Endothelial Cells Enhance Prostate Cancer Metastasis via IL-6→Androgen Receptor→TGF-β→MMP-9 Signals

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Abstract

Although the potential roles of endothelial cells in the microvascules of prostate cancer during angiogenesis have been documented, their direct impacts on the prostate cancer metastasis remain unclear. We found that the CD31-positive and CD34-positive endothelial cells are increased in prostate cancer compared with normal tissues and that these endothelial cells were decreased upon castration, gradually recovered with time, and increased after prostate cancer progressed into the castration-resistant stage, suggesting a potential linkage of these endothelial cells with androgen deprivation therapy. The in vitro invasion assays showed that the coculture of endothelial cells with prostate cancer cells significantly enhanced the invasion ability of the prostate cancer cells. Mechanism dissection found that coculture of prostate cancer cells with endothelial cells led to increased interleukin (IL)-6 secretion from endothelial cells, which may result in downregulation of androgen receptor (AR) signaling in prostate cancer cells and then the activation of TGF-β1/matrix metalloproteinase-9 (MMP-9) signaling. The consequences of the IL-6→AR→TGFβ1→MMP-9 signaling pathway might then trigger the increased invasion of prostate cancer cells. Blocking the IL-6→AR→TGFβ1→MMP-9 signaling pathway either by IL-6 antibody, AR-siRNA, or TGF-β1 inhibitor all interrupted the ability of endothelial cells to influence prostate cancer invasion. These results, for the first time, revealed the important roles of endothelial cells within the prostate cancer microenvironment to promote the prostate cancer metastasis and provide new potential targets of IL-6→AR→TGFβ1→MMP-9 signals to battle the prostate cancer metastasis. Mol Cancer Ther; 12(6): 1–12. ©2013 AACR.

Introduction

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

Emerging evidence indicates that endothelial cells may contribute to the development and progression of prostate cancer (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, endothelial cells are subjected to apoptosis and their numbers decrease. Interestingly, it was shown that the endothelial cells of prostate only, not the endothelial cells 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 prostate cancer, which is frequently accompanied by a high incidence of distant metastasis.

Up-to-date, the focus on the role of endothelial cells in prostate cancer has been in the angiogenesis process. However, the recent reports suggest that there is a mutual interaction of prostate cancer cells and endothelial cells (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 endothelial cells residing in microvascules to affect prostate cancer cells.

In this study, we used multiple in vitro and in vivo strategies to show that, other than their angiogenesis...
functions, endothelial cells can secrete cytokines to inhibit the androgen receptor (AR) function and induce prostate cancer metastasis. The mechanisms by which these endothelial cells contribute to the enhanced metastatic potential of prostate cancer cells were also investigated.

Materials and Methods

Cell lines and coculture experiments

Human umbilical vein endothelial cells (HUVEC), human dermal microvascular endothelial cells (HMEC), LNCaP, C4-2, C81, and CWR22Rv1 cell lines were purchased from the American Type Culture Collection (ATCC). HUVECs were cultured in endothelial cell medium supplemented with growth factors (ATCC) and HMECs were cultured in MCDB131 (Gibco) supplemented with 1 μg/mL hydrocortisone, 10 ng/mL EGF, and 10% 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) were used for coculture and invasion assay, respectively. Cell lines used in these studies were authenticated.

Lentiviral infection

For incorporation of AR-siRNA or scramble control plasmids into prostate cancer cells, lentivirus carrying either control (pLVTHM-scramble) or AR-siRNA (pLVTHM-AR-siRNA) was transduced into HEK293T cells with a mixture of pLVTHM-scramble/pLVTHM-AR-siRNA, psPAX2 (virus packaging plasmid), and pMD2G (envelope plasmid; 4:3:2 ratio) by calcium–phosphate transfection. Culture medium containing virus was collected 32 hours after transfection and filtrated through a 0.4 μm filter to remove cell debris or cells. The collected viruses were added to the target cells in the presence of polybrene (2 μg/mL) to incubate for 24 hours. Cells were refreshed with culture medium and cultured for another 3 days to allow target protein expression. Because the lentiviral vectors express GFP, fluorescence microscopy was used to monitor the infection efficiency via checking the green fluorescence signal.

Cell invasion assay

For in vitro invasion assays, the top chambers of the Transwells were precoated with diluted Matrigel (1:3; BD Biosciences). Before the invasion assays, prostate cancer cells were cocultured with HUVECs (endothelial cells culture medium for control) for 48 hours in Transwell plates. A total of 10^5 prostate cancer cells (in serum-free media) and 10% serum-containing media were plated in the top and bottom chambers, respectively. After 24 to 48 hours of incubation, the cells in the top 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 6 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 was collected from HUVECs culture or HUVECs prostate cancer coculture 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), whereas the IL-6 ELISA Kit (eBioscience) was applied to measure interleukin (IL)-6 level in the conditioned medium. 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) according to the manufacturer’s instructions. One μg of total RNA was subject to reverse transcription using Superscript III transcriptase (Invitrogen). 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'-TATCCTGG-TGGAGTTGTG-3'; antisense, 5'-CAGAGTACATCCTCGCTTCTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-AAATCGGTACATCTCCACGG-3'; antisense, 5'-GTGGCTGG GGCTCTACTTC-3'; CCL5 sense, 5'-ATCCCTATTGCTACTGCCCTC-3'; antisense, 5'-GCCACTGTGTGAAATACTCC-3'; IL-6 sense, 5'-AAATTCGGTACATCCTCGACCG-3'; antisense, 5'-GGAGGTTCAGTTGTGTTCATGC-3'; IL-8 sense, 5'-TG GGACTGTCATG-AATCTGT-3'; antisense, 5'-GCAACACCATCCGCATTTT-3'; E-cadherin sense, 5'-CG AGAGCTACACGTTCAGTG-3'; antisense, 5'-GTCG AGGGAAAAATAGGCGT-3'; TGF-β1 sense, 5'-TTGCT CTACCTCCACAGAGA-3'; and antisense, 5'-TGTTGT GTAGGGCAAGGAC-3'. Expression levels were normalized to the expression of GAPDH RNA.

Western blot analysis

Cells were lysed in cell lysis buffer (50 mmol/L Tris-HCl/pH 7.4; 1% NP-40; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonylfluoride; 1 mmol/L Na3VO4; 1 mmol/L NaF; 1 mmol/L okadac acid; and 1 mg/mL aprozin, leupeptin, and pepstatin). Proteins (20–40 μg) were separated on 8% to 10% SDS-PAGE gel and then transferred onto PVDF membranes (Millipore). After blocking the membranes with 5% fat-free milk in Tris buffered saline with Tween 20 for 1 hour 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 horseradish peroxidase-conjugated secondary antibodies for 1 hour and visualized using the ECL System (Thermo Fisher Scientific).

Hematoxylin and eosin staining

The tissue sections were dewaxed and rehydrated routinely. The sections were stained in hematoxylin for 5 minutes and washed in running tap water for 5 minutes.
Then, the sections were stained in eosin for 30 seconds, 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 conducted as described previously (11). For systematic counting of endothelial cells, 6 ocular measuring fields within a tissue were randomly chosen under a microscope at ×400 magnification. The mean number of human CD31-positive (CD31+) and CD34-positive (CD34+) cells was determined as the endothelial cells count. For AR, TGF-β1, and matrix metalloproteinase 9 (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**

Prostate cancer cells were plated in 24-well plates and transfected with mouse mammary tumor virus (MMTV)-luc containing ARE sequence using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. After transfection, RPMI media containing charcoal-stripped FBS were added with addition of various concentrations of dihydrotestosterone (DHT), 0 (ethanol as vehicle control), 1, and 10 nmol/L, and incubated for 48 hours. pRL-TK was used as internal control. Luciferase activity was measured using Dual-Luciferase Assay (Promega) 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). Twenty mice were injected with prostate cancer cells (10⁶ luciferase-expressing cells with Matrigel, 1:1) and 10 mice were injected without Matrigel as control. Invasion was measured by in vivo Imaging System (IVIS Spectrum, Caliper Life Sciences) 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 hematoxylin and eosin (H&E) and immunohistochemical (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 conducted under the supervision and guidelines of the University of Rochester Medical Center Animal Care and Use Committee.

**Statistical analysis**

The data values were presented as the mean ± SD. Differences in mean values between 2 groups were analyzed by 2-tailed Student t test. P ≤ 0.05 was considered statistically significant.

**Results**

**Endothelial cells/microvascules are increased in prostate cancer compared with normal prostate tissues**

We conducted IHC staining of the human and mouse originated normal prostate/prostate cancer tissues using endothelial cell–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 prostate cancer tissues (Fig. 1A) and TRAMP mouse prostate cancer tissues (Fig. 1B). However, when we compared CD31+ or CD34+ cells in human prostate cancer 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 that the existence of endothelial cells in prostate cancer microenvironment may be linked to prostate cancer progression and can be influenced by androgen deprivation.

**Coculture with endothelial cells enhances invasion ability of prostate cancer cells**

We applied a coculture system to determine whether the presence of endothelial cells could affect prostate cancer cell invasion ability. C4-2 cells were cocultured with endothelial cells (media as control) and the invasion abilities were compared. Because the established human prostate endothelial cells are not available, HUVECs and HMECs were used as 2 different endothelial cells sources as they have been shown to have similar properties compared with the primary human prostate endothelial cells (5) in various endothelial cell–prostate cancer studies (15–17). We found that the invasion abilities of C4-2 cells were increased upon coculture 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 nmol/L DHT condition, which is the human prostate cancer in vivo DHT concentration after ADT (18, 19). Similar results were also obtained when we replaced 1 nmol/L DHT with 10 nmol/L DHT (the human prostate cancer in vivo DHT concentration before ADT; data not shown). Together, results from Fig. 2 suggested that the presence of endothelial cells in the prostate cancer microenvironment might promote prostate cancer invasion before and after ADT in the different prostate cancer cell lines tested.
Endothelial cells coculture mediates downregulation of AR signal in prostate cancer cells

To dissect the potential mechanisms by which endothelial cells enhance the invasion abilities of prostate cancer cells, the activation of several signaling pathways in prostate cancer cells was investigated after coculture with HUVECs/HMECs. Surprisingly, we found significantly decreased AR levels in all prostate cancer cells when cocultured with endothelial cells (Fig. 3A and B and Supplementary Fig. S2). To test whether the downregulation of AR expression is an earlier event than the increased invasion, we carried out time course experiments and found that this AR mRNA downregulation was detected as early as 6 hours after coculture incubation (Fig. 3A and 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 prostate cancer cells were cocultured with endothelial cells, the AR-mediated ARE-luciferase activity was significantly decreased (Fig. 3C), suggesting that both the expression level and AR transactivation activity were decreased upon coculture with endothelial cells.

To investigate whether the downregulation of AR signaling is the key step in mediating the enhanced metastatic potential of prostate cancer cells, invasion abilities of the C4-2 cells were tested after selective knockdown of AR by siRNA strategy. Consistent with previous coculture studies, the knockdown of AR significantly increased C4-2 cells’ invasion (Fig. 3D). However, when we cocultured these AR knocked down prostate cancer cells with HUVECs, we no longer could see the HUVECs effect in promoting prostate cancer invasion ability, indicating that the AR downregulation is critical in triggering endothelial cells-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) versus PC3AR9 cells (with stably transfected human AR; ref. 20). Consistent with the C4-2 cell results, the HUVECs effect in promoting prostate cancer metastasis was shown lower in PC3 cells than in the PC3AR9 cells (Fig. 3E). Together, results from Fig. 3A–E suggest that endothelial cells exert their effect on promoting prostate cancer invasion abilities via
downregulation of AR signaling in the prostate cancer cells.

**IL-6 is a mediator for AR downregulation in endothelial cells-prostate cancer coculture cells**

It was reported that endothelial cells secrete chemokines/cytokines/growth factors to exert their paracrine effect (21). Therefore, we speculated that the endothelial cells effect in enhancing the invasion ability of prostate cancer cells could be through the paracrine effect. We conducted cytokine array to investigate whether the secreted chemokines/cytokines were changed in HUVECs after coculture with prostate cancer C4-2 cells, and as shown in Fig. 4A, we found that 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 coculture with prostate cancer cells (22, 23). The results showed that the levels of several cytokines and chemokines including CCL5 and IL-6 in endothelial cells were increased upon coculture with prostate cancer cells (Supplementary Fig. S1A and S1B).

From these 2 analyses, we speculate that IL-6 and IL-8 are the best possible candidate molecules secreted by endothelial cells to affect the invasion ability of prostate cancer cells. IL-6 has been considered as an important growth-regulatory factor in human prostate cancer (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 prostate cancer (28).

To confirm the above points, we tested the effect of IL-6, IL-4, and IL-8 in downregulating the AR signaling and their ability to increase the invasion ability of prostate cancer 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 effect, and IL-8 failed to change invasion capability of prostate cancer C4-2, C81, and CWR22Rv1 cells (Supplementary Fig. S3D). Also, analysis of IL-6 mRNA and ELISA showed that the coculture of prostate cancer cells significantly increased the IL-6 secretion in endothelial cells (Fig. 4B and C). Western blot analysis and luciferase activity analyses revealed that the IL-6 treatment could downregulate AR expression in androgen-dependent LNCaP cells as well as moderately decrease AR in castration-resistant C4-2, C81, and CWR22Rv1 cells (Fig. 4D). IL-6 treatment also decreased AR transactivation (Fig. 4E). Importantly, adding IL-6–neutralizing antibody in cocultured endothelial cells-prostate cancer cells reversed the HUVECs effects on AR downregulation and the increased invasion abilities of C4-2.
and CWR22Rv1 cells (Fig. 4F and G). Together, results from Fig. 4F and G suggest that IL-6 is the key molecule secreted from endothelial cells to impact the downregulation of AR in prostate cancer cells that results in enhanced prostate cancer cell invasion.

Downregulation of AR results in decreased E-cadherin level with increased TGF-β1 and MMP-9 levels

To dissect the molecular mechanisms by which IL-6–mediated downregulation of AR in prostate cancer led to the increased prostate cancer cell invasion, we examined expression levels of the epithelial–mesenchymal transition (EMT) markers, E-cadherin, N-cadherin, vimentin, and Snail, as 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-cadherin was significantly decreased in prostate cancer cells upon coculture with endothelial cells (Fig. 5A), but we failed to observe an increase in N-cadherin (Supplementary Fig. S4A), which usually accompanies the E-cadherin decrease. We also failed to detect expression changes in the other EMT markers, vimentin and Snail (Supplementary Fig. S4A and S4B). However, while investigating the expressions of TGF-β1 and MMP-9, known as critical molecules in EMT process (30), we found significant increases in prostate cancer cells when cocultured 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 downregulation is essential in mediating increased expressions of these EMT-related molecules in prostate cancer cells.

Figure 3. Endothelial cells downregulate AR signaling in prostate cancer cells. A and B, LNCaP, C4-2, C81, and CWR22Rv1 cells (1 x 10^5/well) were cocultured with HUVECs (medium for control) in Transwell plates (3 μm) for 6, 12, 24 hours. A, Total RNAs were extracted and AR mRNA levels were analyzed by qRT-PCR. B, prostate cancer 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 cocultured with HUVECs (medium for control) in the presence of various concentrations of DHT as indicated. After 24 hours, luciferase activity was measured. D, C4-2 cells, transfected with either AR-siRNA or scramble control, were used in invasion assays as in Fig. 2A. E, PC3 and PC3AR9 cells were used in invasion assay, similar to D. All experiments were repeated 3 times. Data were presented as mean ± SD. **P < 0.05; ***P < 0.01.
It was reported that TGF-β1 upregulates the expression of MMP-9, which is closely associated with tumor invasion (31, 32). Therefore, we treated prostate cancer cells with TGF-β1 inhibitor, SB431542, to test whether the inhibition of the TGF-β1 pathway can block the HUVECs-induced prostate cancer cell invasion. Our data showed that this TGF-β1 inhibitor treatment blocked the increased invasion ability of prostate cancer cells significantly (Fig. 5D), confirming the TGF-β1 role in mediating endothelial cell-increased prostate cancer cell invasion.

We then added IL-6 into the endothelial cell–prostate cancer coculture system to see whether the IL-6 can downregulate AR signaling in prostate cancer cells to alter expressions of TGF-β1 and MMP-9 (Figs. 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 endothelial cell–secreted factor to mediate downregulation of AR signaling and the consequent increases of TGF-β1 and MMP-9 in prostate cancer. Together, results from Figs. 4 and 5 indicated that endothelial cells may influence prostate cancer cell invasion via the IL-6 → 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 endothelial cells to influence prostate cancer invasion.

**In vivo xenografted mice show endothelial cells effect in enhancing prostate cancer metastasis**

To confirm the above in vitro cell lines results showing endothelial cells promote the metastatic ability of prostate cancer cells in vivo, prostate cancer CWR22Rv1 cells were orthotopically implanted, either alone or coimplanted with HUVECs, into the anterior prostates of the nude mice. After injection, the metastatic incidence in these 2
groups of mice was monitored using in vivo imaging system (IVIS). As shown in Fig. 6A, the metastatic incidence of the coimplantation group was significantly increased showing more tumors mainly in the lymph nodes and diaphragm compared with the control group (Fig. 6A–D). The prostate cancer 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, whereas the expressions of TGF-β1 and MMP-9 were increased in the tissues of the coimplantation group mice compared with the control group mouse 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 endothelial cells in prostate cancer 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 microvessels (33). We found that endothelial cell numbers were increased in prostate cancer versus normal tissues and following castration/ADT, compared with before castration/ADT treatment, although further studies using a set of sequential specimens in human tissues are necessary to support these findings.

We showed that endothelial cells may also play an important role in enhancing the metastatic potential of prostate cancer both in vitro and in vivo. These new findings will add insights into endothelial cells contribution to prostate cancer metastasis and emphasize the importance of endothelial cells as a component of the TME.

In mediating endothelial cells role in enhancing the metastatic potential of prostate cancer, we showed that the endothelial cells’ action in enhancing the metastatic ability of prostate cancer was via downregulation of AR, which may challenge the current understanding that AR plays a positive role to promote prostate cancer 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 prostate cancer (39–42), so suggesting the suppressor role of AR in increasing prostate cancer 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 prostate cancer samples after ADT. Cell line studies also showed that ADT causes EMT transition (49). Because the EMT process is highly correlated with metastases (50, 51), these results supported the idea that ADT enhances prostate cancer metastases. ADT with surgical castration was also shown to lead to
Endothelial Cells Increase Metastatic Potential of Prostate Cancer

Figure 6. HUVECs treatment enhances prostate cancer 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 x 10^6 of these cells, either alone or together with HUVECs (10:1 prostate cancer cells:HUVECs), as a mixture with Matrigel, 1:1, total of 20 μL, were orthotopically implanted into the anterior prostates of 8-week-old mice. Tumor growth and metastasis was monitored by examining luminescence using IVIS at 3, 4, 5, and 6 weeks after injection. A, the metastatic incidence shown in 2 groups of mice. B, the imaging data showing primary and metastatic tumors of 2 mice groups. C, the imaging showing diaphragm metastasis. D, H&E and IHC staining of metastatic tumors from diaphragm using antibodies of anti-firefly luciferase antibody (Abcam). E, the imaging showing the ascites metastases obtained from metastatic mouse (left) and primary cultures cells from ascites (right). 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. *, P < 0.05; **, P < 0.01.
increased lymph node (52) or distant (53) metastases. Furthermore, Niu and colleagues (54) found that mice with AR knock down in prostate epithelial cells, developed increased metastatic prostate cancer, with mice dying earlier than in the TRAMP mouse model. Therefore, the results showing AR downregulation in prostate cancer cells upon endothelial cells coculture and increasing prostate cancer metastasis in this study is consistent with these new emerging concepts.

Among several cytokines/chemokines/growth factors identified from endothelial cells to exert paracrine effects to influence prostate cancer 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 prostate cancer metastases, but they might act via different mechanisms and not via downregulation of AR. IL-6 is known to be increased in patients with advanced stages of prostate cancer (55, 56), play an important role in prostate cancer progression (57), and can be secreted from several cell types, including macrophages (58) and adipocytes (59). In this study, we found endothelial cells are another source of IL-6.

VEGF has been suggested as a critical molecule to target endothelial cell–mediated angiogenesis (60, 61). However, none of our results showed significantly increased VEGF levels when cocultured with prostate cancer cells nor increased invasion ability of prostate cancer cells upon addition of VEGF (data not shown).

We found TGF-β and MMP-9 were key molecules mediating IL-6-AR signals to enhance the metastatic potential of prostate cancer. These 2 molecules are known to be the multifunctional factors during diverse physiologic and pathologic 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, immunosuppression, and metastasis growth promotion (63). Zhang and colleagues (64) investigated the TGF-β role in growth and metastasis of the highly metastatic PC-3MM2 human prostate cancer 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 prostate cancer PC3 cell invasion by a urokinase-type plasminogen activator/plasmin-dependent mechanism to play a key role in malignant prostate cancer progression (65). Recently, several studies have shown that TGF-β1 can upregulate 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 2 molecules to block endothelial–promoted prostate cancer metastasis.

We also found E-cadherin decreases in prostate cancer cells upon endothelial cell–prostate cancer coculture, but failed to observe the difference of other EMT markers. It will be interesting to see whether the increase of TGF-β/MMP-9 were due to the E-cadherin level changes or whether these 2 are separate signals.

On the basis of these studies, we believe that development of a combination therapy to block 2 processes, tumor proliferation where AR plays a positive role and metastasis in which AR plays a negative role, is essential. The combination therapy targeting tumor growth (by the classic 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 endothelial cell–mediated IL-6–AR–TGF-β–MMP-9 signaling pathway identified here, to suppress metastasis after classic anti-proliferation therapy to suppress prostate cancer progression, may help us to better battle prostate cancer. Furthermore, in vivo mice studies to test therapeutic approaches need to be conducted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Wang, S.O. Lee, S. Xia, Q. Jiang, L. Li, S. Yeh, C. Chang
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Endothelial Cells Increase Metastatic Potential of Prostate Cancer


Endothelial Cells Enhance Prostate Cancer Metastasis via IL-6 → Androgen Receptor → TGF-β → MMP-9 Signals

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