Proteomic Signatures of Angiogenesis in Androgen-Independent Prostate Cancer

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INTRODUCTION. The observation that angiogenesis, the process of new blood vessel formation, in healthy prostate and early prostate cancer is androgen-dependent gave rise to significant questions on how hypervascularization and increased angiogenesis is also achieved at the molecular level in advanced androgen-independent prostate cancer. The exact paracrine molecular network that is hardwired into the proteome of the endothelial and cancer subpopulations participating in this process remains partially understood.
METHODS. Here, we interrogated the signaling pathways and the molecular functional signatures across the proteome of endothelial cells after interacting with various secretomes produced by androgen-dependent and -independent prostate cancer cells.
RESULTS. We found the significant overexpression ($P < 0.05$) of prominent markers of angiogenesis, such as vonWillebrand factor (vWF) (~2.5-fold) and CD31 (~2-fold) in HUVECs stimulated with conditioned media from the androgen-independent prostate cancer cell line PC3. By mining the proteome of PC3 conditioned media, we discovered a signature of chemokine CXC motif ligands (i.e., CXCL3, CXCL5, CXCL6 and CXCL8) that could potentially coordinate increased angiogenesis in androgen-independent prostate cancer and verified their increased expression ($P < 0.05$) in both in vitro and xenograft models of androgen-independence.
DISCUSSION. Our findings form the basis for understanding the regulation of crucial metastatic phenomena during the transition of androgen-dependent prostate cancer into the highly aggressive, androgen-independent state and provide further insight on potential therapeutic targets of cancer-related angiogenesis. *Prostate 74:260–272, 2014.*
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KEY WORDS: **androgen-independent cancer; prostate cancer angiogenesis; prostate cancer proteomics; chemokines; mass spectrometry**

INTRODUCTION

In the normal prostate and androgen-dependent prostate cancer, it is presumed that formation of novel vasculature within the microenvironment is hormonally regulated [1]. In particular, androgen ablation of nude mice implanted with the androgen-dependent prostate cancer cell line LNCaP reveals that endothelial

Abbreviations: ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs 1; CD31, cluster of differentiation 31; CM, conditioned media; CXCL, chemokine (C-X-C-motif) ligand; CXCR2, CXC chemokine receptor; GRN, granulin; HUVEC, human umbilical vein endothelial cells; IL, interleukin; LC, liquid chromatography; MS/MS, tandem mass spectrometry; NSCLC, non-small cell lung carcinoma; PECAM1, platelet endothelial cell adhesion molecule; RCC, renal cell carcinoma; TBP, tata-binding protein; VEGF, vascular endothelial growth factor; vWF, vonWillebrand factor.

George S. Karagiannis and Punit Saraon contributed equal to this work.

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cells undergo more rapid apoptosis than neoplastic cells and involution of tumor vessels precedes the decrease in tumor mass [2]. Consistently with these observations, androgen ablation therapy in prostate cancer patients leads to increased endothelial cell apoptosis and decreased vascular permeability [3,4]. Moreover, castration in adult rats inflicts vascular regression [5] due to transcriptional downregulation of vascular endothelial growth factor (VEGF) [6], while testosterone replacement restimulates angiogenesis and vascular growth [7].

Advanced prostate cancer is accompanied by transition of an androgen-dependent tumor population to an androgen-refractory one, resulting in poor survival outcome and ineffective response to castration therapy [8–10]. Remarkably though, this transition has been associated with profound increase in tumor vascularization and angiogenesis [11,12]. The transition to an androgen-independent state is usually accompanied by unaltered VEGF levels, as shown in various murine prostate cancer models and human patients [6,13], which cannot adequately explain why these tumors are presented with enhanced vascular density. Collectively, these observations imply that advanced prostate cancers may efficiently recruit a supportive angiogenesis even in the absence of androgens.

In general, angiogenesis represents a prominent hallmark of all cancers [14] and is regulated by a complex dynamic network of both promoting and suppressive factors, whose balance is presumed to shift the angiogenic switch towards hyper- or hypovascularized states [15,16]. Given all the above, our current working hypothesis states that the prostate cancer microenvironment holds an intriguing flexibility to support angiogenesis through different strategies during various stages of progression. In this type of cancer, it seems that the angiogenic switch is initially under the control of hormonal dependence, but later is shifted towards the control of other factors, originating from androgen-independent prostate cancer cells or the surrounding stroma and may effectively act as "recruiter cytokines."

Previous studies have attempted to specify the nature of such recruiter cytokines. For instance, an androgen-dependent cell line (LNCaP), previously shown to induce less angiogenesis when injected in nude mice compared to its androgen-independent counterpart (LNCaP-19) [17], was shown to express significantly higher levels of a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), compared to LNCaP-19 [18]. Since ADAMTS1 serves as inhibitor of angiogenesis [19], its significant downregulation in the androgen-independent state might provide an explanation for the shift of the angiogenic switch towards the promoting phenotype. However, this area is widely unexplored and the cytokine network mediating angiogenesis in androgen-independent prostate cancer remains to be elucidated.

In the current study, we developed co-cultures of endothelial cells with prostate cancer derived conditioned media and investigated protein perturbations in stimulated endothelial cells. Moreover, we attempted to characterize the endothelial recruiter cytokines, which could potentially assist towards the emerging rationale for therapeutic targeting of the prostate angiogenic stroma in combination with androgen deprivation therapy [11,20]. To achieve this, we evaluated proteomic data derived from our previous study [21], whereby proteomic delineation of conditioned media (CM) from androgen-dependent and -independent prostate cancer cell lines had been performed.

MATERIALS AND METHODS

Cell Culture

Cell lines. The human umbilical vein endothelial cell line (HUVEC) was purchased from the American Type Tissue Collection (ATCC, Manassas, VA) and cultured in endothelial cell medium (ScienCell), supplemented with 10% fetal bovine serum (FBS, Thermo Scientific), and endothelial cell growth supplement (EGM) (ScienCell). All experiments with HUVEC cells were carried out within 10 passages of initial cell growth. The human prostate cancer cell lines PC3, DU145, LNCaP, and VCaP were purchased from the ATCC. Cell culture media specified by the ATCC for each of the cell lines were used as follows: Dulbecco's modified Eagle's medium (DMEM) (ATCC) with 10% FBS was used for PC3, DU145, and VCaP; Roswell Park Memorial Institute (ATCC) with 10% FBS was used for LNCaP cells. All cells were maintained at 37°C with 5% CO_2 in a humidified incubator. All experiments were performed within the first five passages from the initiation of all cultures.

Stimulation experiments. Cancer cells (PC3, LNCaP, or LNCaP-SF) were cultured in T-175 cm^2 flasks and allowed to grow in 30 ml of their respective media until they reached 70% confluence. The media was then removed, and cells were washed three times with 20 ml of PBS (Invitrogen). Following the washes, 50 ml of Chinese hamster ovary serum-free medium (Invitrogen) were added to each of the flasks, which were then cultured for 2 days. After further growth, the conditioned medium was collected and centrifuged at 2,000*g* for 5 min to remove cellular debris. The resulting supernatants were collected and used for stimulation of HUVEC experiments. HUVEC cells were

cultured in T-25 flasks until 80% confluence. Cells were then either treated with 5 ml of conditioned media from control (CD-CHO media + 1% FBS + EGM), LNCaP (LNCaP media + 1% FBS + EGM), LNCaP-SF (LNCaP-SF media + 1% FBS + EGM), or PC3 cells (PC3 media + 1% FBS + EGM). After 24 hr, fresh media from each respective condition was added for an additional 24 hr and the HUVEC cells were then detached non-enzymatically, washed twice in PBS, and centrifuged at 1,500*g* for 5 min. Cell pellets were kept at −80°C, until further processing.

Models of androgen-independence. The human prostate cancer LNCaP cell line and the androgen-independent subline LNCaP-SF cells were kindly provided by Dr. Atsushi Mizokami and maintained in DMEM (Wisent, St Bruno, Quebec) medium supplemented with either 10% (v/v) FBS (Hyclone) for LNCaP cells or 10% charcoal stripped FBS (Hyclone) for LNCaP-SF cells at 37°C with 5% CO_2 in a humidified incubator. LuCaP96 and LuCaP96AI xenograft tissues, as previously described [22], were frozen in liquid nitrogen, and then ground into a fine powder using a mortar and pestle. The resulting powdered tissue was lysed using 1% SDS solution followed by sonication on ice for three 30 sec intervals. The samples were then centrifuged at 14,000*g* for 15 min at 4°C, and the resulting supernatant was used for further analysis.

Proteomic Analysis

Sample preparation. Cell pellets were lysed using 300 μl of 0.1% RapiGest (Waters, Inc., Milford, MA) in 25 mM ammonium bicarbonate and sonicated three times. The resulting cell lysates were centrifuged for 15 min, at 15,000*g*, at 4°C and quantified using the BCA assay (Thermo Scientific). Proteins were then heat-denatured for 10 min at 85°C, reduced with 10 mM DTT (Sigma–Aldrich) for 10 min at 70°C, alkylated with 20 mM iodoacetamide (Sigma–Aldrich) for 60 min with shaking in the dark, and were trypsin-digested (Promega) at a ratio of 1:50 (trypsin:protein concentration) overnight at 37°C. The resulting tryptic peptides were reconstituted in 200μl of 0.26 M formic acid in 5% acetonitrile (mobile phase A) buffer.

Strong cation exchange (SCX) liquid chromatograpghy. The samples were subjected to SCX using an Agilent 1100 High Performance Liquid Chromatography (HPLC) system. The HPLC system was connected to a PolySULPHOETHYL A™ column with a 200 Ä pore size and a diameter of 5 μm (The Nest Group, Inc., Southborough, MA). Twelve fractions per replicate were eluted and collected for mass spectrometric analysis. Each fraction was then further C_{18}-extracted using ZipTipC_{18} micropipette tips (Millipore, Billerica, MA) and eluted in 5 μl of Buffer B (90% acetonitrile, 0.1% formic acid, 10% water, and 0.02% trifluoroacetic acid). An additional 80 μl of Buffer A (95% water, 0.1% formic acid, 5% acetonitrile, and 0.02% trifluoroacetic acid) were added to each zipped fraction.

Mass spectrometry (LC-MS/MS). Next, 40 μl of each fraction were injected into an autosampler on the EASY-nLC system (Proxeon Biosystems, Odense, Denmark). Peptides were collected onto a 3-cm C18 column (inner diameter of 200 μm), followed by elution onto a resolving 5-cm analytic C18 column (inner diameter of 75 μm) with an 8 μm tip (New Objective). The HPLC system was coupled online to an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer, using a nano-Electro Spray Ionization (ESI) source (Proxeon Biosystems), in data-dependent mode. The fractions were run on a 55-min gradient, and eluted peptides were subjected to one full MS1 scan (450–1,450 m/z) in the Orbitrap at 60,000 resolution, followed by six data-dependent MS2 scans in the linear ion trap. The following parameters were enabled: monoisotopic precursor selection, charge state screening and dynamic exclusion. In addition, charge states of +1, >4 and unassigned charge states were not subjected to MS2 fragmentation.

Protein identification and data analysis. RAW files were uploaded onto Mascot Daemon (v.2.2) and extract_msn was used to generate DAT and Mascot Generic Files (MGFs). MGF files were further searched on GPM (Global Proteome Machine Manager, version 2006.06.01) against the concatenated non-redundent IPI. Human v.3.62 database with parent and fragment tolerances of 7 ppm to generate X!Tandem files. Next, the DAT and X!Tandem files were merged and searched with Scaffold (Proteome Software, Inc., Portland, OR; v2.0) to generate a file containing protein lists. The final Scaffold files contained normalized spectral counts for each of stimulation conditions (non-stimulated vs. LNCaP-stimulated vs. PC3-stimulated), which were subsequently used as a semi-quantitative measure to compare differential HUVEC protein expression.

Bioinformatics Analyses

Retrieval of proteomic datasets. The LNCaP and PC3 proteomic datasets were retrieved from our previous study [21].

Gene expression meta-analysis using genevestigator. Gene expression meta-analysis was performed as previously described [23].

Protein–protein interaction analysis using STRING. Protein–protein interaction analysis was performed using the STRING visualization tool (http://string-db.org/) [24]. The protein dataset of interest was uploaded into the application using gene identifiers and complete lists of human orthologs were included in the network visualization. Protein–protein interaction networks were algorithmically created based on the following six prediction methods and/or databases; (a) neighborhood, (b) gene-fusion, (c) co-occurrence, (d) co-expression, (e) experiments, and (f) databases. High confidence scores were requested for the output network.

Quantitative PCR

Total RNA was isolated from each cell line using an RNeasy Kit (Qiagen). cDNA was generated from 1 μg of total RNA using the Superscript II cDNA synthesis kit (Invitrogen). Quantitative PCR was conducted using 1× SYBR reagent (Applied Biosystems) and transcript levels of *vWF*, *CD31*, *CXCL3*, *CXCL5*, *CXCL6*, *GRN*, *IL6*, and *IL8* were measured on a 7500 ABI system. All Q-PCR data were normalized to tata-binding protein (TBP) expression. The 5′–3′ forward (F) and reverse (R) primer sequences used are shown in Table I.

Statistical Analysis

Graphs are presented as mean values with standard deviations. The Student's *t*-test was used for parametric tests and $P < 0.05$ was considered significant. All analyses were performed in the SPSS (version 20) environment.

RESULTS

LNCaP and PC3 Prostate Cancer Cells Inflict Diverse Protein Expression Profiles in Endothelial Cells In Vitro

Initially, we sought to verify previously reported observations [11], collectively demonstrating that the status of androgen dependence is involved in differential regulation of angiogenesis in prostate cancer. To achieve so, we stimulated HUVECs with CM derived either from androgen-dependent or androgen-independent prostate cancer cell lines (LNCaP and PC3, respectively). To monitor differential angiogenic responses, we subjected LNCaP-stimulated, PC3-stimulated, and non-stimulated (i.e., control) HUVEC lysates to quantitative proteomic analysis using an LC-MS/MS approach (Fig. 1A), previously developed in our laboratory [25]. This analysis resulted in the identification of 1,737 proteins in all three conditions. We, then, normalized each identified protein's number of spectral counts to the mean number of total spectral counts from all replicates, as previously described [26] (Table SI).

In this normalized data, we used ANOVA to demonstrate statistically significant ($P < 0.05$) protein perturbations across the experimental conditions and successfully identified 81 deregulated proteins (Table SII). However, many of those were identified with only a few spectral counts (Table SII), most probably due to instrumentation limitations (a common problem in most proteomics approaches [27]). A recent study [26] has suggested that the sensitivity and reproducibility of quantitation through spectral counting is significantly enhanced in proteins identified with more than five unique spectra. On this basis, we excluded proteins with mean quantitative value of less than 5 for the triplicates across each experimental group. With this approach, we narrowed down the deregulated proteins to 36 (Fig. 1B; Table SIII).

LNCaP and PC3 Prostate Cancer Cells Inflict Diverse Expression Profiles of Specific Markers of Angiogenesis in Endothelial Cells In Vitro

Of note, vonWillebrand factor (vWF) was found among the significantly ($P < 0.05$) upregulated proteins in both LNCaP- and PC3-stimulated HUVEC compared to the placebo-stimulated HUVEC. The

TABLE I. Forward and Reverse Primer Sequences Used for Quantitative PCR

Gene name	Forward sequence	Reverse sequence
CXCL3	TTCACCTCAAGAACATCCAAAGTG	TTCTTCCCATTCTTGAGTGTGGC
CXCL5	CAGACCACGCAAGGAGTTCATC	TTCCTTCCCGTTCTTCAGGGAG
CXCL6	GGGAAGCAAGTTTGTCTGGACC	AAACTGCTCCGCTGAAGACTGG
GRN	CTCTCCAAGGAGAACGCTACCA	GACTGTAGACGGCAGCAGGTAT
vWF	CCTTGAATCCCAGTGACCCTGA	GGTTCCGAGATGTCCTCCACAT
CD31	AAGTGGAGTCCAGCCGCATATC	ATGGAGCAGGACAGGTTCAGTC
IL6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
IL8	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
TBP	TGTATCCACAGTGAATCTTGGTTG	GGTTCGTGGCTCTCTTATCCTC

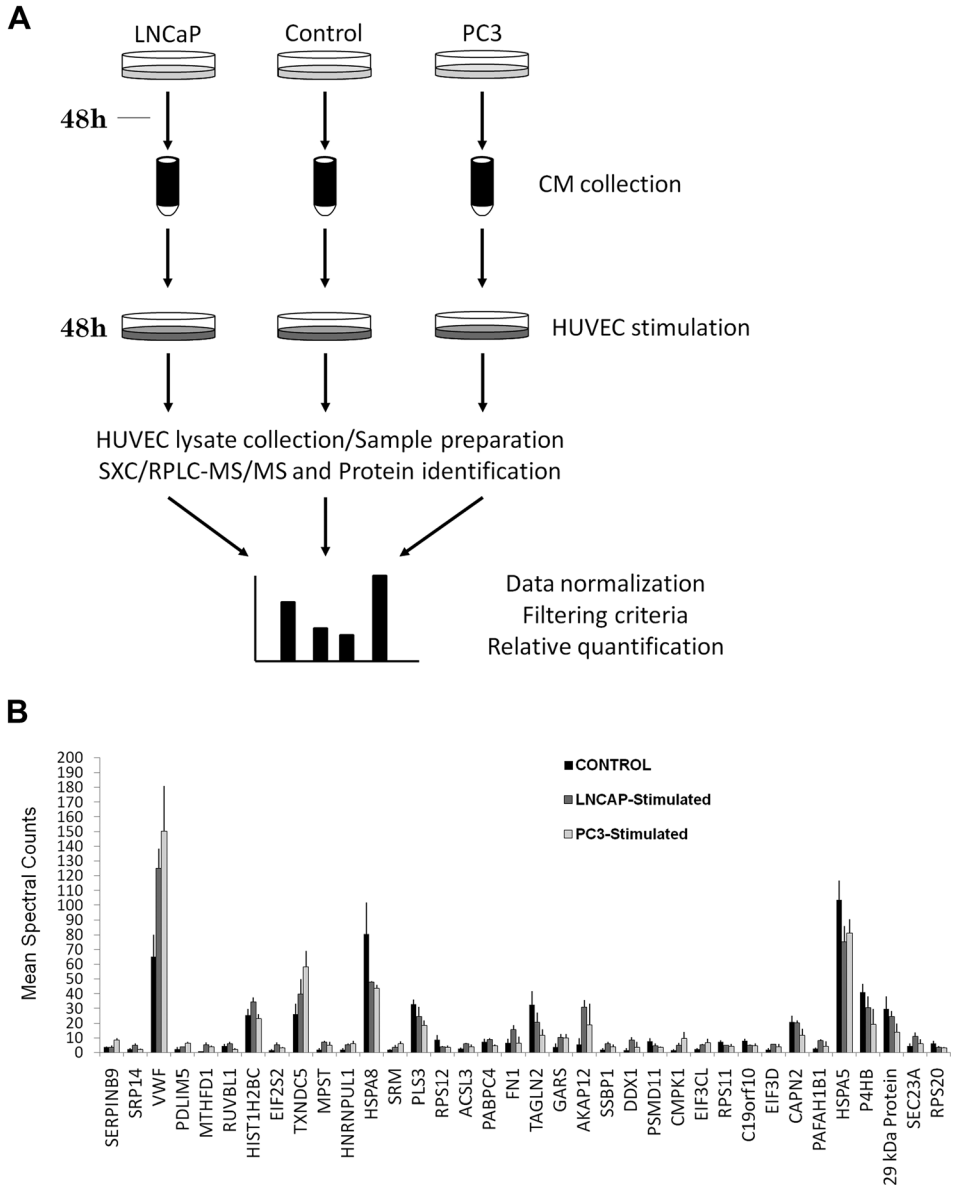

Fig. 1. **A**: Proteomic pipeline for investigation of protein perturbations induced in HUVECs, after stimulation with either LNCaP CM or PC3 CM (serum-free medium served as placebo/control) using an LC-MS/MS approach. See Materials and Methods Section for further details. **B**: The 36 proteins that were differentially secreted in at least one of the three conditions of the proteomic pipeline shown in (**A**) and were identified with a mean of at least five spectral counts in the three replicates.

observed upregulation was approximately 2-fold for the LNCaP- and 2.5-fold for PC3-stimulated HUVEC and was demonstrated with the highest number of mean spectral counts (LNCaP-stimulated, 125.95; PC3-stimulated, 150.15; placebo-stimulated, 65.15) among all the deregulated candidates (Fig. 2A). vWF is a blood glycoprotein required for normal hemostasis, and is constantly produced by endothelial cells, megakaryocytes and subendothelial connective tissue [28]. Therefore, this factor is widely used for determination of relative vascular contribution within tumor microenvironments and its overexpression correlates with tumor hypervascularization [29]. Moreover, a significant difference ($P < 0.05$) in the mean spectral counts of vWF was observed between LNCaP- and PC3-stimulated HUVEC (Fig. 2A). These proteomic observations were accordingly verified in the mRNA level, after reproducing the same experimental platform (Fig. 2B). At the proteome level, a similar pattern of differential regulation was observed for another marker of angiogenesis, the cluster of differentiation 31 (CD31) or platelet endothelial cell adhesion molecule

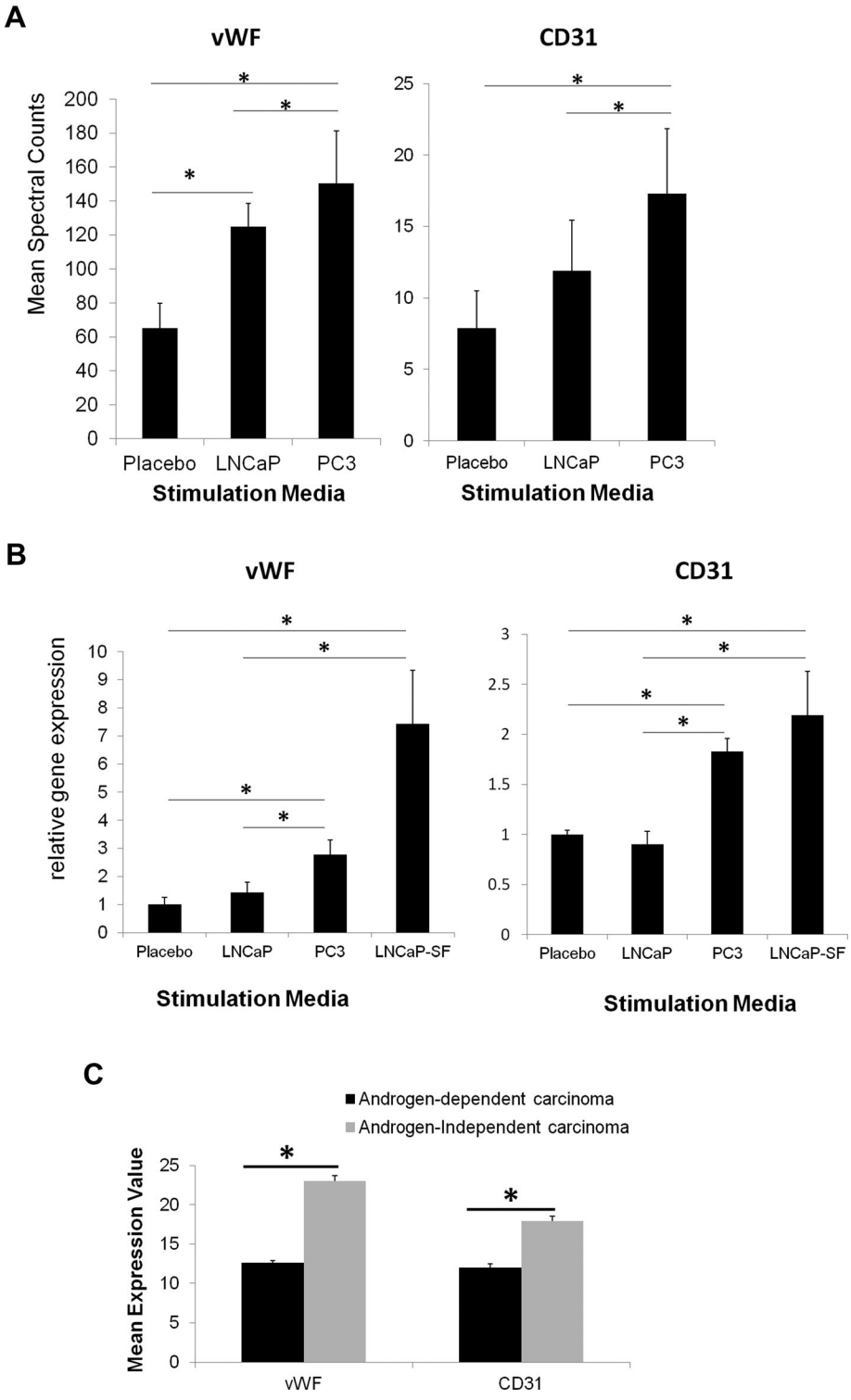

Fig. 2. **A**: Mean spectral counts of vWF and CD31 in LNCaP-, PC3-, and Placebo-stimulated HUVECs. **B**: Gene expression levels of vWF and CD31 in LNCaP-, LNCaP-SF-, PC3-, and Placebo-stimulated HUVECs. **C**: Gene expression meta-analysis, showing mean expression values of vWF and CD31 in androgen-dependent and androgen-independent prostate carcinoma arrays, derived from Genevestigator. *Statistical significance ($P < 0.05$; Student's *t*-test).

(PECAM1) (Fig. 2A), which was also verified in the mRNA level (Fig. 2B). To firmly demonstrate that such angiogenic responses are androgen-independent, we additionally included stimulations of HUVECs with CM from LNCaP-SF, an androgen-independent subline of LNCaP. Interestingly, very similar to PC3 or even more potent angiogenic responses were observed in HUVECs stimulated with such CM (Fig. 2B).

To robustly substantiate that differences in angiogenic potential are particularly invoked by the androgen-dependence status, we took advantage of Genevestigator and performed gene expression meta-analysis to investigate relative expression of the angiogenic markers vWF and CD31 between androgen-dependent and -independent prostate carcinoma arrays. This analysis demonstrated the significant ($P < 0.05$) overexpression of both angiogenic markers in the androgen-independent arrays (Fig. 2C). Collectively, these findings suggest that the androgen-independent PC3 and LNCaP-SF cell lines, in contrast to the androgen-dependent LNCaP, may cause more potent expression of angiogenesis markers in HUVECs.

Proteomic Analysis of LNCaP and PC3 Conditioned Media (CM) Reveals Potential Recruiter Cytokines for Prostate Cancer Angiogenesis

We hypothesized that the androgen-independent cell line PC3, in contrast to LNCaP, might secrete one or more "recruiter cytokines" responsible for the increased angiogenic potential of PC3 on HUVECs. To test this, we utilized published data from our previous study [21], whereby proteomic analysis of CM (secretome analysis) derived from prostate cancer cell lines had been performed using our in-house developed LC-MS/MS approach. Using the minimum-two-peptide-hit criterion, 1,199 proteins were identified in the LNCaP (Table SIV) and 1,448 proteins in the PC3 secretome (Table SV). We, then, aimed to narrow down these datasets to the most promising candidates that could serve as "recruiter cytokines" for prostate cancer neoangiogenesis. To achieve this, we considered previous studies [30–33] that have utilized a comprehensive panel of 44 genes, consisting of 36 cytokines/growth factors known to promote angiogenesis, as well as of 8 cytokines/growth factors known to suppress it (Table SVI). We, then, assessed the mean spectral counts of all 44 cytokines in the LNCaP/PC3 secretome data (Fig. 3A). Interestingly, LNCaP secreted three angiogenesis-promoting cytokines and only one angiogenesis-suppressive, while PC3 secreted 11 and 3, respectively (Fig. 3B).

The identified angiogenesis suppressors (CXCL2, CXCL10, PF4, and TGFB1) were secreted by either LNCaP or PC3 in relatively low amounts (Fig. 3B), indirectly explaining why both of these cell lines produced a positive response of angiogenesis markers in HUVECs. Of the identified angiogenesis-promoting cytokines, VEGF-A and midkine (MDK) were secreted by both cell lines with no significant differences (Fig. 3B). This observation is consistent with previous data depicting that prostate cancer-induced angiogenesis is not influenced by differential regulation of VEGF between androgen-dependent and -independent states [17], and that MDK is equally secreted by both androgen-dependent and androgen-independent cell lines [18]. Interestingly, there was no tumor-promoting cytokine showing high secretion in LNCaP and not in PC3 cells. On the contrary, there were multiple cytokines, specifically CXCL3, CXCL5, CXCL6, GRN, IL6, and IL8 secreted in large amounts by PC3 cells, whereas being entirely absent in the LNCaP CM (Fig. 3B). We, thus, speculated that this cytokine signature could potentially serve as "endothelial-recruiter" in androgen-independent prostate cancer.

Subsequently, we validated this data at the mRNA level. To achieve so, we compared gene expression levels of the recruiter cytokine signature between LNCaP and PC3 cell lines. To surpass the cell line selection biases, we also incorporated two additional prostate cancer cell lines, an androgen-dependent (VCaP) and an androgen-independent (DU145) one, in this analysis. All six cytokines were significantly overexpressed ($P < 0.05$) in the androgen-independent compared to the androgen-dependent cell lines, with the exception of CXCL6 and GRN, which did not show a significant overexpression in the DU145 cell line (Fig. 3C).

The Endothelial Recruiter Cytokine Signature Is Upregulated in Various Models of Androgen-Independent Prostate Cancer

Our data suggest that androgen independent prostate cancer might be particularly related with the secretion of angiogenesis-promoting cytokines, including the CXC motif chemokines CXCL3, CXCL5, CXCL6, and IL8 (also known as CXCL8), as well as IL6. To directly demonstrate association of these potential recruiter cytokines with androgen independence, we calculated gene expression levels of their respective genes in two different prostate cancer models of androgen independence. In the first model (in vitro model), an androgen-independent subline, the LNCaP-SF cell line, had been derived from the parental LNCaP cells after long-term androgen deprivation therapy and 6-month culturing in androgen-deficient culture medium, as previously described [34]. In the second model (xenograft model), castration-sensitive (LuCaP 96) and castration-resistant (LuCaP

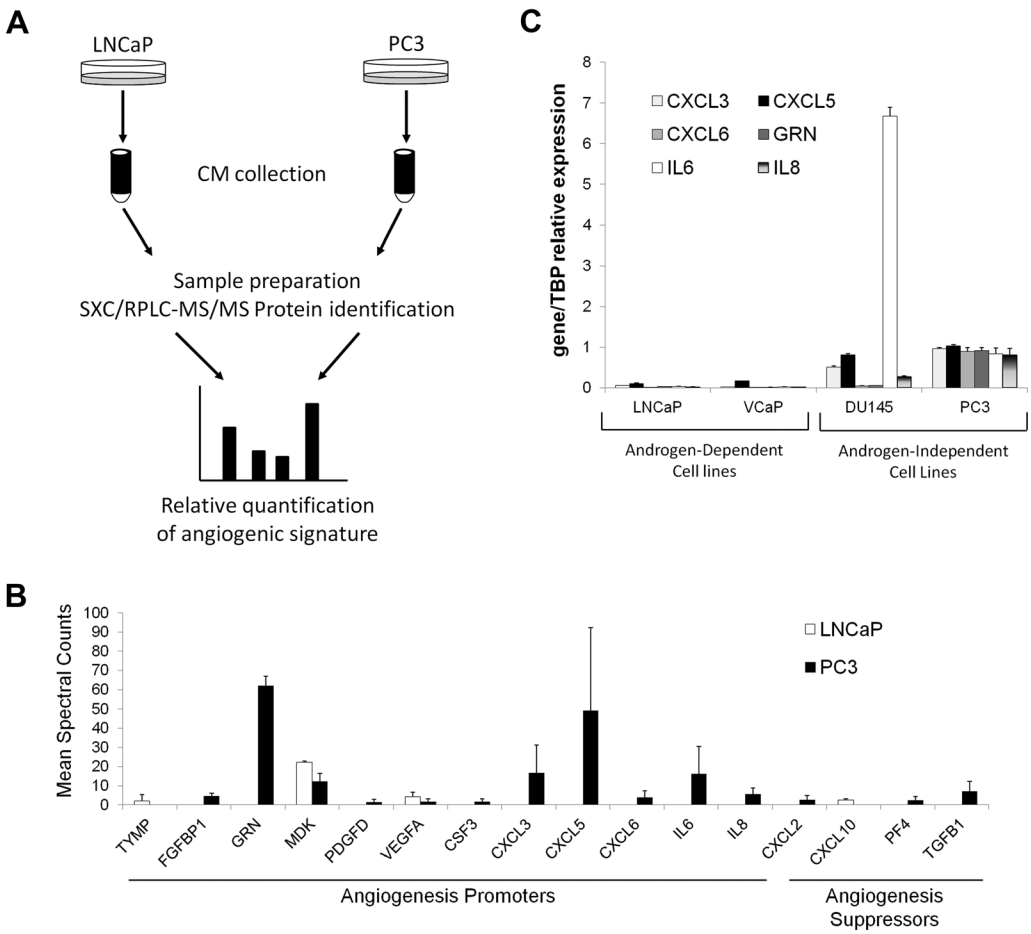

Fig. 3. Proteomic analysis of LNCaP and PC3 conditioned media (CM) reveals potential recruiter cytokines for prostate cancer angiogenesis. **A**: Strategy for quantifying 44 cytokines (potentially involved in cancer angiogenesis) in LNCaP and PC3 CM, obtained from proteomic datasets of our previous work [21]. **B**: Mean spectral counts of the panel of angiogenesis-related cytokines after normalization. **C**: mRNA expression of the six most significantly overrepresented recruiter cytokines of the experiment shown in (**B**) in a set of two androgen-dependent and two androgen-independent prostate cancer cell lines. Data confirm that the recruiter cytokines are indeed expressed by the androgen-independent cell lines.

96AI) strains had been grown from a xenograft mouse model that was generated from a specimen obtained by transurethral resection of the prostate of a patient before documentation of castration-resistant prostate cancer, as previously described [22]. In both the in vitro and the xenograft model, CXCL3, CXCL5, and IL6 were all found significantly overexpressed in the androgen-independent states (Fig. 4A and B). Notably, CXCL3 was ~2- and ~135-fold over-expressed in the androgen-independent compared to the androgen-dependent states in the in vitro and the xenograft model, respectively (Fig. 4A and B). Of the remaining cytokines, a mild overexpression of CXCL6 and GRN was noticed in the androgen-independent state in the xenograft model, but this was not observed in the in vitro model, as well (Fig. 4A and B). IL8 overexpression was not confirmed in any of the two androgen independence models (Fig. 4A and B).

To substantiate the accuracy of these findings, we confirmed these results by gene expression meta-analysis through Genevestigator, for four of the testing cytokines (CXCL3, CXCL5 CXCL6, and IL6). This analysis demonstrated the significant ($P < 0.05$) overexpression of all recruiter cytokines in androgen-independent compared to the androgen-dependent prostate carcinoma arrays (Fig. 4C). Thus, data collectively presented in Figures 3B and C and 4A and B could also be validated through publicly available microarray depositories.

Integrative Pathway Analysis Demonstrates a Functional Association Between the Endothelial Recruiter Cytokine Signature and Angiogenesis

Next, we sought to demonstrate a functional link between the endothelial recruiter cytokines and the

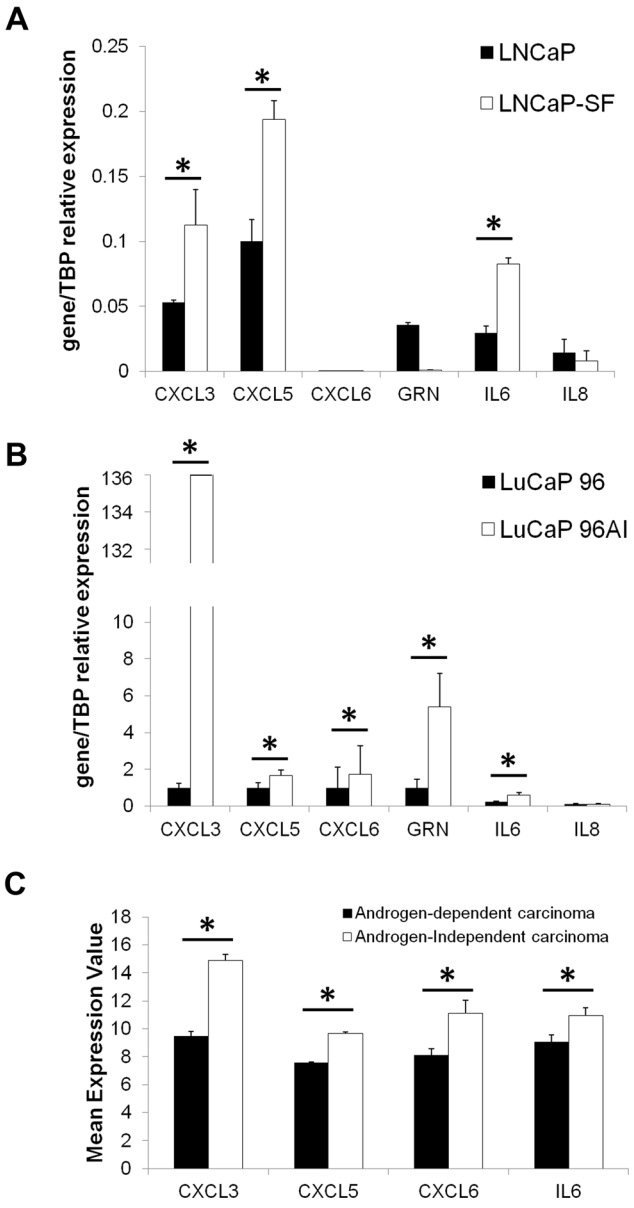

Fig. 4. A panel of genes consisting of C–X–C chemokine and interleukin family members is upregulated in androgen-independent prostate carcinoma. **A**: Relative gene expression of the endothelial recruiter cytokines in the LNCap/LnACaP-SF prostate cancer model of androgen-independence. **B**: Relative gene expression of the six endothelial recruiter cytokines in LuCaP 96/LuACaP 96AI xenograft model. **C**: Gene expression meta-analysis, showing mean expression values of CXCL3, CXCL5, CXCL6, and IL6 in androgen-dependent and androgen-independent prostate carcinoma arrays, derived from Genevestigator. *Statistical significance ($P < 0.05$; Student's *t*-test).

overexpressed angiogenesis markers from the stimulation experiments presented in Figure 1A. To address this issue, we constructed a protein–protein interaction network via STRING tool, using as input the 36 deregulated proteins from HUVEC stimulation (i.e., proteins of the Table SIII) together with CXCL3, CXCL5, CXCL6, IL6, IL8, and GRN. The resulting network produced a cluster of high confidence encompassing all six recruiter cytokines in the middle, which was linked to three independent angiogenic axes in the periphery (Fig. 5A). The serpin-B9 (SERPINB9) and the c-1-tetrahydrofolate synthase (MTHFD1) axes were not further explored in this study, since we found no evidence linking either of them to androgen-independent prostate cancer angiogenesis. However, the third axis (i.e., the Fibronectin/VonWillebrand factor [FN1/vWF] axis), the role of which is widely demonstrated in endothelial cell growth and vascular patterning [35], could successfully validate functional association between the recruiter cytokines and angiogenesis (Fig. 5A).

DISCUSSION

Prostate cancer depends on the process of angiogenesis for both local growth and metastatic dissemination, like most solid tumors [14,36]. In the context of androgen-dependent prostate cancer, this hallmark has been particularly attributed to hormonal regulation of VEGF transcripts [2]. Therefore, the observed hypervascularization and increased angiogenesis have been quite paradoxical in the case of androgen-independent cancer [11,12]. In our study, we proposed that an endothelial recruiter cytokine signature, mostly represented by CXC chemokine family members, is de novo expressed with the transition into the androgen-independent state, and might be responsible for a similar or even higher angiogenic potential than that of the androgen-dependent state (Fig. 5B). The possibility that these recruiter cytokines also exert their angiogenic potential through VEGF remains to be elucidated.

Chemokines are chemotactic cytokines that are involved in the trafficking and activation of a variety of immune cell types, particularly leukocytes. In the prostate, various chemokines have been associated with the induction and progression of prostatitis [37]. In addition, many chemokines encompass pluripotent roles in tumor development, involving the regulation of angiogenesis and metastasis [38]. In this study, we demonstrated that immortalized androgen-independent prostate cancer cells secrete an angiogenesis-promoting cytokine signature, principally consisting of the C-X-C motif chemokines CXCL3, CXCL5, CXCL6, and IL8/CXCL8 as well as IL6 and GRN. Certain members of this signature have been previously linked to the process of neoangiogenesis in the androgen-independent prostate cancer microenvironment and thus verified in the present study. For instance, Moore et al. [39] demonstrated that the

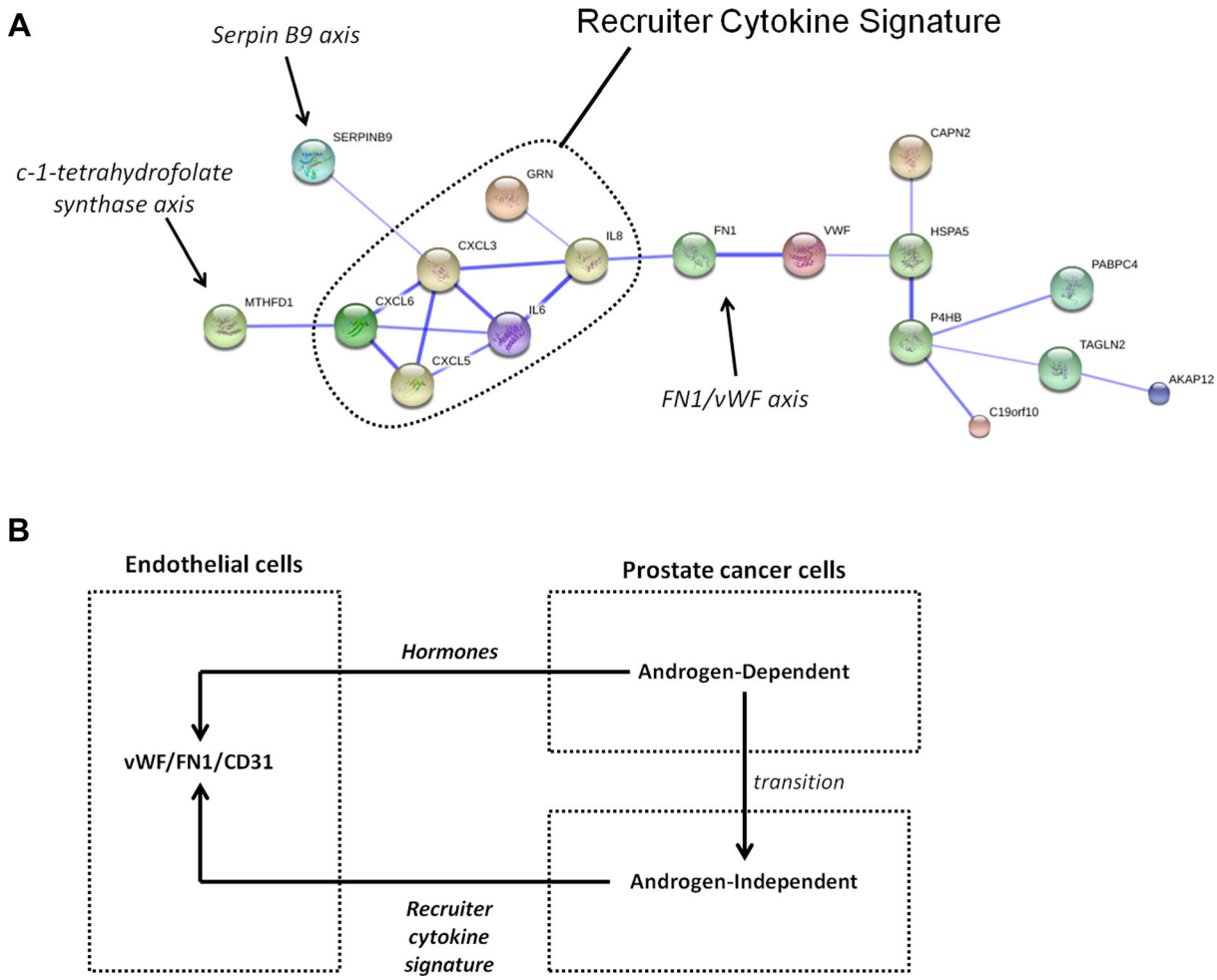

Fig. 5. Proposed functional link between the endothelial recruiter cytokines and the overexpressed markers of angiogenesis. **A**: Protein–protein interaction network (STRING; confidence view) of the list of deregulated proteins from the experiment shown in Figure IA. The panel of the six recruiter cytokines was also included as an input to investigate for potential functional association with the deregulated proteins. Proteins are shown as nodes and interaction as connecting lines between them. **B**: Proposed model. Following transition of prostate cancer from an androgen-dependent to an androgen independent state, an endothelial recruiter cytokine signature is being de novo expressed (CXCL3, CXCL5, CCL6, IL6, IL8, and GRN), which may successfully induce an angiogenic potential to endothelial cells in a paracrine fashion.

tumorigenic potential of the PC3 prostate cancer cell line is partially attributable to the production of the angiogenic chemokine IL8. The same group [39] demonstrated that other androgen-independent prostate cancer cell lines, such as DU145, may exert their angiogenic potential through CXCL1 secretion. However, we noticed an eloquent expression of CXCL3, CXCL5, IL6 and IL8, when we tested our proposed signature expression in DU145 cells (Fig. 3C). Other groups have also reported strong gene expression of CXCL3, CXCL5 and CXCL6 in PC3 and DU145 prostate cancer cell lines [40]. Thus, it seems that the proposed endothelial recruiter cytokine signature represents a generic strategy, with which androgen-independent cancer exert a paracrine shift of the angiogenic switch.

The family of CXC chemokines comprises two distinct groups regarding regulation of angiogenesis. Members of the family lacking the characteristic amino acid motif Glu–Leu–Arg (ELR-) that precedes the first cysteine residue on the primary structure, such as CXCL9 and CXCL10, are angiogenesis-suppressive [41]. On the contrary, CXC chemokine members harboring the ELR motif (ELR+), such as CXCL3, CXCL5, CXCL6 and IL8/CXCL8, are angiogenesis-promoting [41]. Interestingly, when we assessed previously published mass spectrometry data, derived from the androgen-dependent LNCaP and androgen-independent PC3 cells [21], we hardly noticed the presence of ELR- CXC chemokines in their CM (Fig. 3B). This observation may suggest that prostate cancer cells: (a) do not antagonize angiogenesis through ELR- CXC

chemokines, or (b) secrete minimal amounts of ELR-CXC chemokines (i.e., incapable of being detected through LC-MS/MS). In either case, the absence of angiogenesis-suppressive cytokines in the prostate cancer microenvironment may ensure that the shift of the angiogenic switch remains towards the promoting phenotype.

Our data are supported by previous findings of Mestas et al. [42], showing that the chemokines CXCL1, CXCL3, CXCL5, and CXCL8 contribute to increased angiogenesis in renal cell carcinoma (RCC) patients. In addition, this group demonstrated that the disruption of CXC-receptor-2 (CXCR2), through which ELR+ chemokine signaling is transduced, results in decreased angiogenesis and metastatic potential in a CXCR2−/− mouse model of RCC [42]. The targeting of CXCR2 receptor could also inhibit pancreatic cancer cell-induced angiogenesis in vitro settings [43]. Conclusively, the CXCL3/5/8-CXCR2 axis seems to be a popular strategy for solid tumors in regulating their angiogenic responses. Thus, the selective targeting of CXCR2 could serve as an attractive anti-angiogenic therapy.

The regulation of the recruiter cytokine expression is partially understood and remains to be determined. Recently, it has been demonstrated that the ELR+ CXC chemokine balance is destabilized in non-small cell lung carcinoma (NSCLC) through histone post-translation modifications [44]. Since the increased expression and functional activity of these chemokines in the tumor microenvironment is pivotal for NSCLC development and progression, the epigenetic targeting of these pathways could be proposed as an alternative anti-angiogenic therapeutic strategy [44].

The importance of the ELR+ CXC chemokines in the prostate cancer microenvironment should not be underestimated, since recent literature findings suggest alternative mechanisms of their production [45], beyond the currently suggested secretion by androgen-independent prostate cancer cells. Kogan-Sakin et al. [45] have demonstrated that CXCL1, CXCL3, and IL8/CXCL8 could be readily secreted by the cancer-associated stroma, in response to paracrine IL1-secretion from the prostate cancer cells. Along the same lines of evidence, Gallagher et al. [46] demonstrated that CXCL3 ad IL8/CXL8 are significantly upregulated in primary fibroblasts following cocultivation with melanoma cells. Therefore, the expression of our proposed endothelial-recruiter signature could also be deployed by the desmoplastic stroma, in order to inflict an angiogenic response in the prostate cancer microenvironment.

In conclusion, our results form the basis for further investigation on the exact role of CXCLs on the regulation of angiogenesis and neovascularization in the androgen-independent prostate cancer microenvironment. These data further support the notion that targeting the microenvironment may represent a promising therapeutic approach for prostate and possibly other types of cancer.

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