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Research article

Glioma-derived exosome Lncrna Agap2-As1 promotes glioma proliferation and metastasis by mediating Tgf-β1 secretion of myeloid-derived suppressor cells

Yanlong Tian [a], Xiao Gao [a], Xuechao Yang [a,*], Shangjun Chen [b], Yufeng Ren [c]

[a] Department of Pathology, No. 215 Hospital of Shaanxi Nuclear Industry, Xianyang, 712000, Shaanxi, China
[b] Department of Neurosurgery, No. 215 Hospital of Shaanxi Nuclear Industry, Xianyang, 712000, Shaanxi, China
[c] Department of Orthopaedics, No. 215 Hospital of Shaanxi Nuclear Industry, Xianyang, 712000, Shaanxi, China

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ABSTRACT

Background: Glioma (GBM) is the most prevalent malignancy worldwide with high morbidity and mortality. Exosome-mediated transfer of long noncoding RNA (lncRNA) has been reported to be associated with human cancers, containing GBM. Meanwhile, myeloid-derived suppressor cells (MDSCs) play a vital role in mediating the immunosuppressive environments in GBM.
Objectives: This study is designed to explore the role and mechanism of exosomal (Exo) lncRNA AGAP2-AS1 on the MDSC pathway in GBM.
Methods: AGAP2-AS1, microRNA-486-3p (miR-486-3p), and Transforming growth factor beta-1 (TGF-β1) levels were detected by real-time quantitative polymerase chain reaction (RT-qPCR). Cell proliferation, apoptosis, migration, and invasion were detected by 5-ethynyl-2′-deoxyuridine (EdU), flow cytometry, and Transwell assays. E-cadherin, Vimentin, CD9, CD81, and TGF-β1 protein levels were examined using Western blot. Exosomes were detected by a transmission electron microscope (TEM). Binding between miR-486-3p and AGAP2-AS1 or TGF-β1 was predicted by LncBase or TargetScan and then verified using a dual-luciferase reporter assay.
Results: AGAP2-AS1 was highly expressed in GBM tissues and cells. Functionally, AGAP2-AS1 absence or TGF-β1 knockdown repressed tumor cell growth and metastasis. Furthermore, Exo-AGAP2-AS1 from GBM cells regulated TGF-β1 expression via sponging miR-486-3p in MDSCs. Exo-AGAP2-AS1 upregulation facilitated GBM cell growth and metastasis via the MDSC pathway.
Conclusion: Exo-AGAP2-AS1 boosted GBM cell development partly by regulating the MDSC pathway, hinting at a promising therapeutic target for GBM treatment.

Key message: our results discovered a novel mechanism that GBM-derived Exo-AGAP2-AS1 might serve as communication signaling in the tumor immune microenvironment to facilitate GBM progression via mediating TGF-β1 secretion of MDSCs. These findings implied a potential therapeutic approach for GBM.

* Corresponding author. Department of Pathology, No. 215 Hospital of Shaanxi Nuclear Industry, No. 35 Weiyang West Road, Qindu District, Xianyang City, Shaanxi Province, 712000, China.
E-mail address: yangxc406@163.com (X. Yang).

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1. Introduction

As the most prevalent and aggressive primary brain tumor, glioma (GBM) is thought to derive from neuroglia stem or progenitor cells characterized by high morbidity and mortality rate [1,2]. They represent a heterogeneous group of tumors displaying different morphologic, genetic and epigenetic aberrations and an extremely variable response to therapy [3]. GBM has been classified into four subtypes: astrocytoma of grade I and grade II, representing astrocytic tumor, grade III astrocytoma, consisting of anaplastic tumor, and grade IV astrocytoma or glioblastoma multiforme [4,5]. The latter represents the deadliest brain cancer, with high cell heterogeneity and poor prognosis. Moreover, the fact that the incidence of this cancer is increasing with advancing age, peaking at 75–84 years, which is being exacerbated by population growth and aging [6,7]. Clinical, despite substantial progress in multiple conventional treatments, the prognosis of GBM sufferers remains disheartening, especially in the high-grade group [8,9]. Current studies have suggested that immunotherapy is emerging as one of the breakthroughs for cancer therapy and is a powerful clinical strategy for various malignant tumors [10,11], including GBM [12]. In anti-tumor therapies targeting GBM, there have been related articles exhibiting that the immunosuppression induced by myeloid-derived suppressor cells (MDSCs) has a strong ability to tumor escape [13]. Hence, an in-depth understanding mechanism of immunosuppressive cells and factors in the immune microenvironment of GBM is highly desirable for improving the prognosis and survival of patients.

MDSCs represent a large group of immature cells originating from bone marrow, which commonly differentiates into granulocytes, macrophages, or dendritic cells (DCs) under normal physiology [14]. On the contrary, in the context of cancer, tumor microenvironment (TEM) renders MDSCs incapable of differentiation, forming a population of immature heterogeneous cells, which are endowed with robust immunosuppressive activity by different mechanisms [15]. Of interest, MDSC might induce immunosuppression via secretion of TGF-β1, thereby resulting in the failure of tumor immunotherapy and recurrence [16]. In addition, TGF-β is the main regulatory factor of tumor immune escape, which might trigger immune cell dysfunction [17]. In terms of GBM, the pathogenesis of MDSCs involved in tumor progression is far from being addressed.

Recently, numerous laboratories have uncovered that ~90 % of the human genome is actively transcribed, producing non-coding RNAs (ncRNAs) that partake in the modulation of diverse cellular processes [18]. As an important member of non-coding RNAs longer than 200 nt, Long noncoding RNAs (lncRNAs) have been identified as critical regulatory transcripts without or with a limited protein-coding capacity [19]. Dysregulation of multiple lncRNAs has been implicated in tumor growth, metastasis, drug resistance, and immune escape in human cancers [20]. Furthermore, lncRNAs serve as vital regulators in the tumor process by ceRNA networks via sequestering miRNAs away from target mRNAs [21]. For example, a series of lncRNAs, such as BCYRN1 and HANR, were aberrantly expressed and participated in the tumorigenesis of GBM via targeting specific miRNAs [22,23]. As a well-known oncogenic lncRNA, lncRNA AGAP2 antisense RNA 1 (AGAP2-AS1) might accelerate GBM cell growth and metastasis through regulating various miRNAs [24]. Indeed, previous literature has discovered that lncRNA and miRNA partake in the regulation of the MDSCs in the TEM via transcriptional factors-mediated complex regulatory networks [25,26]. However, whether the aberrant expression of AGAP2-AS1 might contribute to MDSC biological characteristics under GBM conditions remains unclear.

Exosomes, extracellular vesicles (30–200 nm), might carry a specific composition of RNA molecules and lipids into target cells by endocytosis [27]. In general, tumor cells might produce more exosomes than normal cells, and tumor cell-derived exosomes have a strong ability to alter the local and distal microenvironments [28]. Recent documents confirmed that ncRNAs might be selectively packed, secreted, and transferred between cells in exosomes and regulate various hallmarks of GBM, containing proliferation, migration, and immune escape [29]. It has been confirmed that tumor-derived exosomes might deliver ncRNAs, particularly miRNAs, to MDSCs and influence their function [30,31]. Herein, publicly available bioinformatics tools found putative binding sites between miR-486-3p and AGAP2-AS1 or TGF-β1 in 293T cells. We aimed to explore whether exosomal AGAP2-AS1 regulates TGF-β1 secretion via sponging miR-486-3p in MDSCs. Our findings verified that GBM-derived exosome AGAP2-AS1 facilitates tumor cell growth and metastasis through modulating TGF-β1 secretion of MDSCs.

2. Materials and methods

2.1. Clinical samples and cell culture

Forty-eight GBM specimens, containing 19 non-metastasis and 24 metastasis, and thirty-three non-tumor brain tissues (NBT) were collected from the No. 215 Hospital of Shaanxi Nuclear Industry with written informed consent from all subjects. Based on the 2007 WHO Classification of Tumors of the Central Nervous System [32], patients were diagnosed with different malignancy grade GBM: 13 grade II, 19 grade III, and 11 grade IV. Immediately after the operation, these specimens were promptly frozen in liquid nitrogen and kept at a stable temperature of −80 °C. This project was approved by the Ethics Committee of No. 215 Hospital of Shaanxi Nuclear Industry [Approval Number: 20220314].

GBM cell lines (U87, #CL-0238; and U251, #CL-0237) and HEK-293 cells were cultivated in corresponding media (Procell, Wuhan, China). Meanwhile, Normal Human Astrocytes (NHA, #CC-2526) were regularly grown in the recommended medium AGM™ BulletKit™ (Lonza, Basel, Switzerland, #CC-3186). All cells were maintained in a cultivation environment of 5 % CO_2 and 37 °C. Besides, myeloid-derived suppressor cells (MDSCs) were obtained as previously described. In short, after being cut into small pieces (1–2 mm^3), tumor samples were digested with type I collagenase (1 mg/mL) and DNase I on a rotating platform. At 2 h after incubation at 37 °C, a single-cell suspension was obtained, followed by incubation with HLA-DR, CD33, CD11b, and CD14 mAbs (eBioscience, San Diego, CA). A flow cytometry (FACSAria, BD Biosciences, Heidelberg, Germany) was used to analyze these stained cells after 30 min.

2.2. RT-qPCR

Trizol reagent (Invitrogen, Paisley Scotland, UK, #10296010CN) was applied for RNA extraction and NanoDrop 2000 system (NanoDrop, Wilmington, DE, USA) for the quality and concentration of RNA samples. After that, the synthesization of cDNA was implemented according to High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA, #4368814) and miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai, China, #B532453-0050). On an Applied Biosystems 7500 Real-Time PCR System, the qPCR analyses were conducted via SYBR Green PCR Kit (Takara, Tokyo, Japan, #RR086B). At last, data were analyzed via the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH or U6. In addition, primer sequences were displayed in Table 1.

2.3. Cell transfection

In short, the oligonucleotides (RiboBio, Guangzhou, China): si-AGAP2-AS1: 5′-AUAAAGCAGGUAACAAGUGGG-3' (sense), 5′-CACUUGUUACCUGCUUUAUAA-3′(antisense); si-TGF-β1: 5′-ACGGAAAUAACCUAGAUGGGC-3' (sense), 5′-CCAU-CUAGGUUAUUUCCGUGG-3′(antisense), miR-486-3p mimic/inhibitor (miR-486-3p/miR-486-3p in) and their controls (si-NC, miR-NC, and miR-NC in), and plasmids (GenePharma, Shanghai, China): pcDNA and pcDNA-TGF-β1 (TGF-β1, NM_000660.7), were collected in this research. Using Lipofectamine 3000 (Invitrogen, #L3000075), 50 nM oligonucleotides and 6 μg plasmids were transfected into GBM cells at 70 % confluence for 48 h, followed by the assessment of the transfection efficiency.

2.4. EdU

After replacing with medium including EdU working solution (20 μM, RiboBio, #C10310-1), 4×10^3 treated cells were incubated for 2 h. Then, these cells were subjected to a 4 % formaldehyde solution fixture for 30 min and 0.5 % Triton-X-100 permeabilization for 20 min. Subsequently, Apollo reaction cocktail was introduced into the cells, which then was stained with DAPI and visualized using a fluorescence microscope.

2.5. Cell apoptosis

Using Annexin V-FITC/PI Apoptosis kit (Beyotime, Shanghai, China, #C1062S), cell apoptosis was analyzed in this experiment. Generally, after being trypsinized and washed, cells were resuspended in 100 μL binding buffer. Following Annexin V-FITC and PI double staining, sample was assessed via FACScan flow cytometer.

2.6. Transwell assay

In general, 24-well transwell chamber (Chemicon, Temecula, CA, USA) or transwell pre-coated Matrigel chamber was respectively utilized for assessing cell migration or invasion. 200 μL of the cell suspension (5×10^4 cells for migration assay, 1×10^5 cells for invasion assay) was located on the upper chamber, and 600 μL of complete medium with 10 % FBS was introduced into the bottom counterparts. 24 h later, migrated or invasive cells in the bottom were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet solution. Next, average cell numbers in 5 randomly selected fields were counted using a microscope (Nikon, Tokyo, Japan, magnification × 100) and Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.7. Western blot

RIPA lysis buffer (Beyotime, #P0013B) including protease inhibitors was used to generate cell or exosome lysis, which then was subjected to 10 % separating gel. After blotting on PVDF membranes (Invitrogen), blots were probed with primary antibodies (Abcam, Cambridge, MA, USA): E-cadherin (1:1000, ab40772), Vimentin (1:1000, ab92547), CD9 (1:1000, ab23105), CD81 (1:1000, ab109201), TGF-β1 (1:2500, 21898-1-AP, Proteintech Group, Rosemont, IL, USA), and GAPDH (1:2500, ab9485) at 4 °C. After being

Table 1
The sequences of primers for RT-qPCR.

Names	Sequences (5′-3′)
AGAP2-AS1: Forward\1	CTTTCCCAAGACCGTCCTCC\1
AGAP2-AS1: Reverse\1	CAGGTAACAAGTGGGGAGCC\1
TGF-β1: Forward\1	TGATGTCACCGGAGTTGTGC\1
TGF-β1: Reverse\1	GTGAACCCGTTGATGTCCACT\1
miR-486-3p: Forward\1	ATAACCGGGGCAGCTCAGT\1
miR-486-3p: Reverse\1	CTGGTGTCGTGGAGTCGG\1
U6: Forward\1	CTCGCTTCGGCAGCACATA\1
U6: Reverse\1	CGAATTTGCGTGTCATCCT\1
GAPDH: Forward\1	CAAATTCCATGGCACCGTCA\1
GAPDH: Reverse\1	GACTCCACGACGTACTCAGC\1

incubated with secondary antibody for 2 h, protein bands were detected with ECL reagent (Millipore, Molsheim, France, # WBULS0500) and analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Exosome detection

Using ExoQuick-TC (System Biosciences, Mountain View, CA, USA, # EXOTC10A-1), exosome isolation from U87 and U251 cells was conducted. Generally, the supernatant was collected and incubated with ExoQuick-TC solution overnight at 4 °C after centrifugation at 3000*g* for 15 min. Following centrifugation two at 1500 g for 30 and 5 min, the supernatant was discarded. After that, PBS was used to re-suspend the exosome pellet. At last, transmission electron microscopy (TEM, Hitachi, Tokyo, Japan) was employed to analyze the micrograph of the isolated exosomes as previously described [31]. Its concentration and size distribution were identified using NTA (Nanosight, Amesbury, UK).

2.9. Detergent and RNase treatments

To validate that AGAP2-AS1 is packed into U87 and U251 cell exosomes, RNase A (20 ng/mL, Invitrogen, # EN0531), 1 % Triton X-100 (Promega, Madison, WI, USA, #H5141), or both were applied to treat GMB cell lines at 37 °C for 10 min, followed by RT-qPCR analysis of AGAP2-AS1 expression.

2.10. Dual-luciferase reporter assay

Based on the analysis of LncBase (https://diana.e-ce.uth.gr/lncbasev3/interactions) and TargetScan (http://www.targetscan.org), the possible binding sites were acquired. For wild-type (WT) luciferase reporter constructs (AGAP2-AS1$_{WT}$ and TGF-β1 3′UTR $_{WT}$), the fragments of AGAP2-AS1 or TGF-β1 3′UTR containing the predicted binding sites with miR-486-3p were cloned into pmirGLO vector (Promega). In parallel, mutant (MUT) constructs (AGAP2-AS1$_{MUT}$ and TGF-β1 3′UTR $_{MUT}$) were obtained using Site-directed gene mutagenesis kit (Takara, #638943). Whereafter, these constructs were co-transfected into 293T cells with miR-486-3p or miR-NC, followed by the analysis of dual-luciferase reporter assay kit (Promega, #E1910).

2.11. ELISA

In this assay, the culture medium of U87 and U251 cells was collected, and the secretion of TGF-β1 was determined using ELISA (Proteintech Group, # KE00002).

2.12. Statistical analysis

GraphPad Prism7 software was employed to analyze the data, which was represented as mean ± standard deviation (SD). A probability value $P < 0.05$ was judged as statistically different. According to data distribution, Student's *t*-test was conducted to compare two groups, and one-way analysis of variance (ANOVA) with Tukey's tests was applied to compare different groups.

3. Results

3.1. AGAP2-AS1 expression was increased in GBM tissues and cells

To investigate the function of AGAP2-AS1 in GBM, its expression pattern was examined in tumor tissues. As shown in Fig. 1A, AGAP2-AS1 was expressed at a high level in 48 GBM samples relative to 33 NBT samples. Remarkably, AGAP2-AS1 was progressively upregulated from grades II to IV (Fig. 1B). Meanwhile, our data presented that the expression of AGAP2-AS1 in the metastasis group (n = 24) was significantly higher than that of the non-metastasis group (n = 19) (Fig. 1C), suggesting that AGAP2-AS1 expression was associated with metastasis in GBM patients. Beyond that, RT-qPCR results further verified that AGAP2-AS1 level was strikingly enhanced in GBM cell lines (U87 and U251) compared with NHA cells (Fig. 1D). Together, these data implied the involvement of

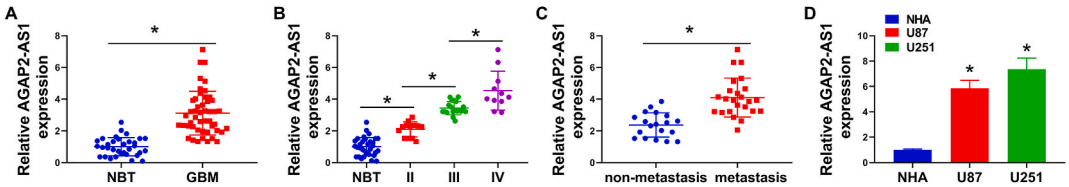

Fig. 1. Expression patterns of AGAP2-AS1 in GBM tissues and cells. (A) RT-qPCR assay was used to measure the expression level of AGAP2-AS1 in 48 GBM tumor tissues and 33 NBT tissues. (B) AGAP2-AS1 expression was determined in GBM patients with high pathological grade III (n = 19) and IV (n = 11) and low grade II (n = 13) using RT-qPCR. (C) AGAP2-AS1 expression was assessed in 19 non-metastasis, 24 metastasis, NHA cells, GBM cell lines (U87 and U251) using RT-qPCR. *$P < 0.05$.

AGAP2-AS1 in GBM progression.

3.2. AGAP2-AS1 silencing repressed GBM cell malignant behaviors in vitro

In order to identify the biological function of AGAP2-AS1 in GBM cells *in vitro*, we knocked down AGAP2-AS1 in U87 and U251 cells. As illustrated in Fig. 2A, AGAP2-AS1 content was significantly decreased in si-AGAP2-AS1-transfected GBM cells versus the control group, suggesting that the knockdown efficiency was successful. After that, EdU assay disclosed that the deficiency of AGAP2-AS1 might obviously hinder GBM cell proliferative ability (Fig. 2B and C). On the other hand, increased cell apoptosis was observed caused by the introduction of si-AGAP2-AS1 in GBM cells (Fig. 2D). Apart from that, transwell assay exhibited that the knockdown of

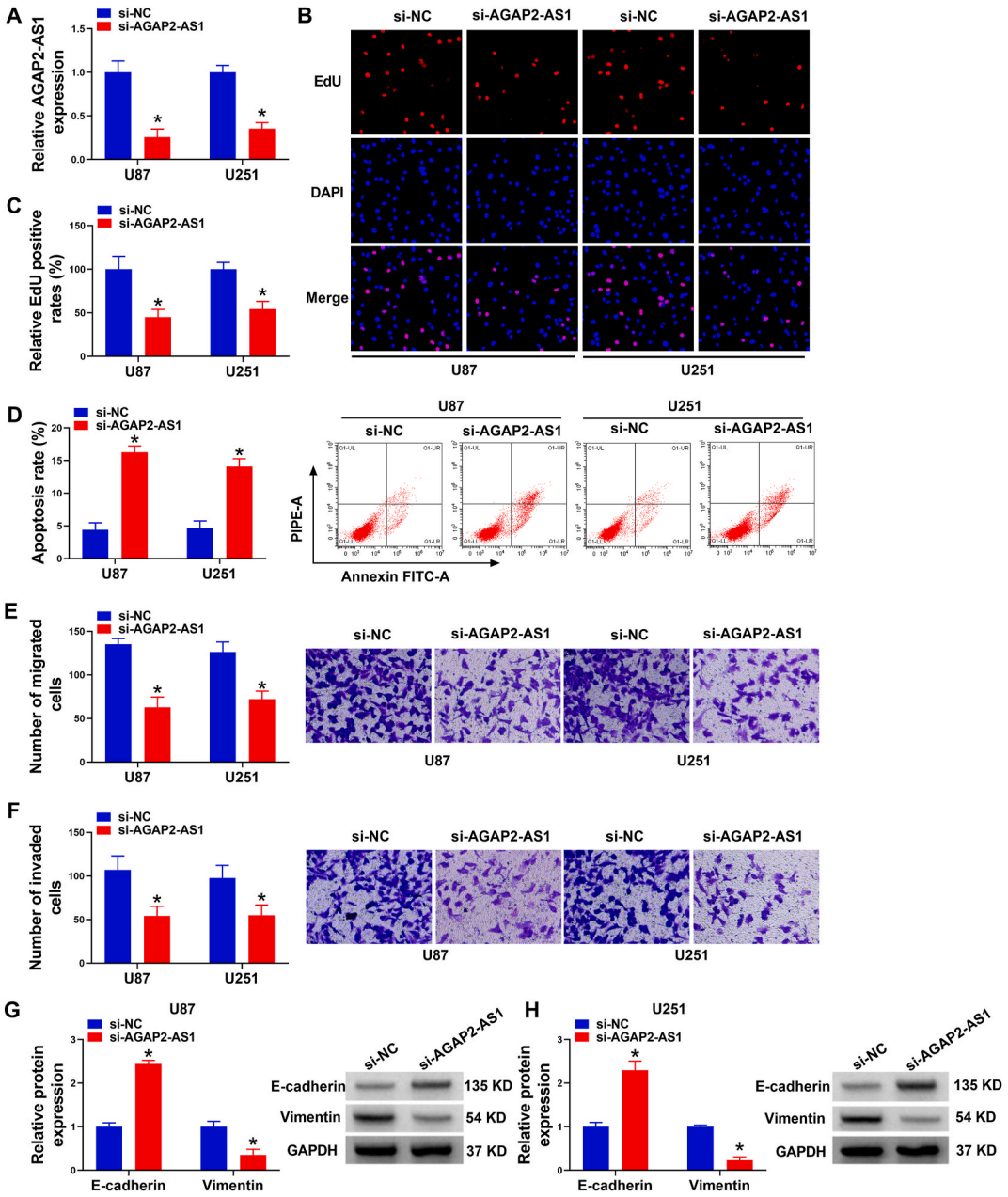

Fig. 2. AGAP2-AS1 promoted GBM cell development *in vitro*. U87 and U251 cells were transfected with si-NC or si-AGAP2-AS1. (A) RT-qPCR analysis of AGAP2-AS1 expression in transfected GBM cells. (B and C) Cell proliferation was assessed using EdU assay in transfected GBM cells. (D) Cell apoptosis rate was examined in transfected GBM cells via flow cytometry assay. (E and F) Cell migration and invasion were tested using Transwell assay in transfected GBM cells. (G and H) EMT-related indicators including E-cadherin and Vimentin were determined using Western blot in transfected GBM cells. $^*P < 0.05$.

AGAP2-AS1 impaired the migration and invasion capability of GBM cell lines (Fig. 2E and F). In addition, Western blot assay was carried out to detect the expression of EMT-related markers E-cadherin and Vimentin. Results displayed that deficiency of AGAP2-AS1 resulted in a significant reduction in E-cadherin protein level and an apparent enhancement in Vimentin level in GBM cells (Fig. 2G and H). Overall, these data indicated that AGAP2-AS1 participated in GBM development by increasing tumor cell proliferation, migration, and invasion.

3.3. *TGF-β1 knockdown blocked GBM cell proliferation, migration, invasion, and induced apoptosis in vitro*

Furthermore, the elevated expression of TGF-β1 in malignant tumors suggested that it is an oncogene [33]. Subsequently, to investigate the role of TGF-β1, *in vitro* loss-of-function analyses were performed in U87 and U251 cells. First of all, the knockdown efficiency of si-TGF-β1 was measured and presented in Fig. 3A. Functionally, the downregulation of TGF-β1 led to a substantial decrease in cell proliferation ability in GBM cells (Fig. 3B). On the contrary, GBM cell apoptosis was facilitated caused by TGF-β1 downregulation (Fig. 3C). Moreover, transwell assay displayed that the abilities of GBM cell migration and invasion were significantly suppressed by TGF-β1 absence (Fig. 3D and E). Additionally, the results from Western blot illustrated that the silencing of TGF-β1 impeded the protein level of E-cadherin and boosted Vimentin protein level in GBM cells (Fig. 3F and G). These findings illustrated that TGF-β1 absence repressed GBM cell development.

3.4. *AGAP2-AS1 exerted oncogenic properties in GBM cells via interacting with TGF-β1*

Subsequently, we further analyzed the influences of AGAP2-AS1 on TGF-β1 expression in GBM cells. As shown in Fig. 4A, the depletion of AGAP2-AS1 might markedly reduce the protein level of TGF-β1 in U87 and U251 cells, which was partially counteracted by the transfection of pcDNA-TGF-β1. After that, the repression of si-AGAP2-AS1 on GBM cell proliferation was effectively overturned by TGF-β1 overexpression (Fig. 4B). Beyond that, AGAP2-AS1 silencing-induced GBM cell apoptosis was partly weakened after pcDNA-

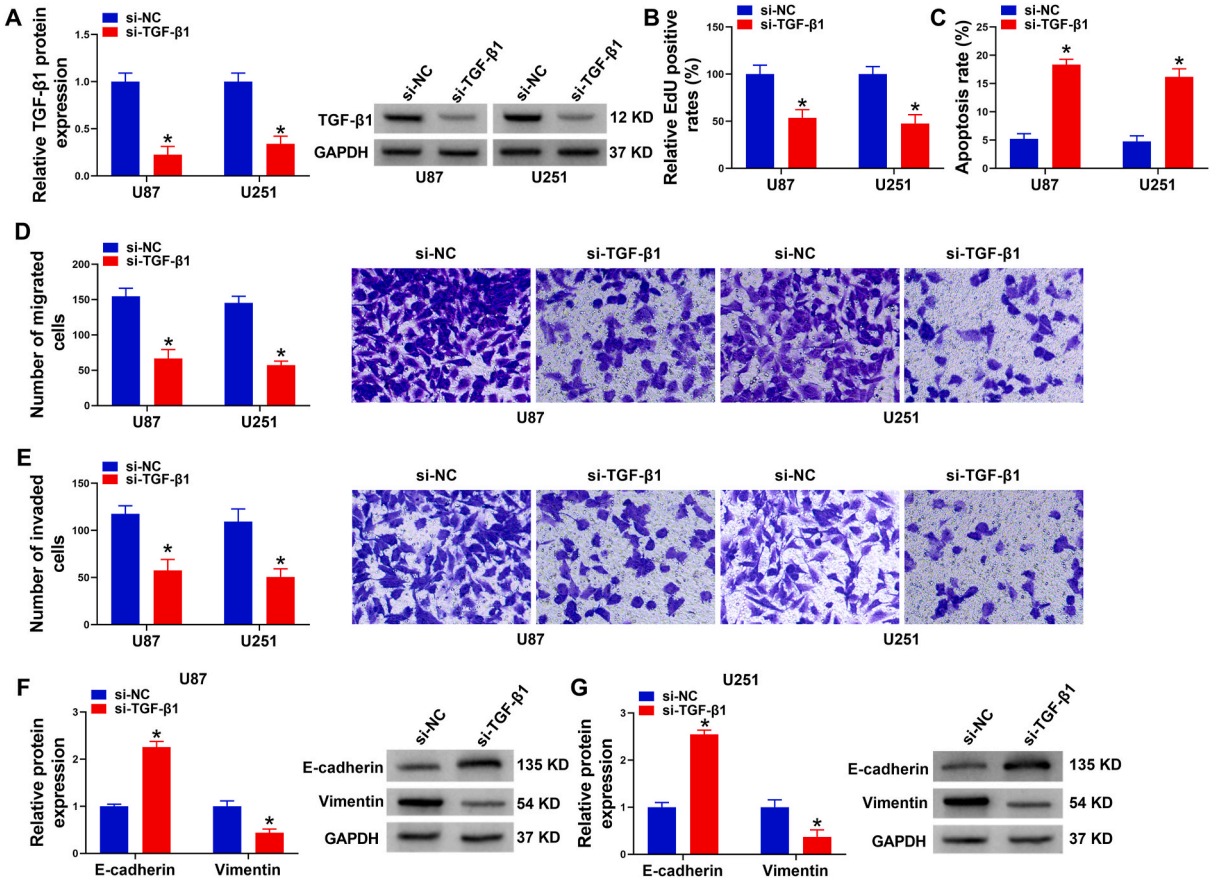

Fig. 3. Effects of TGF-β1 downregulation on GBM cell proliferation, apoptosis, migration, and invasion *in vitro*. U87 and U251 cells were transfected with si-NC or si-TGF-β1. (A) Western blot analysis of TGF-β1 protein level in transfected GBM cells. (B and C) Cell proliferation and apoptosis were assessed using EdU assay and flow cytometry assay. (D and E) Transwell assay was employed to measure GBM cell migration and invasion ability. (F and G) E-cadherin and Vimentin protein levels were measured using Western blot. *$P < 0.05$.

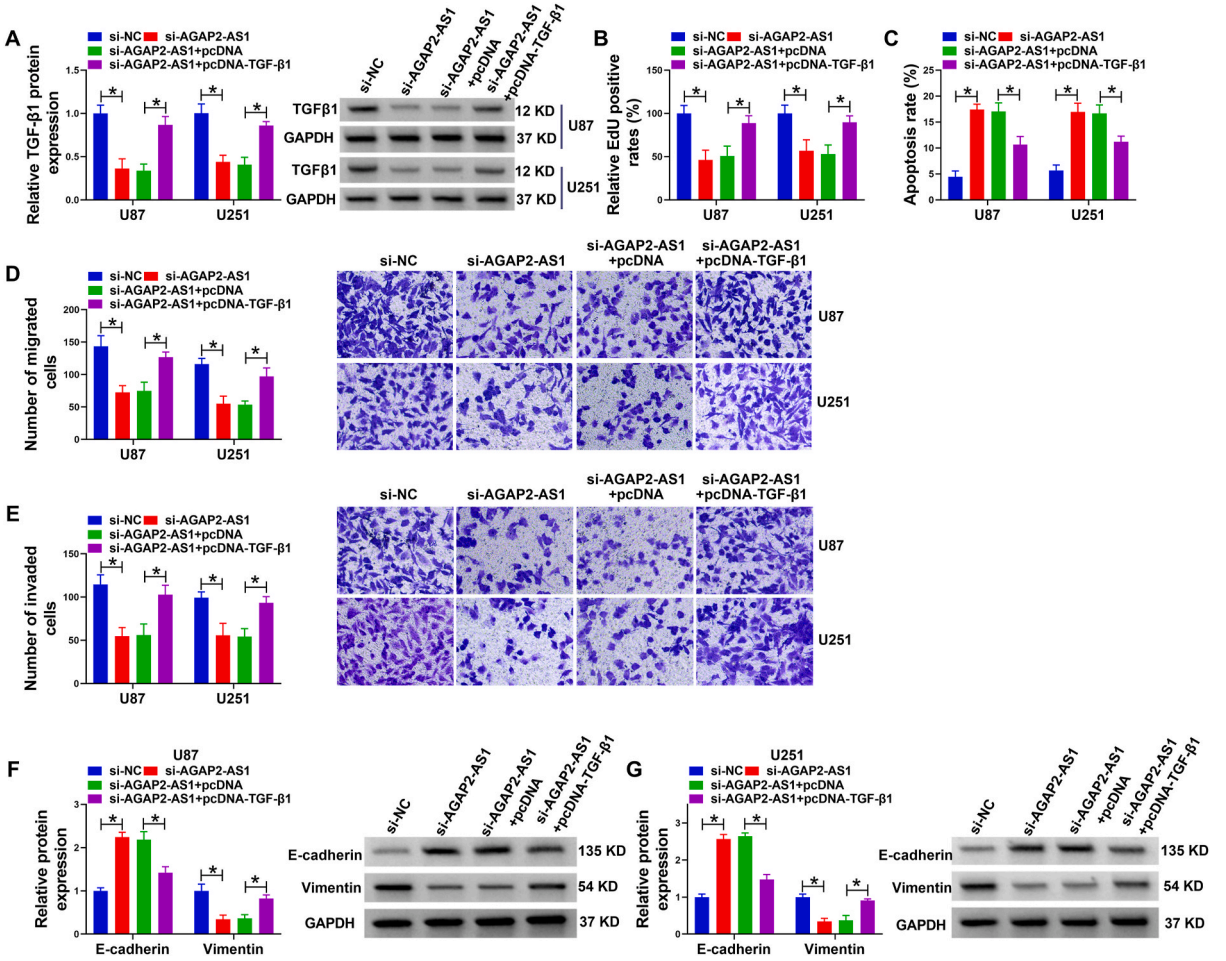

Fig. 4. AGAP2-AS1 might regulate GBM progression via interacting with TGF-β1. U87 and U251 cells were transfected with si-NC, si-AGAP2-AS1, si-AGAP2-AS1+pcDNA or si-AGAP2-AS1+pcDNA-TGF-β1. (A) TGF-β1 protein level was determined using Western blot. (B and C) EdU and flow cytometry assays were applied to examine GBM cell proliferation and apoptosis. (D and E) Transwell analysis of GBM cell migration and invasion ability. (F and G) Western blot assay was used to measure E-cadherin and Vimentin protein levels. $^*P < 0.05$.

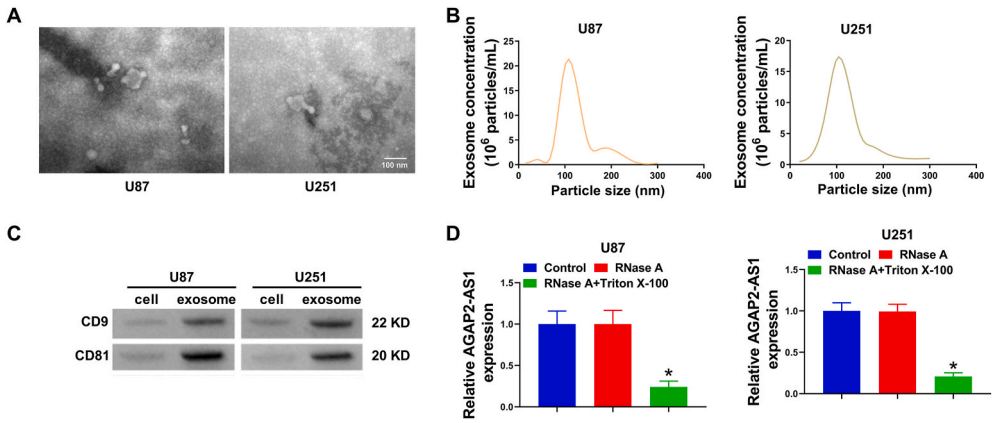

Fig. 5. Exosome-mediated AGAP2-AS1 expression is increased in GBM cells. (A) The representative micrograph of round-shaped vesicles by TEM (scale bars = 100 nm). (B) NTA analysis of the concentration and size distribution of exosomes. (C) CD9 and CD81 protein levels were detected by Western blot in U87 and U251 cells and their corresponding exosomes. (D) AGAP2-AS1 expression was determined by RT-qPCR in U87 and U251 cells treated with control, RNaseA, or RNaseA+Triton X-100. $^*P < 0.05$.

TGF-β1 introduction (Fig. 4C). Besides, transwell assay exhibited that TGF-β1 upregulation might effectively abrogate AGAP2-AS1 deficiency-mediated GBM cell migration and invasion suppression (Fig. 4D and E). Meanwhile, AGAP2-AS1 absence elicited a significant increase in E-cadherin protein expression and a prominent decline in Vimentin level in GBM cells, while these effects were partly reversed by TGF-β1 overexpression (Fig. 4F and G). Altogether, the above-mentioned results elucidated that AGAP2-AS1 might affect GBM cell growth and metastasis via regulating TGF-β1.

3.5. Exosomal AGAP2-AS1 expression is upregulated in GBM cells

Furthermore, in order to explore whether AGAP2-AS1 was secreted by packaging into exosomes, we first isolated exosomes from the U87 and U251 cells. Then, the morphology and phenotypes were identified. As a result, the particles were viewed as vesicles with round or oval membranes under the TEM (Fig. 5A). Meanwhile, NTA analysis discovered that the diameters of most particles were within the range of 20–300 nm (Fig. 5B). Beyond that, Western blot results presented that the protein levels of exosomal markers CD9 and CD81 were significantly elevated in the exosomes from the two GBM cell lines (Fig. 5C). To further verify that AGAP2-AS1 is secreted to the extracellular by the exosome pathway, U87 and U251 cells were treated with RNaseA and Triton X-100. As shown in Fig. 5D, AGAP2-AS1 levels in the RNaseA group were similar to those in untreated group, while AGAP2-AS1 expression was obviously reduced after the treatments with RNaseA and Triton X-100. In summary, these results suggested that AGAP2-AS1 is packaged in GBM cell-derived exosomes.

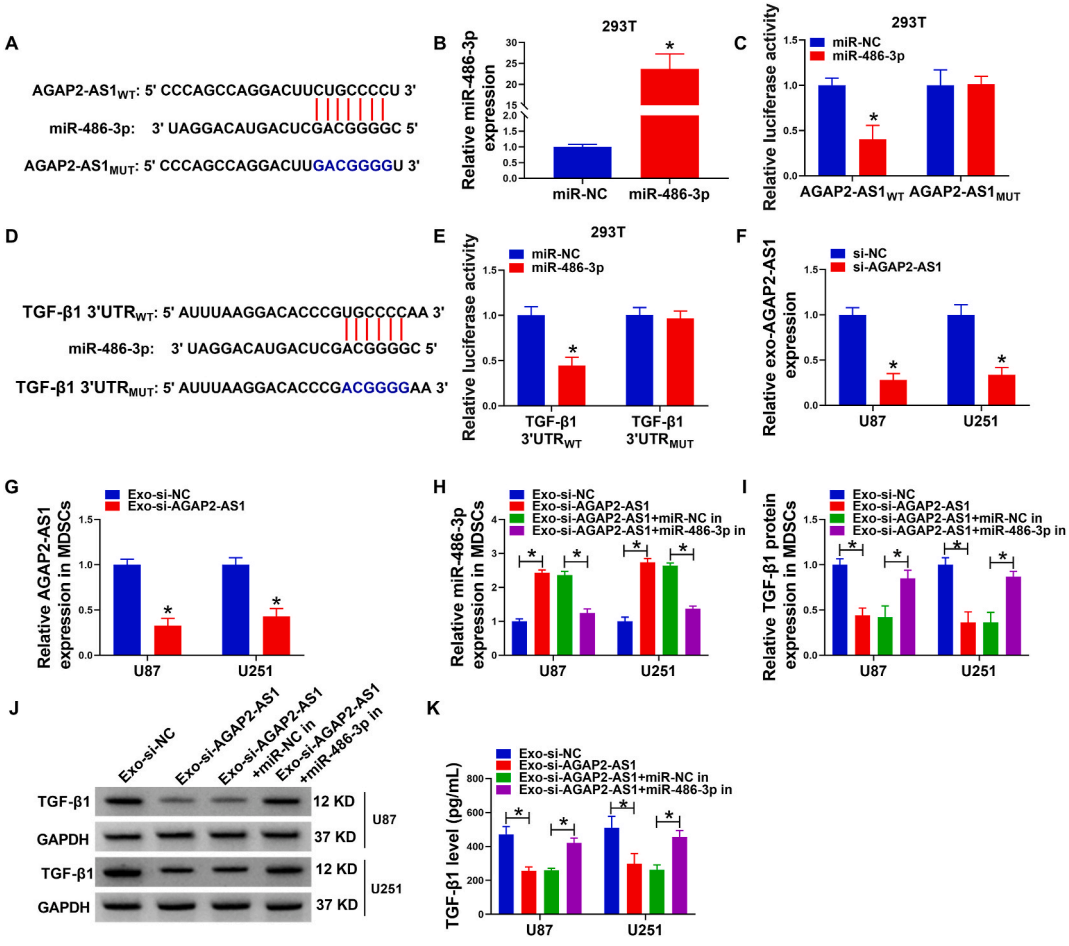

Fig. 6. Validation of exosomal AGAP2-AS1/miR-486-3p/TGF-β1 regulatory axis in MDSCs. (A) Prediction results on LncBase. (B) miR-486-3p expression was examined in 293T cells transfected with miR-NC or miR-486-3p using RT-qPCR. (C) Binding between AGAP2-AS1 and miR-486-3p was validated using a dual-luciferase reporter assay in 293T cells. (D) Prediction results on TargetScan. (E) A dual-luciferase reporter assay was used to confirm the binding between TGF-β1 and miR-486-3p in 293T cells. (F) AGAP2-AS1 level was assessed in U87 and U251 cells transfected with si-NC or si-AGAP2-AS1 using RT-qPCR. (G) Expression of AGAP2-AS1 was examined in MDSCs treated with exosomes from the si-NC or si-AGAP2-AS1-transfected U87 and U251 cells using RT-qPCR. (H–K) MDSCs were incubated with Exo-si-NC, Exo-si-AGAP2-AS1, Exo-si-AGAP2-AS1+miR-NC in, and Exo-si-AGAP2-AS1+miR-486-3p in. (H) miR-486-3p level was measured in treated MDSCs using RT-qPCR. (I and J) TGF-β1 protein level was detected using Western blot in treated MDSCs. (K) ELISA kits determined TGF-β1 secretions in treated MDSCs. *$P < 0.05$.

3.6. Exosomal AGAP2-AS1 positively regulated TGF-β1 expression in MDSCs via sponging miR-486-3p

Next, to further investigate the molecular mechanisms of AGAP2-AS1 affected TGF-β1 in GBM progression, online predicted website LncBase was applied to search for miRNAs containing a potential to interact with AGAP2-AS1. As a result, miR-486-3p was chosen as one candidate with the predicated binding site on AGAP2-AS1 (Fig. 6A). The overexpression efficiency of miR-486-3p in 293T cells was detected and exhibited in Fig. 6B. Later, their binding was verified by using a dual-luciferase reporter assay. Results displayed that the forced expression of miR-486-3p might prominently decrease the luciferase activity of the AGAP2-AS1$_{WT}$ reporter vector, rather than the mutant group in 293T cells (Fig. 6C). MiRNAs can regulate tumorigenesis via regulating target mRNAs expression. For that matter, Targetscan software presented there are binding sites between TGF-β1 and miR-486-3p in 293T cells (Fig. 6D). Then, luciferase assay displayed that miR-486-3p upregulation might reduce the luciferase activity of TGF-β1$_{WT}$ reporter systems, whereas had no significant influence on the mutant groups in 293T cells (Fig. 6E). Besides, RT-qPCR analysis presented that the introduction of si-AGAP2-AS1 might repress the expression level of AGAP2-AS1 in GBM cells (Fig. 6F), implying that the knockdown efficiency is available. After that, we observed that AGAP2-AS1 level was strikingly decreased in MDSCs incubated with exosomes from the si-AGAP2-AS1-transfected U87 and U251 cells compared with the control group (Fig. 6G), indicating the GBM-derived exosomes were internalized by the MDSCs. Subsequently, to explore the function of Exo-AGAP2-AS1 on MDSC development, MDSCs were incubated with Exo-si-NC, Exo-si-AGAP2-AS1, Exo-si-AGAP2-AS1+miR-NC in, and Exo-si-AGAP2-AS1+miR-486-3p in. As shown in Fig. 6H, miR-486-3p level was enhanced by the Exo-si-AGAP2-AS1 group in MDSCs, which was partially abolished by miR-486-3p knockdown. In parallel, the inhibitory role of Exo-si-AGAP2-AS1 on TGF-β1 protein level in MDSCs was effectively mitigated by miR-486-3p absence in MDSCs (Fig. 6I and J). Similar to the Western blot results, ELISA presented that Exo-si-AGAP2-AS1-mediated TGF-β1 secretion reduction was apparently partially attenuated through miR-486-3p downregulation in MDSCs

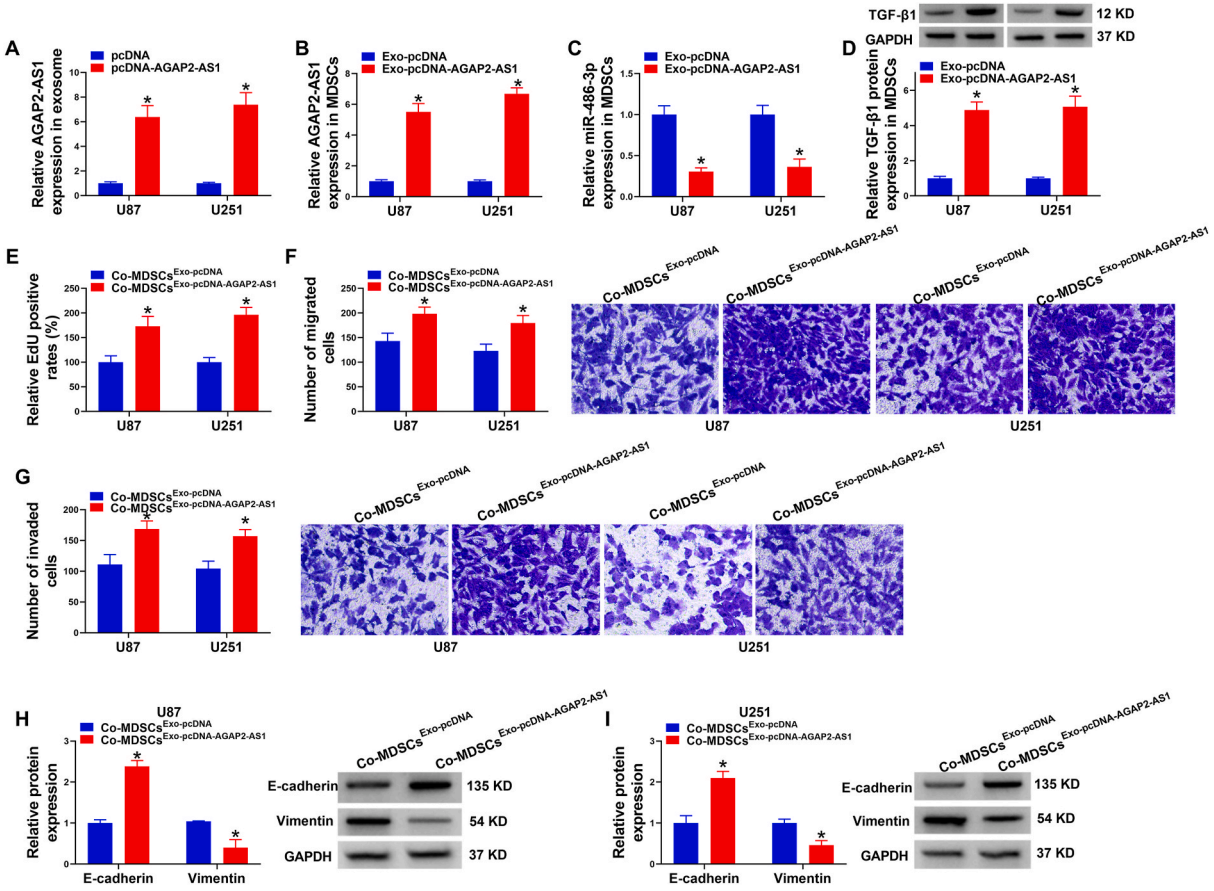

Fig. 7. Exo-pcDNA-AGAP2-AS1 promoted tumor cell proliferation and metastasis. (A) AGAP2-AS1 level was detected in exosomes from the pcDNA or pcDNA-AGAP2-AS1-transfectred U87 and U251 cells by RT-qPCR assay. (B and C) AGAP2-AS1 and miR-486-3p expression was measured using RT-qPCR in MDSCs incubated with Exo-pcDNA or Exo-pcDNA-AGAP2-AS1. (D) TGF-β1 protein level was assessed using Western blot in MDSCs incubated with Exo-pcDNA or Exo-pcDNA-AGAP2-AS1. (E–I) After being incubated with Exo-pcDNA or Exo-pcDNA-AGAP2-AS1, MDSCs were co-cultured with U87 and U251 cells. (E) Cell proliferation was examined in treated GBM cells using EdU assay. (F and G) GBM cell migration and invasion was measured using Transwell assay. (H and I) E-cadherin and Vimentin protein levels were monitored using Western blot in treated GBM cells. *$P < 0.05$.

(Fig. 6K). Collectively, the above data illuminated that exosomal AGAP2-AS1 functioned as a molecular sponge to sequester miR-486-3p from TGF-β1 in MDSCs.

3.7. Exo-pcDNA-AGAP2-AS1 facilitated GBM cell proliferation and metastasis via MDSCs

Some research indicated that tumor exosomal RNAs might boost cancer development via modulating the interaction between tumor cells and MDSCs. Accordingly, we inferred that AGAP2-AS1 might be shuttled from GBM cells to MDSCs via exosomes and further educated MDSCs, which affected tumor cell growth and metastasis. Subsequently, the overexpression efficiency of AGAP2-AS1 in exosomes from U87 and U251 cells was detected and presented in Fig. 7A. Then, AGAP2-AS1 and TGF-β1 contents were clearly elevated in MDSCs incubated with Exo-pcDNA-AGAP2-AS1 versus the Exo-pcDNA group (Fig. 7B and D), whereas miR-486-3p expression was reduced (Fig. 7C). After that, in order to further check the function of GBM-derived exosomes AGAP2-AS1 internalized by the MDSCs, U87 and U251 cells were co-cultured with Co-MDSCs $^{\text{Exo-pcDNA}}$ or Co-MDSCs $^{\text{Exo-pcDNA-AGAP2-AS1}}$. Functional analysis revealed that Co-MDSCs $^{\text{Exo-pcDNA-AGAP2-AS1}}$ might greatly improve the proliferation (Fig. 7E), migration (Fig. 7F), and invasion (Fig. 7G) of U87 and U251 cells when compared with the corresponding control groups. Consistently, increased E-cadherin protein expression and reduced Vimentin protein level in tumor cells were observed in the Co-MDSCs $^{\text{Exo-pcDNA-AGAP2-AS1}}$ group relative to the Co-MDSCs $^{\text{Exo-pcDNA}}$ group (Fig. 7H and I). All of these results concluded that exosomal AGAP2-AS1 boosted GBM cell proliferation and metastasis via MDSCs.

4. Discussion

Nowadays, the complex communication network between cancer cells and neighboring immune cells could shape the outcome of clinical interventions during cancer progression and metastasis [34]. MDSCs are pathologically activated neutrophils and monocytes with potent immunosuppressive activity [35]. Recent documents have suggested that ncRNAs enable MDSCs to interact with cancer cells via secreting cytokines and chemokines, which in turn regulate cancer progression [36,37]. Nevertheless, the biological function of tumor-derived ncRNAs, especially lncRNAs, in MDSCs is poorly defined.

Herein, our findings discovered a new mechanism by which AGAP2-AS1 functions as a communication mediator in the MDSCs. Earlier literature displayed that AGAP2-AS1 might expedite cell proliferation and invasion in different human tumors, containing GBM [38]. Consistent with the former works, AGAP2-AS1 presented enhanced expression in GBM tissues and cell lines. More importantly, the silencing of AGAP2-AS1 might repress GBM cell growth and metastasis *in vitro*. Besides, previous studies have indicated the potential mechanisms capable of stimulating GBM cell growth and tumor progression might be related to the lactate pathway [39,40]. Hence, we inferred that AGAP2-AS1 might be involved in the regulation of the lactate pathway, which we will further explore in the future. Additionally, TGF-β is a multifunctional growth factor involved in tumor growth, invasion, and metastasis [41]. In mammals, the TGF-β family forms a group of three isoforms, TGF-β1, TGF-β2, and TGF-β3, among which TGF-β1 is the most abundantly expressed isoform [42]. As a master immune regulator, TGF-β might act as a tumor promoter via several mechanisms including immunosuppression [43,44]. In this study, our data verified that the downregulation of TGF-β1 might effectively impede the malignant behaviors of GBM cells, in agreement with some previous reports [45,46]. Of interest, rescue experiments exhibited that increased TGF-β1 might partially weaken AGAP2-AS1 absence-mediated GBM cell growth and metastasis repression. Therefore, we further checked the relationship between the two. Regarding the molecular mechanism, the most commonly reported is serving as ceRNAs, lncRNA might hinder miRNA activity, thereby reducing miRNA-mediated repression of target mRNAs [47]. Here, based on bioinformatics analysis, current work discovered that miR-486-3p might interact with AGAP2-AS1 or TGF-β1 in 293T cells, supporting the regulatory network of AGAP2-AS1-miR-486-3p-TGF-β1. Plenty of literature suggested that exosomes, a class of vesicles that can transfer bioactive molecules, are reported to partake in the local and distal intercellular communication between cells. Furthermore, tumor-derived exosomes might transfer a plethora of signals that promote or inhibit immunosuppression of myeloid cell populations [48], containing MDSCs [15,49]. Present work verified that miR-486-3p knockdown might partly abolish the repression of GBM-derived Exo-AGAP2-AS1 downregulation on TGF-β1 contents in MDSCs. In other words, GBM-derived Exo-AGAP2-AS1 might alter the TGF-β1 secretion of MDSCs via sponging miR-486-3p.

To further validate whether this regulatory mechanism might help tumor cells escape immune surveillance, we performed rescue assays. Functional analysis exhibited that GBM-derived Exo-AGAP2-AS1 might intensify GBM cell growth and metastasis via increasing TGF-β1 secretion of MDSCs. It is indisputable that the tumor microenvironment is one of the indispensable components for tumor growth and survival, and within it exists a vast array of tumor-secreted molecules that promote the development of cancerous growth. Exosomes, which are able to stably transfer their molecular carriers to target cells, have been shown to be effective and efficient carriers of intercellular communication between cancer cells and other cells in the tumor microenvironment [50]. Herein, these findings evidence that tumor-derived exosomes secreted by GBM can carry AGAP2-AS1 and deliver it into MDSCs to boost host immunosuppression that expedites tumor progression. Nevertheless, the present work was limited to *in vitro* research, and more *in vivo* experiments about the novel mechanism in GBM using mice models will be conducted in further study. Beyond that, there are two subtypes of MDSCs, monocytes and granulocytes, but we have studied MDSCs as a general type of cell without examining the effect of GBM-derived exosomes on each subtype, separately.

5. Conclusion

In summary, our results discovered a novel mechanism that GBM-derived Exo-AGAP2-AS1 might serve as communication signaling

in the tumor immune microenvironment to facilitate GBM progression via mediating TGF-β1 secretion of MDSCs. These results not only broaden our understanding of how cancer cells mediate immune escape at a distance from the primary tumor, but also provide useful insights for the development of MDSC-targeted therapeutic agents that could significantly improve the efficacy of anti-cancer immunotherapy.

Ethics approval and consent to participate

The present study was approved by the ethical review committee of the No. 215 Hospital of Shaanxi Nuclear Industry[Approval Number:20220314]. Written informed consent was obtained from all enrolled patients.

Consent for publication

Patients agree to participate in this work.

Disclosure of interest

The authors declare that they have no conflicts of interest.

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Data availability statement

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Yanlong Tian: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation. **Xiao Gao:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation. **Xuechao Yang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation. **Shangjun Chen:** Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yufeng Ren:** Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29949.

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