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Original Article

ERα and ERβ oppositely regulated plexin B1 expression and migration of ovarian cancer SKOV-3 cells

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Abstracts: Ovarian cancer has seriously threatened the health of women. Plexin B1 is proved to be an oncogene in various of cancers. However, what is the role of plexin B1 in ovarian cancer was not explored. ERα and ERβ were two main receptors of natural estrogen dominantly secreted by the ovary, which were proved to be involved in several kinds of female-specific cancers. The aim of the study is to explore the role of plexin B1 and elucidate an opposite regulation of ERα and ERβ on plexin B1 expression in ovarian cancer. Plexin B1 and ERα expression were up-regulated in ovarian cancer tissues and human ovarian cancer SKOV-3 cells. Whereas, ERβ expression in ovarian cancer tissues and cells was down-regulated. Over-expression of ERα and ERβ was manipulated to produce $ERα^+$ and $ERβ^+$ SKOV-3 cell lines. Treatment of ERs agonist 17β-estradiol (E_2) significantly elevated the plexin B1 expression and migration of $ERα^+$ SKOV-3 cells. In $ERβ^+$ SKOV-3 cells, plexin B1 expression and cell migration were suppressed by E_2 treatment. When the activities of ERα and ERβ were blocked with ICI182-780 inhibitor, plexin B1 expression and cell migration of $ERα^+$ and $ERβ^+$ SKOV-3 cells were not significantly different from the control. In a conclusion, the results revealed that the biological function of ERα and ERβ in the ovarian carcinogenesis may be antagonistic to each other. Plexin B1 as an oncogene associated with the cell migration was positively regulated by ERα activity but negatively regulated by the activity of ERβ. The activation of ERα in estrogen receptor signaling was a risky factor of ovarian cancer.

Keywords: Plexin B1, ERα, ERβ, ovarian cancer, cell migration

Introduction

IARC in 2012 have disclosed that the average morbidity of patients with ovarian cancer in 184 countries was as high as 6.3/10 0000 [1]. Due to lack of the obvious indicators in the early stage of ovarian cancer, 60-70% patients are diagnosed to be in the advanced stage of ovarian cancer, which is accompanied by metastasis into the peritoneal cavity or even distant organs [2]. The five-year survival of these patients is as low as 28-35% even though the surgical treatment plus chemotherapy [3, 4]. Thus, there is an urgent need to investigate the molecular mechanisms associated with the aggressive growth and metastatic ability of ovarian cancer.

Ovarian cancer tissues and cells are generally estrogen receptors (ERs)-positive [5, 6]. ER signaling pathway initiated by the co-ordinators plays a key role in the reproduction and development of bone and brains. Apart from participation in those important physiological procedures, ER signaling pathway has been revealed to be universally involved in the female-specific cancers, including breast, ovarian, and uterus cancers by regulating the proliferation, cycle, adhesion, migration, and apoptosis of cancer cells [7-9]. Although the significance of ER pathway was not well defined in the process of ovarian cancer, a growing number of clinical reports have clued the fact that the ER pathway was assumed to be involved in the progress of ovarian cancer [10-12]. The ER levels have been suggested to be a bio-marker of ovarian cancer [13]. However, the weakness from clinical data is that they are only statistical data. what is the potential mechanism behind the fact is still unknown. It is well known that the initiation of

ER signaling needed the combination of ERs with its ligands in most case. But, ERs are classically grouped into two isoforms, including ERα and ERβ. In the body of female, the most common natural ER-ligand is estradiol, which was chiefly secreted from the ovary. With a very high homology in the active functional areas, however, the biological function of ERα and ERβ was not meant to be similar to each other [14]. It has been found that ERα and ERβ have generally regulated the same targeted gene in an opposite direction in breast cancer [15-17]. The opposite effect of ERα and ERβ on the ovarian cancer is unknown.

Semaphorins are a large family of either membrane-bound or secreted proteins that were originally described in the nervous system, where these proteins are involved in the establishment of correct neuronal net works [18]. These ligands exert their activities by binding to their high-affinity receptors, neuropilins and/or plexins [18]. Besides the involvement in the neuronal net works, an increasing evidence have indicated that semaphorins and plexins also play critical role in the tumor homeostasis [19]. Previously, our group have reported that semaphorin 4D (sema 4D) was an oncogene of ovarian caner, which was regulated by the activity of ERs [20]. Plexin B1, the receptor of sema 4D, interacts directly with Rho family GTPases through acytoplasmic RhoGTPase binding domain [21]. Rho GTPases play important roles in regulating cell proliferation and migration, suggesting the potential involvement of plexin-B1 in cancer progression and metastasis [22-24].

In the present study, we have disclosed the expression of ERs and plexin B1 expression in ovarian cancer. We have observed an elevated ERα and plexin B1 expression, but a decreasing ERβ expression in ovarian cancer tissues and cells. When ERs signaling was activated in over-expressed ERα SKOV-3 cells, plexin B1 expression was increased and promoted cell migration. In over-expressed ERβ SKOV-3 cells, plexin B1 expression was decreased under the activation of ERs signaling, accompanied by the less cell migration. Our data has proved that the biological function of ERα and ERβ in the ovarian carcinogenesis may be reciprocally antagonistic. Plexin B1 as an oncogene associated with the cell migration was positively regulated by ERα activity, but negatively controlled by the activity of ERβ.

Materials and methods

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium was bought from Hyclon (SH302-43.01B). RNase, DNase and DNA marker (TAKARA) were purchased from Shanghai Bito. Co. Ltd., Shanghai, China. Normal ovarian tissues and ovarian cancer tissues were collected from the patients in Second Affiliated Hospital of Kunming Medical University. All the patients did not receive any medication before surgery. The ovarian cancer tissues were histologically confirmed by two pathologists. The collection of human tissue samples was approved and supervised by the Ethics Committee of Kunming Medical University. Human ovarian cancer SKOV-3 cells and human normal ovarian epithelial IOSE 80 cells were purchased from Yingrun Biological Co. Ltd., Changsha.

Cell culture

SKOV-3 cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, USA), with or without 100 units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA), in a humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37°C.

ERα and ERβ over-expression

The ERα and ERβ highly expressed vectors were constructed as described previously [20]. 293T-pLV-ERα, 293T-pLV-ERβ or blank plasmid were co-transfected SKOV-3 cells with Lenti-Pac HIV Expression Packaging Kit following the manufacturer's instruction (GeneCopoeiat). Transfection efficacy was monitored by an inverted fluorescence microscope. The supernatant was harvested, filtered, and cleared by centrifugation at 500 g for 10 min at 4°C. Three days after infection, 2 mg/mL puromycin was added to the culture media to select the cell populations infected with the lentivirus for 2 weeks. The cell lines transfected with 293T-pLV-ERα and 293T-pLV-ERβ were named ERα$^+$ and ERβ$^+$ SKOV-3 cells, respectively. Cells transfected with blank plasmid were named control (Ctrl) SKOV-3 cells. The over-expression of ERα or ERβ was detected by RT-PCR and western blotting in these three cell lines as described above.

Quantitative real-time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. QRT-PCR was conducted to assess the expression levels of ERα, ERβ, and plexin-B1 using the 2-ΔΔCT method. β-actin was used as an internal standard.

Incubation of E_2 or ICI182-780

ERα$^+$, ERβ$^+$, and Ctrl SKOV-3 cell lines were cultured in 6-well plates containing PRMI 1640 medium and a final 10^{-6} M E_2 solution. After 24 h culture, the cells were harvested to extract the total RNA and proteins. For the ICI182-780 inhibitor treatment, a final concentration of 100 nM ICI182-780 solution was mixed with the three cell lines cultured in PRMI 1640 medium in 6-well plates for 6 h. After that, each well was cultured for another 24 h in the presence of 10^{-6} E_2 solution.

Western blotting

The cells were lysed with RIPA lysis solution (DSL, USA). After total proteins were extracted, a BCA protein assay kit (Pierce, USA) was used to quantify the proteins. Equal protein amounts were mixed with the 4 × loading buffer (Beyotime, China) and then boiled for 5 min for protein denaturation. A total of 15 mg protein from each sample was loaded for 12% SDS-PAGE gel electrophoresis and then transferred to a polyvinylidene fluoride (0.45 mm, PVDF) membrane. Then, the membrane was incubated with ponceau staining solution for 2 min to judge the transfer efficiency of proteins. Once the proteins were proved to have transferred to the membrane successfully, the membrane was incubated with 5% fat-free milk for 30 min. Then, the membrane was incubated with anti-plexin B1 (Abcam, Cat. #13457, Lot #210783), ERα (Abcam, Cat. #22367, Lot #384323), ERβ (Abcam, Cat. #663298, Lot #631005) or β-actin (1:5000, NeoBioscience, Shenzhen, China) antibodies at 4°C overnight. Finally, the membrane was washed and incubated with corresponding HRP conjugated-secondary antibodies at room temperature for 2 h. The bands were visualized using an enhanced chemiluminescence system (ECL, Pierce, Rockford, USA).

Wound healing assay

Cells were seeded in a 6-well culture plate. Then, a sterile 10-ul pipette tip was used to longitudinally scratch a constant-diameter stripe in the confluent monolayer. The medium and cell debris were aspirated away and replaced with 2 ml of fresh serum-free medium. Photographs were taken at 0 h and 24 h after wounding by phase contrast microscopy. Five randomly selected points along each wound were marked, and the horizontal distance between the migrating cells and the initial wound was measured at 24 h.

Immunohistochemistry

Tissue samples were fixed in PBS containing 4% paraformldehyde. The slide was deparaffinaged in dimethylbenzene and followed by rehydration in 80% ethanol. Then, 3% hydrogen peroxide solutions were added to the tissue slides to quench the endogenous peroxidase. After washing with the PBS three times, the slides were incubated with anti-plexin B, ERα or ERβ antibodies overnight at 4°C, then secondary antibody (Dako Co, Glostrup, Denmark) were added and maintained for 2 h at room temperature. Finally, the slides were developed with 3,3-diaminobenzidine (DAB substrate kit for peroxidase; Vector Laboratories) and counterstained with hematoxylin. Images were obtained using an Aperio Scanscope in five randomized visual fields (Aperio Technologies, Vista, CA, USA). The results were separately judged by two pathologists.

Transwell assay

Cell migration was assayed using a Transwell chamber (BD Biosciences) with a polycarbonic membrane (6.5 mm in diameter, 8 μm pore size). Cells are starved 24 h in serum-free medium, and then trypsinized and suspended into the density of 2 × 10^4 cells/mL. 150 μL of the cell suspension was added to the upper chamber, and 400 mL of RPMI 1640 supplemented with 10% FBS was added to the lower chamber. Cells were incubated for 24 h at 37°C, then non-migrating cells on the top surface of membrane were washed twice with PBS. Cells that migrated to the lower surface of the membrane were then fixed with methanol and stained with 20% Giemsa solution for 30 min at 37°C and washed twice with PBS. Then stained cells were observed under an inverted microscope (400 ×) to count the cell number within ten randomly chosen fields and the average number was calculated.

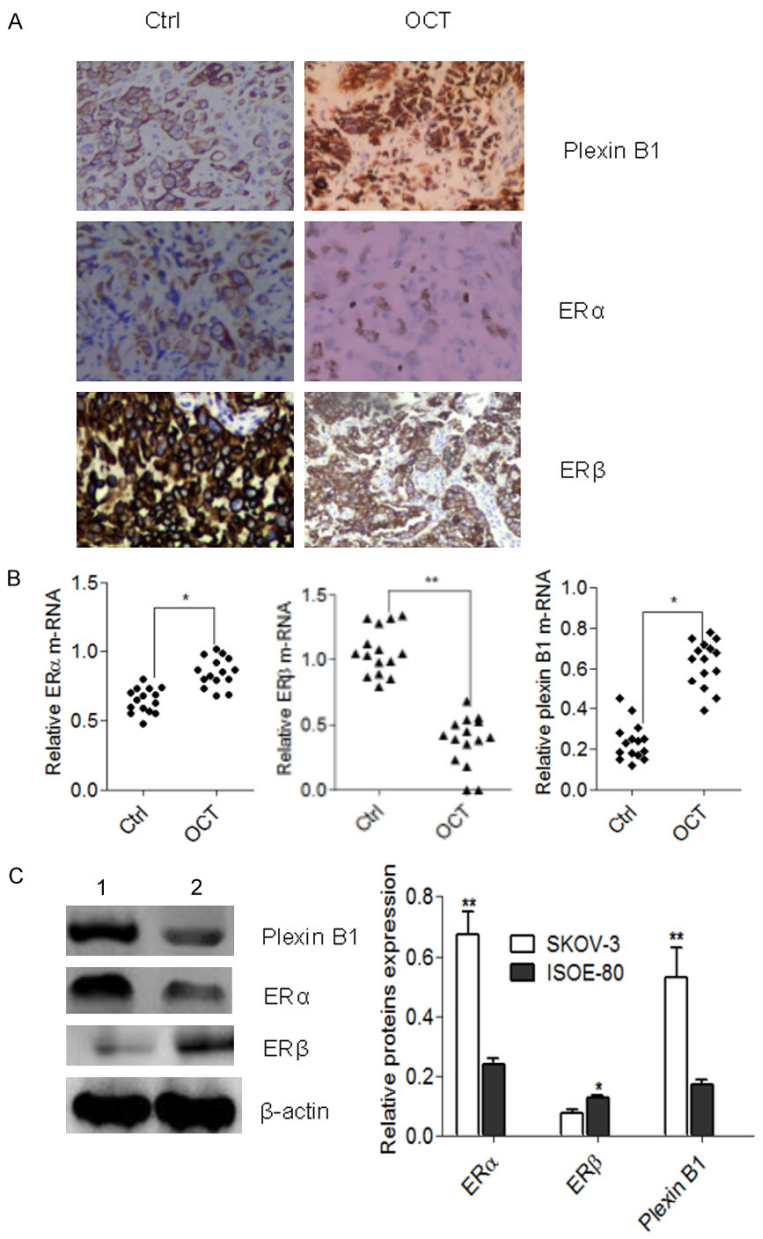

Figure 1. ERs and plexin B1 expression in ovarian cancer tissues (OCT) and cells. A. Proteins expression by immunohistochemical method. B. m-RNA levels by q-RT PCR. After the tissues were excised 12 h, m-RNA levels were quantified (n = 15). β-actin was used as an internal standard. C. Proteins expression by western blotting. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. The cells were harvested after 24 h culture for the analysis. *P<0.05, **P<0.01.

Statistical analysis

Student's t test or one-way ANOVA were used for statistical analysis when appropriate. All statistical analyses were performed using Prime 5.0 software. A two-tailed value of *P*<0.05 was considered statistically significant.

Results

ERα, ERβ, and plexin B1 expression in ovarian cancer tissues and cells

As shown in **Figure 1**, the ERα and plexin B1 proteins were up-regulated in ovarian cancer tissues as compared with those of Ctrl (**Figure 1A**). ERβ protein was found to be down-regulated in the tissues of ovarian cancer (**Figure 1A**). m RNA levels of ERα and plexin B1 in ovarian cancer tissue were also significantly higher than those in Ctrl (P<0.05, **Figure 1B**). ERβ m RNA levels in ovarian cancer tissue were remarkably lower than those in Ctrl. We did not examine ERβ m RNA expression in 2 pathological samples (2/15). The expressive pattern of ERα, ERβ, and plexin B1 proteins in cellular level was similar to that in tissue level (**Figure 1C**). ERβ protein in SKOV-3 cells was declined as compared with that in IOSE-80 cells. Plexin B1 and ERα proteins in SKOV-3 cells were more highly expressed than those in ISOE-80 cells.

Over-expression of ERα and ERβ has not affected plexin B1 expression

As shown in **Figure 2A**, the m-RNA levels of ERα and ERβ were significantly increased via pLV-ERα and pLV-ERβ transfection. An up-regulation of ERα and ERβ protein was correspondingly observed due to the transfection (**Figure 2B**). Plexin B1 m-RNA and protein in both ERα$^+$ and ERβ$^+$ SKOV-3 cells were not significantly different from those in the Ctrl, suggesting that ERs over-expression

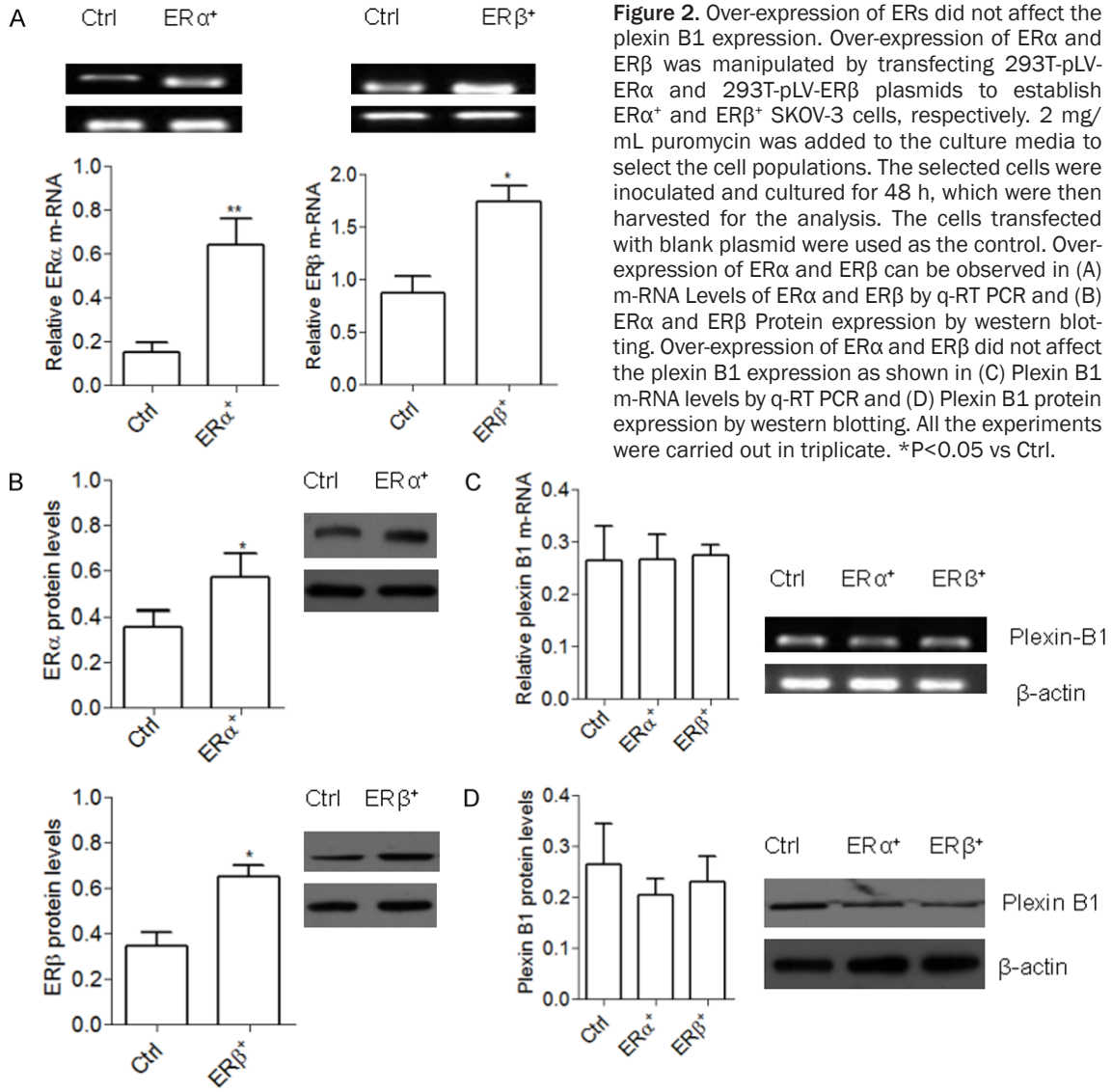

Figure 2. Over-expression of ERs did not affect the plexin B1 expression. Over-expression of ERα and ERβ was manipulated by transfecting 293T-pLV-ERα and 293T-pLV-ERβ plasmids to establish ERα$^+$ and ERβ$^+$ SKOV-3 cells, respectively. 2 mg/mL puromycin was added to the culture media to select the cell populations. The selected cells were inoculated and cultured for 48 h, which were then harvested for the analysis. The cells transfected with blank plasmid were used as the control. Over-expression of ERα and ERβ can be observed in (A) m-RNA Levels of ERα and ERβ by q-RT PCR and (B) ERα and ERβ Protein expression by western blotting. Over-expression of ERα and ERβ did not affect the plexin B1 expression as shown in (C) Plexin B1 m-RNA levels by q-RT PCR and (D) Plexin B1 protein expression by western blotting. All the experiments were carried out in triplicate. *P<0.05 vs Ctrl.

has not affected the plexin B1 expression (**Figure 2C** and **2D**).

ERα and ERβ activation was antagonistic in regulating plexin B1 expression and cell migration

Although ERs expression did not modify plexin B1 expression, it is not causal that ER pathway was not associated with the plexin B1 expression because ER signaling activation in the ER pathway required the presence of the ligands. Therefore, E_2 was co-cultured with the cells to activate ERs signaling. As shown in **Figure 3**, both plexin B1 m-RNA and proteins in ERα$^+$ and ERβ$^+$ SKOV-3 cells have significantly changed. In ERα$^+$ SKOV-3 cells, plexin B1 expression was significantly increased than that in Ctrl (**Figure 3A**). Oppositely, a significant reduction of plexin B1 expression was shown in ERβ$^+$ SKOV-3 cells (**Figure 3A**). Meanwhile, we have observed a negative association of plexin B1 expression with the migration distance. A lower rate of migrating cells was also observed in E_2-treated ERβ$^+$ SKOV-3 cells as compared with those of Ctrl. As shown in **Figure 3B** and **3C**, the migration distance and rate of ERα$^+$ SKOV-3 cells were significantly higher than those of Ctrl and ERβ$^+$ SKOV-3 cells. Moreover, the ERβ$^+$ SKOV-3 cells showed a significantly lower migration distance and rate than Ctrl SKOV-3 cells. The results showed that the plexin B1 expression has been regulated by the activation of ER pathway. ERα and ERβ have oppositely regulat-

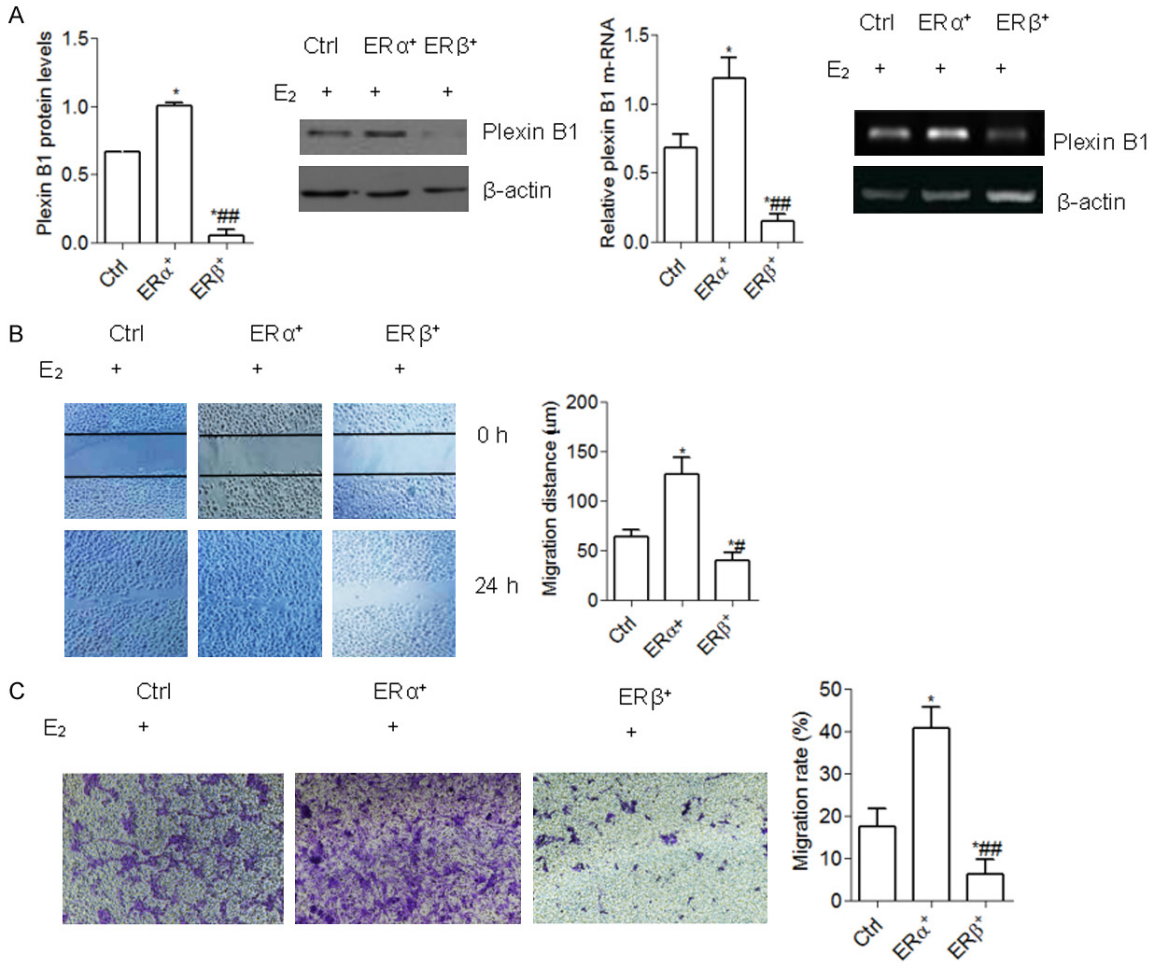

Figure 3. ERα and ERβ activity by E_2-driven have regulated plexin B1 expression and cell migration toward opposite direction. A. Plexin B1 m-RNA and proteins expression. Cells were cultured 24 h in the presence of a final 10^{-6} E_2 solution. Then, the m-RNA and proteins were quantified using q RT PCR and western blotting, respectively. B. Wound healing assay. The distance of cell migration was evaluated. Cells were grown in RPMI 1640 medium containing a final 10^{-6} E_2 solution for 24 h. Scratches were created in a monolayer of confluent cells. Wound healing was observed at 0 and 24 h. Representative scrape lines were photographed and measured using Adobe Photoshop software. C. Transwell assay. Rate of cell migration was evaluated using the transwell assay. The process of cell culture was similar to that of wound healing assay. All the assay was measured in triplicate. *P<0.05 vs Ctrl, ##P<0.01 vs ERα+ SKOV-3 cells.

ed plexin B1 expression. The activation of ERα positively regulated plexin B1 expression, promoting the cell migration. ERβ activity has negatively regulated plexin B1 expression, suppressing the cell migration.

ERα and ERβ inactivation disabled the regulation of plexin B1 expression and cell migration

To further validate the regulation of ERs on the plexin B1 expression and cell migration, cells were pre-incubated with an ERs inhibitor, ICI182780, which can inhibit both ERα and ERβ activity, and then followed by E_2 treatment and detection of plexin B1 expression. The results showed that the levels of plexin B1 proteins in both ERα+ and ERβ+ SKOV-3 cell lines were recovered to Ctrl levels (**Figure 4A**). ERα+ and ERβ+ SKOV-3 cell lines also showed the same statistical m-RNA levels as Ctrl SKOV-3 cells (**Figure 4B**). The regulation of ERs on the plexin B1 expression was completely abolished due to inhibition by ICI182780 inhibitor. When the ER signaling was inhibited, the cell migration of ERα+ and ERβ+ SKOV-3 cell lines was also found to be not significantly different from that of Ctrl SKOV-3 cells (**Figure 4C** and **4D**).

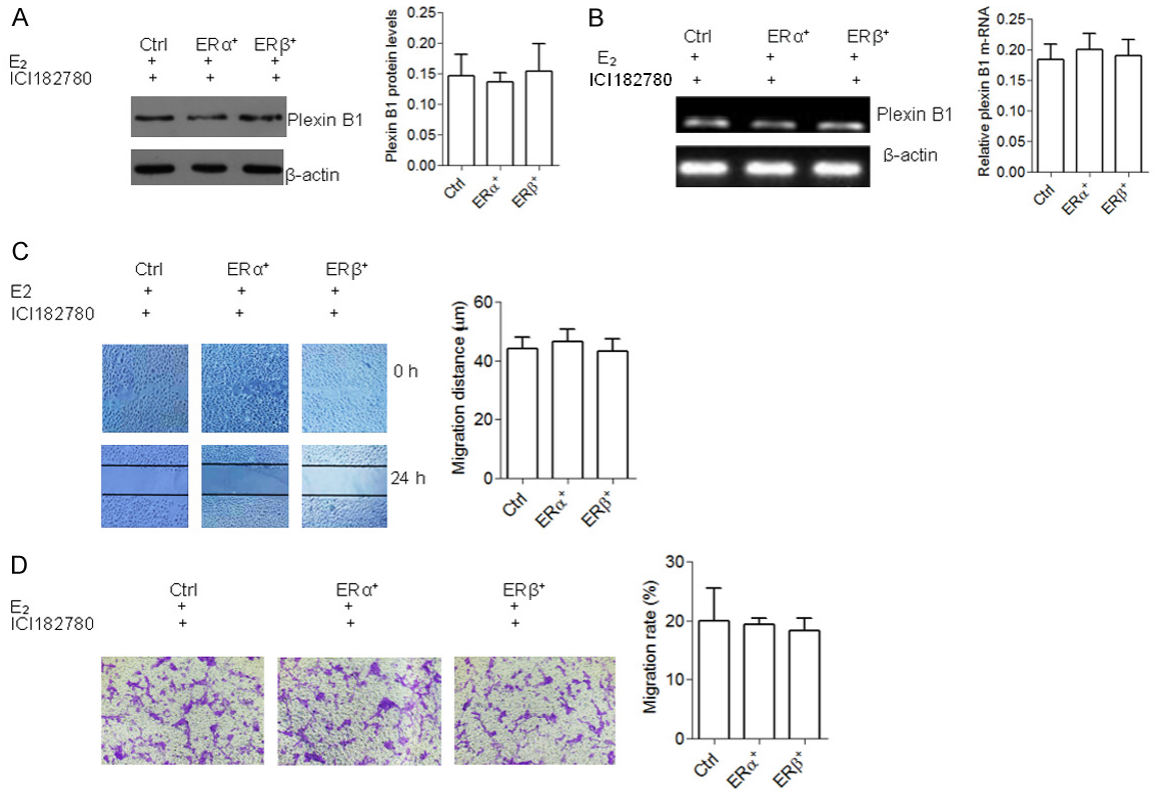

Figure 4. Inhibition of ERα and ERβ activity by ICI182780 inhibitor have abolished the plexin B1 expression and migration toward opposite direction. The cells were cultured in 6-well plates containing PRMI 1640 medium and a final 100 nM ICI182-780 solution for 6 h. Then, a final 10^{-6} M E_2 solutions were mixed and cultured for another 24 h. (A) Western blotting analysis (B) q RT PCR assay. (C) Wound healing assay. (D) Cell migration *assay*. All the assay was measured in triplicate.

Discussion

Recently, the role of sema 4D, via interaction with plexin B1, in activities such as tumor angiogenesis and invasive growth has been disclosed in various types of tumors [25-27]. The oncogene role of sema 4D in ovarian cancers has been also reported by our group [20, 28]. To investigate the role of plexin B1 in ovarian cancer, we continue to detect the plexin B1 expression in ovarian cancer tissues and cells. The results showed that both plexin B1 m-RNA and protein were up-regulated in ovarian cancer tissues and cells, which suggested that plexin B1 may have advanced the progress of ovarian cancer. Given our previous results, the sema 4D/plexin B1 axial signaling may play an important role in the process of ovarian cancer as they have behaved in other types of cancers [25-27]. Although the involvement of ER signaling in the ovarian cancer was assumed, the specific role of ERα and ERβ in the involvement of ovarian cancer was still controversial. Therefore, we have also specially detected the expression of ERα and ERβ in ovarian cancer tissues and cells. It was found that the ERα protein and m-RNA levels in ovarian cancer tissues were significantly increased as compared with those in normal tissues. Whereas, the ERβ protein and m-RNA levels in ovarian cancer tissues was declined versus to the normal tissues. Compared with the normal ovarian cells, an increase of ERα protein and reduction of ERβ protein were also found in ovarian cancer SKOV-3 cells. Our results hinted that the function of ERα and ERβ in the ovarian carcinogenesis may be antagonistic to each other, which was consistent with several previous reports [20, 29-31].

Plexin B1 was the receptor of sema 4D. The sema 4D/plexin B1 axial often jointly functioned in regulating the behavior of cancer cells [25-27]. Sema 4D was revealed to be implicated in E_2-drvien ER signaling in ovarian cancer cells [20]. Therefore, we suspected that plexin

B1 may be one of downstream targets of ER pathway. To prove the speculation, we have over-expressed the ERα and ERβ to obtain the ERα$^+$ and significantly affect the expression of plexin B1. However, ERs play their physiological function *in vivo* depending on the activation from the ligands. Therefore, we were more concerned on the regulation of ERs activation on the plexin B1 expression. When the ER pathway was activated in the presence of E_2, it was observed that ERs exerted effect on the plexin B1 expression. In ERα$^+$ SKOV-3 cells, the plexin B1 expression was significantly increased. Meanwhile, the migration rate and distance of cancer cells was also elevated. Whereas, the above phenomena in ERβ$^+$ SKOV-3 cells were completely adverse to those in ERα$^+$ SKOV3 cells under the activation of ER pathway. Obviously, the differentiation was due to the opposite regulation of the activity of ERα and ERβ. When the ER pathway was suppressed by ICI182780 inhibitor, the plexin B1 expression as well as cell migration of ERα$^+$ and ERβ$^+$ SKOV-3 cells was both declined and comparable to those in Ctrl cells. Since the cytotoxicity of E_2 and ICI182780 on the tested cancer cells was not significant, the expression change of plexin B1 was excluded due to the cell vitality (Supplementary Figure 1). Our results for the first time revealed that the plexin B1 was involved in ovarian cancer and mediated by the ER pathway. The activation of ERα and ERβ has simultaneously but oppositely mediated the plexin B1 expression. Furthermore, the plexin B1 expression was associated with the migration of ovarian cancer cells, thus possibly affecting the aggressive growth and metastatic ability. However, a limitation of the study was that the subtype of ERα and ERβ in regulating plexin B1expression is not identified. Whether ERs activity to regulate the plexin B1 expression and cell migration via sema 4D or not is unclear. Those topics are deserved of further investigation.

Since ERα and ERβ activation driven by E_2 may be antagonistic in the progress of ovarian cancer, a simple detection of ERα and ERβ expressions of patients may be not significant enough in the diagnose of ovarian cancer. We should be more concerned on the ration of ERα/ERβ. The ERα/ERβ value, rather than ERα or ERβ, as the bio-marker of ovarian cancer may be more reasonable. A higher ration of ERα/ERβ may indicate the risk of ovarian cancer. The appellation was also consistent with several previous clinical reports [32, 33]. In the treatment of female illness, the utilization of estrogen drugs should be especially cautious, as such drugs were able to activate the ER pathway. A common utilization of estrogen drugs was in the treatment of menopausal symptom of female. Actually, several meta-analysis and clinical studies have continually reminded us that a long use of hormone replacement therapy may be associated with the risk of ovarian cancer [34-36]. On the basis of our results, we also discourage the long utilization of estrogen drugs in hormone replacement therapy.

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Disclosure of conflict of interest

None.

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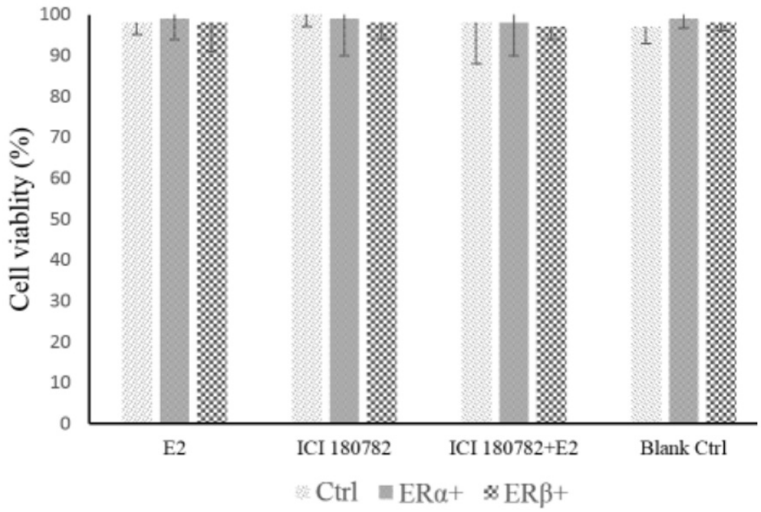

Supplementary Figure 1. The effect of E_2 and ICI 180782 on the cell lines. Cells were seeded in a 96-well dish at a density of $5x10^3$ cells per well and incubated in 1640 containing 10% FBS. After 24 h, the cells were washed with PBS and incubated in 100 µl 1640 medium containing 10 µl Cell Counting Kit-8 solution for 120 min. The absorbance of each well was measured at a wavelength of 450 nm. The relative cell viability was calculated according to a calibration plot.