL chemiluminescence reagent (Millipore, USA) and quantified using ImageJ [19].

Cell transfection

1×10^5 glioma cells were placed in a 6-well plate (Corning, USA) and incubated at the standard condition for 12 h for attachment. miRNA-127-5p mimics, inhibitors, and negative controls were purchased from RiboBio (Guangzhou, China). The full-length sequence of SNX5 was cloned into pcDNA3.1 plasmid (Invitrogen, USA) according to the manufacturer's instruction. Cells were transfected with respective reagents using Lipofectamine™ 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instruction.

Luciferase reporter assays

HEK293T cells (1×10^5 /well) were seeded in a 24-well plate 24h before transfection. CircRNA 0001445 sequence and 3'-UTR of the SNX5 gene were cloned into plasmids carrying luciferase (pmirGLO) (Promega, USA). The mutant circRNA 0001445 or SNX5 reporters were generated by using GeneArt™ Site-Directed Mutagenesis System (Thermo Fisher

Scientific, USA). Wild-type (WT) /mutant (MUT) reporter constructs and miRNA-127-5p mimic/mimic negative control were co-transfected into HEK293T cells using Lipofectamine™ 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. After 48 h transfection, the relative luciferase activity was determined by the Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instruction.

Cell counting kit-8 assay (CCK-8)

CCK-8 assay was performed to determine cell proliferation by using Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instruction. Briefly, after treating with indicated treatments, cells (1×10^5 /well) were placed in a 6-well plate (Corning, USA). 10 μ L CCK-8 reagent was added to each well and incubated for 1 h at 37° C. The cell viability was determined by using the microplate reader Synergy H4 Hybrid Reader (BioTek, USA) at 450 nm absorbance. Each trial was performed with three independent experiments in triplicate.

Wound healing assay

Wound healing assay was performed as previously reported [20]. Briefly, after treating with indicated treatments, glioma cells (1×10^5 /well) were seeded in a 6-well plate (Corning, USA). Sterile 100 μ L pipette tips were used to generate a scratch wound. The floating cells were removed by washing with PBS buffer. 48 h later, the scratches were imaged using an inverted microscope (Nikon, Japan). Each trial was performed with three independent experiments in triplicate.

Cell apoptosis assay

After treating with indicated treatments, glioma cells (1×10^5 /well) were collected by centrifuging at 1500 rpm for 5 min and were washed with PBS buffer solution three times. Cells then were incubated with 5 μ L of PI and 5 μ L of FITC-conjugated Annexin V for 10 min in the dark at room temperature. Lastly, cell apoptosis was determined by ModFit software and FACSCaliber Flow cytometry (BD Bioscience, CA, USA). Each trial was performed with three independent experiments in triplicate.

Transwell invasion assay

After treating with indicated treatments, glioma cells (1×10^5 /well) were seeded in a 24-well transwell chamber for transwell invasion assay as previously described [20]. The invasive cells were stained with 0.5% crystal violet and then were imaged using an

inverted microscope (Nikon, Japan). The invasive cells were quantified in five random fields per well. Each trial was performed with three independent experiments in triplicate.

RNA sequencing

Glioma cells treated with or without EVs were used for RNA sequencing analysis. Total RNA was isolated using the exoRNeasy Midi Kit (Qiagen, USA) according to the manufacturer's instruction. The quality of total RNAs was examined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA sequencing assay and analysis were performed on IlluminaHiSeq 2500 system in Shenzhen Huada Gene Technology Co., Ltd. (Shenzhen, Chian).

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization assay was performed to determine the subcellular distribution of circRNA 0001445 by using Fluorescence *In Situ* Hybridization kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Glioma A172 and U373 cells (2×10^5) were transfected with RFP-labeled pcDNA3.1 vector containing circRNA0001445-overexpressing plasmids using Lipofectamine™ 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. EVs were isolated from circRNA0001445-overexpressing cells and then co-cultured with GFP-labeled glioma cells for 24 h. GFP-labeled glioma cells were washed with PBS solution and fixed with 4% paraformaldehyde for 10 min. Afterward, GFP-labeled glioma cells were permeabilized with 70% ethanol 12 h and washed with PBS solution three times. Cells then were rehydrated in 50% formamide and $2 \times$ SSC for 10 min. The slides were imaged using confocal microscopy (Leica, Germany).

Statistical analysis

Data were analyzed by SPSS 25.0 software (SPSS Inc, Chicago, USA) and were represented as mean \pm SD of three independent biological experiments. Differences between groups were analyzed with Student's t-test or one-way analysis of variance (ANOVA). The statistical significance was defined as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RESULTS

Glioma-derived exosomes promote glioma cell progression and inhibit apoptosis

It has been widely demonstrated that cancer cell-derived exosomes play an essential role in tumor progression

[21, 22]. To investigate the role of exosomes in glioma, EVs were isolated from the supernatant of the culture medium of A172 (A172-EVs) and U373 (U373-EVs) cells. By applying transmission electron microscopy and nanoparticle tracking analysis, the results revealed that EVs displayed a round shape with a double-layer membrane, ranging from 50 to 120 nm in diameter (Figure 1A). Also, the exosomal markers CD9, CD54, and Annexin were positively expressed in A172-EVs and U373-EVs, whereas negatively expressed Calnexin, a negative exosomal marker, as detected by Western blotting assay (Figure 1B). These results together demonstrated that exosomes were successfully isolated in this study. Next, we treated A172 and U373 cells with exosomes derived from A172 (A172-Exo) and U373 cells (U373-Exo), respectively, and then determined cell proliferation, migration, and invasion. As shown in Figure 1C, exosomes notably promoted the proliferation of A172 and U373 cells, as detected by CCK8 assay. In addition, wound healing and transwell assays revealed that glioma cells treated with exosomes showed enhanced migratory ability compared with control cells (Figure 1D). Also, the Transwell invasion assay demonstrated that glioma-derived exosomes significantly promoted the invasion of glioma cells (Figure 1E). Moreover, A172-exo and U373-exo inhibited the apoptosis of glioma cells relative to the control cells (Figure 2A), which was consistent with findings that the protein expression of Bcl-2 was increased while the levels of cleaved-caspase3 and cleaved-caspase9 were decreased in cells treated with exosomes (Figure 2B). Furthermore, the results obtained from the Western blotting assay showed that the protein expressions of N-cadherin, Vimentin, and MMP9 were upregulated, whereas E-cadherin expression was downregulated in glioma cells treated with exosomes (Figure 2C). Collectively, these results suggest that glioma-derived exosomes promote glioma cell progression and inhibit apoptosis.

CircRNA 0001445 is upregulated in glioma cells treated with glioma-derived exosomes

Growing evidence demonstrates that circRNAs packed in cancer cell-derived exosomes are critical for cancer biology [11, 23]. Thus, we sought to identify potential circRNAs that are associated with the role of glioma-derived exosomes. By applying RNA sequencing analysis, we compared the circRNA expression profile between glioma cells treated with exosomes and the control cells treated without exosomes. Notably, the results showed that circRNA 0001445 was the most upregulated circRNA in exosome-treated cells relative to the control cells (Figure 3A). Consistently, RT-qPCR assay showed that the expression of circRNA 0001445 was significantly increased in glioma cells treated with

exosomes (Figure 3B). To determine whether circRNA 0001445 was packaged into exosomes and taken up by recipient glioma cells, exosomes were isolated from RFP-labeled A172 and U373 cells with circRNA 0001445 overexpression, respectively. Then, exosomes were co-cultured with GFP-labeled recipient A172 and U373 cells. As shown in Figure 3C, the red fluorescence signal of circRNA 0001445 was co-localized with the green signal in the cytoplasm of recipient glioma cells, indicating that exosomal circRNA 0001445 could be taken up by glioma A172 and U373 cells. Next, to further investigate the role of circRNA 0001445, A172 and U373 cells were transfected with circRNA 0001445 siRNA (has_circRNA_0001445-si). The results showed that the level of circRNA 0001445 was dramatically down-regulated compared with cells treated with siRNA negative control (NC-si) (Figure 3D). On the other hand, the downregulation of circRNA 0001445 induced by siRNA was reversed by the addition of exosomes in both glioma cell lines (Figure 3E). Taken together, glioma-derived exosomes significantly upregulate the

level of circRNA 0001445 in glioma cells, suggesting that circRNA 0001445 might be an essential factor in regulating glioma progression.

CircRNA 0001445 directly targets miRNA-127-5p

A growing body of evidence suggests that circRNAs, acting as miRNA sponges, exert their biological role through targeting miRNAs, which occurs primarily in the cytoplasm [24, 25]. The fluorescence *in situ* hybridization (FISH) assay demonstrated that circRNA 0001445 was mainly distributed in the cytoplasm of A172 and U373 cells (Figure 4A). Meanwhile, by using the online bioinformatics database TargetScan [26] and StarBase 2.0 [27], we found that there was a binding site of miRNA-127-5p of 3'-UTR of circRNA 0001445 (Figure 4B). To verify this prediction, we performed a luciferase reported assay. The results revealed that the relative luciferase activity was significantly decreased in HEK293T cells co-transfected with miRNA-127-5p mimics and reporter constructs carrying wild-type binding sequence (Figure 4C). No luciferase activity

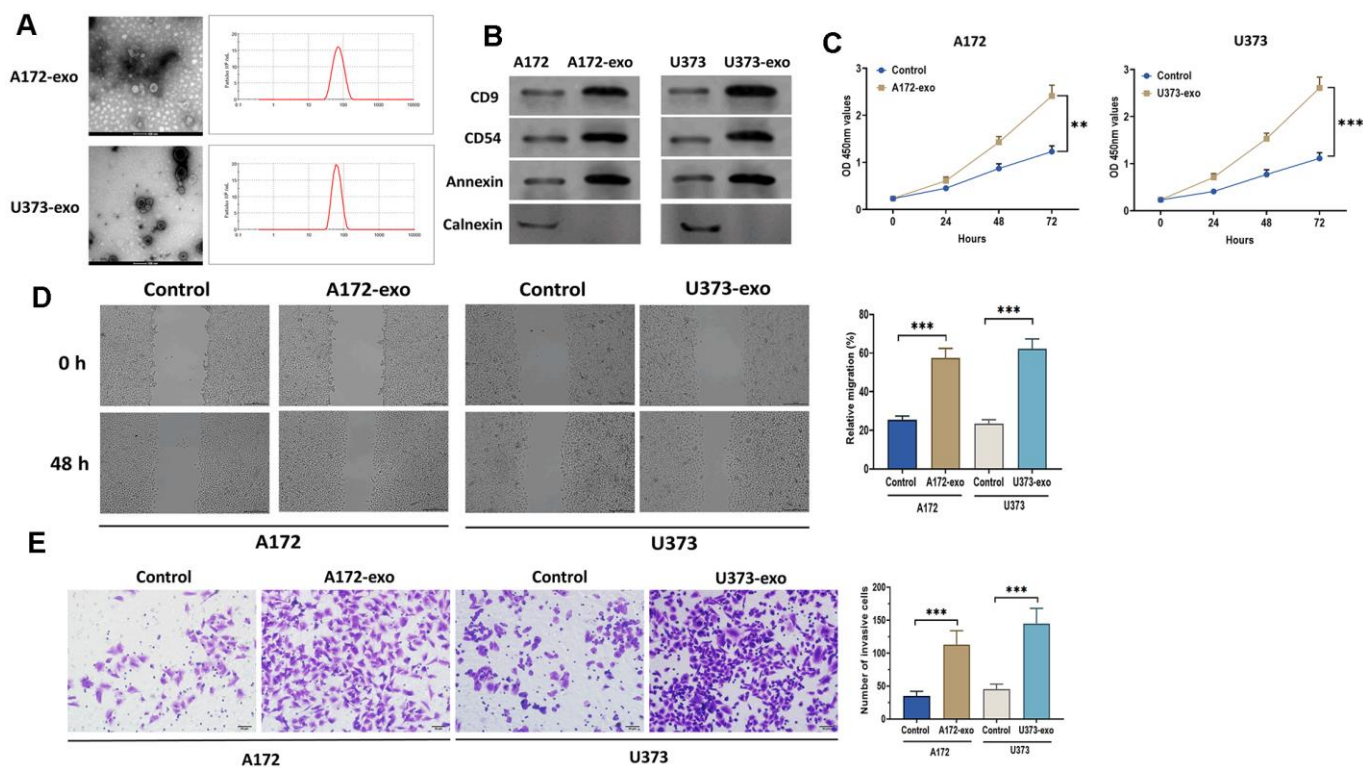


Figure 1. Glioma-derived exosomes promote glioma cell proliferation and migration. (A) The morphology and size distribution of EVs isolated from the supernatant of the culture medium of A172 and U373 cells, as detected by transmission electron microscopy and nanoparticle tracking analysis. scale bar = 100 nm. (B) Protein expressions of exosomal markers CD9, CD54, Annexin, and Calnexin in EVs and glioma cells. (C) Cell proliferation of glioma cells treated with exosomes isolated from A172 or U373 cells, as detected by CCK8 assay. (D) Wound healing assays in glioma cells treated with exosomes isolated from A172 or U373 cells. Scale bar = 200 μ m. (E) Cell migration of glioma cells treated with exosomes isolated from A172 or U373 cells, as detected by Transwell invasion assay. Scale bar = 50 μ m. Data were represented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$.

changes were found in cells co-transfected with miRNA-127-5p mimics and reporter constructs carrying mutant binding site of 3'-UTR of circRNA 0001445. Moreover, the results showed that the expression of miRNA-127-5p was remarkably decreased in glioma cells treated with exosomes. These results together indicate that exosomal circRNA 0001445 acts as a sponge for miRNA-127-5p (Figure 4D).

miRNA-127-5p directly targets SNX5

It has been well-studied that miRNAs play an essential role in various biological processes through targeting 3' UTR of mRNAs, thereby silencing targeting genes [28, 29]. Thus, we applied the same method to predict the target gene of miRNA-127-5p. As shown in Figure 5A, SNX5, a critical regulator in cancers [30–32], had a binding site of miRNA-127-5p in its 3' UTR. This prediction was verified by luciferase reported assay (Figure 5B). Functionally, miRNA-127-5p mimic could significantly downregulate both protein and mRNA expression of SNX5 in glioma cells (Figure 5C, 5D). Intriguingly, glioma cell-derived exosomes increased the protein expression of SNX5 in glioma cells, which was reversed by the addition of miRNA-

127-5p mimics (Figure 5E). Similar results were also found in mRNA expression of SNX5 (Figure 5F). As such, these data suggest that SNX5 was a direct target of miRNA-127-5p.

CircRNA 0001445 promotes glioma proliferation, migration, and invasion through miRNA-127-5p/SNX5 signaling pathway

To further determine whether exosomal circRNA 0001445 plays a role in glioma progression through miRNA-127-5p/SNX5 signaling pathway, we performed a series of functional experiments in A172 cells. Consistent with the data mentioned above, A172-exo and has_circRNA_0001445-si promoted and inhibited glioma cell proliferation, invasion, and migration, respectively (Figure 6A, 6C, 6D). Meanwhile, we found that the combination of A172-exo and has_circRNA_0001445-si did not show a significant role in glioma progression, while this non-effect was switched to positive-effect by the knockdown of miRNA-127-5p (miRNA-127-5p-in) or the overexpression of SNX5 (pcDNA3.1-SNX5) (Figure 6A, 6C, 6D). Furthermore, the opposite results were found in cell apoptosis (Figure 6B). Collectively, these

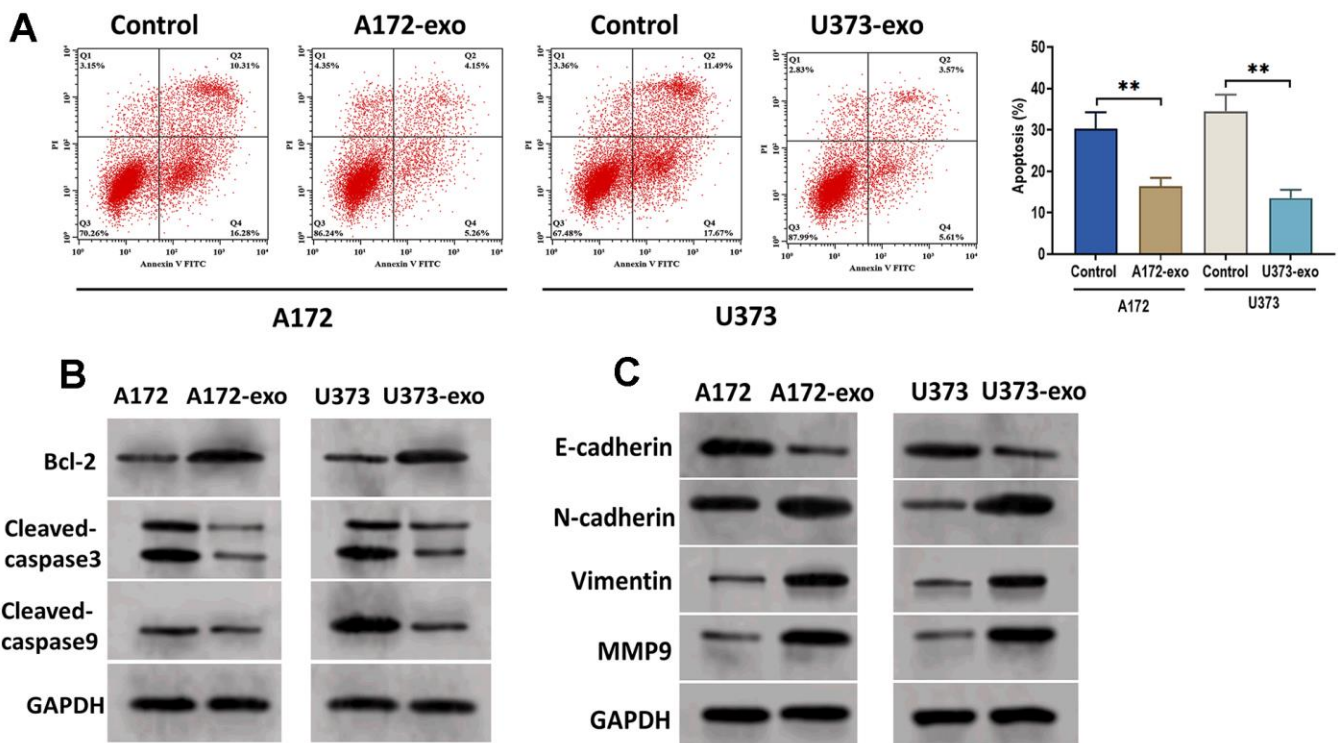


Figure 2. Glioma-derived exosomes inhibit glioma cell apoptosis. (A) Apoptosis of glioma cells treated with exosomes isolated from A172 or U373 cells, as detected by flow cytometry analysis. (B) Protein expression of apoptosis-related proteins Bcl-2, cleaved-caspase3, and cleaved-caspase9. (C) Protein expression of invasion-related proteins E-cadherin, N-cadherin, Vimentin, and MMP9. Data were represented as mean \pm SD. ** $p < 0.01$.

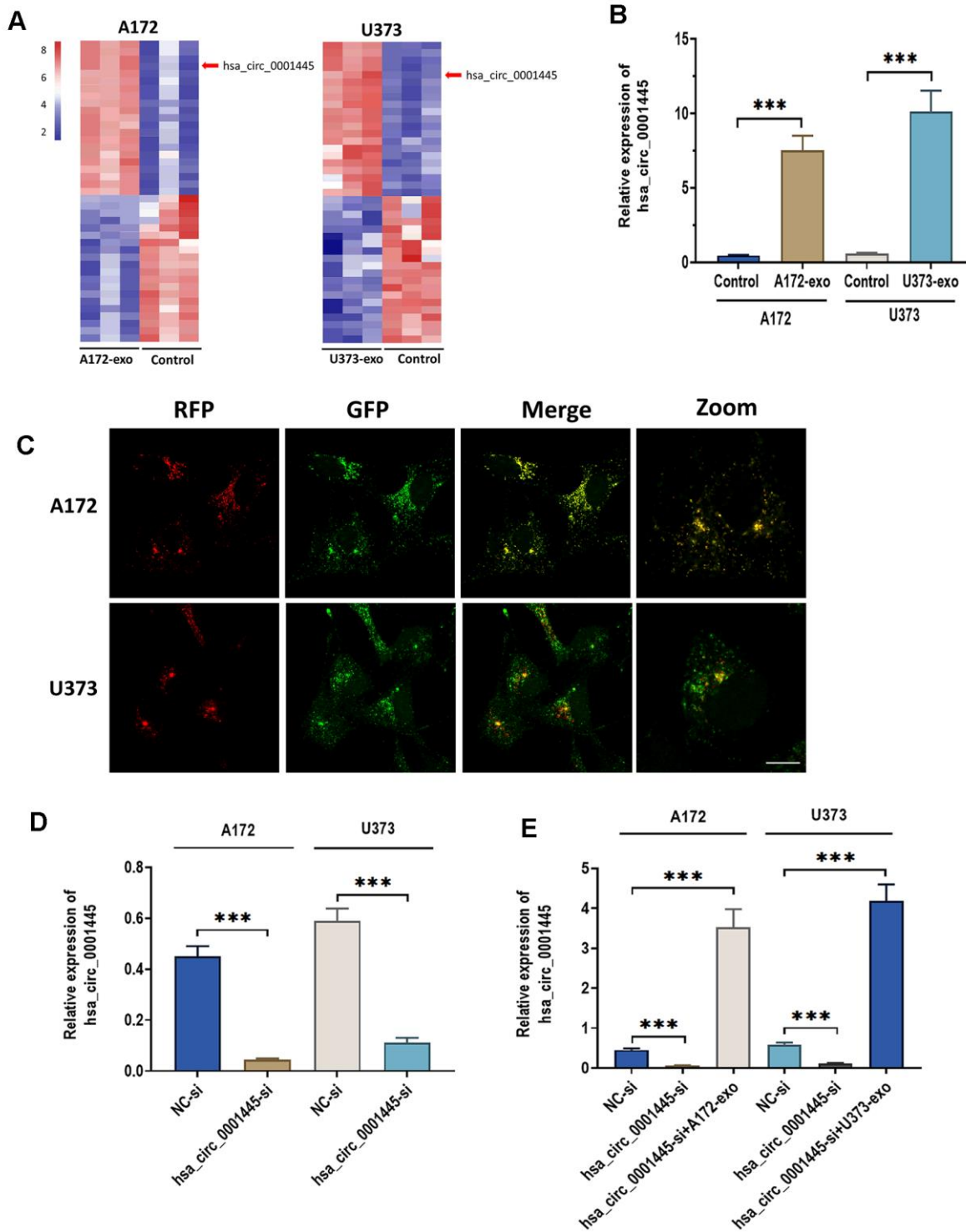


Figure 3. CircRNA 0001445 is taken up and upregulated in glioma cells treated with glioma-derived exosomes. (A) Heat map of the expression profile for differentially expressed circRNAs between glioma cells treated with or without exosomes isolated from A172 or U373 cells, as detected by RNA sequencing. (B) The expression of circRNA 0001445 in glioma cells treated with exosomes isolated from A172 or U373 cells. (C) RFP-labeled exosomal circRNA 0001445 was taken up by GFP-labeled recipient A172 or U373 cells, as illustrated by FISH. Scale bar = 25 μ m. (D) The expression of circRNA 0001445 in glioma cells treated with circPACRGL siRNA (hsa_circ_0001445-si) or siRNA negative control (NC-si). (E) The expression of circRNA 0001445 in glioma cells treated siRNA negative control (NC-si), circRNA 0001445 siRNA (hsa_circ_0001445-si) or hsa_circ_0001445-si plus exosomes isolated from A172 or U373 cells. Data were represented as mean \pm SD. *** $p < 0.001$.

results show that exosomal circRNA 0001445 promotes glioma proliferation, migration, and invasion through the miRNA-127-5p/SNX5 signaling pathway.

DISCUSSION

Exosome-mediated intercellular interaction has been receiving increasing attention due to its essential role in various physiological and pathological processes, including cancers [33, 34]. Exosomes can carry various bioactive molecules, such as miRNAs, circRNAs, and proteins, from donating cells to recipient cells, thereby regulating recipient cells' biological processes [35]. As one of the notable features, the bilayer-lipid membranes of exosomes can protect bioactive cargos from degradation, which plays a critical role in intercellular communication [36]. Growing evidence has been suggested that circRNAs, a novel class of non-coding RNAs (ncRNAs), displayed aberrant expression profiles

in various types of cancers, participating in the regulation of tumorigenesis, progression, metastasis, and drug resistance [37, 38]. Meanwhile, circRNAs derived from exosomes function as signaling molecules to modulate tumor growth and development and act as biomarkers to reflect the malignant characteristics and progression of cancers, indicating the great potential of exosomal circRNAs for cancer diagnosis and prognosis [38]. In this study, we reported that circRNA 0001445 derived from glioma cells is essential for glioma progression, suggesting that exosomal circRNA 0001445 and related signaling pathways may be promising targets for developing treatments of glioma.

There is increasing evidence to suggest that circRNAs often function as sponges of miRNAs to regulate their expressions and functions [39]. In this study, we found that exosomal circRNA 0001445 could dramatically downregulate the expression of miRNA-127-5p and that

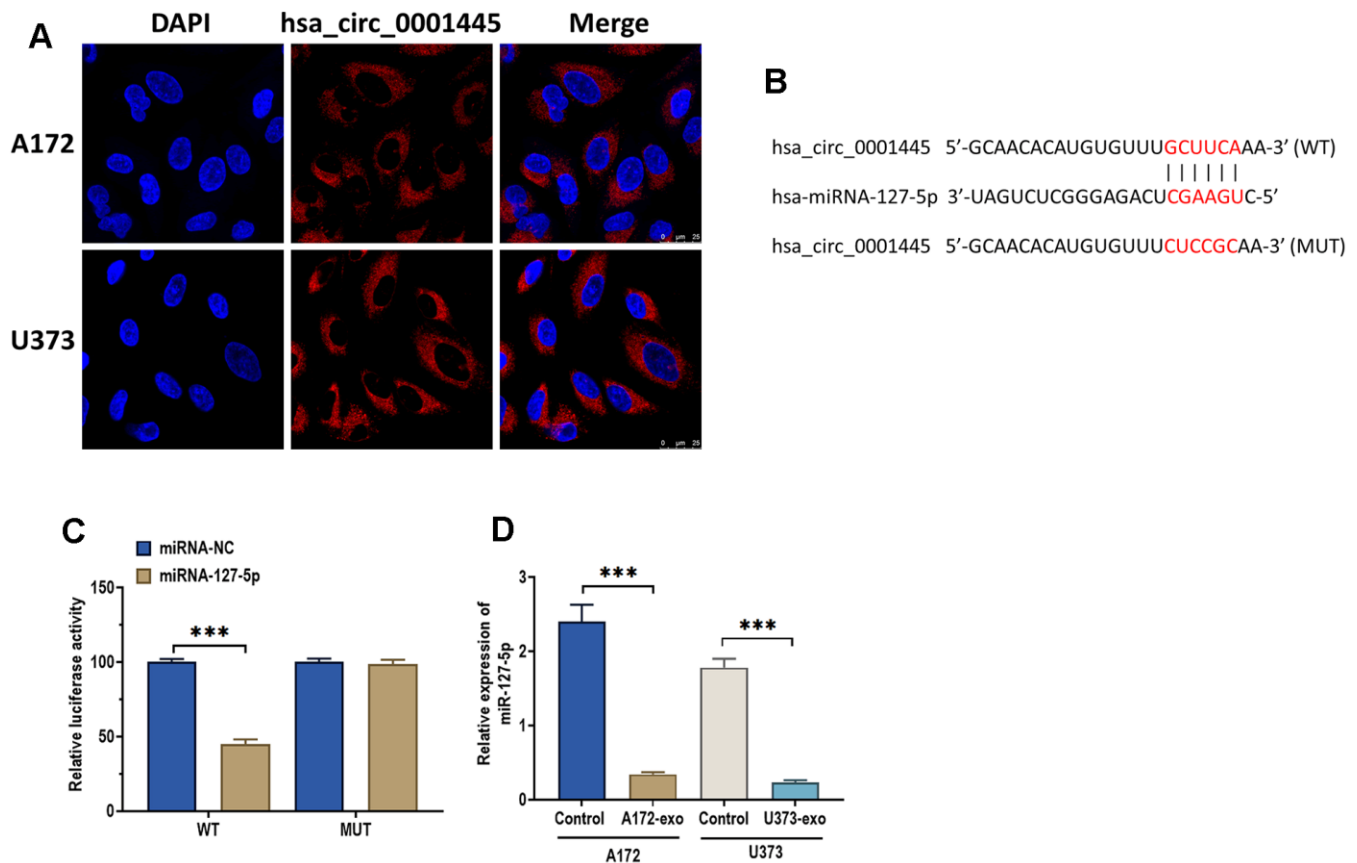


Figure 4. CircRNA 0001445 directly targets miRNA-127-5p. (A) Subcellular distribution of circRNA 0001445 in glioma cells, as detected by FISH assay. Red and blue fluorescence staining represent circRNA 0001445 and nuclei, respectively. Scale bar = 25 μ m. (B) The putative binding sequence of miRNA-127-5p of 3'-UTR of circRNA 0001445, as predicted by online bioinformatics database StarBase and TargetScan. (C) Luciferase reporter assay in HEK293T cells co-transfected with miRNA-127-5p mimics or mimics negative control and reporter constructs carrying wild-type or mutant miRNA-127-5p binding sequence of 3'-UTR of circRNA 0001445. (D) The expression of miRNA-127-5p in glioma cells treated with exosomes isolated from A172 or U373 cells. Data were represented as mean \pm SD. *** $p < 0.001$.

circRNA 0001445 was a direct target of miRNA-127-5p. As a multifunctional regulator, miRNA-127-5p is involved in regulating osteopontin-mediated proliferation of human chondrocytes [40], glucocorticoid-induced osteonecrosis [41], and spinal cord injury [42]. For cancers, miRNA-127-5p participants in cervical cancer progression through Forkhead box D1 (FOXD1) signaling [43]. In the present study, the downregulation of miRNA-127-5p induced by exosomal circRNA 0001445 indicates that miRNA-127-5p may act as an anti-tumor regulator for glioma.

Through bioinformatic analysis and functional experiments, we identified that miRNA-127-5p could directly target SNX5 genes. More importantly, our results revealed that exosomal circRNA 0001445 derived from glioma cells enhanced glioma progression by miRNA-127-5p/SNX5 pathway. SNX5 belongs to the human sorting nexin (SNX) family associated with endocytosis regulation [31]. SNX family is characterized by Phoxhomology (PX) domain that promotes membrane binding through phosphatidylinositol lipid [44]. It has been demonstrated that the SNX family is critical for organelle movement,

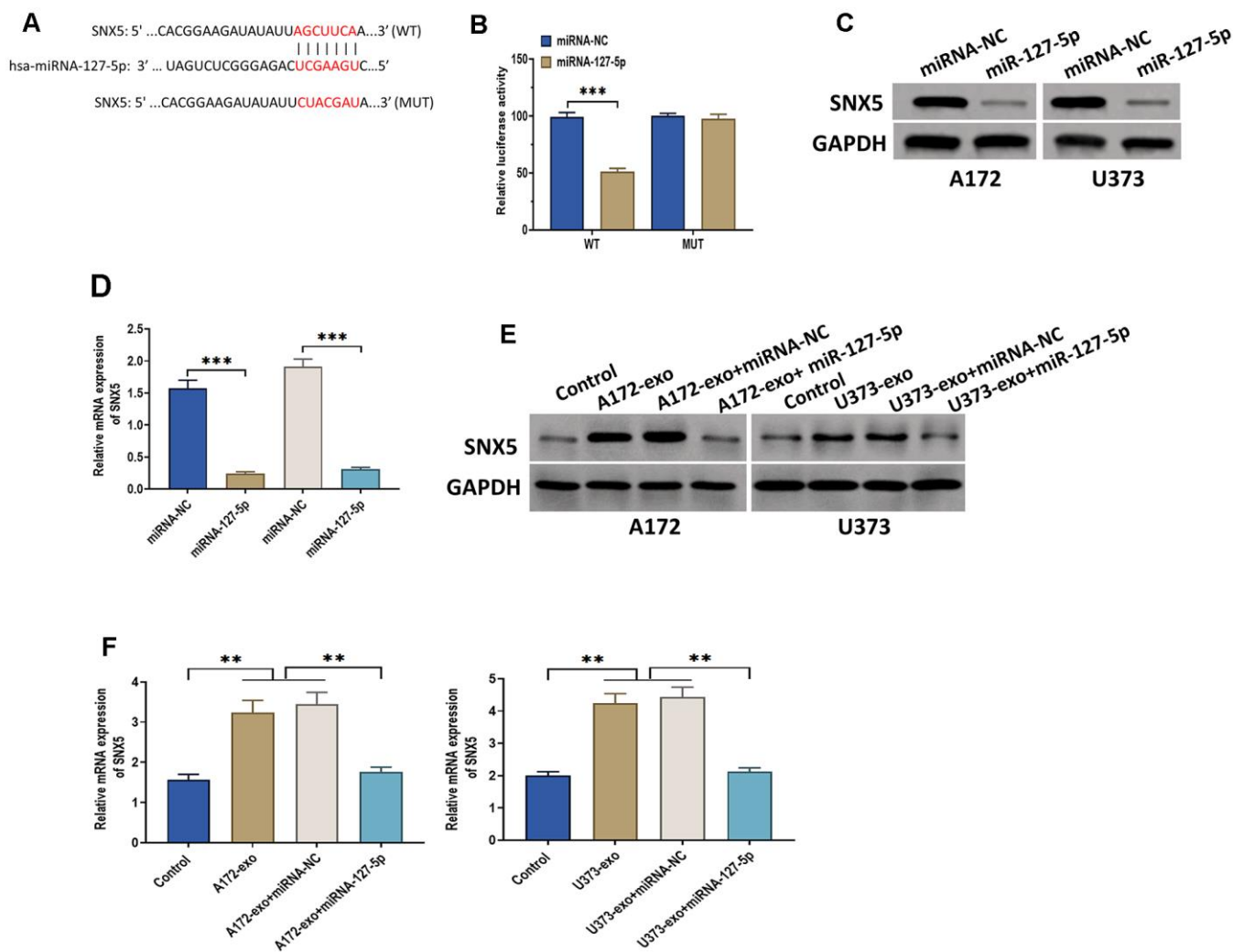


Figure 5. miRNA-127-5p directly targets SNX5. (A) The putative binding sequence of miRNA-127-5p of 3'-UTR of SNX5, as predicted by online bioinformatics database StarBase and TargetScan. (B) Luciferase reporter assay in HEK293T cells co-transfected with miRNA-127-5p mimics or mimics negative control and reporter constructs carrying wild-type or mutant miRNA-127-5p binding sequence of 3'-UTR of SNX5. (C) The protein expression of SNX5 in glioma cells treated with miRNA-127-5p mimics or mimics negative control. (D) The mRNA expression of SNX5 in glioma cells treated with miRNA-127-5p mimics or mimics negative control. (E) The protein expression of SNX5 in glioma cells plus exosomes isolated from A172 or U373 cells, exosomes isolated from A172 or U373 cells plus miRNA-127-5p mimics or mimics negative control. Cells in the control group received no treatment. (F) The mRNA expression of SNX5 in glioma cells treated with exosomes isolated from A172 or U373 cells, exosomes isolated from A172 or U373 cells plus miRNA-127-5p mimics or mimics negative control. Cells in the control group received no treatment. Data were represented as mean \pm SD. ** $p < 0.01$, *** $p < 0.001$.

protein sorting, intracellular signal transduction, as well as transmembrane transport, thereby playing a significant role in tumor progression [45]. Consistently, our data also showed that the upregulation of SNX5 was associated with enhanced glioma progression, indicating the potential of SNX5 as a target for developing glioma treatment.

CONCLUSIONS

Taken together, the results demonstrated that exosomal circRNA 0001445 acts as a pro-tumor regulator in glioma progression and that the effect of circRNA 0001445 was mediated by miRNA-127-5p/SNX5 signaling pathway. This study provides a new

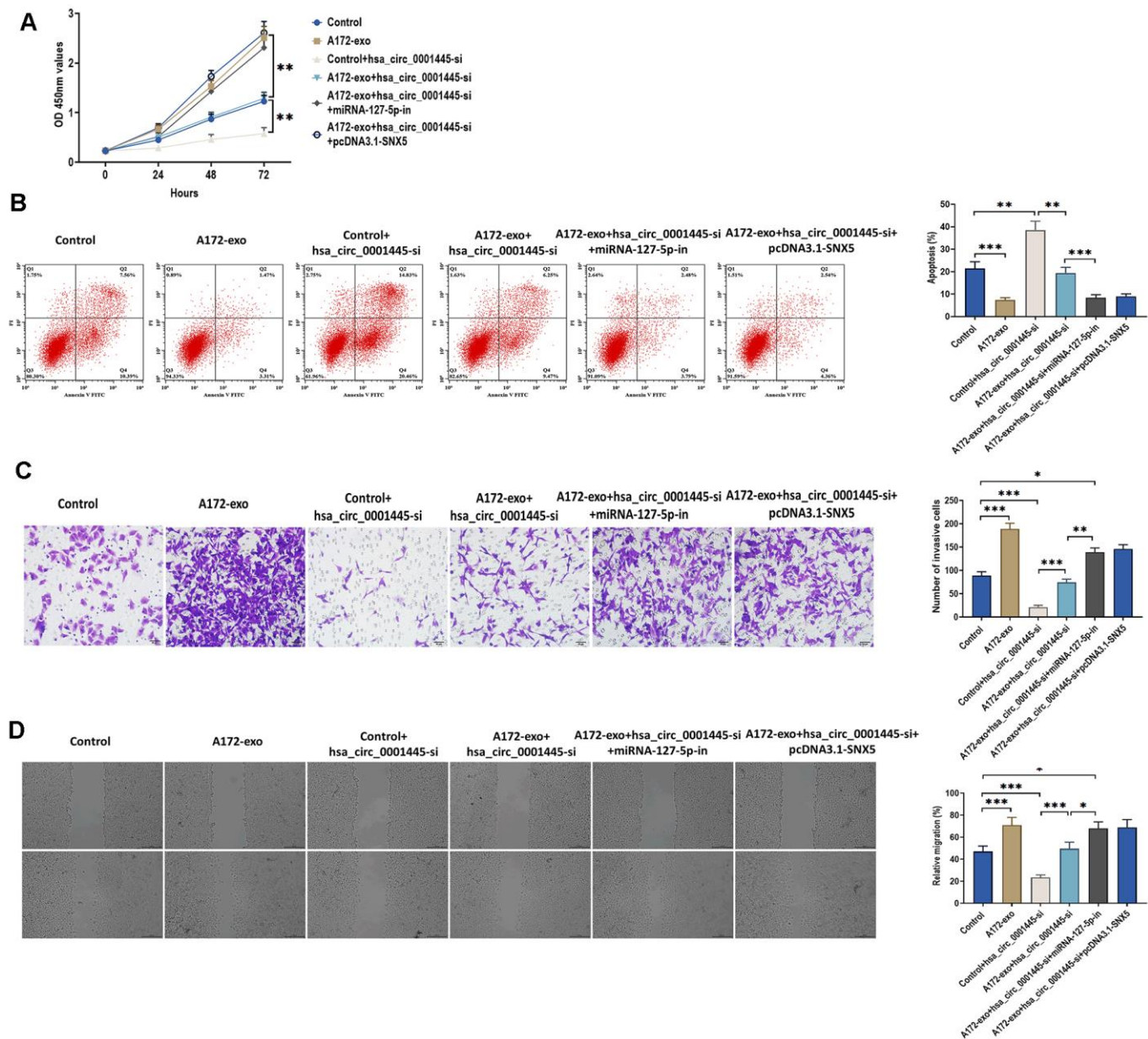


Figure 6. CircRNA 0001445 promotes glioma proliferation, migration, and invasion through miRNA-127-5p/SNX5 signaling pathway. (A) Cell proliferation of A172 cells treated with 1) control (no treatment); 2) exosomes isolated from A172 cells (A172-exo); 3) control plus circRNA 0001445 siRNA (hsa_circ_0001445-si); 4) A172-exo plus hsa_circ_0001445-si; 5) A172-exo and hsa_circ_0001445-si plus miRNA-127-5p inhibitor (miRNA-127-5p-in); and 6) A172-exo and hsa_circ_0001445-si plus pcDNA3.1-SNX5, as detected by CCK8 assay. (B) Apoptosis of A172 cells treated as indicated in (A), as detected by flow cytometry analysis. (C) Cell migration of A172 cells treated as indicated in (A), as detected by Transwell invasion assay. Scale bar = 50 μ m. (D) Wound healing assays in A172 cells treated, as indicated in (A). Scale bar = 200 μ m. Data were represented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

understanding of glioma progression and a promising therapeutic strategy for glioma treatment.

AUTHOR CONTRIBUTIONS

Yonggang Han performed the majority of experiments, gathered and analyzed the data; Yunchao Liu performed the molecular investigations; Baoxu Zhang designed and coordinated the research; Gangfeng Yin wrote the paper.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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