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Endothelial cells enhance prostate cancer metastasis via IL6→androgen receptor→TGFβ→MMP9 signals

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Abstract

While the potential roles of endothelial cells (ECs) in the microvascules of prostate cancer (PCa) during angiogenesis have been documented, their direct impacts on the PCa metastasis remain unclear. We found that the CD31-positive and CD34-positive ECs are increased in PCa compared to the normal tissues and these ECs cells were decreased upon castration, gradually recovered with time, and become increased after PCa progresses into the castration resistant stage, suggesting a potential linkage of these ECs with androgen deprivation therapy. The *in vitro* invasion assays demonstrated that the co-culture of ECs with PCa cells significantly enhanced the invasion ability of the PCa cells. Mechanism dissection found that co-culture of PCa cells with ECs led to increased IL-6 secretion from ECs, which might result in down-regulation of AR signaling in PCa cells, and then the activation of TGF-β/MMP9 signaling. The consequences of the IL-6→androgen receptor→TGFβ→MMP9 signaling pathway might then trigger the increased invasion of PCa cells. Blocking the IL-6→androgen receptor→TGFβ→MMP9 signaling pathway either by IL-6 antibody, AR-siRNA, or TGF-β1 inhibitor all interrupted the ability of ECs to influence PCa invasion. These results, for the first time, revealed the important roles of ECs within the PCa microenvironment to promote the PCa metastasis, and provide new potential targets of IL-6→androgen receptor→TGFβ→MMP9 signals to battle the PCa metastasis.

Keywords

prostate cancer metastasis; endothelial cells; androgen receptor; microvascules; invasion

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Introduction

Once prostate cancer (PCa) progresses into the castration resistant stage, PCa cells may rapidly gain the ability to invade and metastasize to lymph nodes and distant organs (1, 2). The tumor microenvironment (TME) of PCa includes different types of cells such as non-malignant cells, activated fibroblasts, infiltrated macrophages, and other immune cells, as well as microvasculature containing endothelial cells (ECs) (3). How each component of this TME responds to the androgen deprivation therapy (ADT) and contributes to the PCa progression into metastasis is largely unknown.

Emerging evidence indicates that ECs may contribute to the development and progression of PCa (4, 5). Upon ADT, the earliest event is the perturbation of prostatic blood flow (6, 7) and subsequent decreased numbers of microvascules. As a result of this process, ECs are subjected to apoptosis and their numbers decrease. Interestingly, it was shown that the ECs of prostate only, not the ECs of any other organs, respond to ADT. More interestingly, the perturbed microvascules are regenerated rapidly (4, 5) and their numbers are increased in castration resistant PCa (CRPC), which is frequently accompanied by a high incidence of distal metastasis.

Up-to-date, the focus of ECs role in PCa has been in the angiogenesis process. However, the recent reports suggest there is a mutual interaction of PCa cells and ECs (4, 5) and a high number of microvascules may be associated with higher incidence of metastatic cancer (8, 9). Moreover, a higher permeability of the microvascules was observed in the metastatic tumors (10). These results suggest the importance of ECs residing in microvascules to affect PCa cells.

In this study, we used multiple *in vitro* and *in vivo* strategies to demonstrate that, other than their angiogenesis functions, ECs can secrete cytokines to inhibit AR function and induce PCa metastasis. The mechanisms by which these ECs contribute to the enhanced metastatic potential of PCa cells were also investigated.

Materials and Methods

Cell lines and co-culture experiments

Human umbilical vein ECs (HUVECs), human dermal microvascular ECs (HMECs), LNCaP, C4-2, C81, and CWR22Rv1 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HUVECs were cultured in EC medium supplemented with growth factors (ATCC) and HMECs were cultured in MCDB131 (Gibco, Grand Island, NY) supplemented with 1 μg/ml hydrocortisone, 10 ng/ml EGF and 10% fetal bovine serum (FBS). LNCaP, C4-2, C81, and CWR22Rv1 cells were cultured in RPMI 1640 with 10% FBS. Cells were maintained in a humidified 5% CO2 environment at 37°C. Six-well (3 μm) and 24-well (8 μm) transwell plates (Corning, Lowell, MA) were used for co-culture and invasion assay, respectively. Cell lines used in these studies were authenticated.

Lentiviral infection

For incorporation of AR-siRNA or scramble control plasmids into PCa cells, lentivirus carrying either control (pLVTHM-scramble) or AR-siRNA (pLVTHM-AR-siRNA), was transfected into HEK293T cells with a mixture of pLVTHM-scramble/ pLVTHM-AR-siRNA, psPAX2 (virus packaging plasmid), and pMD_2G (envelope plasmid) (4:3:2 ratio) by calcium-phosphate transfection. Culture medium containing virus was collected 32 h after transfection and filtrated through a 0.4 μm filter to remove cell debris or cells. The collected virus were added to the target cells in the presence of polybrene (2 μg/ml) to incubate for 24 hr. Cells were refreshed with culture medium and cultured for another 3 days to allow target

protein expression. Since the lentiviral vectors express green fluorescence protein, fluorescence microscopy was used to monitor the infection efficiency via checking the green fluorescence signal.

Cell invasion assay

For *in vitro* invasion assays, the upper chambers of the transwells were pre-coated with diluted matrigel (1:3) (BD Biosciences, Sparks, MD). Before the invasion assays, PCa cells were co-cultured with HUVECs (ECs culture medium for control) for 48 hrs in transwell plates. 10^5 PCa cells (in serum free media) and 10% serum containing media were plated in the upper and lower chambers, respectively. After 24 to 48 hrs of incubation, the cells in the upper chamber were removed. The insert membranes were fixed in ice cold methanol, stained with crystal violet, and the positively stained cells were counted under the microscope. The numbers of cells were averaged from counting of six random fields. Each sample was run in triplicate and in multiple experiments and values are expressed as mean ± SD.

Cytokine Array and ELISA

Conditioned medium (CM) was collected from HUVECs culture or HUVECs-PCa co-culture and used for cytokine arrays and ELISA analyses. The levels of a selected panel of cytokines were determined using the Human Antibody Array kit (Affymetrix, Santa Clara, CA) while the IL-6 ELISA kit (eBioscience, San Diego, CA) was applied to measure IL-6 level in the CM. The protocols were followed according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNAs were isolated using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. One μg of total RNA was subjected to reverse transcription using Superscript III transcriptase (Invitrogen, Grand Island, NY). Quantitative real-time PCR (qRT-PCR) was conducted using a Bio-Rad CFX96 system with SYBR green to determine the level of mRNA expression of a gene of interest. Primers used were: AR sense, 5′-TATCCTGGTGGAGTTGTG-3′; antisense, 5′-CAGAGTCATCCCTGCTTC-3′; GAPDH sense, 5′-AATGTCACCGTTGTCCAGTTG-3′, antisense, 5 ′-GTGGCTGG GGCTCTACTTC-3′; CCL5 sense, 5′-ATCCTCATTGCTACTGCCCTC-3′, antisense, 5′-GCCACTGGTGTAGAAATACTCC-3′; IL-6 sense, 5′-AAATTCGGTACATCCTCGACGG-3′, antisense, 5′-GGAAGGTTCAGGTTGTTTTCTGC-3′; IL-8 sense, 5′-TGGGGACTGTCTATGAATCTGT-3′, antisense, 5′-GCAACACCATCCGCCATTTT-3′; E-cadherin sense, 5′-CGAGAGCTACACGTTCACGG-3′, antisense, 5′-GTGTCGAGGGAAAAATAGGCTG-3′; TGF-β1 sense, 5′-TTGCTTCAGCTCCACAGAGA-3′, and antisense, 5′-TGGTTGTAGAGGGCAAGGAC-3′. Expression levels were normalized to the expression of GAPDH RNA.

Western Blot Analysis

Cells were lysed in cell lysis buffer (50 mM Tris–HCl/pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na_3VO_4; 1 mM NaF; 1 mM okadaic acid; and 1 mg/ml aprotinin, leupeptin, and pepstatin). Proteins (20-40 μg) were separated on 8–10% SDS/PAGE gel and then transferred onto PVDF membranes (Millipore, Billerica, MA). After blocking the membranes with 5% fat free milk in TBST for 1 hr at room temperature, the membranes were incubated with appropriate dilutions of specific primary antibodies overnight at 4°C. After washing, the blots were incubated with HRP-conjugated secondary

antibodies for 1 hr and visualized using the ECL system (Thermo Fisher Scientific, Rochester, NY).

Hematoxylin and eosin (H&E) Staining

The tissue sections were de-waxed and rehydrated routinely. The sections were stained in hematoxylin for 5 min, and washed in running tap water for 5 min. Then the sections were stained in eosin for 30 sec, dehydrated, and mounted by routine methods. We then examined and photographed at least 10 fields per each slide. The consistent and representative fields were presented in the figures.

Histology and Immunohistochemistry

Prostate tissues were fixed in 10% (v/v) formaldehyde in PBS, embedded in paraffin, and cut into 5-μm sections. Immunostaining was performed as described previously (11). For systematic counting of ECs, six ocular measuring fields within a tissue were randomly chosen under a microscope at 400× magnification. The mean number of human CD31-positive (CD+) and CD34-positive (CD+) cells was determined as the ECs count. For AR, TGF-β1, and MMP-9 quantitation, the German Immunoreactive Score (0–12) was calculated by multiplying the percentage of immunoreactive cells (0%=0; 1–10%=1; 11–50%=2; 51–80%=3; 81–100%=4) by the staining intensity (negative=0; weak=1; moderate=2; strong=3). Scores were considered negative (0–1), weakly positive (2–4), moderately positive (6–8), and strongly positive (9–12).

Luciferase assay

PCa cells were plated in 24-well plates and transfected with MMTV-luc containing ARE sequence using Lipofectamine (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. After transfection, RPMI media containing charcoal stripped FBS were added with addition of various concentrations of DHT, 0 (ethanol as vehicle control), 1, and 10 nM, and incubated for 48 hrs. pRL-TK was used as internal control. Luciferase activity was measured using Dual-Luciferase Assay (Promega, Madison, WI) according to the manufacturer's manual.

In vivo animal studies

Male 6- to 8-week old nude mice were used. CWR22rv1 cells were engineered to express luciferase reporter gene (REN/luc, PPM-Mill/luc) by stable transfection and the positive stable clones were selected and expanded in culture (12). 20 mice were injected with PCa cells (10^6 luciferase expressing cells with Matrigel, 1:1) and 10 mice were co-injected with PCa cells co-cultured with HUVECs (10^5) into the anterior prostate (AP). Metastasis in live mice was monitored using a Fluorescent Imager (IVIS Spectrum, Caliper Life Sciences, Hopkinton, MA) at 6 different time points. After monitoring with the Imager, mice were sacrificed and the metastases in lung, lymph node, and bone were further examined by H&E and IHC staining using anti-firefly Luciferase antibody. To culture metastatic cancer cells from peritoneal ascites, the ascites were collected and immediately diluted into 5 ml PBS before coagulation, were washed 3 times in PBS, and then cultured (13, 14). All animal studies were performed under the supervision and guidelines of the University of Rochester Medical Center Animal Care and Use Committee.

Statistics

The data values were presented as the mean ± SD. Differences in mean values between two groups were analyzed by two-tailed Student's *t* test. $p \leq 0.05$ was considered statistically significant.

Results

ECs/Microvascules are increased in PCa compared to normal prostate tissues

We performed immunohistochemical (IHC) staining of the human and mouse originated normal prostate/PCa tissues using ECs specific antibodies, CD31 and CD34, and found that the CD31+ and CD34+ cell numbers were very low in normal prostate tissues. In contrast, significantly increased CD31+ and CD34+ cells were identified in human PCa tissues (Fig. 1A) and TRAMP mouse PCa tissues (Fig. 1B). However, when we compared CD31+ or CD34+ cells in human PCa C4-2 xenografted tumors, we found that these cells decreased after castration, but gradually increased in tissues of the castration resistant tumors (Fig. 1C). Together, results from Fig. 1A-C suggest the existence of ECs in PCa microenvironment may be linked to PCa progression and can be influenced by androgen deprivation.

Co-culture with ECs enhanced invasion ability of PCa cells

We applied a co-culture system to determine whether the presence of ECs could affect PCa cell invasion ability. C4-2 cells were co-cultured with ECs (media as control) and the invasion abilities were compared. Since the established human prostate ECs are not available, human umbilical vein ECs (HUVECs) and human dermal microvascular ECs (HMECs) were used as two different ECs sources as they have been demonstrated to have similar properties compared to the primary human prostate ECs (5) in various ECs-PCa studies (15-17). We found that the invasion abilities of C4-2 cells were increased upon co-culture with HUVECs (Fig. 2A) and similar results were obtained with LNCaP, C81, and CWR22Rv1 cells (Fig. 2B). The results with HMECs were shown in Supplementary Fig. S1. These tests were done in the presence of 1 nM DHT condition, which is the human PCa *in vivo* DHT concentration after ADT (18, 19). Similar results were also obtained when we replaced 1 nM DHT with 10 nM DHT (the human PCa *in vivo* DHT concentration before ADT) (data not shown). Together, results from Fig. 2 suggested that the presence of ECs in the PCa microenvironment might promote PCa invasion before and after ADT in the different PCa cell lines tested.

ECs co-culture mediates down-regulation of AR signal in PCa cells

To dissect the potential mechanisms by which ECs enhanced the invasion abilities of PCa cells, the activation of several signaling pathways in PCa cells was investigated after co-culture with HUVECs/HMECs. Surprisingly, we found significantly decreased AR levels in all PCa cells when co-cultured with ECs (Fig. 3A, B, and Supplementary Fig. S2). To test whether the down-regulation of AR expression is an earlier event than the increased invasion, we performed time course experiments and found that this AR mRNA down-regulation was detected as early as 6 hrs after co-culture incubation (Fig. 3A, B, and Supplementary Fig. S2), suggesting that altered AR signaling preceded the increased invasion. AR transactivation was also tested in ARE-driven luciferase assay, and as expected, when PCa cells were co-cultured with ECs, the AR mediated ARE-luciferase activity was significantly decreased (Fig. 3C), suggesting that both the expression level and AR transactivation activity were decreased upon co-culture with ECs.

To investigate whether the down-regulation of AR signaling is the key step in mediating the enhanced metastatic potential of PCa cells, invasion abilities of the C4-2 cells were tested after selective knockdown of AR by siRNA strategy. Consistent with previous co-culture studies, the knockdown of AR significantly increased C4-2 cells invasion (Fig. 3D). However, when we co-cultured these AR knocked down PCa cells with HUVECs, we no longer could see the HUVECs effect in promoting PCa invasion ability, indicating that the AR down-regulation is critical in triggering ECs-induced invasion ability of the C4-2 cells.

We also compared the HUVECs influence on the invasion ability of the PC3 cells (lack AR expression), vs PC3-AR9 cells (with stably transfected human AR) (20). Consistent with the C4-2 cell results, the HUVECs effect in promoting PCa metastasis was shown lower in PC3 cells compared to the PC3AR9 cells (Fig. 3E). Together, results from Fig. 3A-E suggest that ECs exert their effect on promoting PCa invasion abilities via down-regulation of AR signaling in the PCa cells.

IL-6 is a key mediator for AR down-regulation in ECs-PCa co-culture cells

It was reported that ECs secrete chemokines/cytokines/growth factors to exert their paracrine effect (21). Therefore, we speculated that the ECs effect in enhancing the invasion ability of PCa cells could be through the paracrine effect. We performed cytokine array to investigate whether the secreted chemokines/cytokines were changed in HUVECs after co-culture with PCa C4-2 cells, and as shown in Fig. 4A, we found the levels of IL-4, IL-6, and IL-8 were increased. We then independently assayed the mRNA levels of all reported and related cytokines/chemokines/growth factors in HUVECs, with or without co-culture with PCa cells (22, 23). The results showed that the levels of several cytokines and chemokines including CCL5 and IL-6 in ECs were increased upon co-culture with PCa cells (Supplementary Fig. S1A and B).

From these two analyses, we speculate that IL-6 and IL-8 are the best possible candidate molecules secreted by ECs to affect the invasion ability of PCa cells. IL-6 has been considered as an important growth-regulatory factor in human PCa (21, 24) and has roles in metastases and morbidity (25). IL-8 has also been reported to be associated with increased metastatic ability of cancer cells (26, 27). In contrast, few reports linked IL-4 to the risk or progression of PCa (28).

To confirm the above points, we tested the effect of IL-6, IL-4, and IL-8 in down-regulating the AR signaling and their ability to increase the invasion ability of PCa cells. As shown in Supplementary Fig. S3C, the AR expression was decreased in C4-2 cells incubated with IL-6, but not with IL-4 or IL-8. In addition, IL-6 could effectively increase invasion, IL-4 had some moderate effects, and IL-8 failed to change invasion capability of PCa C4-2 cells (Supplementary Fig. S3D). Also, analysis of IL-6 mRNA and ELISA demonstrated that the co-culture of PCa cells significantly increased the IL-6 secretion in ECs (Fig. 4B-C). Western blot and luciferase activity analyses revealed that the IL-6 treatment could down-regulate AR expression in androgen-dependent LNCaP cells as well as moderately decreased AR in castration resistant C4-2, C81, and CWR22rv1 cells (Fig. 4D), IL6 treatment also decreased AR transactivation (Fig. 4E). Importantly, adding IL-6 neutralizing antibody in co-cultured ECs-PCa cells reversed the HUVECs effects on AR down-regulation and the increased invasion abilities of C4-2 and CWR22Rv1 cells (Fig. 4F-G). Together, results from Fig. 4F-G suggest that IL-6 is the key molecule secreted from ECs to impact the down-regulation of AR in PCa cells that results in enhanced PCa cell invasion.

Down-regulation of AR resulted in the decreased E-cadherin level with increased TGF-β1 and MMP-9 levels

To dissect the molecular mechanisms by which IL-6 mediated down-regulation of AR in PCa led to increased PCa cell invasion, we examined expression levels of the epithelial-mesenchymal transition (EMT) markers, E-cadherin (E-cad), N-cad, vimentin, and Snail, since the EMT is known to be an important step in the initiation of early dissemination that leads to metastasis (29). Interestingly, we found that the expression of E-cad was significantly decreased in PCa cells upon co-culture with ECs (Fig. 5A), but we failed to observe an increase in N-cad (Supplementary Fig. S4A), which usually accompanies the E-cad decrease. We also failed to detect expression changes in the other EMT markers,

vimentin and Snail (Supplementary Fig. S4A and B). However, while investigating the expressions of TGF-β1 and MMP-9, known as critical molecules in EMT process (30), we found significant increases in PCa cells when co-cultured with HUVECs (Fig. 5A). To further confirm whether TGF-β1 and MMP-9 are the downstream molecules of the AR signaling, we compared their expression in the AR knocked down C4-2 cells and scramble control cells. As shown in Fig. 5B, the expression levels of TGF-β1 and MMP-9 were increased when AR expression was knocked down, indicating that the AR down-regulation is essential in mediating increased expressions of these EMT related molecules in PCa cells.

It was reported that TGF-β1 up-regulates the expression of MMP-9, which is closely associated with tumor invasion (31, 32). Therefore, we treated PCa cells with the TGF-β1 inhibitor, SB431542, to test whether the inhibition of the TGF-β1 pathway can block the HUVECs-induced PCa cell invasion. Our data showed that this TGF-β1 inhibitor treatment blocked the increased invasion ability of PCa cells significantly (Fig. 5D), confirming the TGF-β1 role in mediating ECs-increased PCa cell invasion.

We then added IL-6 into the ECs-PCa co-culture system to see if the IL-6 can down-regulate AR signaling in PCa cells to alter expressions of TGF-β1 and MMP-9 (Fig. 3 and 4). As shown in Fig. 5C, the IL-6 treatment indeed increased expressions of these molecules, and once again confirmed that IL-6 is a critical ECs secreted factor to mediate down-regulation of AR signaling and the consequent increases of TGF-β1 and MMP-9 in PCa. Together, results from Figs. 4-5 indicated that ECs may influence PCa cell invasion via the IL6→AR→TGF-β1→MMP-9 signaling pathway and blocking these signals (either by IL-6 antibody, AR-siRNA, or TGF-β1 inhibitor) interrupted the ability of ECs to influence PCa invasion.

In vivo xenografted mice demonstrate ECs effect in enhancing PCa metastasis

To confirm the above *in vitro* cell lines results showing ECs promote the metastatic ability of PCa cells *in vivo*, PCa CWR22Rv1 cells were orthotopically implanted, either alone or co-implanted with HUVECs, into the APs of the nude mice. After injection, the metastatic incidence in these two groups of mice was monitored using *in vivo* imaging system (IVIS). As shown in Fig. 6A, the metastatic incidence of the co-implantation group was significantly increased showing more tumors mainly in the lymph nodes and diaphragm compared to the control group (Fig. 6A-D). The PCa cells were also detected in the peritoneal ascites fluids in a few cases (Fig. 6E). The morphology of the primary cultured tumor cells isolated from ascites resembled the original CWR22Rv1 cells, indicating that these circulating cells are from the primary tumor site (Fig. 6E). Importantly, we also examined the expression levels of AR, TGF-β1, and MMP-9 in the primary tumors and found that AR expressions were decreased while the expressions of TGF-β1 and MMP-9 were increased in the tissues of the co-implantation group mice compared to the control group mice tissues, which was consistent with our *in vitro* data (Fig. 6F). We used only one mouse model in the *in vivo* animal studies, so further studies are needed to confirm the contribution of ECs in PCa metastasis.

Discussion

The intense neovascularization surrounding tumors suggest their roles not only in supplying nutrients for the continued tumor growth but also in initiating angiogenesis by seeding tumor cells into the blood stream in microvascules (33). We found that EC numbers were increased in PCa vs normal tissues and following castration/ADT, compared to before castration/ADT treatment, although further studies using a set of sequential specimens in human tissues are necessary to support these findings.

We demonstrated that ECs may also play an important role in enhancing the metastatic potential of PCa both *in vitro* and *in vivo*. These new findings will add insights into ECs contribution to PCa metastasis and emphasize the importance of ECs as a component of the TME.

In mediating ECs role in enhancing the metastatic potential of PCa, we showed that the ECs action in enhancing the metastatic ability of PCa was via down-regulation of AR, which may challenge the current understanding that AR plays a positive role to promote PCa progression (34-38). Up-to-date, most of the efforts for decades have applied ADT strategy via suppression of the androgen/AR signaling to battle PCa (39-42), so suggesting the suppressor role of AR in increasing PCa metastasis is novel and challenging. Several recent studies support this idea. The recently published reports on clinical studies suggest that ADT might increase metastases in some patients (43, 44). Increased expressions of the EMT related markers, such as N-cadherin (45), Cadherin-11 (46, 47), and nestin (48), were found in human clinical PCa samples after ADT. Cell line studies also demonstrated that ADT causes EMT transition (49). Since the EMT process is highly correlated with metastases (50, 51), these results supported the idea that ADT enhances PCa metastases. ADT with surgical castration was also demonstrated to lead to increased lymph node (52) or distant (53) metastases. Furthermore, Niu et al (54) found that mice with AR knock down in prostate epithelial cells developed increased metastatic PCa, with mice dying earlier than in the TRAMP mouse model. Therefore, the results showing AR down-regulation in PCa cells upon ECs co-culture and increasing PCa metastasis in this study is consistent with these new emerging concepts.

Among several cytokines/chemokines/growth factors identified from ECs to exert paracrine effects to influence PCa metastasis, we found IL-6 was the strongest candidate molecule and we believe even other cytokines, such as IL-4 and IL-8, might also contribute to enhancing PCa metastases, but they might act via different mechanisms, and not via down-regulation of AR. IL-6 is known to be increased in patients with advanced stages of PCa (55, 56), play an important role in PCa progression (57), and can be secreted from several cell types, including macrophages (58) and adipocytes (59). In this study we found ECs are another source of IL-6.

VEGF has been suggested as a critical molecule to target ECs mediated angiogenesis (60, 61). However, none of our results showed significantly increased VEGF levels when co-cultured with PCa cells nor increased invasion ability of PCa cells upon addition of VEGF (data not shown).

We found TGF-β and MMP9 were key molecules mediating IL-6-AR signals to enhance the metastatic potential of PCa. These two molecules are known to be the multifunctional factors during diverse physiological and pathological processes including development, wound healing, proliferation, and cancer metastasis (62). TGF-β is a growth suppressive cytokine in many normal situations, but becomes an active and important participant in malignant disease functions including angiogenesis, extracellular matrix deposition, immuno-suppression, and metastasis growth promotion (63). Zhang et al (64) investigated the TGF-β role in growth and metastasis of the highly metastatic PC-3MM2 human PCa cells and found that TGF-β signaling enhanced tumor angiogenesis by regulating IL-8 expression in tumor cells. TGF-β1 was also shown to enhance PCa PC3 cell invasion by an uPA/plasmin-dependent mechanism to play a key role in malignant PCa progression (65). Recently, several studies have shown that TGF-β1 can up-regulate MMP-9 expression and activity in other cells, such as human skin (66), corneal epithelial cells (67), and brain astrocytes (62). These results, together with our current findings may allow us to develop a

new therapeutic approach based on targeting these two molecules to block ECs-promoted PCa metastasis.

We also found E-cad decreases in PCa cells upon ECs-PCa co-culture, but failed to observe the difference of other EMT markers. It will be interesting to see whether the increase of TGF-β/MMP9 were due to the E-cad level changes or if these two are separate signals.

Based on these studies, we believe that development of a combination therapy to block two processes, tumor proliferation where AR plays positive role, and metastasis in which AR plays negative roles, is essential. The combination therapy targeting tumor growth (by the classical ADT) and angiogenesis (by blocking VEGFR tyrosine kinase) has been attempted (68). A therapy targeting bone and brain metastasis has also been suggested (69). Maybe in the near future, development of an effective therapeutic strategy to interrupt the ECs mediated IL6→AR→TGFβ→MMP9 signaling pathway identified here, to suppress metastasis after classical anti-proliferation therapy to suppress PCa progression, may help us to better battle PCa. Further *in vivo* mice studies to test therapeutic approaches need to be performed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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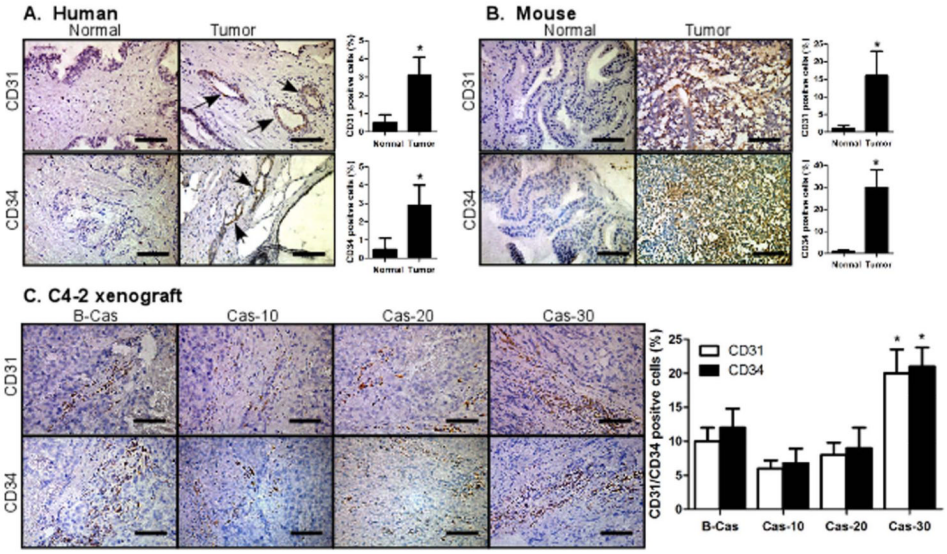

Fig. 1. Endothelial cells/microvasucles are increased in PCa vs normal tissues

(A-B) Immunohistochemical staining of normal prostate and prostate tumor tissues. (A) Human patient tissues and (B) TRAMP mouse tissues. Human normal prostate and prostate tumor tissues were obtained from young (about 30 years of age) normal organ donors and patients with radical resection of prostate cancer, respectively, at the Shanghai First People's Hospital (Shanghai, China). TRAMP tumors were obtained from 28 wks old TRAMP mice (B6 background). (C) Immunohistochemical staining of C4-2 orthotopic xenografted tumor tissues, before and after castration. C4-2 cells were orthotopically injected into APs of mice. After tumors developed, mice were castrated and castration resistant tumors allowed to re-develop. Mice were sacrificed 10, 20, and 30 days after castration and tumors were obtained. Tumors from some mice before castration were used as controls. Tumor tissues were immunohistochemically stained with CD31 and CD34 antibodies. Black arrows represent CD31/CD34 positive cells.

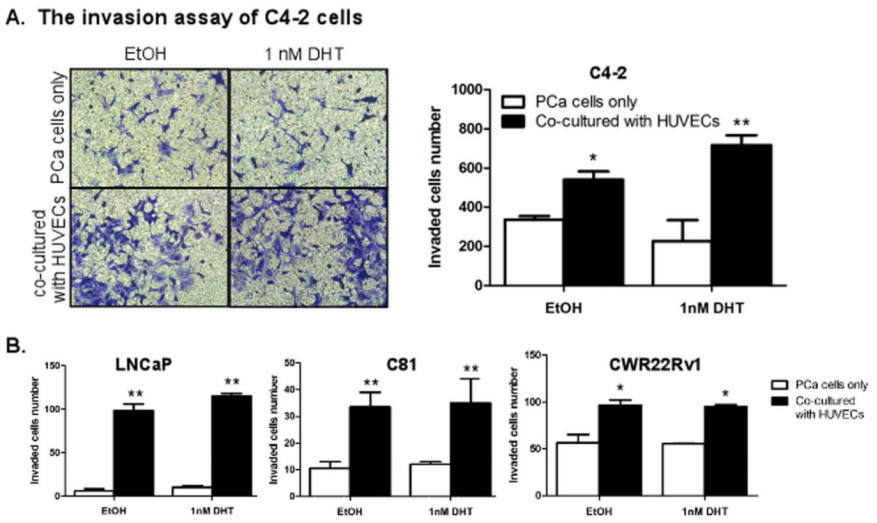

Fig. 2. Endothelial cells enhance PCa cells invasion *in vitro*

(A) C4-2 cells (1×10^5/well) were co-cultured with HUVECs and invasion assay performed in the presence of 0 (EtOH) and 1 nM concentrations of DHT. Invaded cells were stained with toluidine blue and the positively stained cells were counted from six random fields. (B) LNCaP, C81, and CWR22Rv1 cells were used for invasion assay, similar to (A). Results are expressed as mean ± SD. Different numbers between two groups were analyzed by two-tailed Student's *t* test. * $p < 0.05$, ** $p < 0.01$.

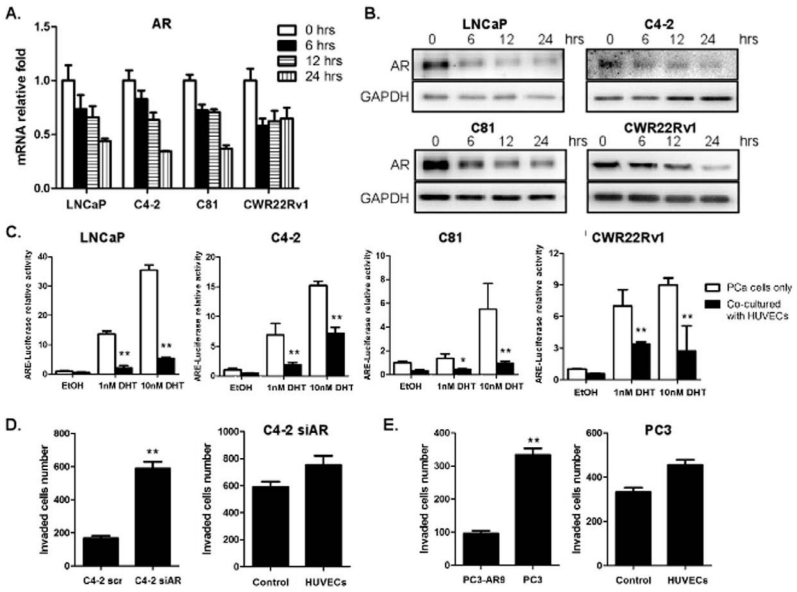

Fig. 3. Endothelial cells down-regulate AR signaling in PCa cells

(A-B) LNCaP, C4-2, C81 and CWR22Rv1 cells (1×10^5/well) were co-cultured with HUVECs (medium for control) in transwell plates (3 μm) for 6, 12, 24 hrs. (A) Total RNAs were extracted and AR mRNAs level was analyzed by qRT-PCR. (B) PCa cell extracts were obtained for Western blot analysis for AR expression. (C) LNCaP, C4-2, C81 and CWR22Rv1 cells were transfected with MMTV-luc containing ARE and co-cultured with HUVECs (medium for control) in the presence of various concentrations of DHT as indicated. After 24 hrs, luciferase activity was measured. (D) C4-2 cells, transfected with either AR-siRNA or scramble control, were used in invasion assays as in 2A. (E) PC3 and PC3AR9 cells were used in invasion assay, similar to (D). All experiments were repeated three times. Data were presented as mean ± SD. (*$p < 0.05$, **$p < 0.01$).

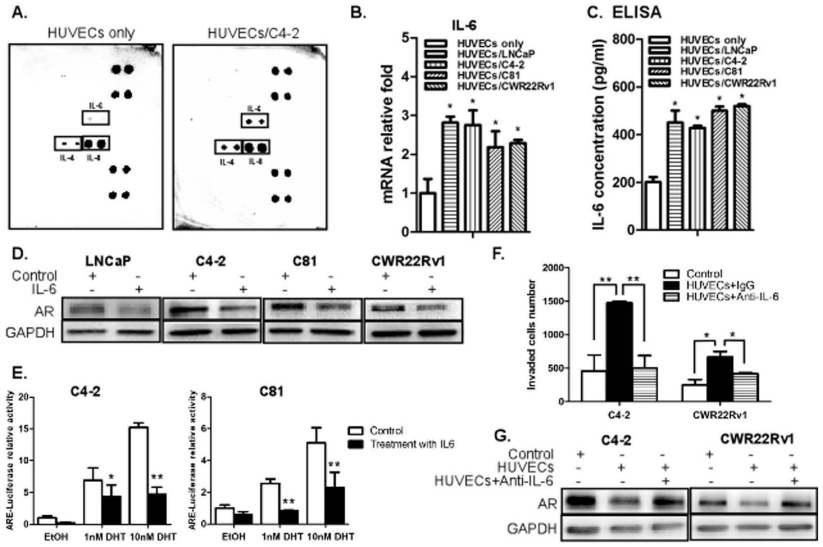

Fig. 4. IL-6 is a potential mediator to down-regulate AR signaling, which in turn, enhances invasion ability of PCa cells

(A) Cytokine array analysis. HUVECs were co-cultured with or without C4-2 cells for 2 days and the conditioned media (CM) were collected for cytokine array analysis. (B) qPCR analysis showing mRNA levels of IL-6. Total RNAs were extracted from HUVECs, either with or without co-culture with PCa cells, cDNAs obtained by reverse transcriptase reaction, and the mRNA levels of IL-6 were analyzed. (C) IL-6 ELISA assay result demonstrating increase in IL-6 secretion in PCa cells upon co-culture with HUVECs. HUVECs were co-cultured with LNCaP, C4-2, C81 and CWR22Rv1 cells for 2 days and the CM were collected for ELISA. (D) LNCaP, C4-2, C81 and CWR22Rv1 cells (1×10^5/well) were treated with 20 ng/ml of human recombinant IL-6 (Prospec Bio) for 6 hrs. PCa cell extracts were used for Western blot analysis for AR expression. (E) C4-2 and C81 cells were transfected with MMTV-luc containing ARE. After transfection, cells were treated with 20 ng/ml of IL-6 for 24 hrs in the presence of various concentrations of DHT as indicated, and luciferase activity was measured. (F) C4-2 and CWR22Rv1 cells were co-cultured with HUVECs in the presence of anti-IL-6 neutralizing antibody (R&D systems) (normal IgG as control) for 2 days and invasion assay was performed. (G) PCa cell extracts were obtained under the similar conditions as in (F) and used for the Western blot analysis for AR. Data are mean ± SD. (*$p < 0.05$, **$p < 0.01$).

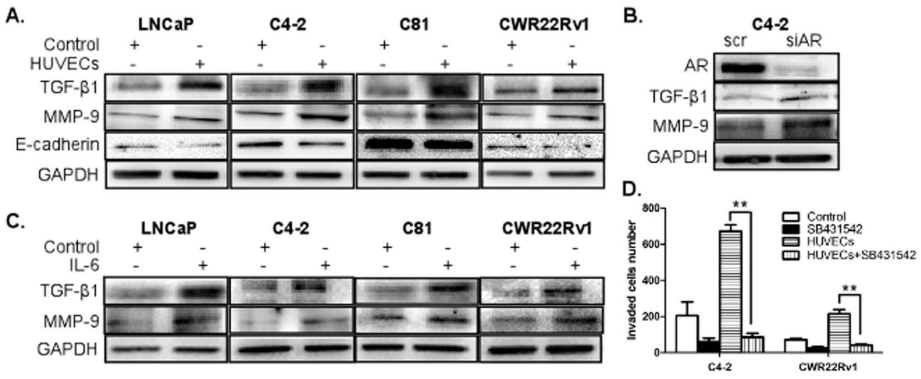

Fig. 5. Down-regulation of AR signals resulted in decrease in E-cadherin level, but increase in TGF-β1 and MMP-9

(A) LNCaP, C4-2, C81, and CWR22Rv1 cells (1×10^5/well) were co-cultured with HUVECs (medium for control) for 48 hrs in transwell plates. PCa cell extracts were obtained for Western blot analyses using antibodies for TGF-β1 (Santa Cruz Biotechnology), MMP-9 (Abcam) and E-cadherin (MAB1838, R&D systems) expressions. (B) C4-2 cells were infected with lentivirus carrying AR-siRNA or scrambled control. Cell extracts were obtained for Western blot analysis for the expression analysis of the indicated molecules. (C) LNCaP, C4-2, C81, and CWR22Rv1 cells (1×10^5/well) were treated with 20 ng/ml of IL-6 for 48 hrs. PCa cell extracts were obtained for Western blot analyses for TGF-β1 and MMP-9 expressions. (D) C4-2 and CWR22Rv1 cells were co-cultured with HUVECs (medium for control) in the presence of TGF-β1 inhibitor, SB431542 for 2 days and invasion assays were performed.

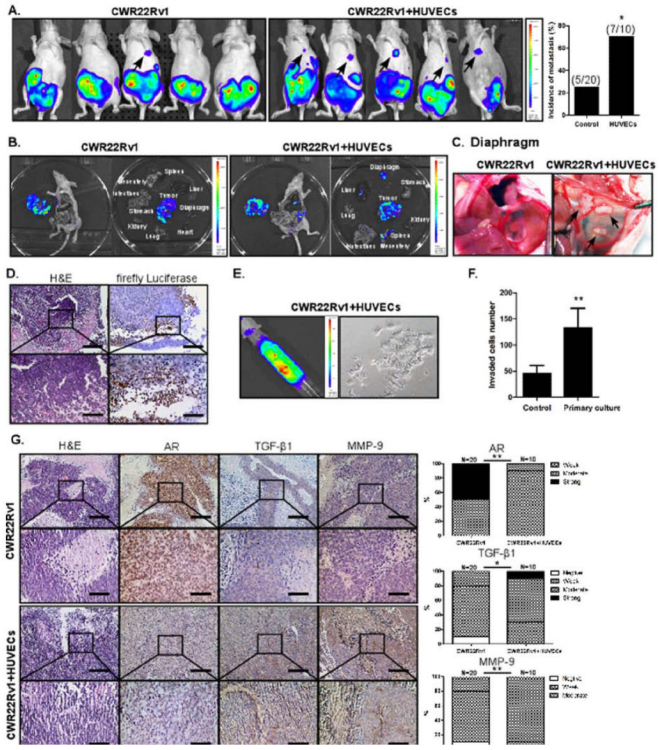

Fig. 6. HUVECs treatment enhances PCa metastasis in orthotopic xenografted mice

CWR22Rv1 cells were transfected with luciferase (Luciferase-pcDNA3, Addgene), stable clones were selected, and their luciferase activity was confirmed before injection. 1×10^6 of these cells, either alone or together with HUVECs (10:1 PCa cells:HUVECs), as a mixture with Matrigel, 1:1, total of 20 μl, were orthotopically implanted into the APs of 8 wks old mice. Tumor growth and metastasis was monitored by examining luminescence using IVIS at 3, 4, 5, and 6 wks after injection. (A) The metastatic incidence shown in two groups of mice. (B) The imaging data showing primary and metastatic tumors of two mice groups. (C) The imaging demonstrating diaphragm metastasis. (D) H&E and IHC staining of metastatic tumors from diaphragm using antibodies of anti-firefly Luciferase antibody (Abcam). (E) The imaging demonstrating the ascites metastases obtained from metastatic mouse (left panel) and primary cultures cells from ascites (right panel). (F) Invasion assay of primary cultured CWR22Rv1 from ascites (parental CWR22Rv1 cells as control). (G) H&E and IHC staining of primary and metastatic tumors using antibodies of AR, TGF-β1, and MMP-9.