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Gallic acid mediates tumor-suppressive effects on osteosarcoma through the H19-Wnt/β-catenin regulatory axis

Fengxiang Pang [a,b,1], Shouchang Ding [a,b,1], Nan Li [a,b], Zhipeng Li [a,b], Nannan Tian [a,b], Chuanjian Shi [c], Fengwei Zhang [a,b], Yongxin Mai [a,b], Jinfang Zhang [d,*], Junyan Wang [e,**]

[a] Key Laboratory of Orthopaedics and Traumatology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, 510405, China
[b] The First Clinical Medical College, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, 510405, China
[c] School of Pharmaceutical Sciences, Southern Medical University,Guangzhou, Guangdong, 511458, China
[d] Shenzhen Hospital (Futian) of Guangzhou University of Chinese Medicine, China
[e] School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, 510006, China

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ABSTRACT

Background: Osteosarcoma (OS) is the most common primary malignancy in bone tissues, and effective therapeutics remain absent in clinical practice. Traditional Chinese medicines (TCM) have been used for thousands of years, which provide great insights into OS management. Gallic acid (GA) is a natural phenolic acid enriched in various foods and herbs. Several pharmacological activities of GA such as anti-oxidation and anti-inflammation have been well-established. However, its biological function in OS remains not fully understood.

Methods: The potential anti-cancer properties of GA were evaluated in 143 B, U2OS and MG63 cells. Its effects on cell growth, cell cycle, apoptosis and migration were examined in these OS cells. The lncRNA H19 and Wnt/β-catenin signaling were detected by qPCR, luciferase activity and Western blotting assays. The *in vivo* effect of GA on tumor growth was investigated using an orthotopic mouse model.

Results: In the present study, GA was found to suppress the tumor growth *in vitro via* inducing cell cycle arrest and apoptosis in OS cells, and inhibit the invasion and metastasis as well. Using the orthotopic animal model, GA was also found to suppress tumorigenesis *in vivo*. Long noncoding RNA (lncRNA) H19 was demonstrated to be down-regulated by GA, and thus disrupted the canonical Wnt/β-catenin signaling in OS cells. Furthermore, the ectopic expression of H19 rescued the GA-induced suppressive effects on tumor growth and metastasis, and partially reversed the inactivation of Wnt/β-catenin signaling.

Conclusions: Taken together, our results indicated that GA inhibited tumor growth through an H19-mediated Wnt/β-catenin signaling regulatory axis in OS cells.

The translational potential of this article: The information gained from this study provides a novel underlying mechanism of GA mediated anti-OS activity, suggesting that GA may be a promising drug candidate for OS patients.

1. Introduction

Osteosarcoma (OS) is a rare type of bone tumor that usually develops in teenagers [1–3]. It mainly localizes in the diaphysis of long bones, especially in areas of large joints of limbs, e.g. distal femur, proximal tibia, and humerus [1,2,4]. As a primary bone tumor, OS is an aggressive malignancy with highly invasive and metastatic potential. Even with the advances in the neoadjuvant chemotherapy and surgical techniques, the survival rate of primary OS patients was just 60%; and for the patients with relapse or migration, the survival rate was lower than 30% [1].

Abbreviations: OS, osteosarcoma; TCM, traditional Chinese medicine; GA, gallic acid; LncRNAs, long noncoding RNAs; pH19, H19 overexpression plasmids; pBabe, the empty lasmids; qRT-PCR, Quantitative reverse-transcription polymerase chain reaction; RIPA, Radio Immunoprecipitation Assay; IHC, Immunohistochemistry; PI, propidium iodide; Myc, Cellular-myelocytomatosis viral oncogene; CD44, cluster of differentiation 44; Oct3/4, POU class 5 homeobox 1.

* Corresponding author. Shenzhen Hospital (Futian) of Guangzhou University of Chinese Medicine.
** Corresponding author. School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou 510006, China.
E-mail addresses: zhangjf06@gzucm.edu.cn (J. Zhang), wangjunyan@gzucm.edu.cn (J. Wang).
[1] Fengxiang Pang and Shouchang Ding contributed equally to this paper.

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Therefore, it is urgently needed to develop effective strategies for OS. As an indispensable part of Chinese culture, traditional Chinese medicines (TCM) have been used for centuries in China. Mounting evidence demonstrate that herb medicines have great advantages in preventing or treating cancers, which bring bright insights for developing novel therapeutic strategies against malignancy [5]. Gallic acid (GA) is a kind of phenolic acid abundantly found in various foods and herbs [6,7]. Substantial evidence demonstrates that GA plays significant roles in multiple malignancy treatment *via* suppressing cell viability, inducing apoptosis, or inhibiting migration [8,9]. GA was also reported to inhibit the migration and invasion of OS cells [10]. However, this is only a phenomenal observation lack of detailed mechanism. Considering the clinical potential of GA in cancer treatment, more studies are needed to clarify its function in OS and the underlying mechanism.

Long noncoding RNAs (lncRNAs), which are usually more than 200 nucleotides in length, have been considered as important and powerful regulators in various biological activities and disease progressions [11–13]. Accumulating studies revealed that the dysregulated lncRNAs play important functional roles in cancer biology [14,15]. The lncRNA H19, encoded by an imprinted gene, was frequently found to be up-regulated in multiple cancers, and promoted tumorigenesis and metastasis [14,16]. In OS, abnormal elevation of H19 was closely associated with poor tumor-grade, high-risk clinical stage and deep tumor invasion, and shorter survival time [17,18]. All these reports suggest that H19 may be a potential diagnostic biomarker or therapeutic target for OS patients.

In the present study, we systematically investigated the anti-cancer effects of GA on OS and it was found that GA could inhibit tumor growth and metastasis *in vitro* and *in vivo*. Mechanically, GA induced the down-regulation of H19, and led to the disruption of Wnt/β-catenin signaling. Therefore, our findings demonstrated that GA was able to suppress OS tumorigenesis through an H19-mediated Wnt/β-catenin signaling regulatory axis, suggesting that GA may be a promising candidate against OS in clinical practice.

2. Materials and methods

2.1. Cell culture

The human OS cell lines including 143 B, MG63 and U2OS cells were cultured in DMEM (Gibco, Carlsbad, CA) with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% Penicillin-Streptomycin (Hyclone, Paschng, Austria) in humidity at 37 °C, 5% CO_2. GA was purchased from Aladdin and dissolved in DMSO for usage.

2.2. Cell viability assays

Cell viability analysis was performed according to the previous report [19]. Briefly, OS cells were seeded into 96-well plates and maintained overnight. Then GA with gradient concentrations from 0 to 200 μM were added, and further incubated for 24, 48, and 72 h. The cell viability was detected by Cell Counting Kit-8 (Beyotime, Shanghai, China) examination, and the absorbance was measured at 450 nm using a Hybrid Multi-Mode Microplate Reader (Tecan, Switzerland). All the experiments were performed in triplicates.

2.3. Colony formation assays

The colony formation was examined according to our previous study [20]. The 143 B and U2OS cells were seeded into 6-well plates, and incubated with 150 μM GA for two weeks. The colonies were fixed and stained with 0.5% crystal violet for 20min. The colonies were captured and quantified by ImmunoSpot analyzers (CTL).

2.4. Flow cytometry examination

As previously reported [21], the 143 B and U2OS cells were seeded into 6-well plates, and incubated with 150 μM GA for 48 h. Then the cells were labeled by Annexin V-FITC/PI double staining kit (KeyGEN, Nanjing, China), and monitored by flow cytometry (Beckman, Pasadena, CA). For cell cycle analysis, cells were seeded and incubated with 150 μM GA, and then monitored by the Cell Cycle Detection Kit (KeyGEN, Nanjing, China) for flow cytometry examination.

2.5. Wound healing and transwell assays

The 143 B and U2OS cells were seeded into 6-well plates overnight, then incubated with 150 μM GA for 48 h, and cell metastasis and cell invasion were detected. As mentioned before [20], the monolayer cells were carefully scratched by a sterile tip, and incubated with 150 μM GA in DMEM with 1% FBS. With 48 h-treatment, the wound areas were estimated by Image J. For transwell assays, GA-treated cells and control cells were seeded in the upper chamber with serum-free medium, and 10% FBS medium was added into the lower chamber. Then they were incubated for 24 h, and the cells on the upper chamber were gently scraped. The cells stayed on the lower membrane surface were fixed with methanol for 20min and stained with 0.1% crystal violet. Images were taken by Multifunctional Cell Imaging Microplate reader (BioTek, USA) and invasion rates were recorded by Gene 5.

2.6. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted by Trizol (Invitrogen, Carslbad, CA, USA), and cDNA was reversely transcribed using PrimeScript™ RT Reagent Kit (TaKaLa, Japan). The quantitative PCR examination was conducted in triplicates on a LightCycler 480 system (Roche, Basel, Switzerland) using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher, USA). The primer sequences for real-time PCR examination were listed in Table 1. The housekeeping gene GAPDH was used as control, and relative expression levels of candidate genes were calculated *via* the $2^{-\Delta\Delta Ct}$ methodology.

2.7. Luciferase activity assay

Cells were seeded on 24-well plate and transfected with TOPflash plasmids using Lipofectamine 3000 according to our previous study [22]. With GA treatment for 48 h, the luciferase activity was detected using Bright-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) on a Hybrid Multi-Mode Microplate Reader.

2.8. Western blotting

Total protein was extracted by Radio Immunoprecipitation Assay (RIPA) buffer, and the nuclear and cytoplasmic fractions were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (Invent). The supernatant protein was collected by centrifugation and quantified by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Then the proteins were separated by 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, MA, USA). Blocked with 5% fat-free milk, the membranes were probed with the following antibodies β-catenin (1:2000; Cell Signaling Technology, USA), Lamin B1 (1:2000; Cell Signaling Technology, USA) and GAPDH (1:2000; Cell Signaling Technology, USA) for overnight. Subsequently, they were incubated with the HRP-labelled secondary antibodies (Millipore, USA), and exposed by the FluorChem R system (ProteinSimple, San Jose, CA, USA) with the chemiluminescence (ECL, Hangzhou, China). Lamin B1 and GAPDH were

Table 1
The primer sequences used in this study.

	Forward	Reverse
β-catenin	CCGTTCGCCTTCATTATGGA	GGCAAGGTTTCGAATCAATCC
CCND1	CTGGAGGTCTGCGAGGAACA	CCTTCATCTTAGAGGCCACGAA
CD44	TCAGAGGAGTAGGAGAGAGGAAAC	GAAAAGTCAAAGTAACAATAACAGTGG
c-Myc	TTCGGGTAGTGGAAAACCAG	CAGCAGCTCGAATTTCTTCC
OCT3/4	TCGAGAACCGAGTGAGAGGC	CACACTCGGACCACATCCTTC
Axin2	CTTTCGCCAACCGTGGTT	GGATCGCTCCTCTTGAAGGA
H19	TGCTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGC
Linc-ROR	CTGGCTTTCTGGTTTGACG	CAGGAGGTTACTGGACTTGGAG
HOTTIP	CCTAAAGCCACGCTTCTTTG	TGCAGGCTGGAGATCCTACT
TINCR	TGTGGCCCAAACTCAGGGATACAT	AGATGACAGTGGCTGGAGTTGTCA
HULC	TTCACCAGGCTGATAGTCCG	ACACGTCCTTCCCATAAACCC
GAPDH	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA

used as the internal control of nuclear protein and cytoplasmic protein, respectively.

2.9. H19 overexpression plasmid and cell transfection

The H19 overexpression plasmid (pH19) was generated as previously reported [23] and the empty plasmids (pBabe) were used as control. The OS cells were seeded in six-well plates and they were transfected with pH19 and pBabe by using Lipofectamine 3000 (Invitrogen) respectively.

2.10. Osteosarcoma intra-tibia tumor-bearing model

The intra-tibia tumor-bearing model was applied for the *in vivo* experiments [24]. Female Balb/c-nude mice (4–5 weeks old) were purchased from the Laboratory Animal Center, Southern Medical University. All experimental procedures were approved by the Ethics and Animal Research Committee of Southern Medical University Guangzhou, China. 143 B cells (1×10^6/100 μl) were injected into the medullary cavity of the right tibia of mice, and animals were randomly assigned into two groups (n = 10). Group 1 was intragastric injection (i.g.) with 50 mg/kg GA; and group 2 was administrated with vehicle buffer. Animals were treated every day for 4 weeks, and tumor size was measured every 7 days. Tumor volume was calculated according to the following formula: volume = $(W1 \times W2^2)/2$, where W1 = major diameter (mm) and W2 = minor diameter (mm) of tumors.

2.11. Immunohistochemistry (IHC) examination

Tumor specimens were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (5 μm) were incubated with the Ki-67 antibody (Calbiochem, Darmstadt, Germany) and β-catenin antibody for 4 h. Visualization was achieved by using the 3,3′-diaminobenzidine substrate (Dako, Denmark) followed by counterstaining with hematoxylin. The images were taken and quantified with Image J.

2.12. TUNEL staining

Deparaffinized and rehydrated sections were subjected to permeabilized by 0.5% Triton X-100 (Cat. NO.: T109027, Aladdin, Shanghai, China) and incubated with normal goat serum (Cat. NO. G9023, Sigma–Aldrich, USA) for 1 h. The detection of apoptotic cells in tumor tissues was performed using an In Situ Cell Death Detection Kit (Cat. NO.: 11684817910, Roche, Basel, Switzerland) according to the manufacturer's instructions, and the nucleus was counterstained with DAPI. The images were captured using a 3DHistech (Pannoramic MIDI, Hungary) and quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.13. Statistical analysis

All experiments were repeated in triplicates. Data were presented as mean ± SD. Statistical differences between each group were compared using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. GA significantly suppressed cell viability in OS cells

As mentioned before, GA has been reported to suppress tumor growth in multiple cancers [9]. To validate its anti-cancer effects on OS, three OS cell lines including 143 B, MG63 and U2OS were treated with series concentration of GA. The cell viability was examined and the results showed that GA exhibited significant anti-tumor activity with the concentration from 50 to 200 μM in a dose- and time-dependent manners in 143 B cells (Fig. 1A), MG63 cells (Fig. 1B) and U2OS cells (Fig. 1C). The IC50 value of GA was nearly 150 μM in the three OS cells for the 48-h treatment. We therefore selected 150 μM for further investigation. The subsequent colony formation also displayed fewer and smaller colonies in GA treated 143 B and U2OS cells (Fig. 1D and F), further confirming its anti-cancer potential.

3.2. GA induced cell cycle arrest and apoptosis in OS cells

We next investigated the effects of GA on cell cycle and apoptosis. The GA-treated 143 B and U2OS cells were subjected to the cell cycle and apoptosis analyses. As shown in Fig. 2, more cells in the G2/M phase were observed in GA-treated 143 B (Fig. 2A) and U2OS cells (Fig. 2B) compared with their respective controls. Moreover, an increased percentage of apoptotic cells was also found in GA-treated 143 B cells (Fig. 2C) and U2OS cells (Fig. 2D). These data indicated that GA inhibited OS cell proliferation *via* inducing apoptosis and cell cycle arrest.

3.3. GA inhibited the invasion and metastasis of OS cells

The inhibitory effects of GA on invasion and metastasis in OS cells were also examined, and the data of wound healing revealed the bigger scratch areas in GA treated cells (Fig. 3A and D), indicating the inhibitory effect of GA on migration. The further transwell examination also showed less invaded cells in GA-treated groups (Fig. 3E and F), which was consistent with the wound healing results.

3.4. GA suppressed the Wnt/β-catenin signaling in OS cells

Considering that the canonical Wnt/β-catenin signaling plays important roles in tumorigenesis, we wondered whether this signaling could participate in this GA-mediated anti-cancer activity. As shown in Fig. 4A, the luciferase activities of the Wnt/β-catenin signaling reporter TOPflash were significantly suppressed by GA in both 143 B and U2OS cells. The total expression of critical transcriptor β-catenin was examined and it was found that β-catenin expression was reduced by GA in 143 B cells while remained consistent in U2OS cells (Fig. 4B). As well known,

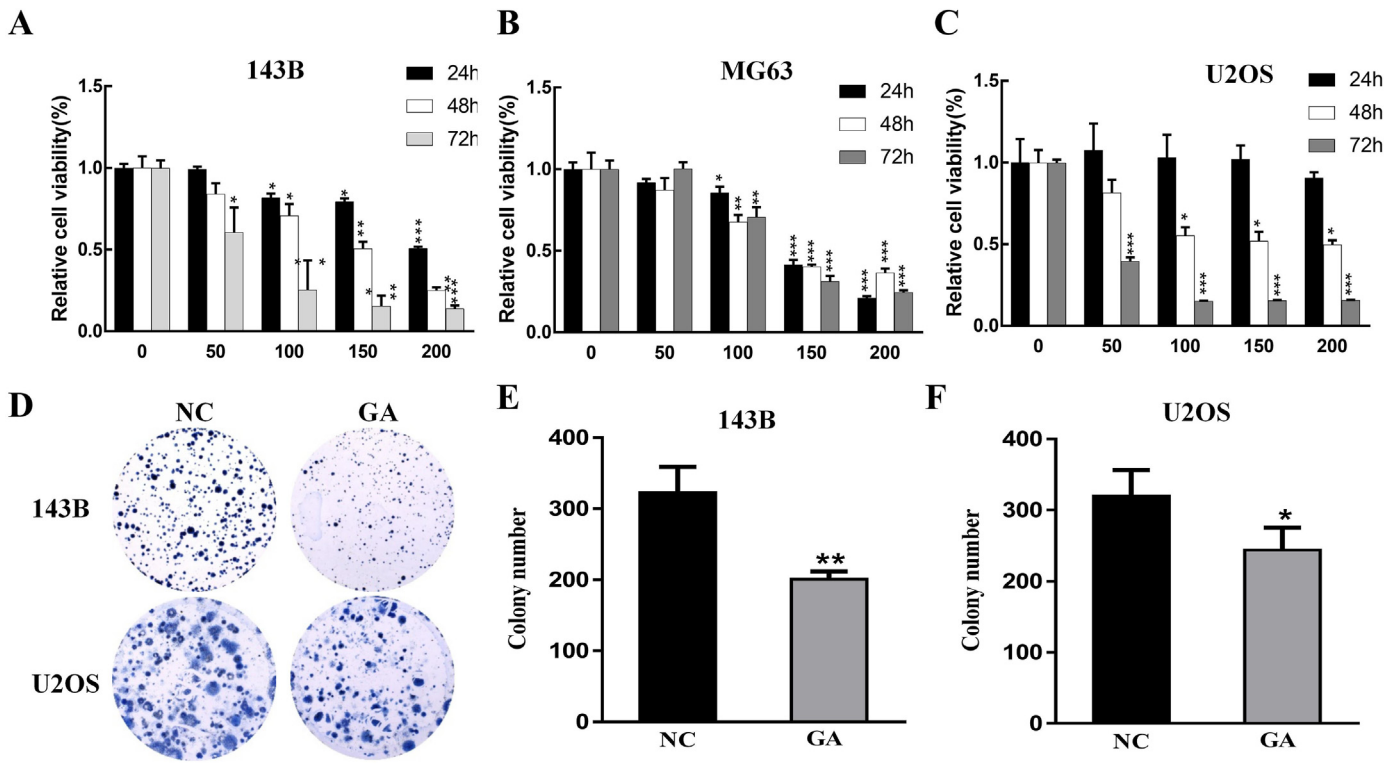

Fig. 1. GA inhibited the cell viability in OS cells. A-C, 143 B (A), MG63 (B) and U2OS (C) cells were treated with serial concentrations of GA, and the effects of GA on cell viability were measured by cell counting Kit-8 examinations at 24, 48 and 72 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* 0. D-F, 143 B and U2OS cells were treated with 150 μM GA for 14 days and the colony formation was examined. *, $P < 0.05$; **, $P < 0.01$; *vs* NC.

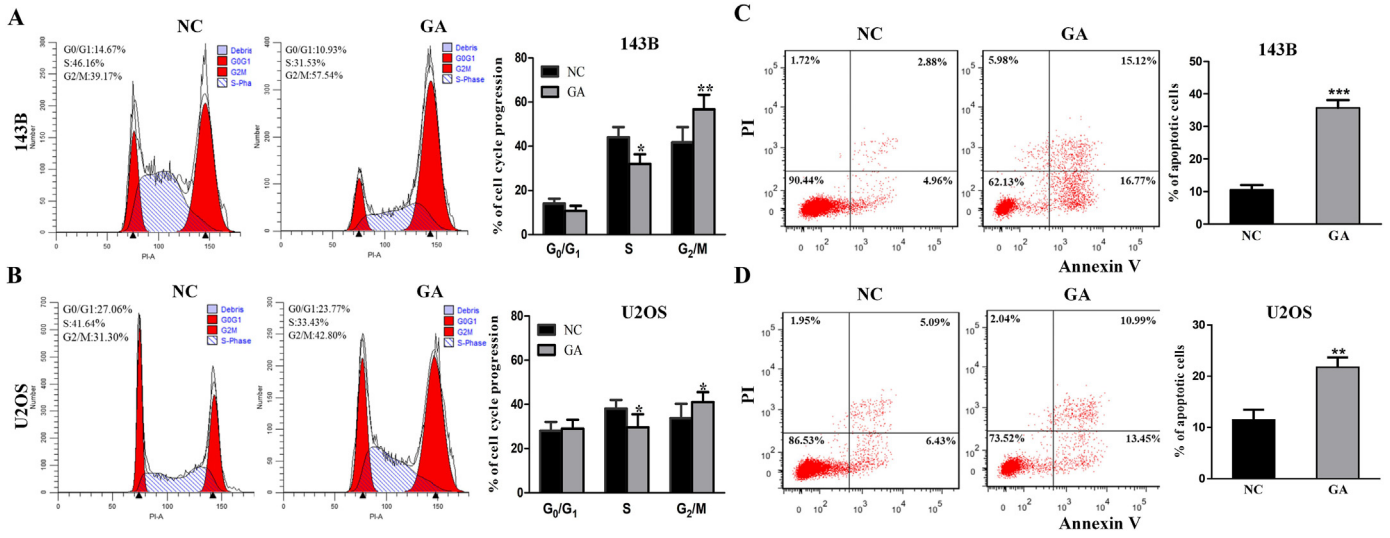

Fig. 2. GA induced cell cycle arrest and apoptosis in OS cells. A-B, 143 B (A) and U2OS (B) cells were treated with 150 μM GA for 48 h, then harvested and subjected for cell cycles analyses. C-D, 143 B (C) and U2OS (D) cells were incubated with 150 μM GA for 48 h, and the apoptotic cells were examined by Annexin V-FITC and PI double staining. Left panel, the results of one such assay; Right panel, statistical analyses of three independent experiments (mean ± SD). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* NC.

the activation of canonical Wnt/β-catenin signaling mainly depends on the nuclear transfer and accumulation of the β-catenin. We thereby further analyzed the protein level of intranuclear β-catenin and intracytoplasmic β-catenin. It was found that β-catenin expression was suppressed in the nucleus, while the cytoplamic β-catenin remained consistent in the two GA-treated OS cells (Fig. 4C). Furthermore, several downstream target genes of Wnt/β-catenin signaling were examined and these genes including C-myc, CD44, Cyclin-D1 and Oct3/4 were all down-regulated with GA treatment (Fig. 4D and F). These data indicated that GA induced the inactivation of Wnt/β-catenin signaling in OS cells.

3.5. GA suppressed tumor growth and migration of OS cells in vivo

We next examined the *in vivo* role of GA in the tumorigenesis using an orthotopic animal model. 1×10^6 143 B cells were injected into the medullary cavity of the right tibia of nude mice, and GA (50 mg/kg) was

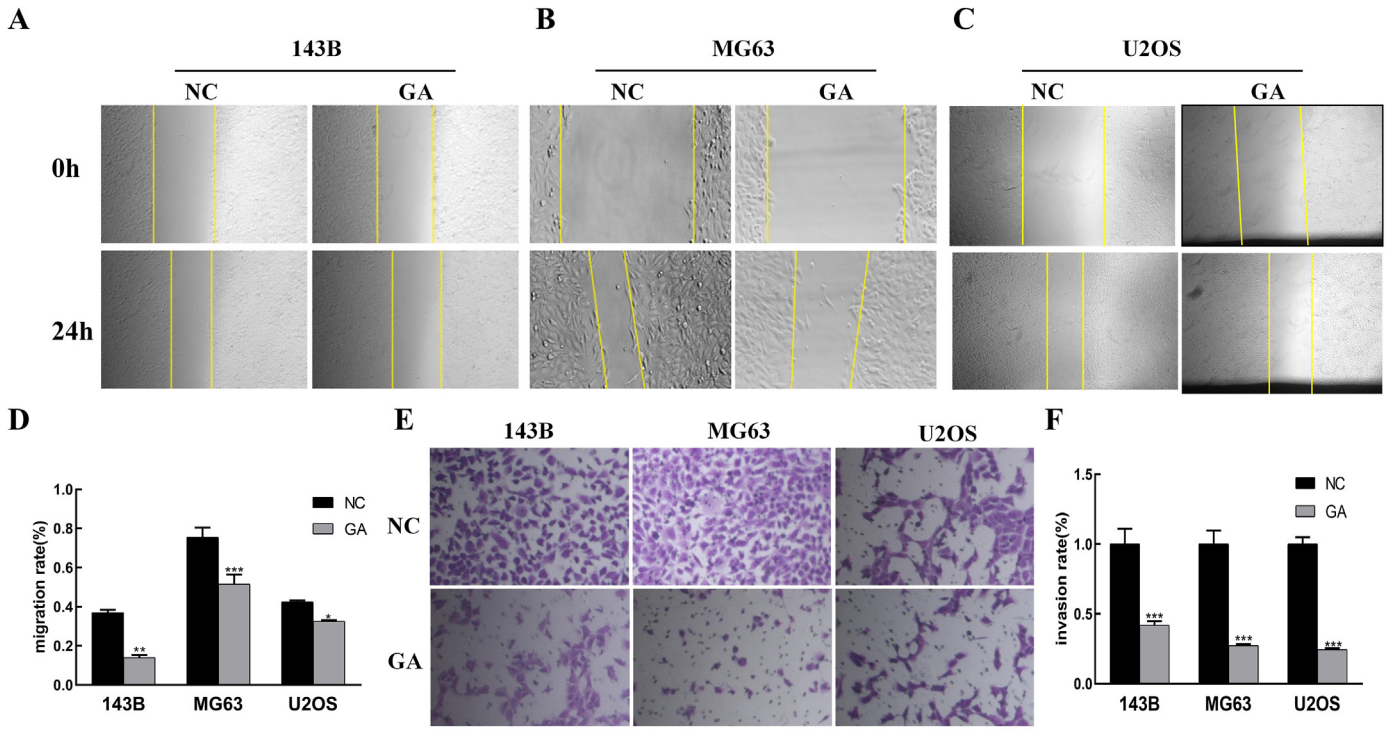

Fig. 3. GA inhibited the invasion and metastasis of OS cells. A-C, 143 B (A), MG63 (B) and U2OS (C) cells were treated with 150 μM GA for 48 h, and wound-healing migration was assayed. D, the semi-quantitative examination of wound-healing areas was in the three cell lines. E-F, transwell invasion of the GA-treated cell lines and the quantitative assays were examined. Data are presented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$; *vs* NC.

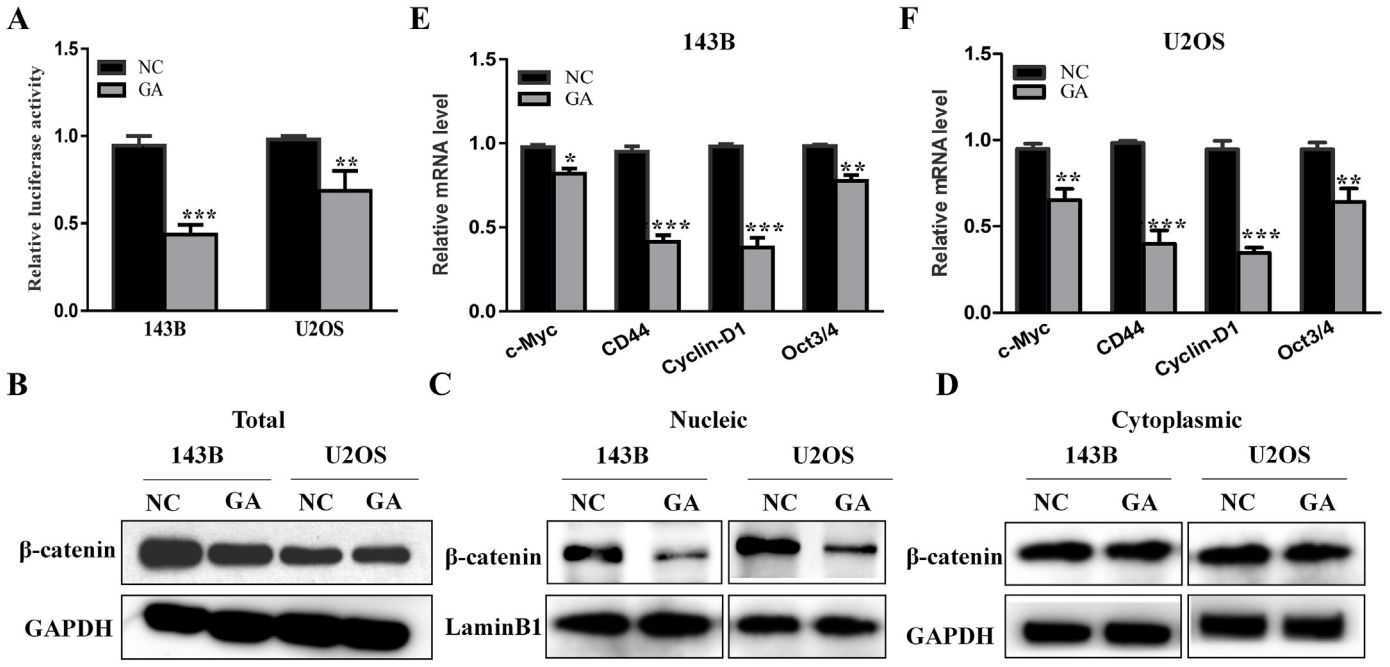

Fig. 4. GA suppressed the Wnt/β-catenin signaling in OS cells. A, After transfected with the luciferase reporter TOPflash, the two OS cells were treated with 150μM GA for 48h, and the luciferase activities were measured. B, The total β-catenin expression in GA-treated cells was determined by Western blotting. C-D, The expression of cytoplasmic β-catenin and intranuclear β-catenin were examined at protein level. Lamin B1 (nuclear expression) and GADPH (cytoplasmic expression) were used as the loading controls. E-F, The expression of several downstream targets of Wnt/β-catenin pathway was examined by qRT-PCR assays. *, P <0.05; **, P <0.01; ***, P< 0.001; *vs* NC.

intragastrically administrated once a day. Strikingly, the smaller tumor burden was observed in the GA-treated groups (Fig. 5A and B), and a significant reduction in tumor growth (Fig. 5C) and weight (Fig. 5D) were also found in these treated animals. The further H&E staining showed that GA administration significantly suppressed tumor growth and metastasis *in vivo* (Fig. 5E and F). By IHC staining, the decreased expression of cell proliferative marker Ki-67 and β-catenin were exhibited in GA treated groups (Fig. 5G). Using Tunnel staining, more positive

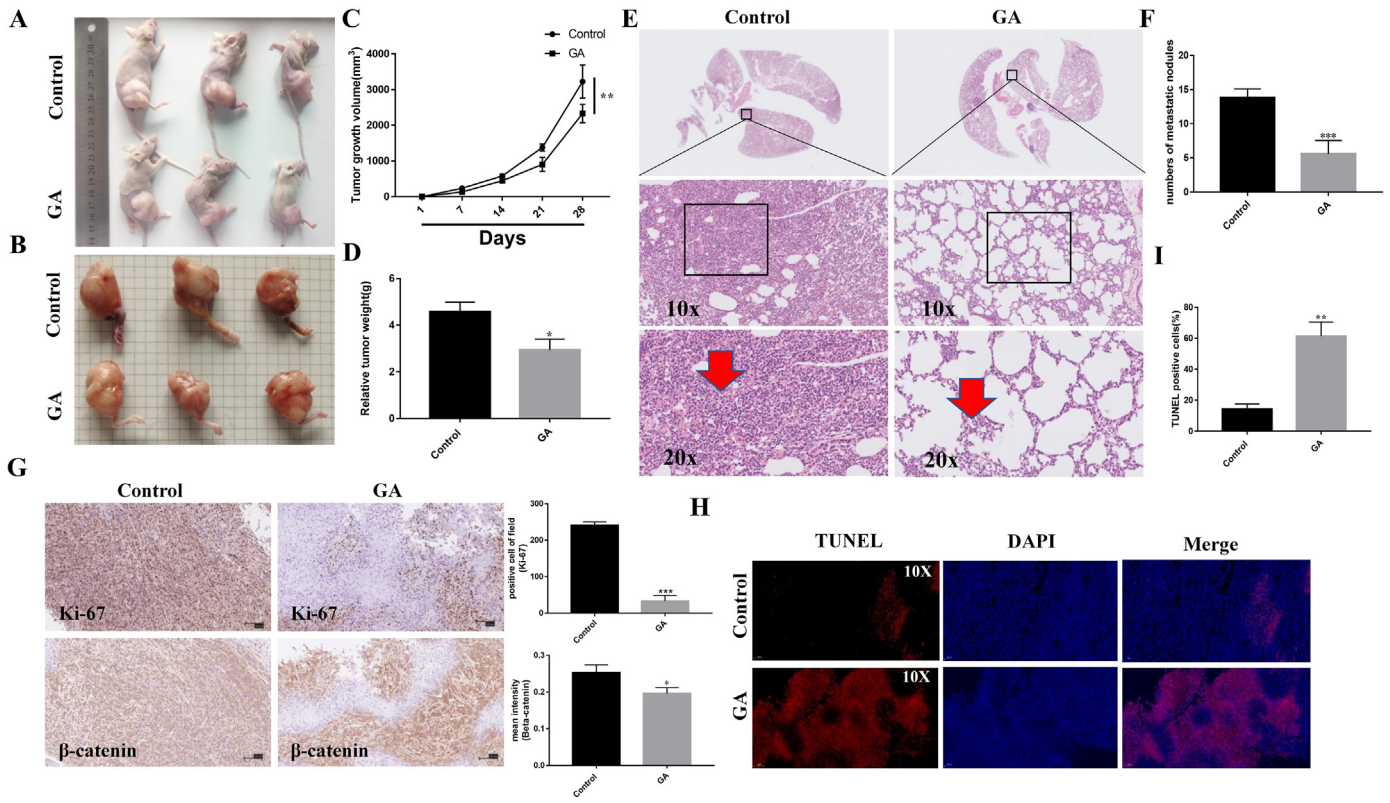

Fig. 5. GA reduced the tumor growth and metastasis of OS cells *in vivo*. 143 B cells were injected into the medullary cavity of the right tibia of nude mice, and GA was administrated with intragastric injection. A-B, The representative images showed smaller tumors in GA treated groups. C-D, The growth curve of tumor volumes (C), and the tumor weight (D) were measured. E-F, H&E staining for the lung tissue and quantitatively analyzed. G, The immunofluorescence of Ki-67 and β-catenin stained sections followed by counterstaining with DAPI. H–I, The apoptotic cells in tumor specimens were assessed by using TUNEL assays and quantitatively analyzed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* control group.

cells were observed in the specimens derived from GA-treated animals, suggesting that GA promoted the apoptotic cells *in vivo* (Fig. 5H and I).

3.6. GA suppressed H19 expression in OS cells

Considering that lncRNAs have been considered as significant regulators in various cell activities and disease progression, we hypothesize some lncRNAs may be involved in this process. Several lncRNAs such as HOTTIP, H19, linc-ROR, TINCR and HULC, which have been reported to mediate tumor growth *via* Wnt/β-catenin signaling, were analyzed and the results showed that among these lncRNAs, H19 was dramatically down-regulated by GA in 143 B (Fig. 6A) and U2OS (Fig. 6B) cells.

3.7. GA inhibited OS cells proliferation via the H19/β-catenin signaling

To further clarify whether H19 participates in GA-regulated anti-OS activity, the rescue study was further designed. We generated H19 overexpressing plasmids (pH19), and H19 was obviously up-regulated in this pH19 transfected cells (Fig. 7A). Subsequently, the H19-overexpressing cells were treated with GA and the results of cell viability and colony formation showed that the enforced expression of H19 partially attenuated the GA-induced suppressive effects (Fig. 7B and D). The inhibitory effects of GA on Wnt/β-catenin signaling were also examined, and it was found that the suppressive luciferase activity was partially promoted by H19 enforced expression (Fig. 7E). We also found that the GA-induced suppressive expression of nuclear β-catenin was partially reversed by H19 overexpression (Fig. 7F), and the decreased downstream targets were also partially reversed as well (Fig. 7G), suggesting H19 elevation partially alleviated the GA-induced the inactivation of Wnt/β-catenin signaling in OS cells.

3.8. GA suppressed metastasis through inhibiting H19 expression in OS cells

We further examined the effects of ectopic expression of H19 on the GA-induced metastatic suppression. The results showed that H19 overexpression alleviated the inhibitory effect of GA on wound healing (Fig. 8A and B) and transwell metastasis (Fig. 8C and D), suggesting H19 overexpression successfully rescued the GA-induced the inhibitory migration potential.

4. Discussion

As the most common type of cancer in bone tissues, OS is characterized with distant metastasis and multi-drug resistance, which make OS highly malignant with poor prognosis. Till now, amputation surgery is the principal therapy strategy in clinical practices [25]. However, it brings huge burdens and discomforts to individual and society. The development of innovative, targeted therapies is imperative and of high clinical significance. Definitely, TCM has been used in China for thousands of years, and now it is gaining global popularity. Increasing evidence demonstrated that numerous medicinal plants are beneficial to cancer patients and certainly merit further investigation in clinical trials [26,27]. GA is a naturally-derived plant flavone that is abundant in dietary substances and traditional medicine herbs. Many previous studies indicated that GA served as a significant inhibitor for cancer progression. For instance, GA suppressed cell viability of prostate cancer and colorectal cancer by inducing cell cycle arrest and apoptosis [28,29]. In the present study, we systematically investigated the anti-tumor activities and underlying mechanism of GA in OS. The results revealed that GA suppressed the tumorigenesis and metastasis *via* the H19-Wnt/β-catenin

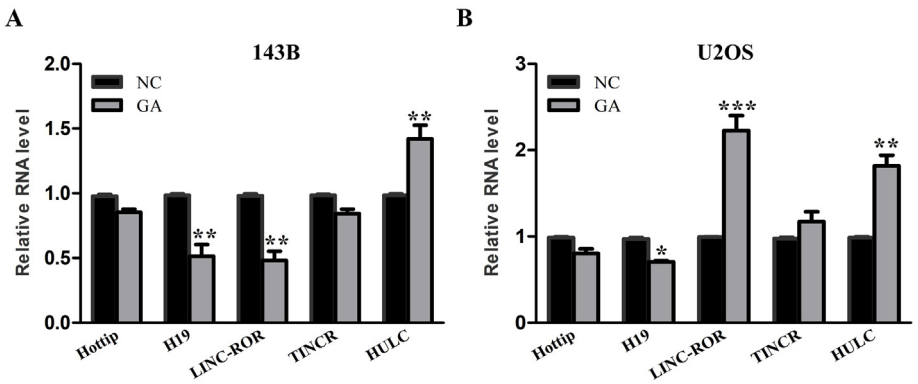

Fig. 6. H19 was the most changeable candidate in GA-treated OS cells. A-B, 143 B (A) and U2OS (B) cells were incubated with GA for 48 h, and several lncRNAs were chosen to examine their expression profiles by qRT-PCR assays. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* NC.

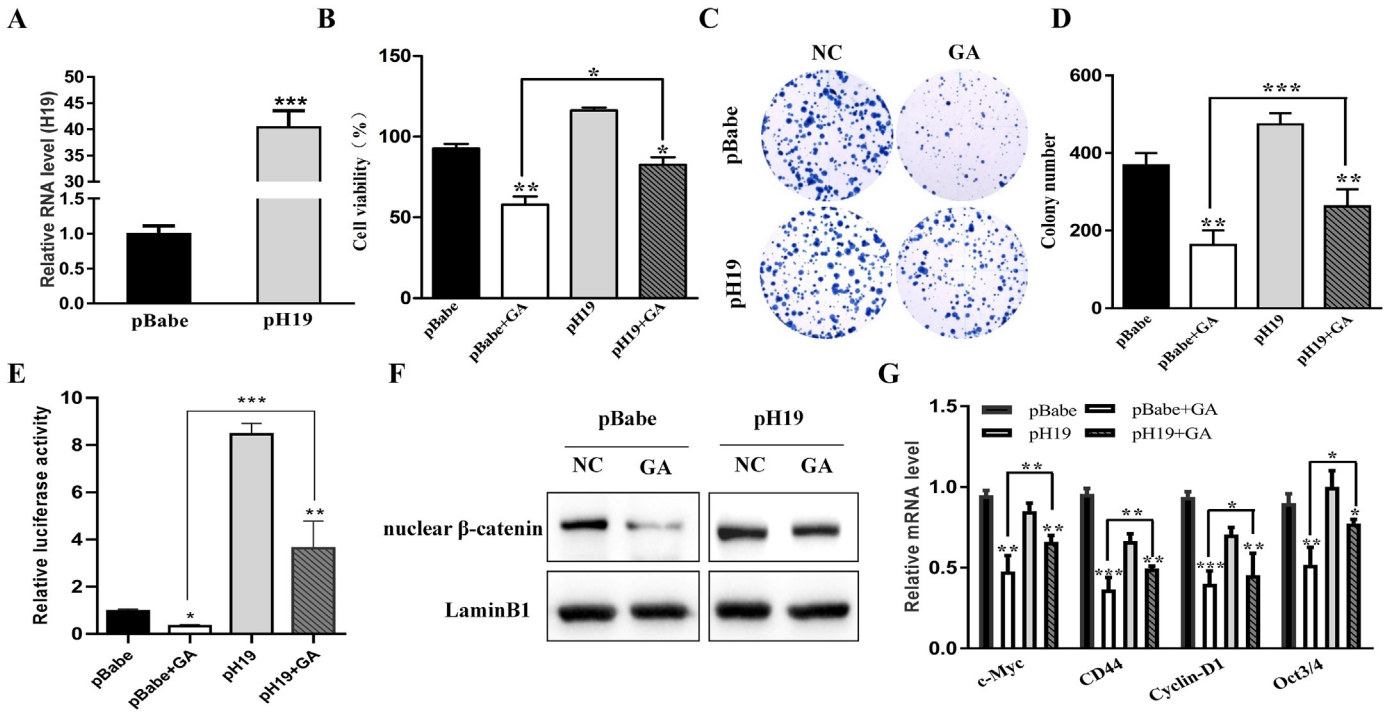

Fig. 7. GA suppressed cell proliferation in OS cells *via* H19-Wnt/β-catenin regulatory axis. A, The expression of H19 was measured in the H19-overexpressing 143 B cells by qRT-PCR examination. ***, $P < 0.001$; *vs* pBabe. B, The cell viability was examined in GA treated the H19-overexpressing 143 B cells. *, $P < 0.05$; **, $P < 0.01$. C-D, The colony formation was measured with GA treatment in H19-overexpressing OS cells. **, $P < 0.01$; ***, $P < 0.001$. E, The luciferase activity of Topflash was examined in H19-overexpressing 143 B cells with GA treatment. F, The luciferase activity and protein level of nuclear β-catenin was examined by Western blotting after treatment with GA in H19-overexpressing 143 B cells. G, Several downstream target genes of Wnt/β-catenin signaling were examined by qRT-PCR examination after GA treatment in H19-overexpressing 143 B cells. *, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$.

regulatory axis, suggesting that GA may be a potential candidate for OS patients.

To examine the *in vitro* anti-cancer function of GA in OS, the cells were treated with GA and the results of cell viability and colony formation demonstrated that GA played the anti-proliferative activity in OS cells. The further flow cytometry examination revealed the cell cycle arrest and apoptosis in GA-treated OS cells. We therefore considered that GA suppressed tumor growth partially *via* the induction of cell cycle arrest and apoptosis, which was consistent with previous reports on prostate cancer and colon cancer [30,31]. Although several reports have illustrated that GA inhibited the migration and motility in cancer cells such as leukemia, non-small cell lung cancer, gastric cancer and oral cancer [32–35], our results firstly demonstrated that GA significantly suppressed the invasion and metastasis of OS cells. Based on these reports and our results, we believe GA has great potential to be developed as a therapeutic drug candidate against OS in clinical practice.

Carcinogenesis is a multi-factorial process that involves various regulatory factors and signaling pathways. The signalings such as Wnt/β-catenin, Hedgehog, Notch, and PI3K/Akt/mTOR are all established to participate in the development of OS [36]. Among them, the classical Wnt/β-catenin signaling is one of the most important pathways in tumorigenesis [37,38]. Aberrant activation of Wnt/β-catenin signaling is a common event in tumor progression. In human OS, this signaling was closely associated with tumor metastasis, chemotherapy resistance and poor prognosis, suggesting disruption of the Wnt/β-catenin signaling may be a potential therapeutic intervention for OS patients [36,37]. Previous studies have demonstrated that GA suppressed the activation of Wnt/β-catenin signaling in colorectal cancer and melanoma [39,40]. So

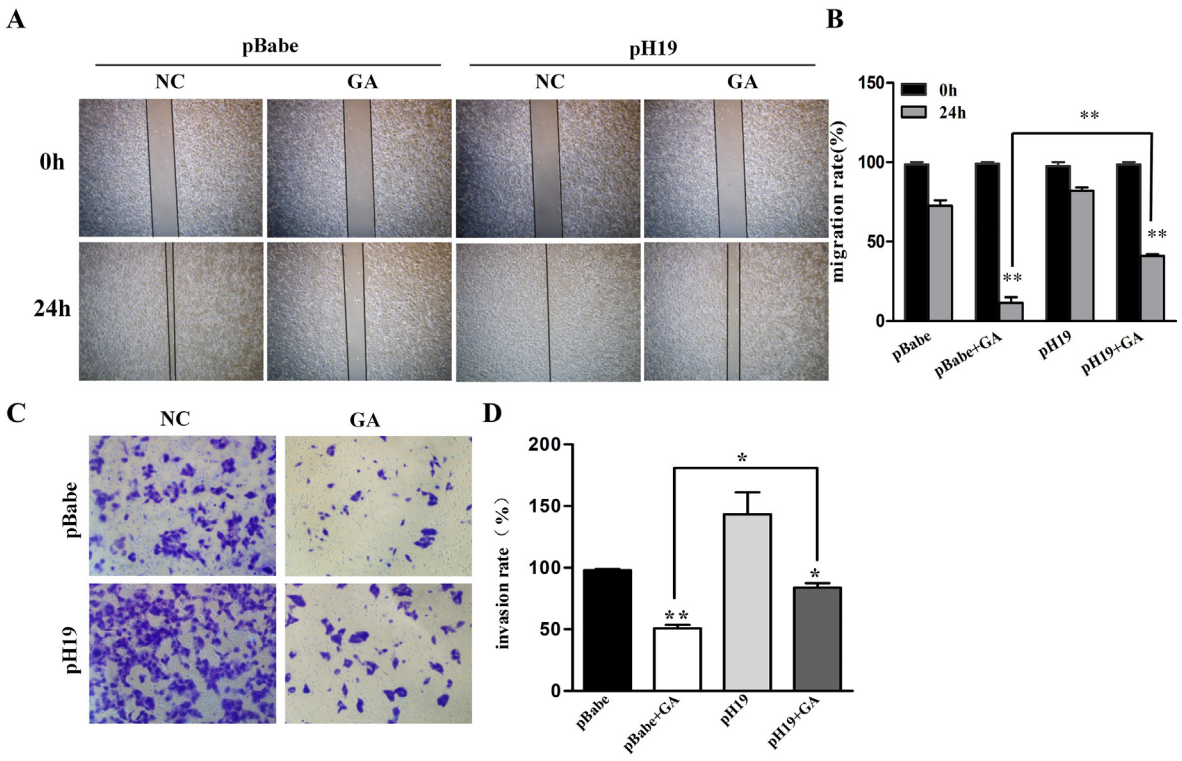

Fig. 8. H19 overexpression partially reversed the GA-induced inhibitory effects on metastasis in OS cells. A-B, wound-healing migration of GA-treated H19 overexpressing cells and the quantitative assays were examined. C-D, transwell invasion of the GA-treated H19 overexpressing cells and the quantitative assays were examined. Data are presented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

we supposed that Wnt/β-catenin signaling might participate in the GA-induced anti-tumorigenesis in OS. The suppressive effects of GA on the luciferase activity of Wnt signaling reporter TOPflash and the expression of β-catenin and several downstream target genes indicated that GA induced the inactivation of Wnt/β-catenin signaling in OS cells. Interesting, the total expression of β-catenin was decreased by GA in 143 B cells while it remained consistent in U2OS cells. However, the intranuclear β-catenin expression was reduced in both cells. As far as we know, the canonical Wnt/β-catenin signaling is triggered mainly through the nuclear transfer and accumulation of β-catenin. We therefore speculated that GA induced the inactivation of Wnt/β-catenin signaling in OS cells possibly *via* disrupting the β-catenin translocation from the cytoplasm to the nucleus and led to the suppressive nuclear accumulation.

LncRNAs are a kind of important regulators involved in diverse biological processes, and they play important roles in tumorigenesis such as cell proliferation, apoptosis and metastasis [41]. Their emergence brings new insights into the investigation of Chinese medicinal molecular pharmacology. The lncRNA H19, one of the most well-known imprinted genes, is located on human chromosome 11 and it is transcribed only from the maternally inherited allele [14]. During the past twenty years, a broad spectrum of studies has been conducted to evaluate the function of H19 in carcinogenesis. More and more emerging studies have shown that H19 is associated with risk of various cancers *via* promoting cancer initiation, progression and metastasis, highlighting H19 as an oncogenic regulator in tumorigenesis [14,17,18]. Moreover, it was demonstrated that H19 promoted tumor development, migration and chemoresistance *via* activating Wnt signaling in several cancers such as glioma, pancreatic cancer, colorectal cancer [42–44]. Our previous study also validated that H19 activated Wnt/β-catenin signaling, and thus resulted in the methotrexate (MTX) resistance in colorectal cancer [45]. In the present study, H19 as well as Wnt/β-catenin signaling were found to be suppressed by GA in OS cells. The rescue studies also indicated that elevated expression of H19 partially attenuated the GA-induced anti-cancer activities, and partially reversed the GA-induced inactivation of Wnt/β-catenin signaling in OS cells. These results suggest that H19 is a really therapeutic target of GA in OS cells. Of course, there are some limitations in this study. For instance, the detailed mechanism underlying how GA mediated H19 expression and whether miR-675, encoded by H19, was also involved in the GA-mediated anti-OS activity remain elusive, and more experiments are needed to address in our future study.

Based on all these results, we concluded that GA played the anticancer function in OS cells *via* suppressing cell proliferation, inducing apoptosis, and cell cycle arrest, and inhibiting metastasis. H19 was found to be significantly suppressed by GA, and thus induced the disruption of Wnt/β-catenin signaling in OS cells, which provide novel insights into cancer management through a synergistic intervene between conventional pharmacotherapy and molecular target therapeutics.

Data availability

The main data are included in this manuscript. All data are available from the corresponding author on reasonable request.

Author statement

Conception and design of study: J.Y.Wang, J.F.Zhang; acquisition of data: F.X.Pang, S.C.Ding, Z.P.Li, N.N.Tian; analysis and/or interpretation of data: C.J.Shi, F.W.Zhang, N.Li, Y.X.Mai. Drafting the manuscript: F.X.Pang, J.F.Zhang, S.C.Ding, Z.P.Li; revising the manuscript critically for important intellectual content: J.Y.Wang.

Conflict of interest

A conflict of interest occurs when an individual's objectivity is potentially compromised by a desire for financial gain, prominence, professional advancement or a successful outcome. The Editors of the

Journal of Orthopaedic Translation strive to ensure that what is published in the Journal is as balanced, objective and evidence-based as possible. Since it can be difficult to distinguish between an actual conflict of interest and a perceived conflict of interest, the Journal requires authors to disclose all and any potential conflicts of interest.

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