Silencing of Estrogen receptor β promotes the invasion and migration of osteosarcoma cells through activating Wnt signaling pathway

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Research article

Abstract

Background This study aimed to evaluate the specific roles of Estrogen receptor β (ERβ) on the invasion and migration of osteosarcoma (OS) cells, and explore the regulatory mechanisms relating with Wnt signaling pathway. Methods The expression of ERβ was detected on human OS tissues by quantitative real-time PCR and immunohistochemistry. U2-OS cells were transfected with siRNA-ERβ (si-ERβ) to downrgulate ERβ, and treated with FH535 to inhibit Wnt signaling. The migration and invasion ability was detected by scratch and transwell assay, respectively. The expression of β-catenin, MMP-7 and MMP-9 was detected by Western blot. Subcutaneous tumor-bearing model was established by injection of U2-OS cells into mice, and the tumor volumes were measured. Orthotopic transplantation model was established by transplantation of tumor tissues into the liver of mice, and the metastatic tumors were counted. Results ERβ was downregulated in human OS tissues and U2-OS cells. The transfection of si-ERβ significantly increased the scratch healing rate, the number of invasion cells, and the expression of β-catenin, MMP-7 and MMP-9 in U2-OS cells. The injection of si-ERβ-transfected U2-OS cells into mice significantly increased the subcutaneous tumor volume, the expression of β-catenin, MMP-7 and MMP-9, and the number of metastatic tumors in liver tissues. The promoting effects of si-ERβ on the invasion and migration of U2-OS cells were significantly reversed by FH535 in vitro and vivo. Conclusions Silencing of ERβ promotes the invasion and migration of OS cells via activating Wnt signaling pathway.

Background

Osteosarcoma (OS) is a common malignant bone tumor that usually develops in teenagers [1]. It is estimated that the incidence of OS is 4 million/year worldwide, with a peak at the age of 15-19 years [2]. The clinical outcomes of patients with metastatic OS are extremely poor [3]. The 5-year survival of localized OS is about 65%-70%, while the 5-year survival of metastatic OS is only less than 20% [4]. The discovery of novel therapeutic targets against metastatic OS are urgently needed.

Estrogen receptor β (ERβ), also known as nuclear receptor subfamily 3 group A (NR3A2) is an important transcription factor that involved in the occurrence and development of cancers [5]. ERβ has been considered as a potential therapeutic target in cancers, which can significantly inhibit the proliferation of diverse cancer cell lines, such as colon cancer SW480 cells [6], breast cancer MCF-7 cells [7], prostate cancer DU145 cells [8], and OS U2-OS cells [9]. Note worthily, ERβ also exerts an obvious inhibitory role on the invasion and migration of cancer cells. It has been reported that ERβ1 significantly inhibits the migration and invasion of breast cancer cells, as well as the tumor formation in mice [10,11]. ERβ1 inhibits the invasion and migration of breast cancer cells in vitro, and the invasion, dissemination and micrometastasis of breast cancer cells in vivo [12]. In addition, a pervious study has proved that ERβ significantly promoted the migration and invasion of OS cells [9]. However, the regulatory mechanisms of ERβ on OS are not fully revealed.

Wnt signaling pathway is a β-catenin-dependent extracellular pathway that involved in a multitude of cellular processes, such as proliferation, apoptosis, differentiation, and migration [13]. The inhibition of Wnt signaling pathway has been proved to promote the migration and invasion of OS cells by various studies. For examples, the upregulation of naked cuticle homolog 2 (NKD2), a negative regulator of Wnt signaling pathway decreases the migration and invasion ability of OS cells in vitro, and inhibits the tumor metastasis in vivo [14]. The transfection of β-catenin siRNA decreases the invasion ability of OC cells through downregulating membrane type-1 matrix metalloproteinase (MT1-MMP) [15]. In addition, a previous study has proved that Erb-041, an ERβ agonist inhibits skin photocarcinogenesis in mice though downregulating Wnt signaling pathway [16]. However, the specific regulatory relationship between ERβ and Wnt signaling pathway on OS is still unclear.

In this study, ERβ was silenced by siRNA-ERβ (si-ERβ). The specific roles of si-ERβ on the migration and invasion of U2-OS cells were evaluated in both vitro and vivo. In addition, the regulatory mechanisms of ERβ relating with Wnt signaling pathway were investigated. Our findings may provide a novel therapeutic target, and a new insight into the underlying mechanisms for the treatment of metastatic OS.

Methods

Clinical specimens

A total of 24 patients (11 male and 13 female; 14-51 years old) histologically diagnosed as OS (14 distal femur and 10 proximal tibia) were screened from our hospital between January 2016 and January 2018. Paraffin-embedded OS tissues, and adjacent normal tissues were collected form these patients prior to administering any treatment. This study was approved by the local Institutional Review Board, and informed consents were obtained from all subjects.

Immunohistochemistry (IHC)

Paraffin-embedded tissues were sliced at 5 μm, dewaxed in xylene, dehydrated with graded ethanol, incubated in 0.3% H_2O_2 for 15 min, and incubated in 10 mM ethylene diamine tetraacetic acid (EDTA) for 15 min under microwave irradiation. The sections were blocked with 10% bovine serum albumi (BSA) for 30 min, and incubated with primary antibody (anti-ERβ, 1:100, Cell Signaling, USA) for 3 h at 37°C. Then the sections were washed with PBS for 5 times, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 1000, Cell Signaling) for 1h at 37°C. Followed by staining with diaminobenzidine (DAB), dehydration with graded ethanol and vitrification with dimethylbenzene, positive stained cells (yellow-brown or brown) were observed under microscope (Olympus, Japan), and counted in five randomly selected fields.

Cell culture and treatments

Human OS cell line U2-OS and human osteoblast cell line hFOB1.19 (preserved in our laboratory) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1%

penicillin-streptomycin. Cells were maintained in a humidified incubator containing at 37°C with 5% CO2.

U2-OS cells in logarithmic growth phase were randomly divided into four groups: si-ERβ, U2-OS cells transfected with si-ERβ (Santa Cruz Biotechnology, USA) for 48 h; NC-ERβ, U2-OS cells transfected with siRNA-negative control-ERβ for 48 h; si-ERβ + FH535, U2-OS cells transfected with siRNA-ERβ and treated with 20 μmol/L FH535 (an inhibitor of Wnt signaling) (Sigma, USA) for 48 h; Blank, U2-OS cells without transfection and treatment. Cell transfection was performed by using lipfectamine 2000 (Thermo Fisher Scientific, USA) according to the manufacturer's instruction.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from specific tissues, and cells by using RNApreppure tissue kit (TIANGEN, China), and RNApreppure cell kit (TIANGEN), respectively. cDNA was synthesized by using PrimeScript RT reagent kit (Takara, China). The special primers were used as followed: ERβ-F, 5'-GCCGCCCCATGTGCTGAT-3'; ERβ-R, 5'-GGACCCCGTGATGGAGGACTT-3'; β-catenin-F, 5'-TGAGGACAAGCCACAAGATTAC-3'; β-catenin-R, 5'- TCCACCAGAGTGAAAAGAACG-3'. GAPDH was used as an internal control (GAPDH-F, 5'-GAGTCAACGGATTTGGTCGT-3'; GAPDH-R: 5'-TTGATTTTGGAGGGATCTC-3'). The PCR program included 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20s, and 72°C for 34s. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method [17].

Western blot

Cells of different groups were lysed in RIPA Lysis buffer (Thermo Fisher Scientific). Total proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidenefluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in Tris Buffered Saline Tween (TBST) for 1 h, and incubated with primary antibody (anti-ERβ, -MMP-7, -MMP-9, and -β-catenin; 1: 100, Cell Signaling) overnight at 4°C. After washed with TBST for three times, the membrane was incubated with HRP-conjugated secondary antibody (1:1000, Abcam, England) for 2 h at 25°C. The protein bands were visualized and quantified using a Gel Imaging System (Thermo Fisher Scientific).

Immunofluorescence

Cells of different groups were fixed in 4% paraformaldehyde for 20 min at 4°C, and permeated in 0.1% Triton X-100 (MP Biomedicals, USA) for 5 min. Then cells were blocked with 5% BSA for 30 min, and incubated with primary antibody (anti-β-catenin, 1:100, Abcam) overnight at 4°C. After washed with PBS for 5 times, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (1:500, Abcam) for 1h at 37°C. Followed by staining with DAPI (4,6-diamino-2-phenylindole), positive stained cells (green fluorescence) were observed under fluorescence microscope (Olympus).

Scratch assay

Cells of different groups were seeded at a density of 0.5×10^6/well in 6-well plates, and cultured overnight (more than 90% confluence). A wound track at approximately 5 mm was scored in each dish with a pipette head, and the debris was removed by 3 times of washing with phosphate buffer saline (PBS). After 48h of culturing, the scratch healing state was observed under microscope (Olympus).

Transwell assay

Transwell assay was performed by using transwell chambers (Corning, USA). Cells of different groups were seeded at a density of 0.1×10^5/μL in the upper chamber (pre-coated with Matrigel). A total of 600 μL DMEM containing 100 ng/mL stromal cell-derived factor-1 (SDF-1) were placed in the lower chamber. After incubated at 37°C for 24 h, cells on the upper chamber were removed with cotton swabs. Cells on the lower chamber were fixed in formaldehyde for 30min and stained with 0.1% crystal violet for 20min. Positive stained cells were observed under microscope (Olympus).

Establishment of subcutaneous tumor-bearing model and orthotopic transplantation model in mice

Four-week-old specific pathogen free (SPF) mice (male, 20-25g) were purchased from the Medical College of Shanghai Jiaotong University (Shanghai, China). Mice were feeding at 25°C and 50% humidity with free access to water and food. A total of 100μl U2-OS cells in different groups (si-ERβ, NC-ERβ, si-ERβ + FH535, and Blank) were subcutaneously injected into the posterior limb of each mouse at a density of 0.1×10^8 cells/ml (subcutaneous tumor-bearing model). Mice were killed by cervical dislocation, and the tumor volumes were measured by vernier caliper every 5 days. After the injection for 20 days, small pieces of tumor tissues were transplanted into the liver of healthy mice (orthotopic transplantation model). Five weeks later, the metastatic tumors in liver tissues were counted, and observed by Hematoxylin-Eosin (HE) staining. All animal experiments were approved by the local Institutional Review Board.

HE staining

The liver tissues of mice were fixed in 10% formaldehyde, and embedded in paraffin. The tissue sections at 5μm were dewaxed in xylene, rehydrated in graded ethanol, and stained with Hematoxylin for 5 min and Eosin (Beyotime) for 2 min. After dehydrated with graded ethanol and vitrificated with dimethylbenzene, the tissues were observed under microscope (Olympus).

Statistical analyses

All experiments were performed in triplicate, and all data were expressed as mean ± Standard Deviation (SD). Statistical analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL). Comparison between different groups was determined by Student's t test (two groups) and one-way ANOVA (more than two groups). A p-value less than 0.05 was considered to be significantly different.

Results

ERβ was downregulated in human OS tissues

The expression of ERβ was detected in OS tissues of 24 OS patients. qRT-PCR showed that the expression of ERβ was significantly lower in OS tissues than in adjacent normal tissues (P < 0.001) (Fig 1A). In addition, IHC showed that the positive cell rate was significantly lower in OS tissues than in adjacent normal tissues (27.1 ± 1.49% vs. 73.0 ± 1.40%, P < 0.001) (Fig 1B)

ERβ was downregulated in U2-OS cells

The expression of ERβ was detected in U2-OS and hFOB1.1 cells. qRT-PCR and Western blot showed that the expression of ERβ was significantly lower in U2-OS cells than in hFOB1.1 cells at both mRNA and protein level (P < 0.001) (Fig 2A and B). Then si-ERβ was used to silence ERβ in U2-OS cells. As shown in Fig 2C and D, the expression of ERβ in U2-OS cells was significantly inhibited by si-ERβ at both mRNA and protein level (P < 0.01). No significantly different on the expression of ERβ was observed between blank and NC-ERβ group (Fig 2C and D).

si-ERβ transfection activated Wnt signaling pathway

In order to evaluate the regulatory relationship between ERβ and Wnt signaling pathway, the expression of β-catenin was detected. Western blot showed that the expression of β-catenin was significantly higher in U2-OS cells than in hFOB1.1 cells at protein level (P < 0.01) (Fig 3A). U2-OS cells in si-ERβ group exhibited significantly higher expression of β-catenin than those in NC- ERβ and blank group (P < 0.001). However, the expression of β-catenin in si-ERβ-transfected U2-OS cells was significantly inhibited by the intervention of FH535 (P < 0.01) (Fig 3B). In addition, immunofluorescence showed that si-ERβ transfection significantly increased the fluorescence intensity of β-catenin in U2-OS cells, and promoted the nuclear aggregation of β-catenin. The intervention of FH535 significantly decreased the fluorescence intensity of β-catenin in si-ERβ-transfected U2-OS cells, and inhibited the nuclear aggregation of β-catenin (Fig 3C).

si-ERβ transfection promoted the migration of U2-OS cells

The effect of si-ERβ on the migration of U2-OS cells was evaluated. Scratch assay showed that U2-OS cells in si-ERβ group exhibited significantly higher scratch healing rate than those in NC-ERβ and blank group (72.47 ± 3.19% vs. 33.56 ± 3.42% and 31.47 ± 3.19, P < 0.001). The intervention of FH535 significantly decreased the scratch healing rate in si-ERβ-transfected U2-OS cells (32.47 ± 2.19 vs. 72.47 ± 3.19% , P < 0.001) (Fig 4A and B).

si-ERβ transfection promoted the invasion of U2-OS cells

The effect of si-ERβ on the invasion of U2-OS cells was evaluated. Transwell assay showed that the number of invasion cells was significantly higher in si-ERβ group than in NC-ERβ and blank group (76.11 ± 2.97% vs. 39.41 ± 1.89% and 35.51 ± 3.84, P < 0.001). The intervention of FH535 significantly decreased the number of invasion cells in si-ERβ-transfected U2-OS cells (36.11 ± 2.97 vs. 76.11 ± 2.97% ,

P < 0.001) (Fig 5A and B). In addition, Western blot showed that the expression of MMP-7 and MMP-9 in U2-OS cells was significantly higher in si-ERβ group than in NC-ERβ and blank group (P < 0.001). The intervention of FH535 significantly decreased the expression of MMP-7 and MMP-9 in si-ERβ-transfected U2-OS cells (P < 0.001) (Fig 5C and D).

si-ERβ transfection promoted the growth and metastasis of OS tumors in mice

In order to further evaluate the pro-migratory and -invasive effects of si-ERβ in vivo, subcutaneous tumor-bearing model was established in mice. As shown in Fig 6A and B, the tumor volumes in mice of different groups were all significantly increased in a time-dependent manner. The tumor volumes were significantly higher in si-ERβ group than in NC-ERβ and blank group, beginning from the 10th day (P < 0.001). The intervention of FH535 significantly decreased the tumor volumes in mice injected with si-ERβ-transfected U2-OS cells (P < 0.001) (Fig 6A and B). In addition, Western blot showed that the expression of β-catenin, MMP-7 and MMP-9 in tumor tissues was significantly higher in si-ERβ group than in NC-ERβ and blank group (P < 0.001). The intervention of FH535 significantly decreased the expression of β-catenin, MMP-7 and MMP-9 in tumor tissues of mice injected with si-ERβ-transfected U2-OS cells (P < 0.001) (Fig 6C and D). Furthermore, the metastatic ability of si-ERβ-transfected U2-OS cells was evaluated in a mouse model of orthotopic transplantation. As shown in Fig 6E and F, more metastatic tumors were observed in the liver tissues of mice in si-ERβ group than in NC-ERβ and blank group (P < 0.001). The intervention of FH535 significantly decreased the number of metastatic tumors in the liver tissue of mice injected with si-ERβ-transfected U2-OS cells (P < 0.001) (Fig 6E and F).

Discussion

ERβ is an estrogen-regulated transcription factor that plays a critical role in the progression of cancers [18]. A large number of pervious studies have proved that ERβ is downregulated in diverse cancers, such as breast cancer [19], ovarian cancer [20], prostatic cancer [21], and colon cancer [22]. In this study, the expression of ERβ was detected in both human OS tissues and U2-OS cells. We found that the expression of ERβ was significantly lower in OS tissues than in adjacent normal tissues, and significantly lower in U2-OS cells than in hFOB1.1 cells. Our findings are just consistent with pervious studies, and illustrate that ERβ is downregulated in OS.

The inhibition of cell invasion and migration is an important anti-tumor manifestation of ERβ on cancers [23,24]. In this study, the invasion and migration abilities of si-ERβ-transfected U2-OS cells were evaluated. We found that the transfection of si-ERβ into U2-OS cells significantly increased the scratch healing rate and number of invasion cells. These findings are just consistent with a pervious study that the knockdown of ERβ significantly increases the migration and invasion abilities of U2-OS cells [9]. In order to further identify the pro-migratory and -invasive effects of si-ERβ in vivo, subcutaneous tumor-bearing model and orthotopic transplantation model were established in mice. We found that the injection of si-ERβ-transfected U2-OS cells significantly increased the subcutaneous tumor volume, and the number of metastatic tumors in liver tissues of mice. These phenomena indicate that si-ERβ promotes the growth

and metastasis of OS tumors in vivo, which are consistent with pervious studies on animal models of breast cancer. It has been reported that exogenous ERβ expression significantly inhibits the growth of MCF-7 tumor xenografts in mice, and tumors are only observed in 2/6 mice injected with MCF-7-ERβ [25]. MDA-MB-231 cells are disseminated away from the injection site of zebrafish at 5th day post-injection, while ERβ1-expressing MD-MB-231 cells remain at the primary site [12]. In addition, we also found that si-ERβ significantly increased the expression of MMP-7 and MMP-9 in U2-OS cells and OS tumors of mice. Since MMP-7 and MMP-9 are positively associated with tumor metastasis [26,27], the upregulated MMP-7 and MMP-9 contribute to the promoting effects of si-ERβ on the invasion and migration of U2-OS cells in vitro and on the growth and metastasis of OS tumors in vivo.

The anti-tumor mechanisms of ERβ are complex, which related with diverse regulatory factors, such as E-cadherin [10], epidermal growth factor receptor (EGFR) [28], transforming growth factor β (TGFβ) [29], p53-upregulated modulator of apoptosis (PUMA) [30], nuclear factor-kB/B-cell lymphoma-2 (NF-kB/BCL-2) and phosphatidylinositol-3 kinase/Akt (PI3K/Akt) [9]. In this study, the regulatory relationship between ERβ and Wnt signaling pathway was evaluated on U2-OS cells. We found that the si-ERβ significantly upregualted β-catenin in U2-OS cells, which indicates that the downregulation of ERβ activates the Wnt signaling pathway in U2-OS cells. The activation of Wnt signaling pathway contributes to promoting the invasion and migration of OS cells. Pervious studies have proved that the upregulation of NKD2 (a negative regulator of Wnt signaling) and the transfection of β-catenin siRNA can both decrease the migration and invasion abilities of OS cells through inhibiting Wnt signaling pathway [14,15]. Therefore, we suspect that si-ERβ may promote the invasion and migration of U2-OS cells through activating Wnt signaling pathway. This hypothesis is further confirmed by the intervention of FH535. We found that the intervention of FH535 significantly decreased the scratch healing rate, the number of invasion cells, and the expression of MMP-7 and MMP-9 in U2-OS cells transfected with si-ERβ. In addition, β-catenin was also upregulated in OS tumors of mice injected with si-ERβ-transfected U2-OS cells. The intervention of FH535 in mice injected with si-ERβ-transfected U2-OS cells significantly decreased the subcutaneous tumor volume, the expression of β-catenin, MMP-7 and MMP-9, as well as the number of metastatic tumors in liver tissues. These results further illustrate that the promoting effects of si-ERβ on the growth and metastasis of OS tumors in mice may attribute to the activation of Wnt signaling pathway.

Conclusions

In conclusion, si-ERβ significantly promoted the invasion and migration of U2-OS cells in vitro, and the growth and metastasis of OS tumors in vivo. The promoting effects of si-ERβ on OS metastasis was closely related with the activation of Wnt signaling pathway. ERβ might be a potential therapeutic target for metastatic OS. However, this study is limited in si-ERβ. Further researches on the specific roles and regulatory mechanisms of ERβ overexpression on OS metastasis are still needed.

Abbreviations

osteosarcoma (OS)

siRNA-ERβ (si-ERβ)

Declarations

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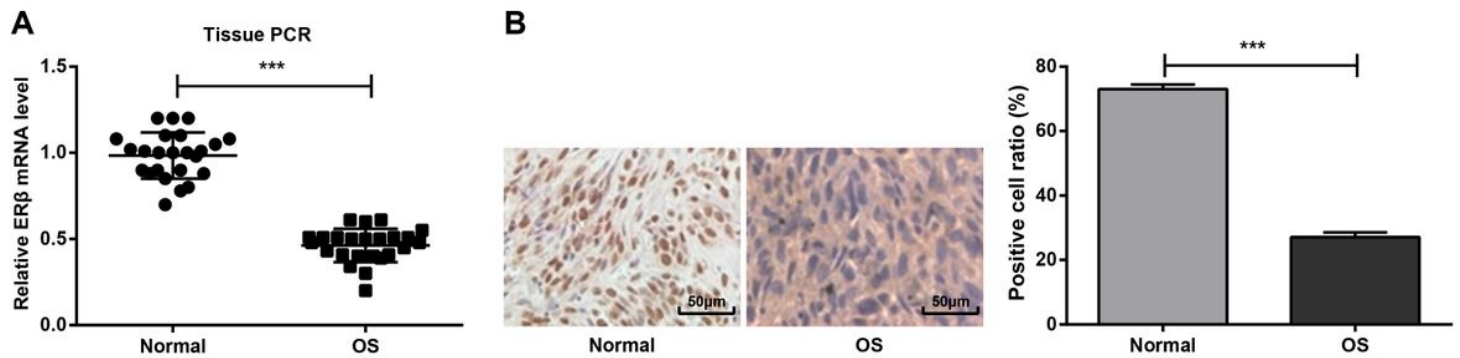
Figures

Figure 1

The expression of Estrogen receptor β (ERβ) in osteosarcoma (OS) and adjacent normal tissues of 24 OS patients detected by quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC). A) relative expression of ERβ at mRNA level (qRT-PCR); B) positive stained cells. ***, P < 0.001.
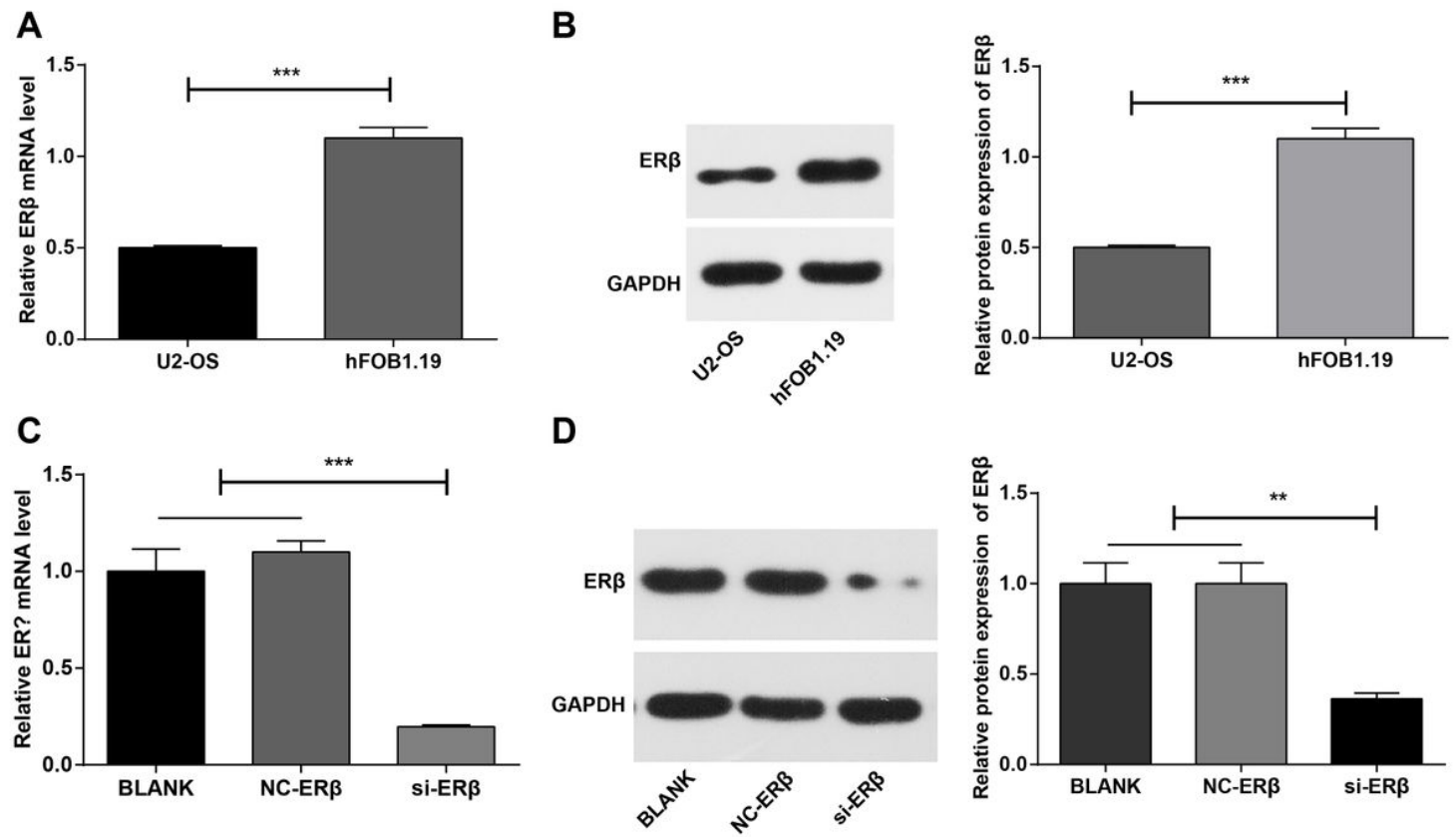

Figure 2

The expression of Estrogen receptor β (ERβ) in U2-OS and hFOB1.1 cells detected by quantitative real-time PCR (qRT-PCR) and Western blot. A and C) relative expression of ERβ at mRNA level (qRT-PCR); B and D) relative expression of ERβ at protein level (Western blot). si-ERβ, U2-OS cells transfected with siRNA-ERβ for 48 h; NC-ERβ, U2-OS cells transfected with siRNA-negative control-ERβ for 48 h; Blank, U2-OS cells without transfection. **, P < 0.01; ***, P < 0.001.
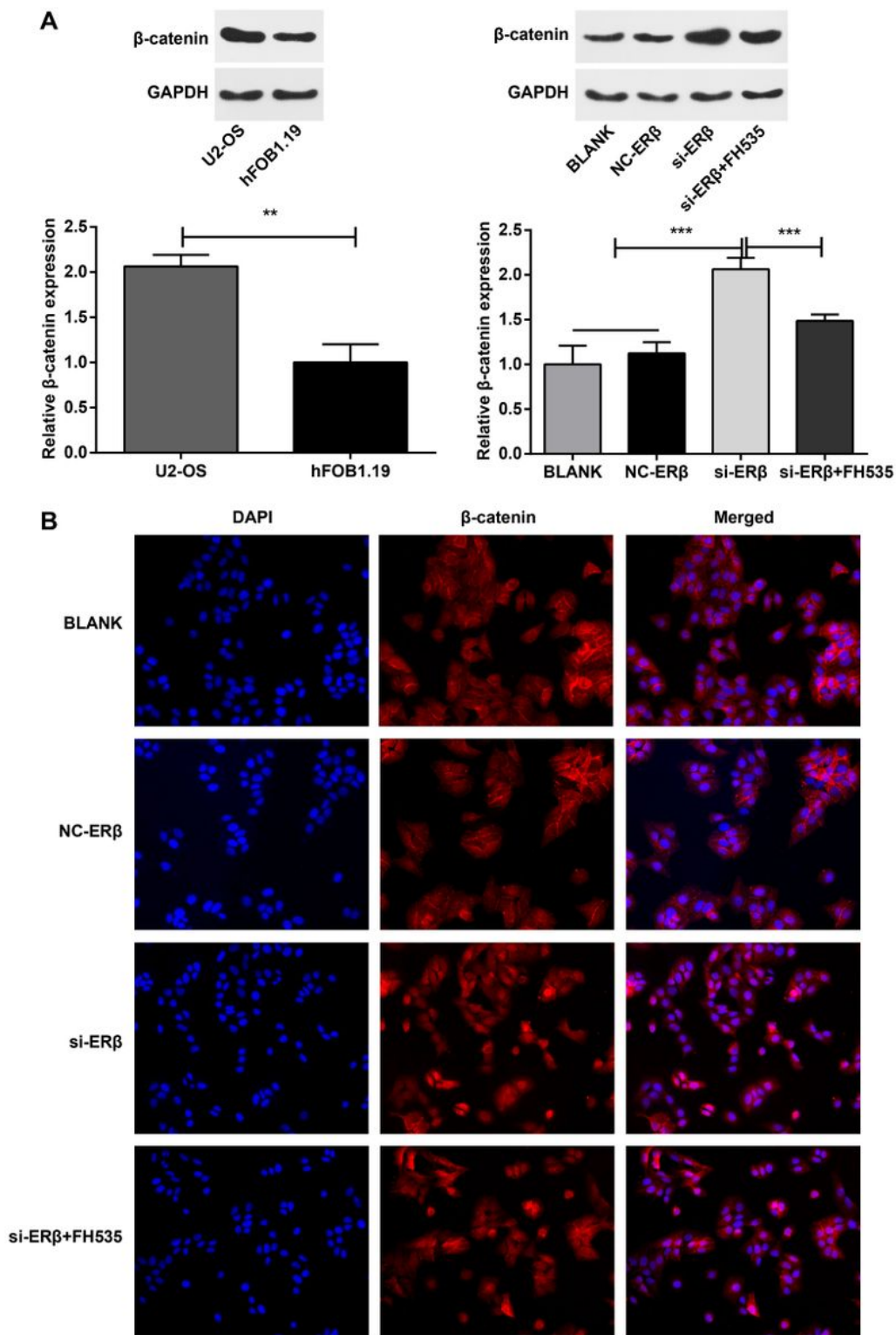
Figure 3

The expression of β-catenin in U2-OS and hFOB1.1 cells detected by Western blot and Immunofluorescence. A and B) relative expression of β-catenin at protein level (Western blot); C) positive stained cells. si-ERβ, U2-OS cells transfected with siRNA-ERβ for 48 h; NC-ERβ, U2-OS cells transfected with siRNA-negative control-ERβ for 48 h; si-ERβ + FH535, U2-OS cells transfected with siRNA-ERβ and

treated with 20 μmol/L FH535 for 48 h; Blank, U2-OS cells without transfection and treatment. **, P <
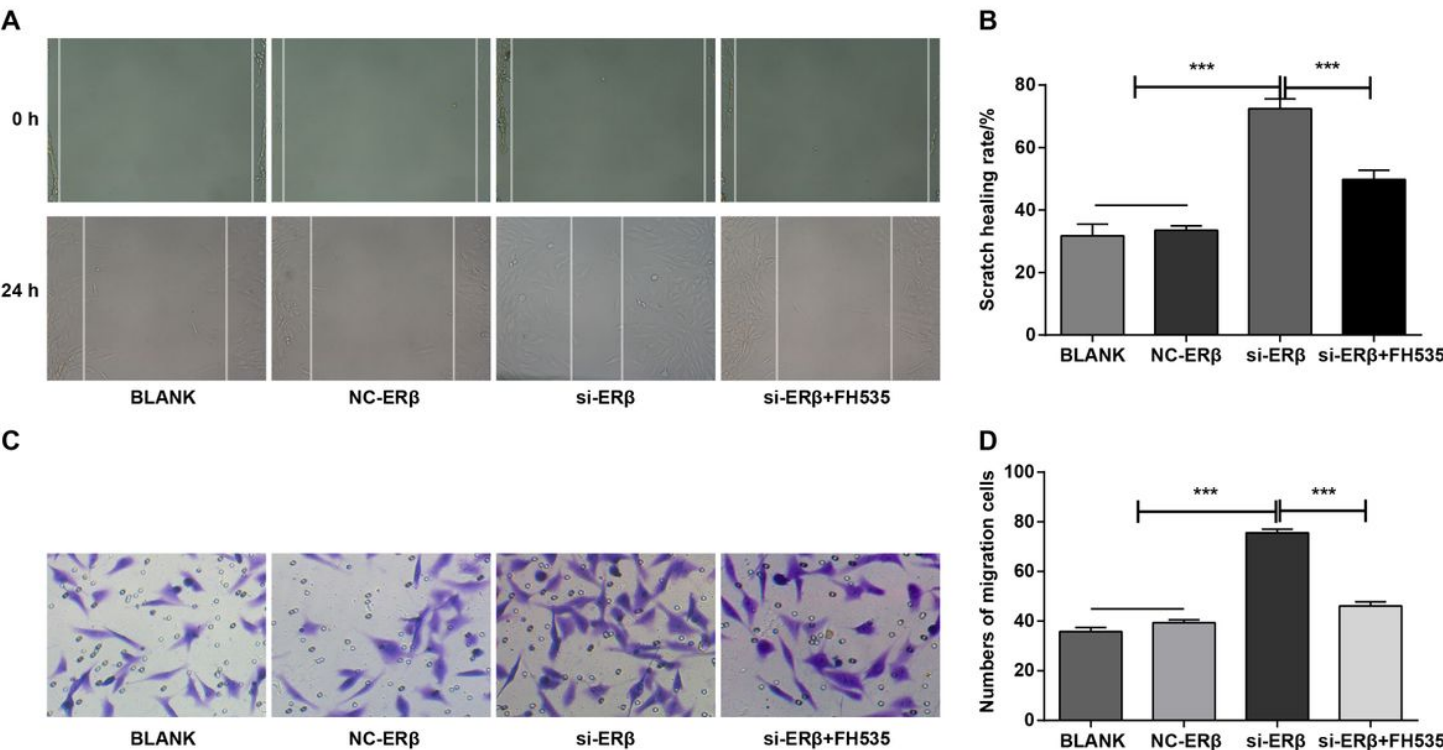0.01; ***, P < 0.001.

Figure 4

The migration of U2-OS cells detected by Scratch assay. A) scratch healing under microscope; B) scratch
healing rate. si-ERβ, U2-OS cells transfected with siRNA-ERβ for 48 h; NC-ERβ, U2-OS cells transfected
with siRNA-negative control-ERβ for 48 h; si-ERβ + FH535, U2-OS cells transfected with siRNA-ERβ and
treated with 20 μmol/L FH535 for 48 h; Blank, U2-OS cells without transfection and treatment. ***, P <
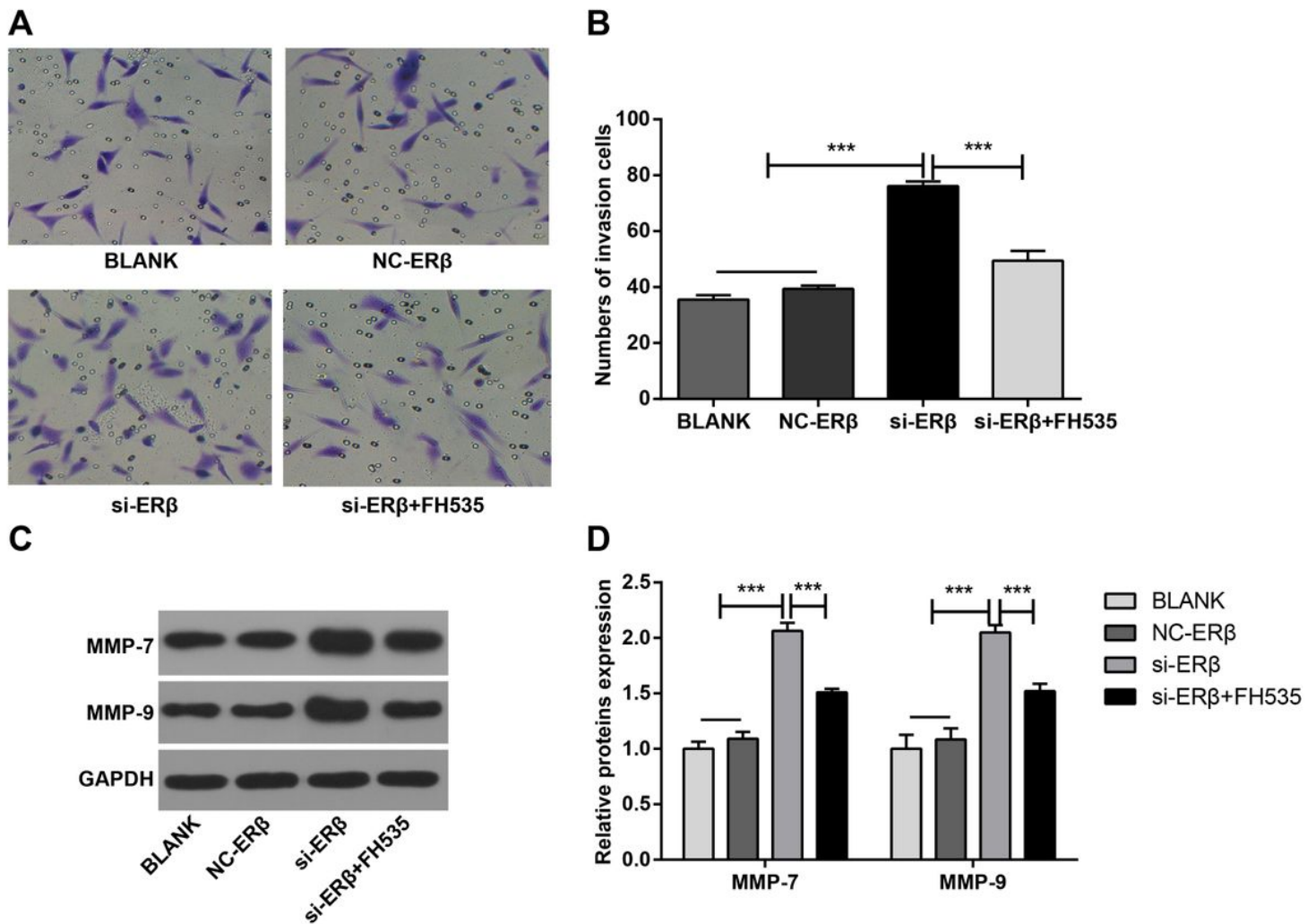0.001.

Figure 5

The invasion of U2-OS cells detected by Transwell assay and Western blot. A) invasion cells under microscope; B) number of invasion cells; C) protein brands of Western blot; D) relative expression of MMP-7 and MMP-9 at protein level (Western blot). si-ERβ, U2-OS cells transfected with siRNA-ERβ for 48 h; NC-ERβ, U2-OS cells transfected with siRNA-negative control-ERβ for 48 h; si-ERβ + FH535, U2-OS cells transfected with siRNA-ERβ and treated with 20 μmol/L FH535 for 48 h; Blank, U2-OS cells without transfection and treatment. ***, P < 0.001.
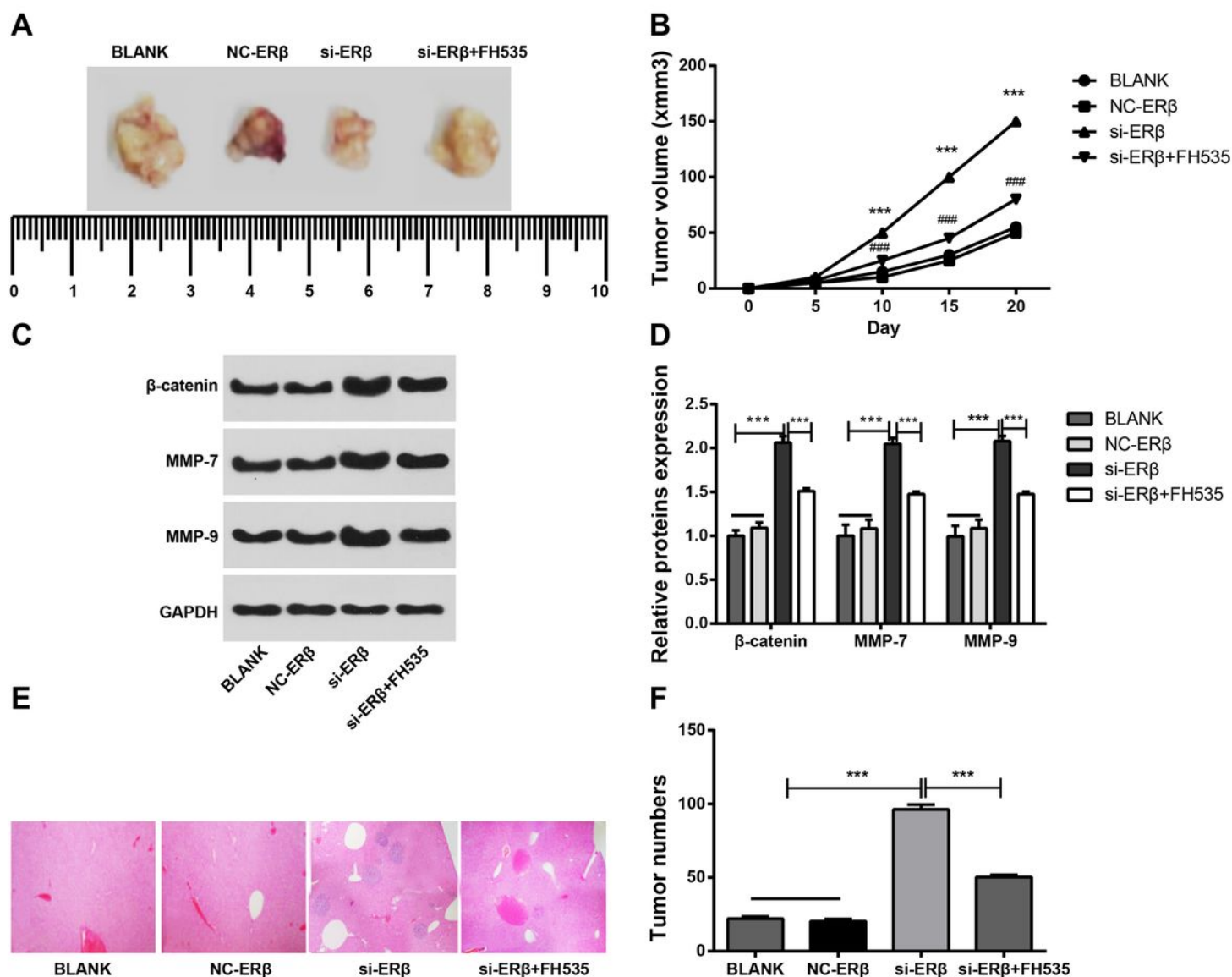
Figure 6

The growth and metastasis of osteosarcoma (OS) tumors in mice. A) subcutaneous tumors under naked eye; B) subcutaneous tumor volumes at different time points; C) protein brands of Western blot; D) relative expression of β-catenin, MMP-7 and MMP-9 at protein level (Western blot); E) metastatic tumors in the liver tissues of mice under microscope (HE staining) and naked eye; F) the number of metastatic tumors. si-ERβ, U2-OS cells transfected with siRNA-ERβ for 48 h; NC-ERβ, U2-OS cells transfected with siRNA-negative control-ERβ for 48 h; si-ERβ + FH535, U2-OS cells transfected with siRNA-ERβ and treated with 20 μmol/L FH535 for 48 h; Blank, U2-OS cells without transfection and treatment. ***, P < 0.001 vs. NC-ERβ and blank; ###, P < 0.001 vs. si-ERβ.

Supplementary Files

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- NC3RsARRIVEGuidelinesChecklist.docx