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ORIGINAL ARTICLE – CANCER RESEARCH

G-protein-coupled estrogen receptor suppresses the migration of osteosarcoma cells via post-translational regulation of Snail

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Abstract

Background Emerging evidences show that G-protein-coupled estrogen receptor (GPER) can regulate the progression of various cancers, while its roles in the progression of osteosarcoma (OS) are not well illustrated.

Methods The expression of GPER in OS cells and tissues were checked. Its roles in cell migration and expression of Snail was checked by use of its agonist G-1.

Results We found that the expression of GPER in OS cells and tissues were lower than that in their corresponding controls. OS patients with higher levels of GPER showed increased overall survival rate (OS) as compared with the lower ones. The activator of GPER (G-1) or overexpression of GPER can inhibit the migration and invasion of OS cells and downregulate mesenchymal markers. G-1 can rapidly decrease the expression of Snail, one powerful epithelial–mesenchymal transition transcription factor (EMT-TF). Overexpression of Snail can attenuate the suppression effects of G-1 on migration of OS cells, suggesting that Snail was involved in GPER-regulated migration of OS cells. Mechanically, G-1 rapidly decreased the protein of Snail but had no effect on its mRNA expression. This was because G-1 can decrease the protein stability of Snail. Further, G-1 increased the expression of FBXL5, which can trigger the proteasome-mediated degradation of Snail. Knockdown of FBXL5 can reverse G-1-induced downregulation of Snail in OS cells.

Conclusion Activation of GPER can suppress the migration and invasion of OS cells via FBXL5-mediated post-translational down regulation of Snail. It suggested that targeted activation of GPER might be a potent potential therapy approach to overcome the metastasis of OS patients.

Keywords GPER · Snail · EMT · Osteosarcoma · FBXL5

Introduction

Osteosarcoma (OS), a common primary malignancy of the bone, typically occurs in children and adolescents (Anderson 2016). The age-standardized incidence of OS is about five per million cases per year in the United States (Kansara et al. 2014). The combination of surgery and chemotherapy is the major treatment approach for OS patient, with the overall survival rates for non-metastatic OS about 50–70% (Chou et al. 2008). Metastasis is the major reason for cancer-associated deaths (Daw et al. 2015). The metastasis has been found in statistically up to 23% of OS patients when they were diagnosed (Hattinger et al. 2015). The overall 5-year survival rate of OS patients with metastasis was only about 20% (Meazza and Scanagatta 2016). It indicated that targeted inhibition of factors promoting metastasis of OS cells would be great helpful for OS treatment.

Estrogenic signaling has been suggested to regulate the progression of various hormone-related or no related cancers including OS (Fic et al. 2015; Salvatori et al. 2009). It has been reported that estrogen can downregulate epidermal growth factor receptor (EGFR) and then promote the differentiation of human OS U2OS cells (Salvatori et al. 2009), while 17β-estradiol can regulate cell proliferation, colony formation, migration, and invasion of human OS MG-63 cells via an estrogen receptor (ER)-independent manner (Fang et al. 2015). In addition, ERβ can suppress the growth of OS cells by regulating integrin, IAP, NF-kB/BCL-2 and PI3K/Akt signal pathway (Yang et al. 2017). Estrogen-related receptor α (ERRα) can participate in the transforming growth factor-β (TGF-β)-induced

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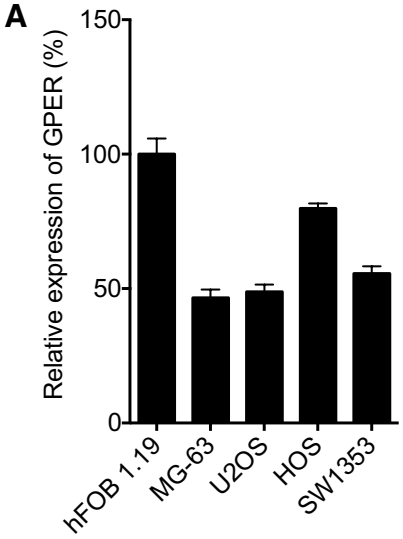
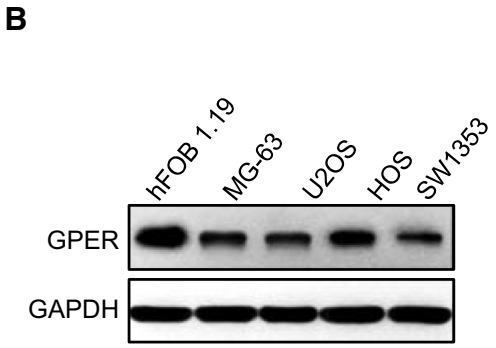
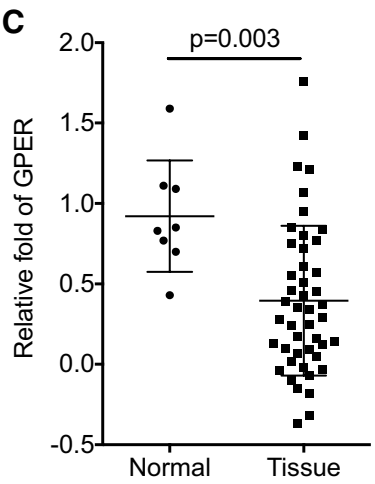
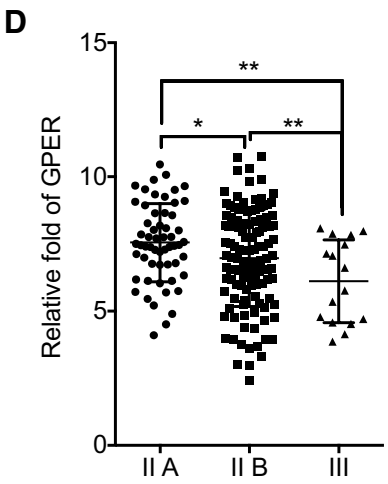
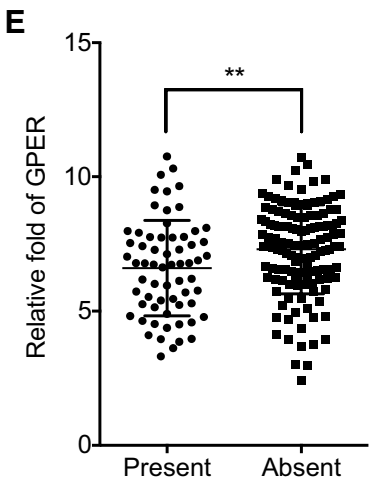
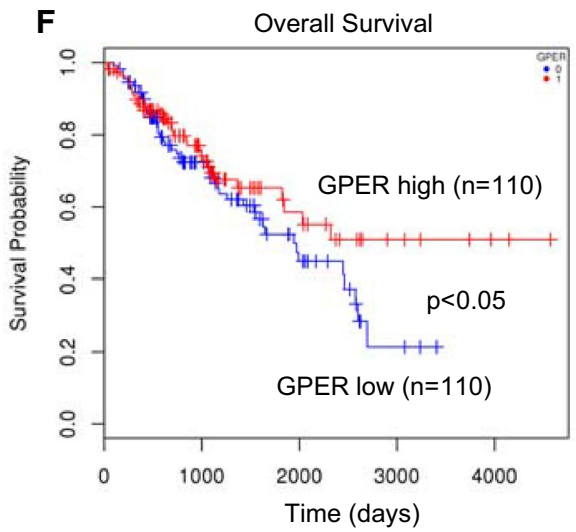

A
Relative expression of GPER (%)
150
100
50
0
hFOB 1.19
MG-63
U2OS
HOS
SW1353

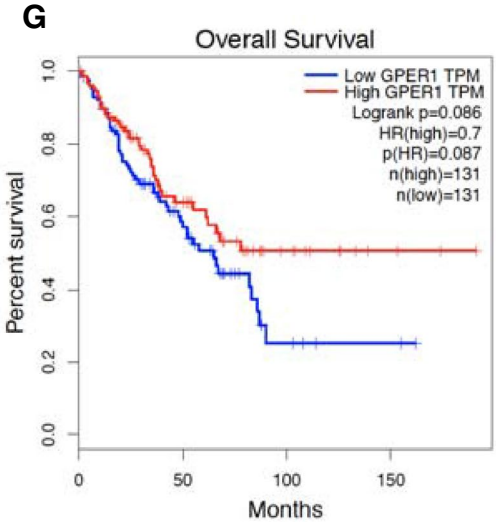

B
hFOB 1.19
MG-63
U2OS
HOS
SW1353
GPER
GAPDH


C
p=0.003
Relative fold of GPER
2.0
1.5
1.0
0.5
0.0
-0.5
Normal
Tissue


D
**
*
**
Relative fold of GPER
15
10
5
0
II A
II B
III


E
**
Relative fold of GPER
15
10
5
0
Present
Absent


F
Overall Survival
GPER high (n=110)
p<0.05
GPER low (n=110)
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
3000
4000
Time (days)


G
Overall Survival
Low GPER1 TPM
High GPER1 TPM
Logrank p=0.086
HR(high)=0.7
p(HR)=0.087
n(high)=131
n(low)=131
Percent survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
Months

◂**Fig. 1** The expression of GPER in OS cells and tissues. The mRNA (**a**) and protein (**b**) expression of GPER in OS and human osteoblastic hFOB 1.19 cell was measured by qRT-PCR and western blot analysis, respectively; **c** the mRNA expression of GPER in OS and adjacent normal tissues based on data available from Oncomine database; the mRNA expression of GPER on different clinical stages (**d**) or presence or absence of distant metastasis (**e**) of OS patients based on data available from LinkedOmics; the overall survival (OS) in OS patients with high or low expression of GPER was plotted by the Kaplan–Meier method based on data available from LinkedOmics (**f**) or GEPIA (**g**). Data are presented as mean values ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$

epithelial–mesenchymal transition (EMT) of OS cells (Chen et al. 2017). ERRα can confer the methotrexate resistance of OS cells via attenuation of reactive oxygen species (ROS) production and P53-mediated apoptosis (Chen et al. 2014). These data suggested that estrogenic signals can regulate the progression of OS.

G-protein-coupled estrogen receptor 1 (GPER) is a member of the G-protein receptor superfamily which can bind E2 to initiate short term, non-genomic, signaling events (Maggiolini and Picard 2010). GPER has been reported to regulate the proliferation, migration, invasion and drug resistance of various cancer cells (Molina et al. 2017). However, its expression and roles on the progression of OS are still not illustrated. Our present study found that overexpression or activation of GPER can suppress the migration and invasion of OS cells. Further, activation of GPER can decrease the protein stability of EMT-related transcription factor Snail via FBXL5-mediated proteasome degradation.

Materials and methods

Cell line and culture

The human osteosarcoma cell line MG-63, U2OS, HOS, and SW1353 cells were purchased from the Institute of Cell Bank/Institutes for Biological Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Clark Bioscience, Richmond, VA, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL), at 37 °C with 5% CO_2. Human fetal osteoblastic cell line hFOB1.19 was also purchased from the Institute of Cell Bank/Institutes for Biological Sciences (Shanghai, China) and cultured according to the previous studies (Meng et al. 2018; Yen et al. 2007).

Chemicals and reagents

All inhibitors including LiCl and cyclohexane (CHX) were purchased from Sigma-Aldrich (Sigma, Victoria, BC, Canada). The pcDNA3.1 (Vector), pcDNA/GEPR, and pcDNA/Snail were purchased from Addgene (Cambridge, MA, USA). Small interfering RNAs (siRNA) for Snail or FBXL5 and scrambled control (si-NC) were from Ribobio (Guangzhou, China). G-1 was purchased from Selleck Chemicals (Houston, TX, USA). The maximal final concentration of dimethyl sulfoxide in the medium was 0.1%, which did not exhibit toxicity.

Western blot analysis

The proteins were extracted using lysis buffer (keyGEN) for 30 min on ice. After centrifugation, the supernatant was harvested. The protein concentration was tested using BCA protein assay. Then 50 µg of total proteins were separated using 10% SDS-PAGE gels. The proteins were then transferred to polyvinylidene fluoride membranes at 60 V for 2 h at 4 °C and blocked with 5% milk in PBST. The membranes were further incubated with primary antibodies (all antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C. After being incubated with the matched secondary antibody for 2 h at room temperature, the blot was revealed with enhanced chemiluminescence Western blotting detection reagent (GE Healthcare). GPADH was used as the loading control for normalization.

Database analysis

The levels of GPER in OS and adjacent normal tissues were analyzed using ONCOMINE gene expression array datasets (http://www.oncomine.org), an online cancer microarray database. The overall survival (OS) in OS patients with high or low expression of GPER was plotted by the Kaplan–Meier method using GEPIA, one newly developed interactive web server for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects (Tang et al. 2017). The associations between GPER and clinical characteristics were analyzed using LinkedOmics database (http://www.linkedomics.org), which are web-based tools to deliver fast and customizable functionalities based on TCGA data.

Cell proliferation assay

The cell proliferation was tested using MTT assay according to previous studies (Azmi et al. 2015). Briefly, 5×10^3 cells per well were seeded into 96-well micro-titer culture plates (Corning, USA). After treatment, 20 µL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL in PBS) was added into each well and further incubated for 2 h. The plates were evaluated using Tecan plate reader (TECAN, Durham, NC) at 595 nm.

In vitro migration and invasion assay

We used wound-healing assay to evaluate the roles of GPER on in vitro migration of OS cells according to the previous study (Bustos et al. 2017). Briefly, cells were scratched using a 10 µL pipette tip and cultured in medium containing 1% FBS. The images were acquired using a Samsung I310W camera at the indicated time periods. The fractional closure was measured using Image J software. The in vitro invasion assay was conducted using chambers with 8-µm pores (BD Biosciences, San Jose, CA, USA) pre-coated with Matrigel (BD Biosciences). Cells (4×10^4) diluted in 300 µL of serum-free medium were added into the upper chamber. The lower chamber was added with 600 µL of 10% serum medium. At the end of experiments, invaded cells were fixed, washed, and stained with 0.6 mL of 0.1% crystal violet solution. The number of invaded cells were counted at five fields per membrane for each group using an IX71 inverted microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR (qRT-PCR)

RNAs were extracted using the Total RNA Purification Kit® (Norgen, Biotek Corp) according to the protocols. The cDNA was synthesized using High Capacity RNA-to-cDNA™ Kit (Applied Biosystems®). The expression of interested genes was tested using the following primers: Snail, 5′-GAC CAC TAT GCC GCG CTC TT-3′ (forward) and 5′-TCG CTG TAG TTA GGC TTC CGA TT-3′ (reverse); GAPDH, 5′-GAC TCA TGA CCA CAG TCC ATG C-3′ (forward) and 5′-AGA GGC AGG GAT GAT GTT CTG-3′ (reverse). The comparative cycle threshold method ($2^{-\Delta\Delta C_T}$) was used to calculate the relative fold change of target gene transcript. GPADH was used as the loading control for normalization. The experiment was conducted three times independently.

Statistical analysis

All data are presented as mean ± standard deviation and analyzed using SPSS software (version 19.0, IBM, Chicago, IL, USA). The difference between two groups was analyzed using the Student's *t* test. The value of *p* less than 0.05 was considered as statistical significant.

Results

The expression of GPER in OS cells and tissues

We first checked the expression of GPER in various OS cell lines and human osteoblastic hFOB 1.19 cells using

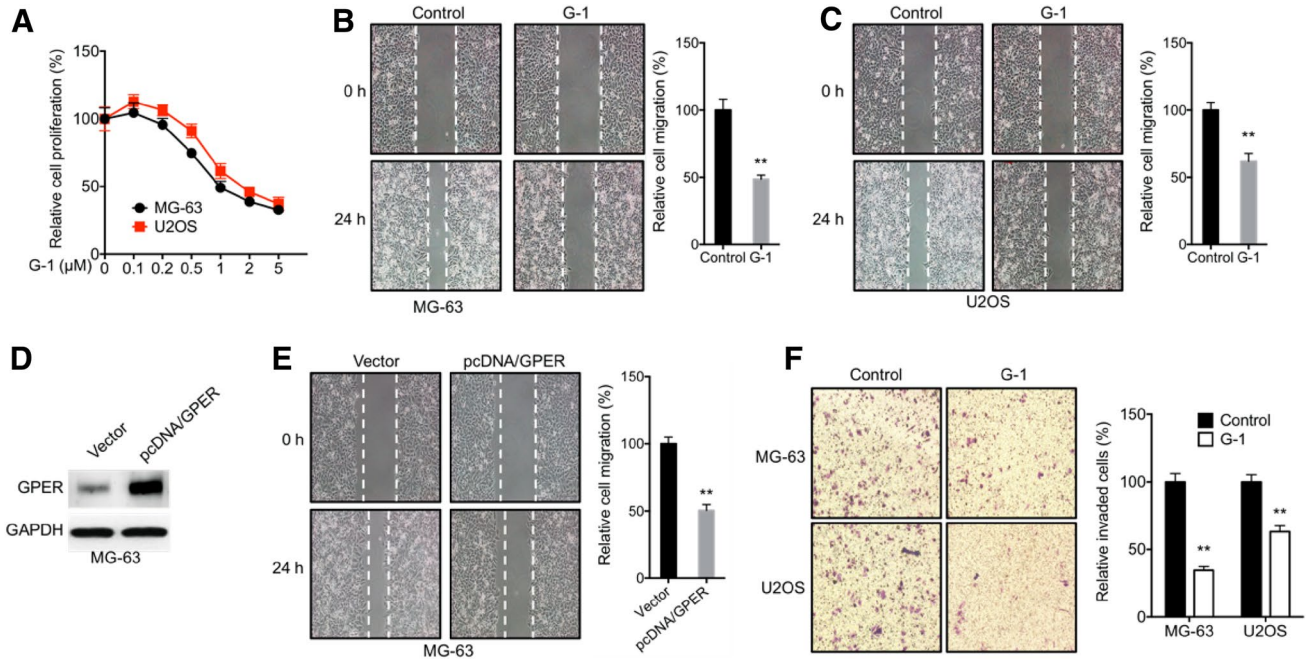


Fig. 2 Activation of GPER inhibited the migration and invasion of OS cells. **a** MG-63 or U2OS cells were treated with increasing concentrations of G-1 for 48 h, cell proliferation was tested; MG-63 (**b**) or U2OS (**c**) cells were treated with or without 200 nM G-1 for 24 h, the migration was tested by wound-healing assay; MG-63 cells were transfected with pcDNA (vector) of pcDNA/GPER for 24 h, the expression of GPER was tested by western blot analysis (**d**), the migration was tested by wound-healing assay (**e**); **f** MG-63 or U2OS cells were treated with or without 200 nM G-1 for 24 h, the in vitro invasion was tested by transwell analysis. Data are presented as mean values ± SD of three independent experiments. $^{**}p<0.01$ compared with control

qRT-PCR. Results showed the expression of GPER in human OS MG-63, U2OS, HOS, and SW1353 was significantly decreased as compared with that in human osteoblastic hFOB 1.19 cells (Fig. 1a). The down regulation of GPER in human OS cells was confirmed by the results of western blot analysis (Fig. 1b). We then evaluated the expression of GPER in OS tissues. The data of Barretina Sarcoma revealed that the expression of GPER in OS tissues was significantly lower than that in adjacent normal tissues (Fig. 1c). Furthermore, the data from LinkedOmics showed that clinical stage (Fig. 1d), distant metastasis (Fig. 1e) and GPER expression were independent prognostic factors for OS patients ($p < 0.05$). In addition, data analysis using LinkedOmics (Fig. 1f) or GEPIA (Fig. 1g) indicated that OS patients with higher levels of GPER showed increased overall survival rate (OS) as compared with the lower GPER OS patients. These results showed that GPER is decreased in OS cells and tissues and correlated with better prognosis of OS patients.

Activation of GPER inhibited the migration and invasion of OS cells

To investigate the potential roles of GPER on the progression of OS, we treated MG-63 cells with increasing concentrations of G-1, the specific activator of GPER. The results showed that G-1 greater than 1 μM can significantly decreased the proliferation of MG-63 and U2OS cells (Fig. 2a). We then evaluated the potential effects of 200 nM G-1, which had no effect on proliferation of either MG-63 or U2OS cells, on in vitro cell migration and invasion. The data showed that 200 nM G-1 can significantly suppress the wound healing of both MG-63 (Fig. 2b) and U2OS (Fig. 2c) cells. Further, overexpression of GPER (Fig. 2d) can also suppress the wound healing of MG-63 cells (Fig. 2e). Transwell analysis showed that G-1 at 200 nM can suppress the in vitro invasion of both MG-63 and U2OS cells (Fig. 2f). These data showed that activation of GPER inhibited the migration and invasion of OS cells.

Activation of GPER regulated the expression of mesenchymal markers and Snail

We further checked the markers related to cell migration and invasion in OS cells treated with G-1 or transfected with pcDNA/GPER. We found that G-1 treatment can decrease the expression of fibronectin and vimentin in both MG-63 and U2OS cells (Fig. 3a). Consistently, overexpression of GPER also decreased the expression of fibronectin and vimentin in both MG-63 and U2OS cells (Fig. 3b). These data suggested that GPER can regulate the expression of mesenchymal markers in OS cells. We further evaluated the expression EMT-TF including Snail, Slug, Zeb1, and Twist in G-1-treated MG-63 cells. Our data showed that G-1 can decrease the expression of Snail, while not others, in MG-63 cells (Fig. 3e). Further, G-1 can decrease the expression of Snail via a dose-dependent manner in MG-63 cells (Fig. 3f). These results showed that activation of GPER can regulate the expression of mesenchymal markers and Snail.

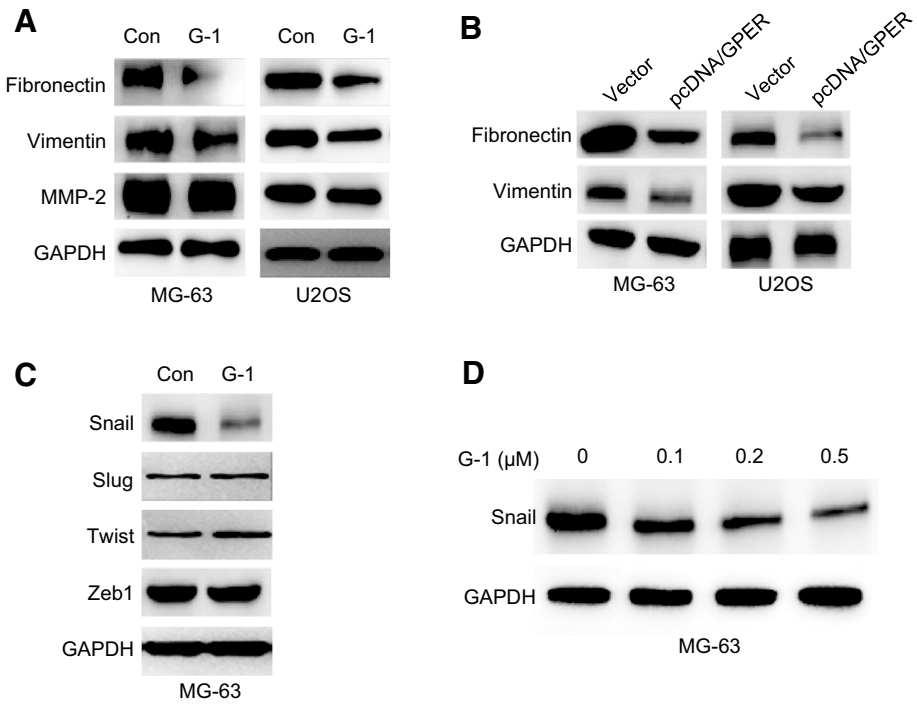


Fig. 3 Activation of GPER regulated the expression of mesenchymal markers and Snail. **a** MG-63 or U2OS cells were treated with or without 200 nM G-1 for 24 h, the expression of mesenchymal markers was tested by western blot analysis; **b** MG-63 or U2OS cells were transfected with pcDNA (vector) or pcDNA/GPER for 24 h, the expression of mesenchymal markers was tested by western blot analysis; **c** MG-63 cells were treated with or without 200 nM G-1 for 24 h, the expression of EMT-TFs was tested by western blot analysis; **d** MG-63 cells were treated with increasing concentrations of G-1 for 24 h, the expression of Snail was tested by western blot analysis

Snail-mediated GPER-regulated migration and invasion of OS cells

As a powerful EMT-TF, Snail can promote the in vitro and in vivo migration and invasion of cancer cells (Brabletz et al. 2018). We then overexpressed Snail in MG-63 cells and further treated cells with G-1. Wound-healing assay showed that overexpression of Snail can attenuate G-1 suppressed migration of MG-63 cells (Fig. 4a). Transwell assay confirmed that Snail also partially reversed G-1-suppressed in vitro invasion of MG-63 cells (Fig. 4b). Western blot analysis showed that overexpression of Snail can attenuate G-1 downregulated fibronectin and vimentin in MG-63 cells (Fig. 4c). These results indicated that Snail is involved in GPER-regulated migration and invasion of OS cells.

GPER post-translationally regulated the expression of Snail

We then investigated the mechanisms responsible for GPER-regulated expression of Snail. Both G-1 (Fig. 5a) and GPER overexpression (Fig. 5b) had no significant effect on the expression of Snail mRNA in either MG-63 or U2OS cells. However, G-1 can rapidly decrease the protein expression of Snail in MG-63 cells science treatment for 2 h (Fig. 5c). By treating cells with CHX, we found that G-1 can significantly decrease the protein half-life of Snail in MG-63 cells (Fig. 5d). These results indicated that GPER can decrease the protein stability of Snail and then downregulate its expression in OS cells.

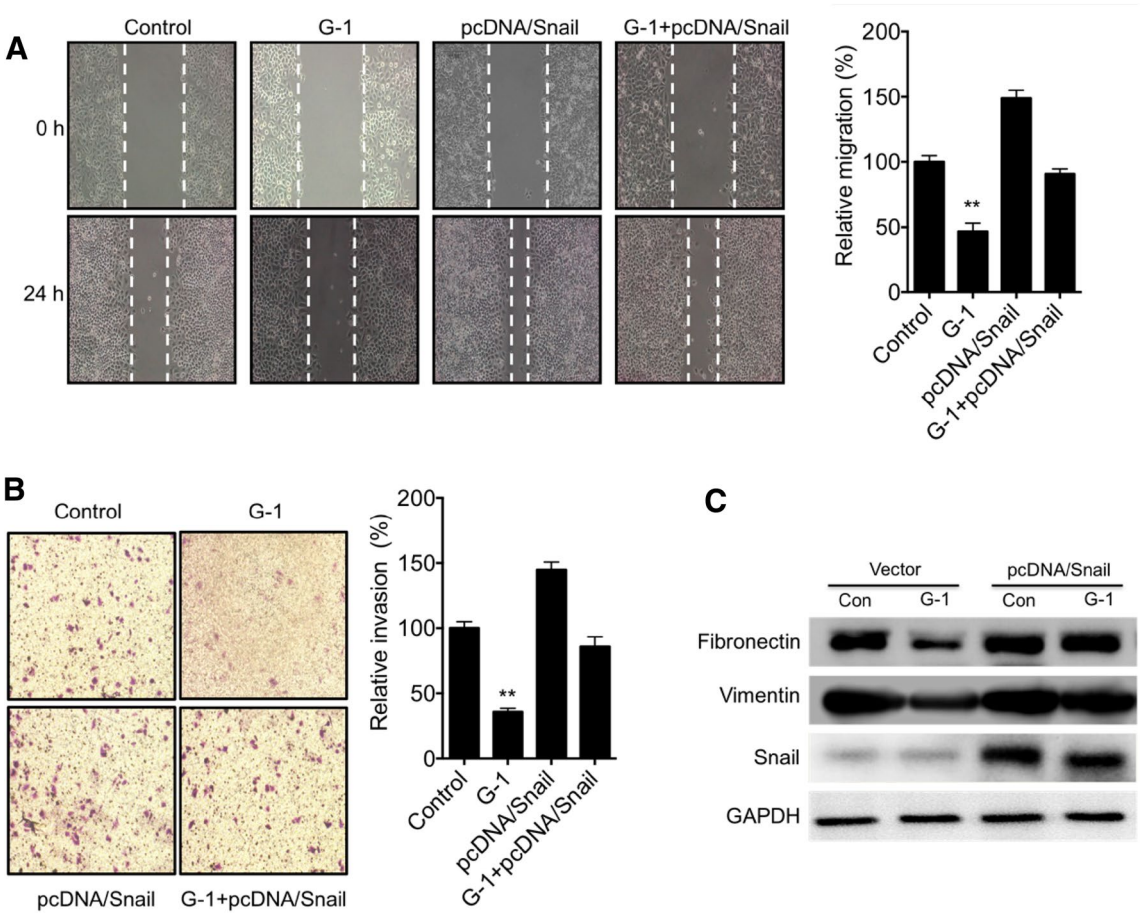


Fig. 4 Snail-mediated GPER regulated migration and invasion of OS cells. MG-63 cells transfected with pcDNA (vector) or pcDNA/Snail were further treated with or without 200 nM G-1 for 24 h, the migration was tested by wound-healing assay (**a**), the in vitro invasion was tested by transwell analysis (**b**), the expression of fibronectin, vimentin, and Snail was checked by western blot analysis (**c**). Data are presented as mean values ± SD of three independent experiments. $^{**}p < 0.01$ compared with control

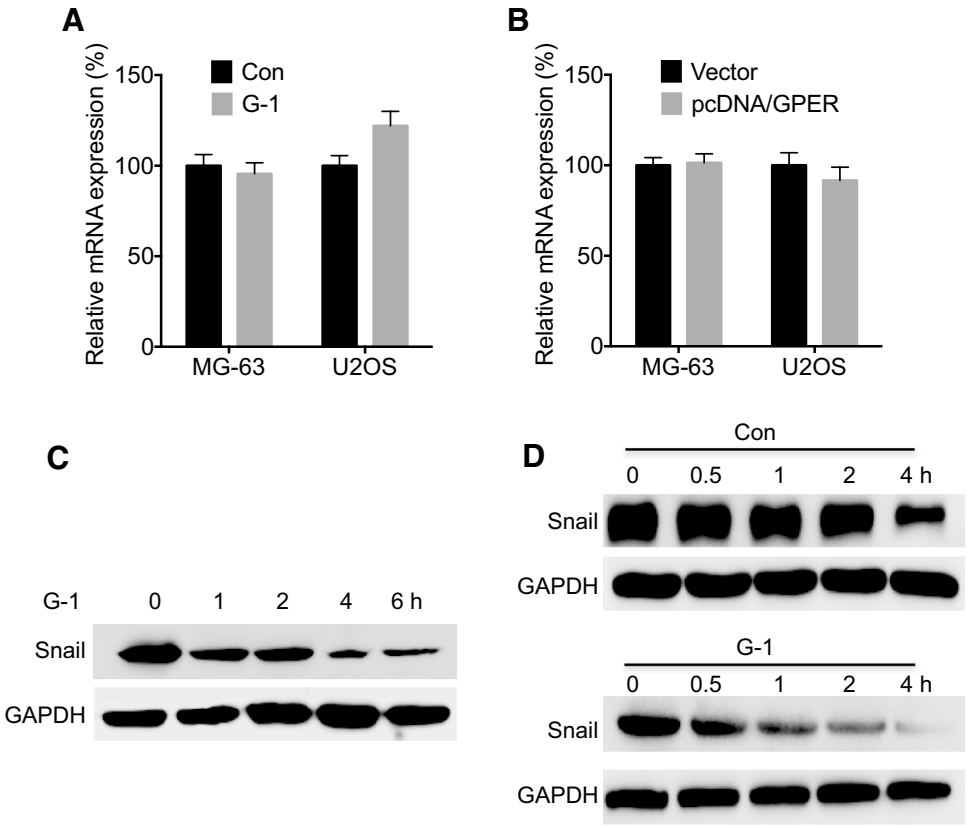


Fig. 5 GPER post-translationally regulated the expression of Snail. **a** MG-63 or U2OS cells were treated with or without 200 nM G-1 for 24 h, **b** MG-63 or U2OS cells were transfected with pcDNA (vector) or pcDNA/GPER for 24 h; **c** MG-63 cells were treated with 200 nM G-1 for the indicated time periods; **d** MG-63 were pre-treated with or without 200 nM for 30 min, and then further treated with CHX for the indicated time periods. Data are presented as mean values ± SD of three independent experiments

FBXL5 was involved in GEPR-regulated stability of Snail protein

It has been reported that GSK3β can bind to and phosphorylate Snail at two consensus motifs and then lead the degradation of Snail protein (Zhou et al. 2004). However, we found that G-1 had no effect on the phosphorylation and total levels of GSK3β in MG-63 cells (Fig. 6a). Further, the GSK3β inhibitor LiCl also had no effect on G-1-decreased expression of Snail in MG-63 cells (Fig. 6b). These suggested that GSK3β was not involved in GPER-regulated protein stability of Snail. FBXL5 (Vinas-Castells et al. 2014), FBXL14 (Vinas-Castells et al. 2010), and FBXO11 (Zheng et al. 2014) can regulate the protein stability of Snail. We found that G-1 treatment can significantly increase the expression of FBXL5, while not FBXL14 or FBXO11, in both MG-63 and U2OS cells (Fig. 6c). We then knocked down the expression of FBXL5 in MG-63 cells (Fig. 6d). The data showed that knockdown of FBXL5 can reverse G-1-suppressed expression of Snail in MG-63 cells (Fig. 6e). These data indicated that FBXL5 was involved in GEPR-regulated stability of Snail protein.

Discussion

Although few studies indicated that estrogen signals can regulate the progression of OS via multiple downstream signals (Svoboda et al. 2010), there was no study concerning the expression and potential roles of GPER on the OS development including metastasis. Our present study showed that GPER is decreased in OS cells and tissues and correlated with better prognosis of OS patients. Activation of GPER can inhibit the migration and invasion of OS cells via suppression of Snail, one powerful EMT-TF. Mechanically, GPER can decrease the protein stability of Snail via increasing the expression of FBXL5.

GPER was identified as a non-nuclear estrogen receptor protein and initially considered as an 'orphan receptor' (Filardo et al. 2000). Later, Revankar et al. (2005) confirmed that 17β-estradiol is the natural ligand of GPER. It has been reported that about 20% of human cancers are related to the alteration of GPER (Filardo 2018). The role of GEPR in the cancer progression is still a matter of debate. GPER can suppress the proliferation of estrogen receptor-negative breast cancer cells in vitro and in vivo (Wei et al. 2014). Using GPER-knockout mice, Marjon et al. (2014) found that tumors from GPER-1-knockout mice were histologically of a lower grade and showed fewer lung metastases than wildtype mice. However,

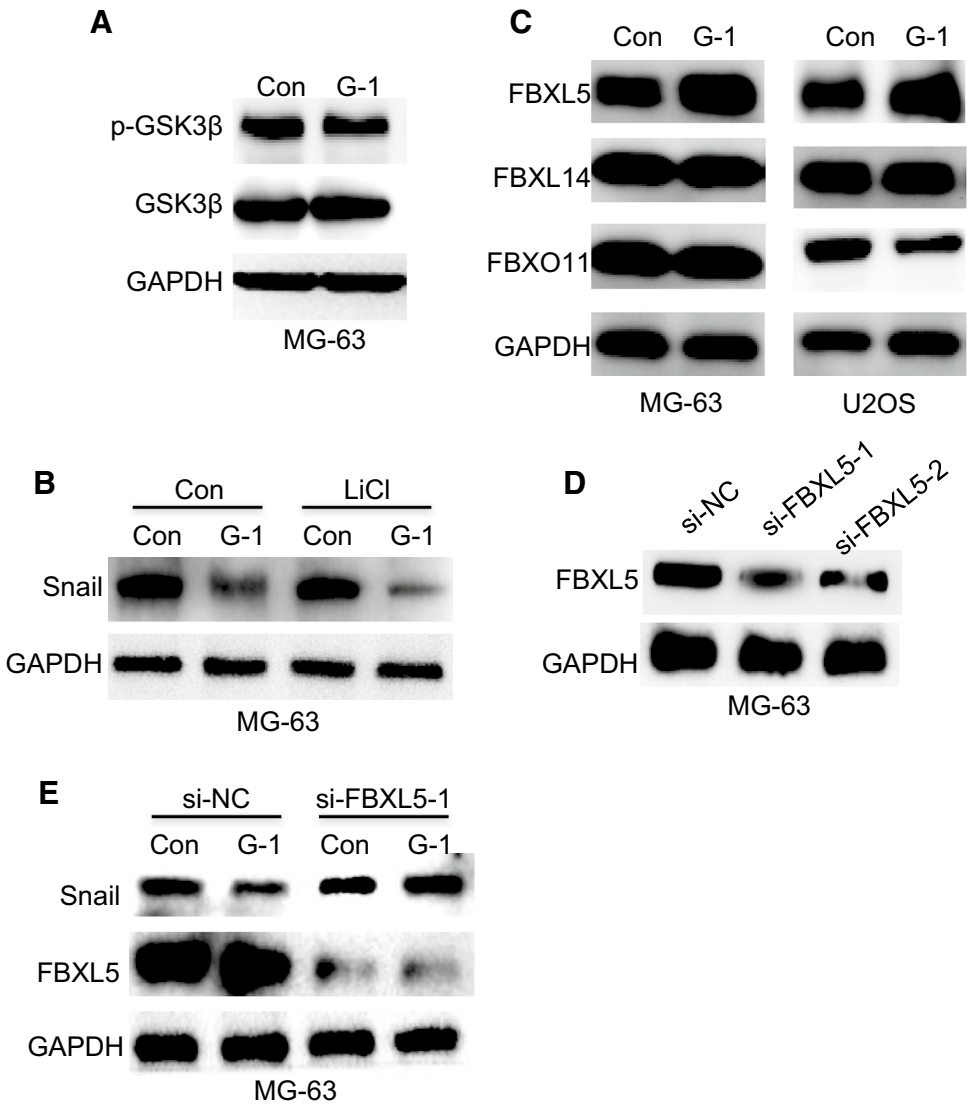


Fig. 6 FBXL5 was involved in GEPR-regulated stability of Snail protein. **a** MG-63 cells were treated with or without 200 nM G-1 for 30 min, the phosphorylation and expression of GSK3β was checked; **b** after pre-treated with or without GSK3β inhibitor LiCl for 90 min, MG-63 cells were further treated with or without 200 nM G-1 for 24 h, the expression of Snail was checked; **c** cells were treated with or without 200 nM G-1 for 24 h, the expression of protein was checked by western blot analysis; **d** MG-63 cells were transfected with si-NC or si-FBXL5-1/-2 for 24 h, the expression of FBXL5 was checked by western blot analysis; **e** MG-63 cells were transfected with si-NC or si-FBXL5-1 for 12 h and then further treated with or without 200 nM G-1 for 24 h, the expression of Snail was checked

GPER activation can stimulate proliferation and migration/invasiveness in renal cell adenocarcinoma cells (Feldman et al. 2016). In our present study, we found that the expression of GPER in OS cell and tissue was decreased in OS cells and tissues and correlated with better prognosis of OS patients. G-1 can inhibit the proliferation of OS cells at concentrations greater than 1 μM. G-1 at concentration of 200 nM, which had no effect on cell proliferation, can inhibit the migration and invasion of OS cells. This was consistent with recent study that activation of GPER can suppress the migration and angiogenesis of triple negative breast cancer (Liang et al. 2017) and lung cancer (Zhu et al. 2016) cells. Our study, together with published data, suggested that GPER might be a potent potential therapy target for OS treatment.

Our data indicated that FBXL5-mediated stabilization of Snail is involved in GPER-suppressed migration and invasion of OS cells. Snail has been characterized as a key EMT regulator (Nieto 2002) to repress the expression of E-Cad by binding to the E-Box site in its promoter (Zhou et al. 2004). In addition, recent studies suggested that Snail is also involved in chemoresistance of OS cells (Fang et al. 2016). Our data showed that activation of GPER can inhibit the expression of Snail, while overexpression of Snail can attenuate G-1-suppressed cancer cell migration and invasion. In addition, G-1 decreased the stability of Snail via upregulation of FBXL5 in OS cells. FBXL5 is a Snail ubiquitin ligase which can promote its polyubiquitination and affect its protein stability and function by impairing DNA binding (Vinas-Castells et al. 2014). We found that GPER can increase the expression of FBXL5 and knockdown of FBXL5 can attenuate GPER-suppressed expression of Snail. These data suggested that FBXL5/

Snail is involved in GPER-regulated migration and invasion of OS cells.

In conclusion, we have demonstrated that activation of GPER can suppress the migration and invasion of OS cells via inhibition of Snail. Further, GPER can regulate the protein stability of Snail via modulating the expression of FBXL5. Our study indicated that activation of GPER might be a potent potential therapy approach for OS treatment.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Research involving human participants and/or animals No human or animal study.

Informed consent Informed consent was obtained from all individual participants included in the study.

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