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THE RON RECEPTOR TYROSINE KINASE POSITIVELY REGULATES ANGIOGENIC CHEMOKINE PRODUCTION IN PROSTATE CANCER CELLS

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Abstract

Overexpression of the Ron receptor tyrosine kinase has recently been shown in a wide variety of human cancers. However, no studies have examined Ron receptor expression or function during prostate tumorigenesis. We report here that Ron is highly expressed in human prostate adenocarcinoma and metastatic lymph nodes compared to normal prostate or benign prostate hyperplasia. Furthermore, we show that Ron is overexpressed in PC-3 and DU145 prostate cancer cell lines, and that levels of angiogenic chemokines produced by prostate cancer cells positively correlates with Ron expression. Knockdown of Ron in PC-3 or DU145 cells results in a significant decrease in angiogenic chemokine production and is associated with decreased activation of the transcription factor NF-kappaB. Moreover, exogenous overexpression of Ron in LNCaP cells is sufficient to induce a significant increase in angiogenic chemokines that can be abrogated by inhibition of NF-kappaB signaling. Given that the function of angiogenic chemokines is important in the development of new blood vessels, we also examined the ability of Ron to modulate endothelial cell migration. Our data show that knockdown of Ron in prostate cancer cells results both in significantly less endothelial cell chemotaxis compared to Ron-expressing cells *in vitro* as well as in reduced tumor growth and decreased microvessel density following orthotopic transplantation into the prostate *in vivo*. In total, our data suggest that the Ron receptor is important in modulating prostate tumor growth by modulating angiogenic chemokine production and subsequent endothelial cell recruitment.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths among men, and one in six men will be diagnosed with this disease during his lifetime. While current treatment modalities such as a radical prostatectomy or radiation therapy can prove to be beneficial in the short-term for early-stage prostate cancer, recurrence is common. Once prostate cancer cells metastasize, the mortality rate dramatically increases (Pound *et al.*, 1999). Therefore, to

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more successfully treat prostate cancer, a greater understanding of the biological and physiological mechanisms that are involved in regulating prostate tumor growth and metastasis is needed.

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is a critical factor in tumor growth and metastasis. This process provides the essential nutrients to nourish the growing tumor and is also important in removing waste products from the tumor. Moreover, studies have shown that prostate tumor angiogenesis positively correlates with increased tumor growth and progression and is associated with decreased mean survival time (Lissbrant *et al.*, 1997). Without prostate tumor angiogenesis, the tumor would not be able to grow and metastasize, therefore factors involved in tumor angiogenesis are promising targets for prostate cancer therapeutics (Craft and Harris, 1994; Jimenez *et al.*, 2006).

One such potential target is the Ron receptor tyrosine kinase. Ron is the only other member of the Met family of cell surface receptors, and has been implicated in several human cancers (Chen *et al.*, 2000; Maggiora *et al.*, 1998; O'Toole *et al.*, 2006; Thomas *et al.*, 2007). The Ron receptor is expressed preferentially in macrophages and epithelial cells (Leonis *et al.*, 2007; Wagh *et al.*, 2008). The Ron ligand, hepatocyte growth factor-like protein (HGFL), is predominantly expressed in hepatocytes and is secreted into the circulation working primarily in an endocrine fashion (Bezerra *et al.*, 1998; Bezerra *et al.*, 1993). Binding of HGFL to Ron leads to receptor phosphorylation and activation, which then results in the regulation of a wide variety of downstream signaling pathways implemented in multiple cellular processes including cellular proliferation, migration, branching morphogenesis, and cell scattering (Leonis *et al.*, 2007; Wagh *et al.*, 2008). Interestingly, we have shown previously that in a mouse model of breast cancer, mice deficient in Ron exhibit reduced mammary tumor formation with a decrease in tumor microvessel density, suggesting that Ron plays a role in tumor angiogenesis (Peace *et al.*, 2005). The role of the Ron receptor in prostate tumor growth and angiogenesis however, is unknown.

Chemokines are a group of small molecules ranging in size from 8 to 10 kDa that are classified into four groups based on conserved cysteine residues near the amino terminus. Chemokines are important in several biological processes, including the inflammatory response, and also in certain diseases such as chronic inflammation and atherosclerosis. Additionally, a family of chemokines called angiogenic chemokines have been shown to be important regulators of neovascularization. Angiogenic chemokines share conserved cysteine residues separated by any amino acid (CXC) followed by a glutamate, leucine, arginine (ELR+) motif near the amino terminus. The ELR motif is critical for the pro-angiogenic functions of these chemokines (Keeley *et al.*, 2008). CXC ELR+ chemokines, including CXCL1, CXCL5, CXCL8, act as chemoattractants for endothelial cells. Conversely, angiostatic CXC chemokines such as CXCL10, that lack the ELR+ motif, act in an opposite manner (Rollins, 1997). The balance between angiogenic and angiostatic chemokines that is present in normal cells becomes deregulated in highly proliferative cancer cells, resulting in increased angiogenic CXC chemokine production and subsequent increased tumor angiogenesis (Strieter *et al.*, 2006). Angiogenic chemokines, produced and

secreted by prostate cancer cells, form a gradient and promote endothelial cell migration from existing blood vessels by binding to their receptor, CXCR2 (Shen and Lentsch, 2004). This process results in the formation of new blood vessels from pre-existing vessels, that integrate into the tumor (Waugh *et al.*, 2008). CXCL8 in particular, has been shown to be a strong inducer of endothelial cell migration through binding to the CXCR2 receptor (Shen *et al.*, 2006; Waugh *et al.*, 2008).

Increased angiogenic chemokine production has been implicated in the pathogenesis of prostate cancer (Balbay *et al.*, 1999; Greene *et al.*, 1997; Hepburn *et al.*, 1997). When prostate cancer cells are injected into severe combined immunodeficiency (SCID) mice, tumor growth can be inhibited by blocking antibodies against CXCL1 and CXCL8, thus demonstrating the significance of angiogenic chemokines in prostate tumor growth (Moore *et al.*, 1999). Additionally, in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse model of prostate cancer, mice lacking the CXCR2 receptor have decreased prostate tumor size and decreased prostate tumor angiogenesis, further signifying the importance of angiogenesis in prostate tumor growth (Shen *et al.*, 2006). Interestingly, inhibiting the NF-kappa B (NF-κB) transcription factor in PC-3 prostate cancer cells results in decreased angiogenic chemokine production (Shen and Lentsch, 2004), suggesting NF-κB is a critical regulator of angiogenic chemokine production in these cells.

To investigate the significance of Ron in prostate cancer, we examined the expression and function of this receptor in relation to prostate tumor formation and angiogenesis. Our studies show that Ron is highly expressed in human prostate cancers and that Ron expression levels are associated with the production of angiogenic chemokines in prostate cancer cells. We also show that overexpression of Ron in LNCaP cells is sufficient to induce CXCL8 production and that this expression is dependent, at least in part, on NF-κB activation. Coordinately, Ron inhibition in PC-3 or DU145 cells results in a significant decrease in angiogenic chemokine production and a Ron knockdown in DU145 cells lead to decreased NF-κB activity. This regulation by Ron is biologically important in the regulation of endothelial cell migration *and in vivo* by impacting prostate tumor vascularization.

MATERIALS AND METHODS

Immunohistochemistry on Human Prostate Cancer Specimens

Immunohistochemistry was performed on tissue microarrays (Cat. # IMH-303 Imgenex, San Diego, CA; Cat. # TMA1202-4 Chemicon, Millipore, Billerica, MA; Cat. # 75-4063, Zymed Carlsbad, CA) or from tissue samples obtained from the University of Cincinnati Cancer Center Tissue Bank. Tissue staining was performed as previously described (Peace *et al.*, 2005). Two antibodies were used for Ron immunohistochemistry which provided similar results (1:50 Ron α, BD Transduction Laboratories, San Diego, CA, or 1:50 Ron C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Samples with no primary antibody or an IgG control antibody served as negative controls. Scoring the percent of positive specimens for Ron and the mean intensity of Ron staining was performed as previously described (O'Toole *et al.*, 2006) with the mean intensity of epithelial staining obtained by multiplying the relative intensity score (0–3) by the percentage of epithelial cells staining positive for Ron.

Flow Cytometry

Flow cytometric analyses were performed on prostate cells as follows: 1×10^6 cells were fixed in 2% formaldehyde and resuspended in 1ml 70% ethanol. Cell were permeabilized in PBS/BSA plus 0.5% TritonX-100, treated with primary antibody (1:100 Ron, C-20) or controls in PBS/BSA/TritonX-100 for 1 hour, and were subsequently incubated in phycoerythrin (PE)-conjugated anti-rabbit secondary antibody (0.5 ug, Molecular Probes, Carlsbad, CA). After washing, cells were resuspended in 1ml water for flow analysis. The samples were analyzed using a Coulter Epics XL (Beckman Coulter, Miami, FL). Expression of Ron was graded as no detectable expression (+/−), low expression (+), intermediate expression (++), or high expression (+++) based on mean fluorescent intensity (MFI). A MFI of less than 40 was considered no expression, 40–60 as low expression, 60–80 as intermediate expression, and over 80 as high Ron expression.

Quantitative Real-Time PCR

RNA was isolated from the cell lines indicated and was used to generate cDNA using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. To measure chemokine expression, quantitative real-time PCR analyses were performed utilizing SYBR green incorporation (Applied Biosystems, Foster City, CA) with the following primers: CXCL8 Forward: 5'-TTG GCA GCC TTC CTG ATT TC-3' and Reverse: 5'-TGA GAG TGA TTG AGA GTG GAC CA-3'; Met (Yoshida *et al.*, 2002), $VEGF_{165}$ (Collado *et al.*, 2007), and CXCL5 (Sawa *et al.*, 2008). Gene expression values were normalized to 18S Forward: 5'-AGT CCC TGC CCT TTG TAC ACA-3' and Reverse: 5'-GAT CCG AGG GCC TCA CTA AAC-3' as an internal control. Relative gene expression results are reported.

Immunoprecipitations, Western Analysis and Kinase Assays

For immunoprecipitations, 1 milligram of total cellular lysate in 1ml PBS containing protease inhibitor (Complete Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN) was incubated with 5μg of primary antibody (Ron C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for one hour at 4°C. Protein G-agarose beads were added and complexes were incubated overnight at 4°C. Immunocomplexes were then collected and subjected to Western analysis as previously described (Zinser *et al.*, 2006). Primary antibodies used were Ron C-20 (1:400), phospho-Tyrosine (4G10 1:1000, Upstate, Billerica, MA), phospho-Ron (Y1238/1239; 1μg/ml, R&D Systems, Minneapolis, MN), IκBα (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Met (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Actin C4 (1:40,000; received as a gift from Dr. James Lessard at Cincinnati Children's Hospital Medical Center, Cincinnati, OH). Peroxidase-conjugated secondary antibodies were applied, membranes were developed using ECL Plus Western Detection Reagent (GE Healthcare, Piscataway, NJ), and protein bands were detected by autoradiography. Kinase assays were performed as previously described (Zinser *et al.*, 2006) with 1 milligram of protein lysate utilized for immunoprecipitations with the Ron α antibody and with myelin basic protein as the substrate. Samples were separated by SDS-Page and the gels were fixed, dried and imaged on a phosphoimager (Typhoon Trio, GE Healthcare, Piscataway, NJ).

Chemokine Production and Enzyme-Linked ImmunoSorbent Assay

Cells were plated in a 24-well tissue culture dish in complete media. After reaching ~80% confluency, serum-free media was added (Defined Keratinocyte Media, Gibco, Carlsbad, CA). Supernatants were collected over time and chemokine levels were determined by Enzyme-Linked ImmunoSorbent Assays (ELISAs) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Crystal violet assays were performed by fixing cells in 10% neutral buffered formalin for 15 minutes, and then incubating cells with 0.1% crystal violet in 25% methanol for 30 minutes. The cells were washed, air-dried, and 500μl of DMSO was added. After shaking 30 minutes, the absorbance was read at 570nm. Relative cell number was plotted by calculating the fold change in cell growth from 0 hours to 72 hours and normalizing the values to one cell line set at 1.0. The relative level of chemokine production between cell lines was calculated with the amount of chemokines produced normalized to the relative cell number observed during the time course of the experiment (Figure 3A and B). LNCaP, 22RV1 and PC-3 cells all grew to similar extents and the chemokine values were not adjusted. However, DU145 cells grew significantly faster during the experimental observation period (Figure 3D) and the relative chemokine values for these cells were decreased accordingly by the change in cell number.

NF-κB Activity

To obtain cells with a stable knockdown of Ron, cells were infected with either a lentivirus or a retrovirus containing a Ron-specific shRNA or a nonsense shRNA as a control (constructs were purchased from Open Biosystems; Ron shRNA catalog #RHS3979, RHS1764; viruses were made at the Cincinnati Children's Hospital viral vector core). Stable populations were then selected with 1μg/ml puromycin. 1×10^5 cells were plated in triplicate in Minimum Essential Medium (MEM) containing 5% FBS. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with either a NF-κB reporter (pNF-κBluc) or empty vector (pTAL-luc) construct and a control plasmid expressing Renilla (pRL-TK). 24 hours after transfection, the cells were lysed and subjected to a dual-luciferase assay according to manufacturer's protocol (Dual-Luciferase Reporter Assay System, Promega, Madison, WI). Samples were read using the GloMax® 96 Microplate Luminometer with Dual Injectors (Promega, Madison, WI). NF-κB electrophoretic mobility shift assays were performed as previously described (Nikolaidis *et al.*, 2009; Shen and Lentsch, 2004).

Endothelial Cell Migration

Ron knockdown or control cells were plated in triplicate in Minimum Essential Medium (MEM) containing 5% FBS. At 70% confluency, cells were serum-starved, and 48 hours later supernatant was collected and was used as the chemoattractant for endothelial cells (HUVECs) in a transwell migration assay. For the migration assay, 1×10^5 HUVECs were plated in the top chamber of 8.0μm transwells (Corning Costar Corporation, Cambridge MA) in endothelial cell growth media (Mediatech, Inc., Manassas, VA). The following day, cells were washed with PBS, and serum-free MEM was added. For inhibition of CXCR2, 300μM of SB225002 (Calbiochem, Gibbstown, NJ) was added to HUVECs. The chemoattractant was diluted 1:10 in serum-free MEM, added to the bottom of the 24-well

plate, and HUVEC cells were allowed to migrate for 5 hours. The number of live cells on the bottom of the transwell was measured using an MTT (3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide) assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO) with absorbance read at 570nm.

Ron Overexpression

LNCaP prostate cancer cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a pCI-neo vector (Promega, Madison, WI) containing human Ron cDNA or with pCI-neo as a control. Cells were selected with G418 and stable cells were plated at 3×10^4 cells per well in 6-well plates, grown to 70% confluency, and serum-starved for 24 hours. Cells were treated with 100ng/ml of recombinant HGFL (R&D Systems, Minneapolis, MN) in serum-free media or left untreated. 50μM of Bay 11-7082 (Calbiochem, San Diego, CA) was added to cells for 48 hours to examine NF-κB inhibition. Supernatant was collected at 48 hours and analyzed by ELISA.

Orthotopic Injections and CD31 Staining

PC-3M-luc2 cells (5×10^5, Caliper Life Sciences, Hopkinton, MA), a PC-3 cell line containing stable luciferase expression, or PC-3M-luc2 cells with a stable Ron knockdown, were orthotopically injected into dorsolateral prostates of 11 week old nude mice. Tumors were harvested 7 weeks post-injection and tumor mass was recorded. The tumors were fixed and processed for histological analyses. CD31 staining was performed as previously described (Loh *et al.*, 2009). Tissues were incubated with an anti-CD31 antibody (1:500, Dako, CA, USA) and DAB enhanced liquid substrate system (3,3'-diaminobenzidine tetrahydrochloride-Sigma, Mo, USA) was used for detection. Microvessel density was calculated as previously described (Peace *et al.*, 2005).

Statistical Analysis

Data are expressed as mean ± standard error. Statistical significance comparing different experimental groups was determined by Student's t-test for pair wise comparisons, or ANOVA for comparison of multiple groups using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Differences between groups were accepted as significant when $p<0.05$.

RESULTS

Ron is overexpressed in human prostate cancer specimens

Ron expression has been grossly examined in a variety of human cancers including prostate cancer, although there has been no published reports analyzing relative Ron expression during prostate tumorigenesis (O'Toole *et al.*, 2006; Wang *et al.*, 2007). To determine the degree of Ron overexpression throughout the progression of prostate disease, we analyzed a series of human prostate tissue arrays for Ron expression by immunohistochemistry. Figure 1 shows representative Ron staining observed in normal prostate tissue, prostate adenocarcinoma and in metastatic prostate disease. Table 1 documents the percent of Ron positive tissues per category as well as the mean intensity of Ron expression observed. Of note, high Ron expression was observed in the benign prostate hyperplastic tissue compared to normal prostate tissue with an even further increase in Ron expression detected in prostate

adenocarcinoma tissue. In addition, all three lymph nodes analyzed with prostate metastases exhibited high Ron expression.

Ron is highly expressed in PC-3 and DU145 human prostate cancer cells

To determine the expression of Ron in human prostate cells, Western analyses were performed. As shown in Figure 2A, Western analysis demonstrates high Ron expression in PC-3 and DU145 cells and little Ron expression in 22RV1 and LNCaP cells. Interestingly, PC-3 and DU145 cells are two androgen-independent prostate cancer cell lines derived from metastatic prostate cancers. Further analysis of Ron expression (Supplemental Figure S1A) shows PC-3 cells express high amounts of Ron, while the non-invasive CA-HPV-10 cells derived from a primary human prostate tumor express an intermediate amount of Ron, and the non-transformed, immortalized prostate cell line PZ-HPV-7, has very little Ron expression. These results correlate with the pattern of Ron expression observed in the human prostate tumor tissue arrays shown in Figure 1 and Table 1.

Ron is active in PC-3 and DU145 cells

In many tumors overexpressing Ron, a high degree of receptor phosphorylation is frequently observed (Maggiora *et al.*, 1998; Peace *et al.*, 2001; Wang *et al.*, 2007; Zhou *et al.*, 2003). To determine if Ron is phosphorylated and serves as an active kinase in prostate cancer cells, we performed Western analyses following immunoprecipitation with a Ron-specific antibody. As shown in Figure 2B, Ron is tyrosine phosphorylated in PC-3 and DU145 cells suggesting Ron is active in these cell lines. We also analyzed PZ-HPV-7 (a low Ron-expressing cell line) cells for tyrosine phosphorylation of Ron following Ron immunoprecipitation. Compared to PC-3 and DU145 cells, PZ-HPV-7 cells have minimal tyrosine phosphorylation of Ron (Figure 2B). To further determine the activity of Ron, we performed kinase assays on Ron immunoprecipitated from PC-3 or DU145 cells. Utilizing myelin basic protein as an exogenous substrate, we observed that both PC-3 and DU145 cells exhibit Ron-dependent kinase activity (Figure 2C). Interestingly, similar results were observed both with cells cultured in media containing serum and with cells under serum-deprivation conditions (data not shown). We have also demonstrated that these prostate cancer cells do not endogenously produce the Ron ligand, HGFL (data not shown), suggesting that Ron may be constitutively phosphorylated and active in the absence of ligand in these prostate cancer cell lines.

Prostate cancer cells with high Ron expression also produce high levels of angiogenic chemokines

To determine if prostate cancer cells with high Ron expression also produce relatively high levels of angiogenic chemokines, we analyzed the prostate cancer cell lines LNCaP, 22RV1, DU145 and PC-3 for the production of the angiogenic chemokines, CXCL8 (Figure 3A), CXCL1, (Figure 3B) and CXCL5 (Figure 3C) by ELISA analysis of culture supernatant. LNCaP and 22RV1 cells, which have low Ron expression, produce relatively low levels of CXCL8 when compared with the two high Ron-expressing prostate cancer cells, DU145 and PC-3. Neither LNCaP nor 22RV1 cells have detectable levels of CXCL1 or CXCL5. To determine whether the changes in chemokine production did not simply reflect changes in

cell growth among the various cell lines, the growth of all the cell lines was monitored over the time course of the experiment. As shown in Figure 3D, the change in cell growth over time was similar in LNCaP, 22RV1 and PC-3 cells, although the DU145 cells showed an increase in cell number by 72 hours. Given this increase in cell number, the relative levels of chemokines from DU145 cells are shown, which have been normalized to cell number for Figures 3A–C. Similar to protein expression, LNCaP and 22RV1 cells had undetectable levels of CXCL8 (Figure 3E) and CXCL5 mRNA (Figure 3F), although these cells had the highest levels of VEGF mRNA (Figure 3G).

Ron inhibition in PC-3 or DU145 cells leads to decreased production of angiogenic chemokines but not of VEGF or the angiostatic chemokine CXCL10

We have shown that Ron expression correlates with angiogenic chemokine production in prostate cancer cells, and next sought to determine the impact of Ron inhibition on chemokine production. PC-3 cells were transfected with either Ron-specific siRNA (Ron siRNA) or non-specific scrambled siRNA (Non siRNA). A significant loss of Ron mRNA was observed by 48 hours post-transfection (Figure 4A). To examine if loss of Ron expression impacted chemokine production in these cells, 30 hours following transfection, the cells were placed into serum free media and supernatant was collected at specific intervals. During this time frame, there was not significant change in cell number between control PC-3 cells and Ron knockdown PC-3 (Figure 4E). There are significant decreases in the angiogenic chemokines CXCL8 (Figure 4B), CXCL5 (Figure 4C), and CXCL1 (Figure 4D) secreted by Ron-knockdown PC-3 cells compared to control cells. Production of vascular endothelial growth factor (VEGF) and the angiostatic chemokine CXCL10 were also examined. PC-3 cells secrete relatively low levels of these factors (VEGF, Figure 3G) and there was no change in production in either CXCL10 or VEGF with Ron loss (data not shown). To complement our studies and to determine the specificity of Ron inhibition in another cell line, Ron expression was also knocked down in DU145 cells by infection with either a nonsense shRNA construct (shNon) as a control, or a Ron shRNA construct (shRon). As shown in Figure 5A, Ron expression was efficiently depleted in the Ron shRNA infected cells, which was associated with a significant decrease in CXCL8 production compared to control infected cells (Figure 5B). No change in cell proliferation was observed between DU145 control cells or DU145 Ron knockdown cells (Figure 5C).

Ron inhibition blocks NF-κB activity in DU145 cells

NF-κB has been shown previously to be an important regulator of angiogenic chemokine production in prostate cancer cells (Shen and Lentsch, 2004; Wilson *et al.*, 2008). To examine whether Ron regulates angiogenic chemokine production by impacting NF-κB activation, we examined NF-κB activity in control (shNon) and Ron knockdown (shRon) DU145 cells. Cells were transfected with a NF-κB reporter construct and a control plasmid. After 24 hours, cells were lysed and analyzed for NF-κB activity utilizing a dual-luciferase reporter assay system. Figure 5D shows a significant decrease in NF-κB reporter activity in the Ron knockdown DU145 cells compared to control cells, which is consistent with the decreased angiogenic chemokine production observed in Figure 5B. Consistent with the decrease in NK-κB activity, a corresponding increase in the NF-κB inhibitory protein, IκBα, was observed in the Ron knockdown DU145 cells compared to the control cells (Figure 5A).

To further support modulations in NF-κB activity, electrophoretic mobility shift assays were performed as previously described (Nikolaidis *et al.*, 2009; Shen and Lentsch, 2004). Ron loss in DU145 cells leads to a decrease in NF-κB (p65 and p50) DNA binding activity (Supplemental Figure S2).

Prostatic Ron expression is important to induce endothelial cell migration

We have shown that Ron is an important mediator of angiogenic chemokine production in prostate cancer cells. Next, we wanted to determine the functional impact of Ron signaling on the chemotactic migration of endothelial cells. Human umbilical vein endothelial cells (HUVEC) previously shown to express the CXCR2 receptor and respond to angiogenic chemokines, were utilized (Li *et al.*, 2003). Serum-free cell culture supernatant was collected from DU145 stable Ron knockdown (shRon) or control (shNon) cells. The culture supernatant was then applied as a chemoattractant for HUVECs in a transwell migration assay. Figure 5E demonstrates that the loss of Ron in DU145 cells leads to a significant reduction in endothelial cell migration. Previous studies have shown that endothelial cell migration to PC-3 cell culture supernatant can be partially blocked by inhibiting the angiogenic chemokine receptor, CXCR2, on the endothelial cells (Shen *et al.*, 2006). Similarly, endothelial cell migration to DU145 cell culture supernatant can also be significantly reduced by inhibition of CXCR2 (Supplemental Figure S2C),

Ron overexpression is sufficient to induce CXCL8 production in LNCaP prostate cancer cells through a mechanism dependent on NF-κB signaling

We have shown that Ron inhibition leads to a diminution of angiogenic chemokine production by PC-3 and DU145 prostate cancer cell lines. To examine whether Ron overexpression in prostate cancer cells is sufficient to induce angiogenic chemokine production, Ron was stably overexpressed in LNCaP cells (Figure 6A). In Ron-overexpressing LNCaP cells, we observed significantly increased CXCL8 both at the protein (Figure 6B) and RNA (Figure 6C) level compared to control transfected cells. Moreover, this induction in CXCL8 is further enhanced when these cells are treated with the Ron ligand, HGFL (Figure 6B). However, there was not a statistical difference between HGFL treated and untreated groups. Interestingly, Figure 6D demonstrates that this induction of CXCL8 could be abrogated with the NF-κB pathway inhibitor Bay 11-7085.

Ron expression promotes prostate tumor microvessel density

To examine a role for Ron in tumor vascularization *in vivo*, PC-3 cells with a stable knockdown of Ron (Figure 7A) or control cells were orthotopically injected into the prostates of nude mice. Seven weeks after injection, the prostates were harvested and prostate tumor mass (Figure 7B) and microvessel density was determined (Figure 7C and D). Compared to control cells, Ron knockdown cells not only formed smaller tumors but the overall microvessel density per equal area of tumor was significantly less.

Ron knockdown in DU145 cells does not affect Met expression

To examine the relationship between Ron and Met in prostate cancer cells, real-time PCR analysis was performed for Met in PC-3, DU145, 22RV1 and LNCaP cells. PC-3 cells have

the highest levels of Met, DU145 and 22RV1 cells have intermediate levels, and LNCaP cells have minimal Met expression (Supplemental Figure S3A). Met levels are similar to the expression pattern of Ron in these cells (Figure 2A). To determine whether a Ron knockdown in prostate cancer cells alters Met expression, we examined DU145 control cells or DU145 Ron knockdown cells for Met expression. Ron knockdown does not alter Met levels either at the RNA (Supplemental Figure S3B) or protein level (Supplemental Figure S3C).

DISCUSSION

Ron overexpression has been associated with several different human cancers, including prostate cancer (O'Toole *et al.*, 2006). Our studies show for the first time that Ron overexpression may play an important role in human prostate cancer progression by promoting the production of angiogenic chemokines. This regulation of angiogenic chemokines by Ron is functionally significant because the amount of chemokines produced and secreted impacts endothelial cell migration. Given that the levels of angiogenic chemokines and extent of endothelial cell migration are critical steps in angiogenesis (Waugh *et al.*, 2008), our data suggest that the Ron receptor may be an important mediator of prostate tumor angiogenesis.

In this report, we have shown that Ron expression is increased in human benign hyperplastic prostate tissue and is further increased in human prostate adenocarcinoma relative to normal prostate tissue (Figure 1 and Table 1). Similar results were observed in human pancreatic tissue wherein minimal Ron expression was detected in the normal pancreatic ducts or in early pancreatic intraepithelial neoplasia, although Ron was highly expressed in the more advanced pancreatic disease (Thomas *et al.*, 2007). This consistent expression pattern suggests that Ron may be playing a role in the progression to advanced disease. While with our limited sample size we did not find Ron expression to correlate with either prostate cancer grade or Gleason score, there are several gene expression array studies demonstrating Ron expression is progressively higher in metastatic prostate cancer compared to prostate carcinoma, and Ron in prostate carcinoma being more highly expressed than in the normal prostate (Dhanasekaran *et al.*, 2005; Lapointe *et al.*, 2007). In addition, multiple studies have indicated that Ron overexpression examined by microarray analyses also correlates with hormone-refractory/androgen-independent prostate cancers (Best *et al.*, 2005; Tomlins *et al.*, 2007; Varambally *et al.*, 2005). These studies support our observations that Ron is highly expressed in prostate cancer adenocarcinoma and in metastatic prostate cancer, and minimally expressed in normal or benign prostate specimens.

An analysis of immortalized human prostate cell lines revealed a similar trend to that found in human prostate specimens, wherein Ron expression increased from the non-transformed, immortalized cells (PZ-HPV-7), to cells derived from benign prostate tumor (CA-HPV-10), to cells derived from metastatic prostate (PC-3 and DU145). Interestingly, the two androgen-independent prostate cancer cell lines, PC-3 and DU145 have high Ron expression, while 22RV1 and LNCaP express little to no Ron (Figure 2A and Table 2). Similar to Ron, the related receptor tyrosine kinase Met, has recently been shown to be upregulated in prostate cancer (Verras *et al.*, 2007). Our analysis of Met expression in

prostate cancer cell lines showed a similar trend to Ron expression (Supplemental Figure S3A). Interestingly, androgen receptor signaling was shown to negatively regulate Met expression at the transcriptional level in androgen-dependent prostate cancer cells. Additionally, LNCaP xenografts in castrated SCID mice had upregulated Met expression as compared to intact mice, and Met expression levels were inversely correlated with androgen receptor expression (Verras *et al.*, 2007). These data suggest that Met signaling may play a role in androgen-independent prostate cancer. Based on the progressive increase of Ron expression observed in prostatic hyperplasia and prostate adenocarcinoma compared to normal prostate tissue, the high expression of Ron observed in the three metastatic lymph nodes analyzed, and increased Ron expression in androgen-independent cell lines, it is interesting to speculate that the Ron receptor may also play an important role in androgen-independent prostate cancer.

Based on kinase assays and Western analyses performed in serum-free and serum-containing conditions, our data suggest Ron exhibits constitutive receptor phosphorylation and kinase activity in PC-3 and DU145 cells (Figure 2B and 2C). Immunohistochemical staining of human tumor specimens and an analysis of tumor lysates from breast and colon tissues has shown that Ron overexpression is associated with a high degree of receptor phosphorylation (Maggiora *et al.*, 1998; Zhou *et al.*, 2003). Our studies are also consistent with overexpression studies performed in NIH/3T3 cells wherein the overexpression of Ron was sufficient to induce activity of this receptor (Peace *et al.*, 2001). While we have observed constitutive Ron activity in cell lines following overexpression, the requirement of HGFL in Ron expressing tumors in vivo has not yet been established. Interestingly, we have not observed endogenous expression of HGFL in any of the prostate cell lines used in this study, although *in vivo*, it is important to note that HGFL is widely available in the prostate microenvironment due to the high circulating levels of this protein and may provide a functional role in tumor promotion (Bezerra *et al.*, 1998; Bezerra *et al.*, 1993). Consistent with this idea, a recent report has shown that exogenous overexpression of HGFL in Ron expressing breast tumors leads to increased tumor growth and a broadened pattern of metastatic dissemination suggesting that HGFL is an important contributor to metastases (Welm *et al.*, 2007).

We demonstrate that Ron expression in prostate cell lines correlates with the expression levels of a select group of CXC ELR+ chemokines, in particular CXCL-8, -5 and -1. The prostate cancer cell lines PC-3 and DU145, which exhibit the highest Ron expression, produce the highest levels of these angiogenic chemokines (Figure 3). We demonstrated that PC-3 cells transiently transfected with a Ron-specific siRNA resulted in significantly decreased production of CXCL8, CXCL5, CXCL1 (Figure 4). Further, the diminution of Ron expression was specific for this group of chemokines, as there were no alterations observed in the CXC ELR- chemokine CXCL10 or in the production of VEGF. Similarly, Ron inhibition in DU145 cells led to decreased CXCL8 (Figure 5B) demonstrating the regulation of chemokine production by Ron in multiple prostate cancer cell lines. In support of the significance of Ron expression in the induction of angiogenic chemokines, we showed that exogenous Ron expression in LNCaP cells was sufficient to induce the production of CXCL8 (Figure 6). These studies are the first to implicate the Ron receptor tyrosine kinase

in the production of angiogenic factors in epithelial cancer cells, and suggest that this receptor may play an important role in regulating tumor angiogenesis.

With respect to angiogenesis, previous studies have linked the significance of angiogenic chemokines to the migration and chemoattraction of endothelial cells and to angiogenesis *in vivo* (Addison *et al.*, 2000; Balbay *et al.*, 1999; Hepburn *et al.*, 1997; Koch *et al.*, 1992; Salcedo *et al.*, 2000). Importantly, we have also shown that modulation of Ron, which is overexpressed in a variety of tumor types, has a dramatic impact on endothelial cell recruitment (Figure 5E). Similar to a mouse model of breast cancer (Peace *et al.*, 2005), we have also demonstrated a decrease in prostate tumor size correlating with decreased tumor microvessel density when Ron-deficient cells are orthotopically transplanted into prostates of nude mice (Figure 7).

Mechanistically, our data show that Ron is an important regulator of NF-κB activity in DU145 and LNCaP cells. Knockdown of Ron in DU145 cells resulted in decreased NF-κB activity, and increased IκBα levels compared to control cells. These findings correlate with the observed decreases in chemokine production and endothelial cell migration (Figure 5). In addition, we also demonstrated that the production of CXCL8 induced by Ron overexpression in LNCaP cells was blocked by treatment with a pharmacological inhibitor of NF-κB signaling (Figure 6D). This data is consistent with published reports documenting the requirement of NF-κB signaling for the production of CXC ELR+ chemokines in PC-3 cells and suggests that Ron signaling has a positive impact on NF-κB regulation in prostate cancer cell lines (Shen and Lentsch, 2004).

In support of our studies with Ron and angiogenesis, the Met receptor has also been implicated in tumor angiogenesis. Interestingly, Met and Ron have similar expression patterns in prostate cancer cells (Figure 2A and Supplemental Figure S3A). Treatment of small cell lung carcinoma cells and non-small cell lung carcinoma cells with the small molecule inhibitor for Met, PHA665752, in mouse xenograft experiments led to a reduction in tumor size and a dramatic reduction in tumor angiogenesis (Puri *et al.*, 2007). Similarly, Met activation by its ligand, hepatocyte growth factor (Bezerra *et al.*), has been shown to induce CXCL8 expression in esophageal squamous cell carcinoma cells and several reports have shown that serum levels of HGF are correlated with CXCL8 production (Ren *et al.*, 2005). These studies support the contention that the Ron receptor tyrosine kinase may play an important role in the production of angiogenic chemokines that promote tumor growth and angiogenesis. Therefore, targeting Ron may be useful therapeutically in a wide variety of cancers, including the treatment of prostate cancer, by impacting tumor angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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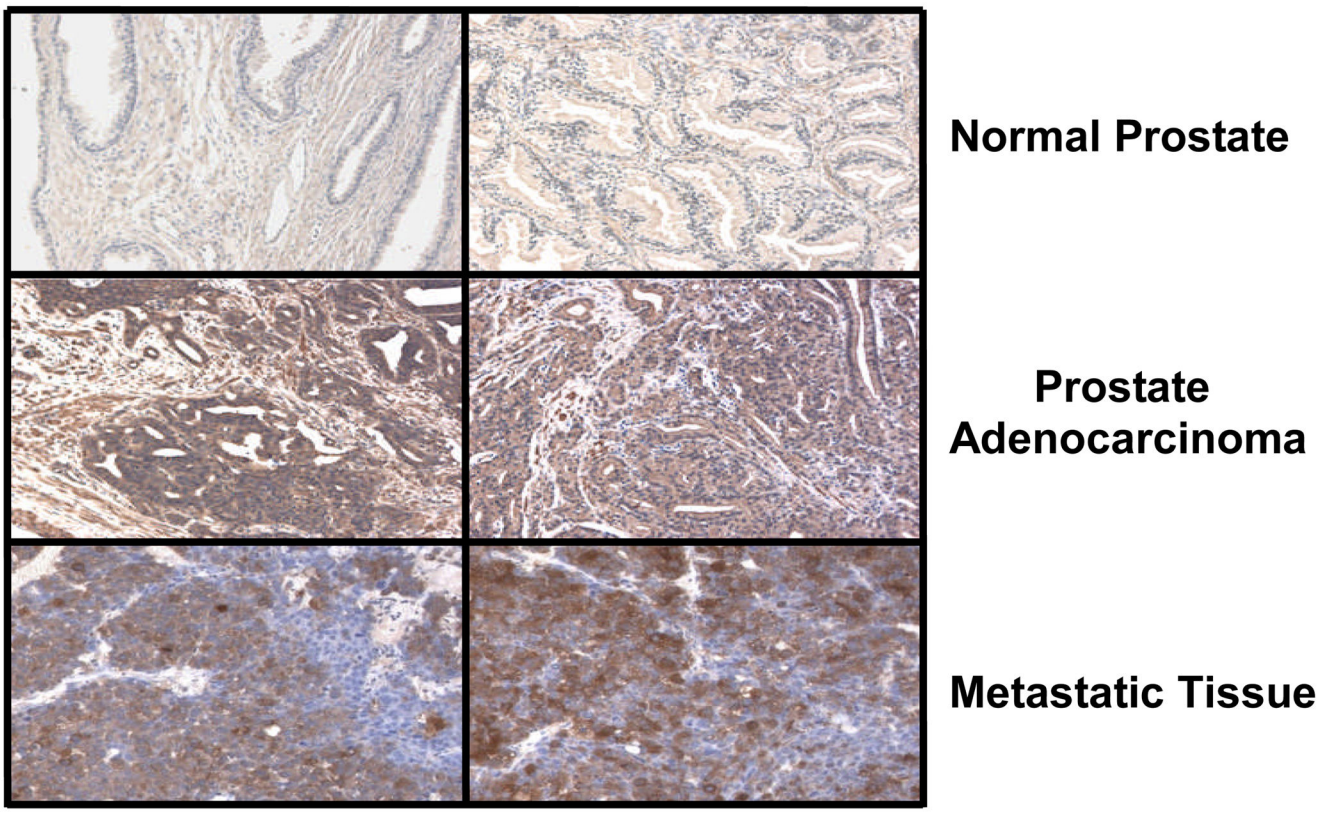

Figure 1. Ron is highly expressed in human prostate cancer specimens

Increased Ron expression is observed in prostate cancer and metastatic tissue compared to normal prostate tissue. Representative stained specimens of normal prostate, prostate adenocarcinoma, and metastatic lymph node from advanced prostate cancer are depicted.

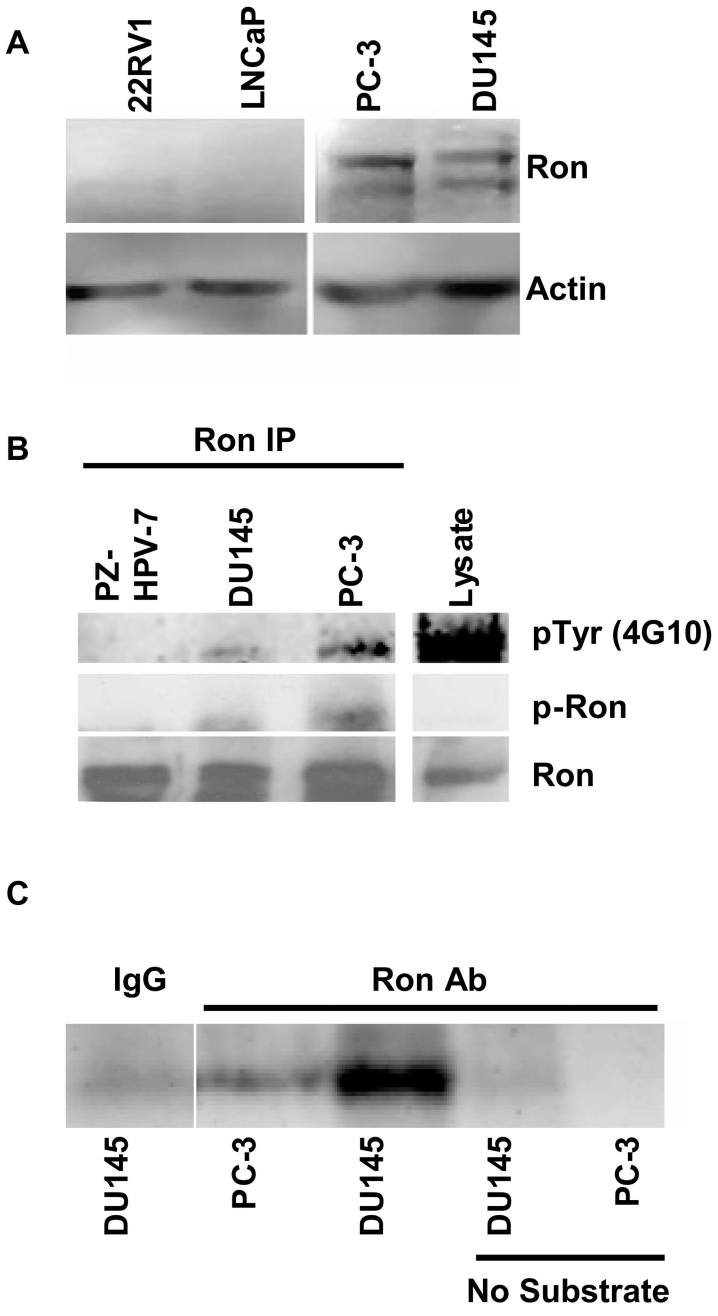

Figure 2. Ron receptor expression and activation in human prostate cells

A, Ron expression in human prostate cells. Ron is highly expressed in PC-3 and DU145 prostate cancer cells compared to 22RV1 and LNCaP cells as determined by Western analysis. **B**, and **C**, Ron is tyrosine phosphorylated and is an active kinase in DU145 and PC-3 cells but not in the non-transformed prostate cell line PZ-HPV-7. **B**, PZ-HPV-7, DU145 or PC-3 cells were immunoprecipitated with an antibody against Ron and were subjected to Western analysis with a phospho-tyrosine or a phosphorylated Ron (Tyrosine 1238/1239) antibody. A lysate control is shown representing 20% of the total input. **C**,

DU145 or PC-3 cells were immunoprecipitated with a Ron antibody (or with IgG as a control), and a kinase assay was performed with myelin basic protein as substrate. Controls included reactions that did not include substrate.

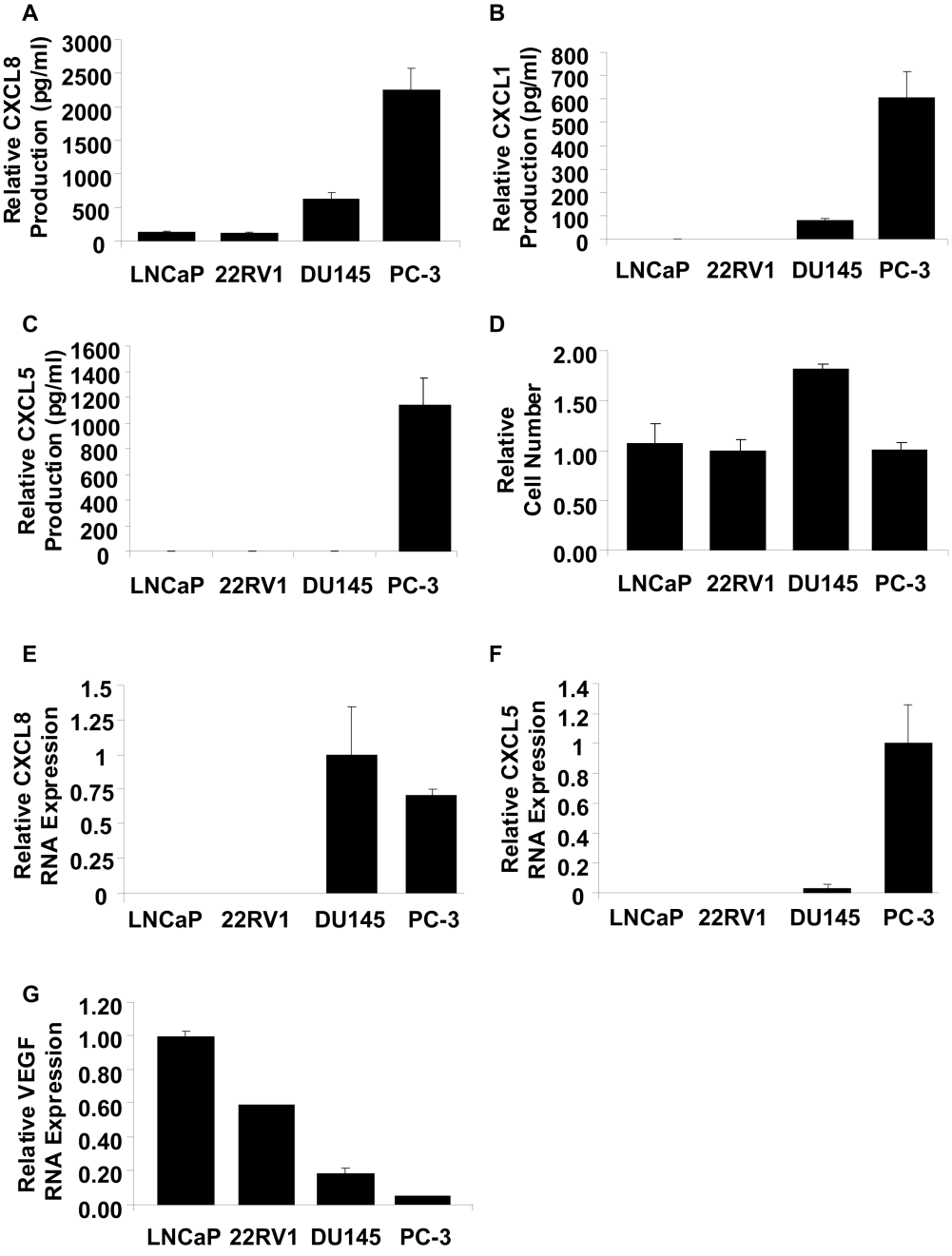

Figure 3. DU145 and PC-3 prostate cancer cells with high Ron expression produce high amounts of angiogenic chemokines

A, B, and **C**, Levels of CXCL8, CXCL1, and CXCL5 in prostate cancer cells. LNCaP, 22RV1, DU145 and PC-3 cells were placed in serum free media for 72 hours and supernatant was used to determine levels of CXCL8 (Figure 3A), CXCL1 (Figure 3B), and CXCL5 (Figure 3C) by ELISA. Only DU145 and PC-3, which overexpress Ron, produce CXCL8 and CXCL1 while limited to no expression of these chemokines is observed in LNCaP and 22RV1 cells. Only PC-3 cells had detectable levels of CXCL5. Similar levels of

all three chemokines were also observed at 96 hours (data not shown). **D**, DU145 cells grow at a greater rate than LNCaP, 22RV1, and PC-3 cells. 72 hours after plating cells, crystal violet assays were performed to determine changes in cell number over time. LNCaP, 22RV1 and PC-3 cells all had similar rates of cell growth over 72 hours, while DU145 had a higher growth rate. The relative levels of chemokines in **A–C** reflects a normalization to cell number for the DU145 cells (see Materials and Methods). **E, F** and **G**, RNA levels of CXCL8, CXCL5 and VEGF in prostate cancer cells. Similar to results as determined by ELISA, quantitative real-time PCR analyses confirmed that both DU145 and PC-3 cells have high levels of CXCL8 (Figure 3E), while PC-3 cells are the only cell line that had detectable levels of CXCL5 (Figure 3F). Conversely, DU145 and PC-3 have low levels of VEGF compared to LNCaP and 22RV1 cells (Figure 3G).

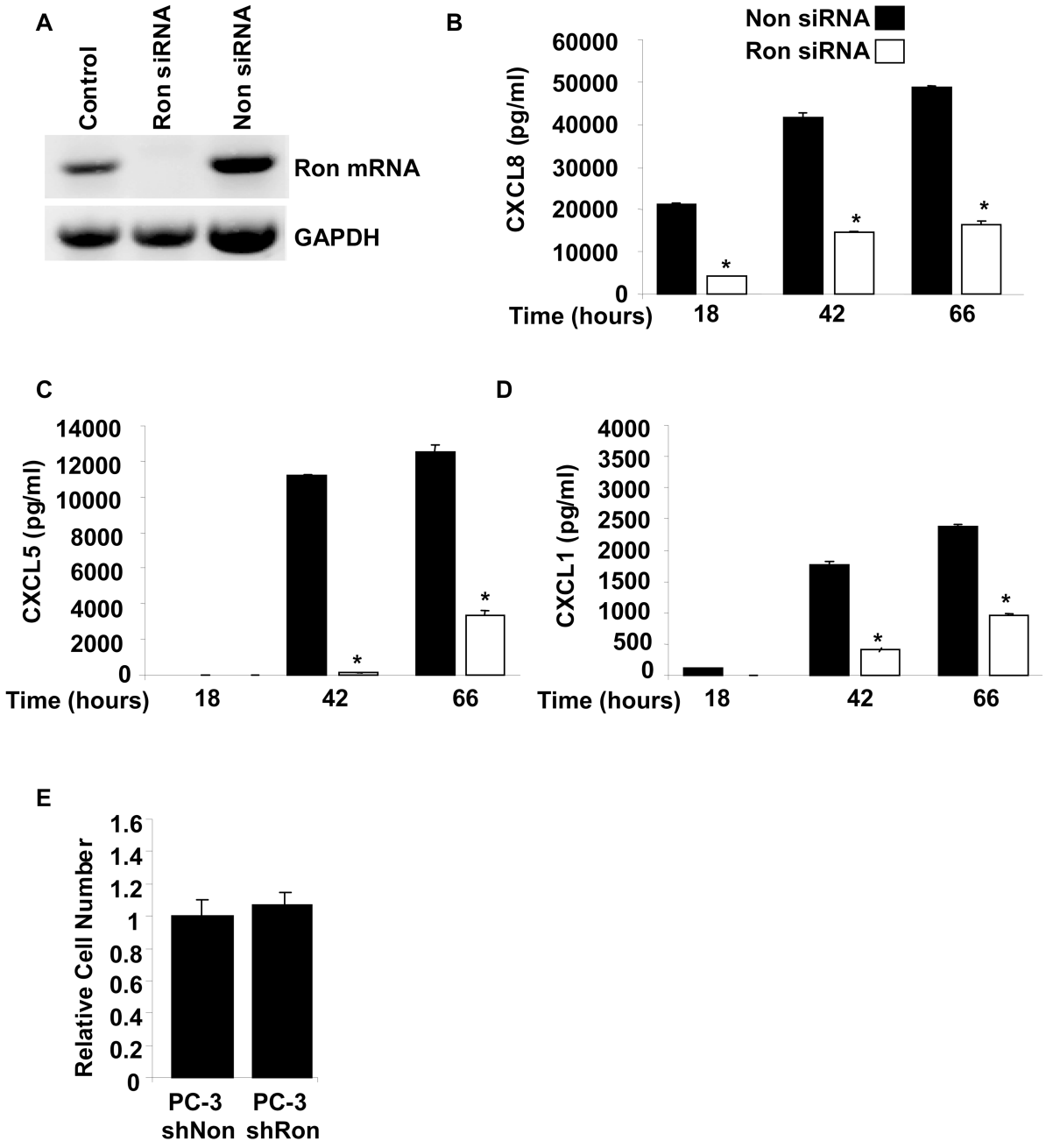

Figure 4. Knockdown of Ron in PC-3 cells leads to decreased levels of angiogenic chemokine production

PC-3 cells were either untransfected (Control), transfected with a Ron-specific siRNA (Ron siRNA), or transfected with a control nonsense siRNA (Non siRNA). **A**, Ron expression levels were determined by RT-PCR 48 hours after transfection. **B, C** and **D**, A significant decrease in production of CXCL8 (Figure 4B), CXCL5 (Figure 4C) and CXCL1 (Figure 4D) was observed in the Ron siRNA treated cells compared to nonsense siRNA treated cells. **E**, No change in cell growth was detected between the PC-3 cells with a knockdown of Ron

compared to control PC-3 cells during the course of the experiments as judged by similar crystal violet staining. Data are expressed as means ± SE. Experiments were performed in triplicate and a representative experiment is shown. *$p<0.05$ compared to nonsense siRNA treated group.

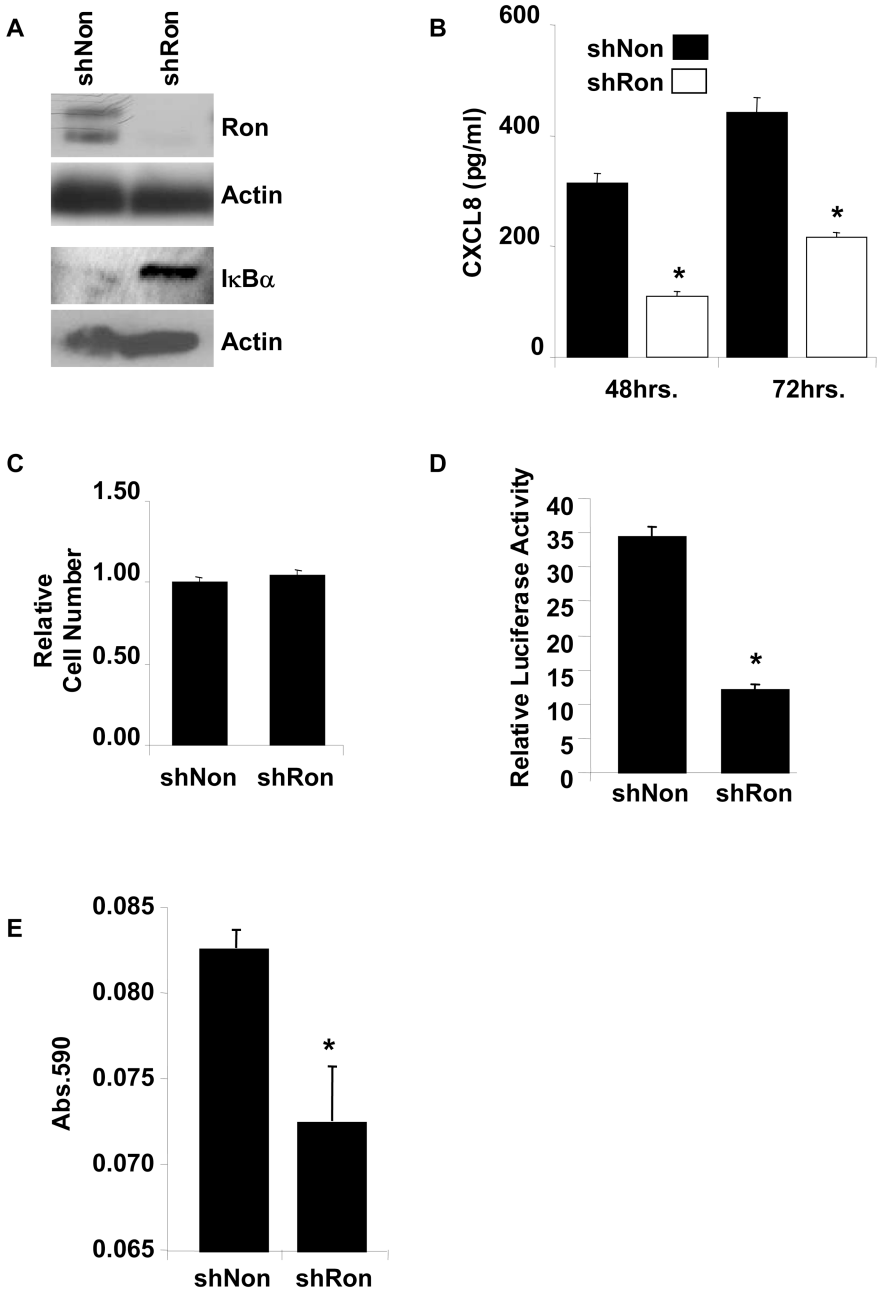

Figure 5. Ron knockdown in DU145 cells leads to decreased chemokine production, NF-κB activity, and endothelial cell chemoattraction

A, DU145 cells were stably infected with either control shRNA (shNon) or a Ron-specific shRNA (shRon) and examined for Ron and IκBα expression (Figure 5A). **B**, DU145 cells with a loss of Ron produce significantly less CXCL8 compared to control cells. **C**, DU145 cells with a Ron knockdown have similar growth rates as control DU145 cells as determined by crystal violet analyses. **D**, DU145 Ron knockdown cells have decreased NF-κB activity compared to control cells. **E**, Supernatants from Ron knockdown DU145 cells exhibit less

endothelial cell chemotaxis compared to supernatants from control cells. Data are expressed as means ± SE. Experiments were performed in triplicate and a representative experiment is shown. *$p<0.05$ compared to shNon group.

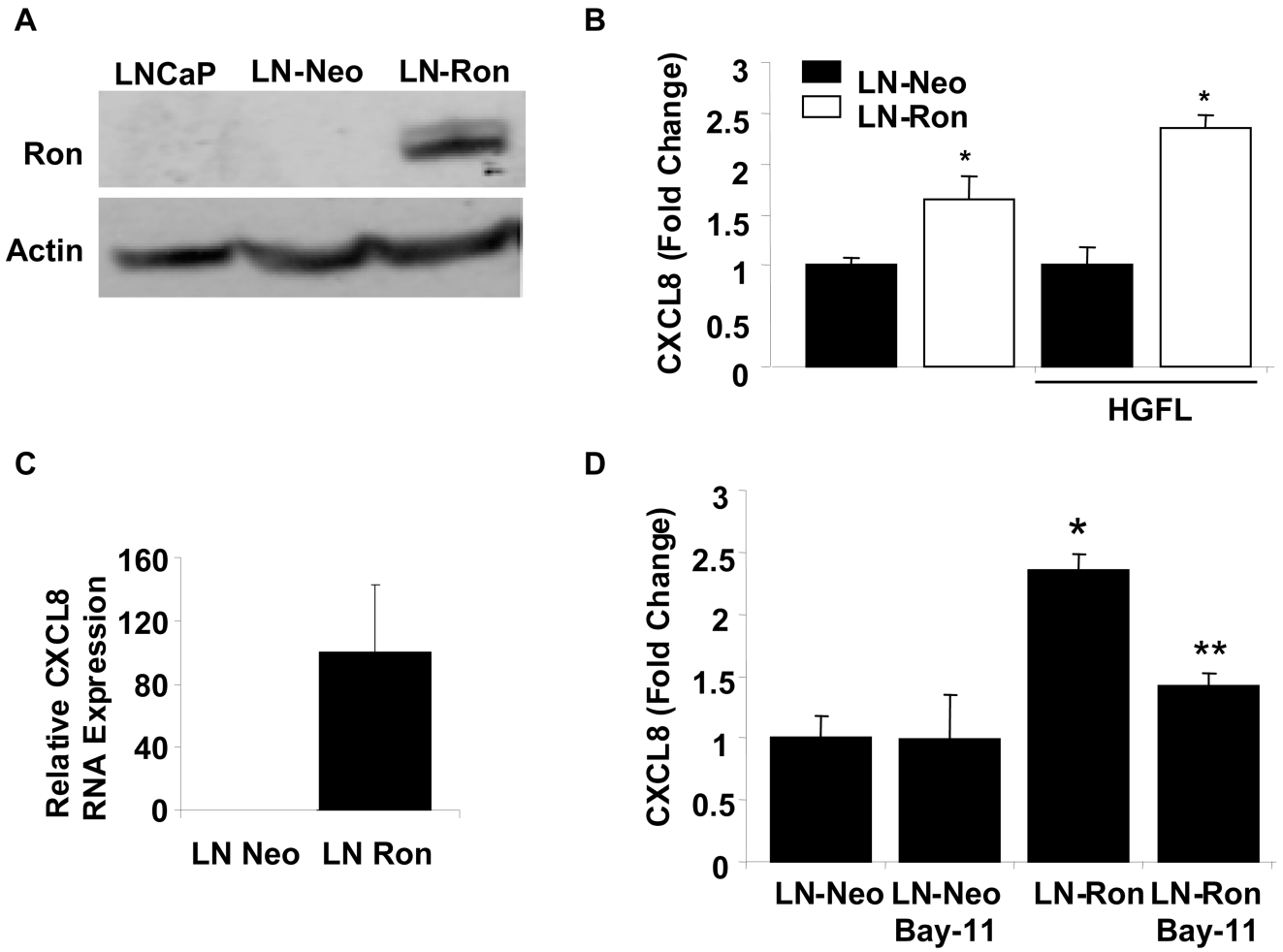

Figure 6. Ron overexpression leads to increased CXCL8 production through a mechanism dependent on NF-κB

A, LNCaP cells were stably transfected with a Ron-overexpression plasmid (LN-Ron) or a vector control plasmid (LN-Neo). **B**, Ron overexpression alone, or Ron overexpression with the addition of HGFL is sufficient to induce CXCL8 protein production. **C**, Ron overexpression in LNCaP cells results in increased CXCL8 RNA levels as determined by qRT-PCR. **D**, Increased CXCL8 production by Ron overexpression in LNCaP cells can be abrogated by treatment with the NF-κB pathway inhibitor, Bay 11-7082 (50μM). Data are expressed as fold change ± standard error over the corresponding LN-Neo group. Experiments were performed in triplicate and were reproducible. Representative experiments are shown. *$p<0.01$ compared to the corresponding LN-Neo group. **$p<0.01$ compared to LN-Ron group.

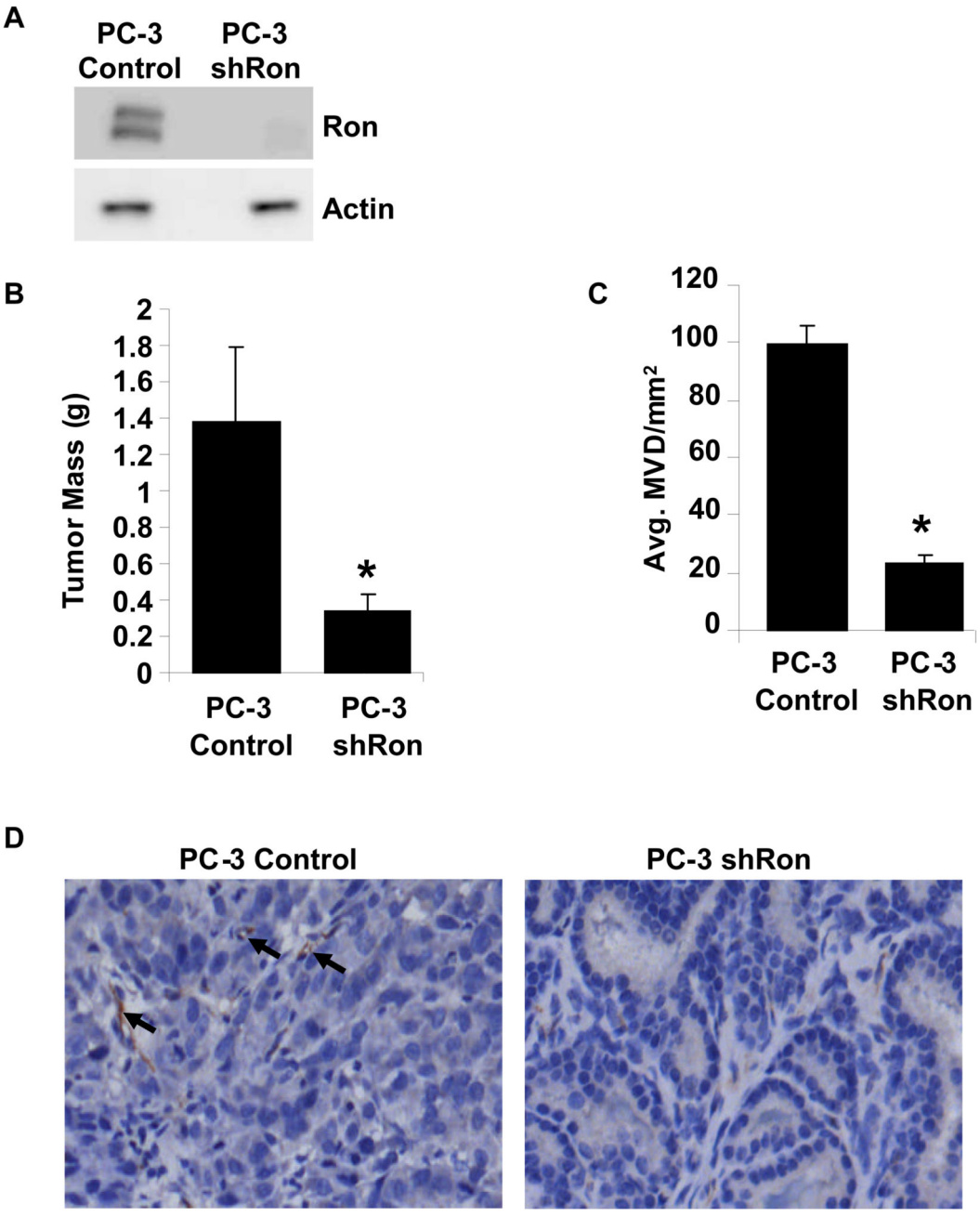

Figure 7. Ron expression promotes prostate tumor cell growth and microvessel density *in vivo*

A, PC-3 cells were stably infected with Ron shRNA and analyzed for Ron expression by Western analysis. **B**, Seven weeks post orthotopic injection into the prostate, PC-3 Ron knockdown cells form significantly smaller tumors compared to control PC-3 cells. **C** and **D**, Tissue sections from tumors were stained for CD31 to examine microvessel density. **C**, Ron knockdown PC-3 tumors have decreased tumor vasculature as determined by CD31 staining. **D**, Representative pictures of CD31 staining (arrows depict positive areas) are depicted from both control PC-3 tumors and PC-3-Ron knockdown tumors (original

magnification 200×). Data are expressed as means ± SE. *p<0.05 compared to PC-3 Control group.

Table 1

Ron expression in human prostate specimens

Prostate Tissue	% Positive	Mean Intensity	Intensity Range
Normal Prostate	12.5% (1/8)	50	0–160
Benign Prostatic Hyperplasia	74% (14/19)	143	0–180
Prostate Adenocarcinoma	86% (38/44)	156	20–300
Metastatic Adenocarcinoma	100% (3/3)	285	270–300

The percent of human prostate specimens that stained positive for Ron expression and the mean intensity of Ron staining is shown. The number of Ron expressing samples over the total number of samples examined is shown in parentheses.

Table 2

Ron expression in human prostate cell lines.

Cell Line	Ron Expression	Mean Fluorescent Intensity
DU145	+++	>80
PC-3	++	60–80
CA-HPV-10	+	40–60
LNCaP	+	40–60
22RV1	+/−	20–40
PZ-HPV-7	+/−	20–40

Ron is highly expressed in PC-3 and DU145 prostate cancer cells compared to CA-HPV-10, LNCaP, 22RV1, and PZ-HPV-7 prostate cells as determined by Western Analysis and Mean Fluorescent Intensity of Ron staining based on Flow cytometry.