UNIVERSITY OF CINCINNATI

Date: 2-Apr-2010

I, Megan Thobe, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Cell & Molecular Biology

It is entitled:

The Ron Receptor Tyrosine Kinase in Prostate Cancer

Student Signature: Megan Thobe

This work and its defense approved by:

Committee Chair: Susan Waltz, PhD

Arthur Buckley, PhD

Susanne Wells, PhD

Alex Lentsch, PhD

Karen Knudsen, PhD
The Ron Receptor Tyrosine Kinase in Prostate Cancer

A dissertation submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

in the Department of Cancer and Cell Biology
of the College of Medicine
by

Megan N. Thobe

B.S. Miami University

April 2, 2010

Committee Chair, Advisor : Susan E. Waltz, Ph.D.
Abstract

Prostate cancer is a leading cause of cancer related death among men in the United States, and despite current therapies, once the cancer progresses and metastasizes, the mortality rate is dramatically increased. Therefore, advances in our understanding of prostate cancer signaling pathways involved in prostate tumor progression are critical for the development of new therapeutic options. Prostate tumor angiogenesis has been shown to be critical for prostate tumor growth and metastasis, and the production of angiogenic chemokines is important for tumor vascularization. Angiogenic chemokines are a group of cytokines that act as chemoattractants for endothelial cells, and have been implicated in the pathogenesis of prostate cancer. A receptor tyrosine kinase termed Ron has also been implicated in several human cancers, including prostate cancer.

In our studies, we sought to determine the impact of Ron receptor signaling in prostate cancer cells on the production of angiogenic chemokines. Our data show that Ron is highly expressed in human prostate cancer specimens compared to normal prostate tissue, and is also highly expressed in PC-3 and DU145 prostate cancer cell lines. Interestingly, Ron expression correlated with angiogenic chemokine production in prostate cancer cells. Inhibition of Ron in PC-3 or DU145 cells resulted in decreased angiogenic chemokine production, with no effect on VEGF or on the angiostatic chemokine CXCL10. The impact of Ron on the production of angiogenic chemokines is at least partially dependent on the activation of the NF-kappaB transcription factor, which has previously been shown to regulate angiogenic chemokine production in prostate cancer cells. Additionally, overexpression of Ron in LNCaP prostate cancer cells is sufficient to induce production of the angiogenic chemokine CXCL8, and these levels
can be abrogated using an inhibitor of NF-kappaB. Furthermore, compared with control
PC-3 cells, Ron-knockdown PC-3 cells orthotopically transplanted into the prostates of
nude mice resulted not only in decreased prostate tumor growth, but also in decreased
prostate microvessel density, further validating our hypothesis that Ron is indeed a
regulator of prostate tumor angiogenesis. These data are further discussed in Chapter 3.

We also sought to determine the role of the Ron receptor tyrosine kinase in vivo,
by utilizing the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mouse
model. We found that prostate tumors taken from TRAMP mice have overexpression of
Ron compared to wild-type mouse prostate. Furthermore, TRAMP mice crossed with
mice lacking functional Ron exhibited decreased prostate tumor size correlating with
reduced prostate vasculature. Utilizing cell lines derived from TRAMP mouse prostates
lacking functional Ron or cells derived from TRAMP mouse prostates expressing Ron,
we found Ron-deficient TRAMP cells to be less viable than Ron-expressing TRAMP
cells. Interestingly, there is no difference in apoptosis between cell lines, however our
data suggests Ron-expressing TRAMP cells have a survival advantage. These results are
discussed in further detail in Chapter 4.

Taken together, our studies demonstrate for the first time that Ron receptor
signaling is critical for angiogenic chemokine production in prostate cancer cells.
Additionally, Ron is necessary for prostate tumor growth and angiogenesis in vivo, and is
a regulator of cellular viability in cells derived from TRAMP mouse prostates. These
studies provide novel insight into how the Ron receptor impacts prostate cancer, and
enhances our understanding of Ron receptor signaling in tumor angiogenesis.
Copyright Notice

1.) Chapter 1 contains an original review article:


2.) Chapter 3 contains an original research article:

Acknowledgements

I would like to acknowledge my advisor Dr. Susan Waltz for all of her support and guidance throughout my graduate studies. She has been instrumental in my progression as a research scientist and has taught me not only about experiment design and interpretation of data, but how to truly think like a scientist in all aspects. I also owe her special thanks for her patience, especially with helping me write manuscripts, making figures for papers and presentations, and putting together posters. She has also been extremely encouraging in discussions we had about what I want to do post-graduation, which was tremendously helpful. Susan has been a great mentor, both scientifically and personally, and has given me many opportunities to become a better researcher. I look forward to many years of collaborating and keeping in touch.

I would also like to thank my thesis committee members for their support and guidance. Dr. Arthur Buckley, Dr. Karen Knudsen, Dr. Alex Lentsch, and Dr. Susanne Wells have all been extremely involved and helpful in my graduate studies. I give thanks to Dr. Wells for being an excellent mentor when I first started out in cancer research. Her enthusiasm, love for her work, and her entire laboratory environment was what influenced me to join graduate school, and for that I am grateful. Dr. Wells and Dr. Lentsch have been especially helpful in guiding me towards the next step in my career.

I would like to especially thank some of the past and present members of the Waltz laboratory, including: Jerilyn Gray, Devikala Gurusamy, Dr. Aaron Marshall, Purnima Wagh, Dr. Nikolaos Nikolaidis, William Stuart, Dr. Rishikesh Kulkarni, Dr. Becca McClaine, Dr. Karen Huezo, Dr. Sara Meyer, Dr. Glendon Zinser and Dr. Mike
Leonis. They have not only offered me good critiques and advice on a scientific level, but have become some of my closest friends, and have been wonderful to work with.

I would like to acknowledge the Department of Defense (project # PC060821) for funding for three years. Also, I would like to acknowledge past and present member of the Cancer and Cell Biology graduate program for their support, especially Barbara Carter, Dr. Robert Brackenbury, Dr. Peter Stambrook and Dr. Wallace Ip.

Finally, I would like to give many special thanks to my friends and family for their unwavering support. My parents have always been by my side and have been very helpful both mentally and emotionally. They have gone far beyond to ensure my happiness and productivity during these years were maximized, and I could not have done this without their love and support. I would also like to thank my fiancé Timmy, who is also a graduate student in the Cancer and Cell Biology program. He has undoubtedly been my “rock” the past five years, both in the lab and at home. His encouragement, support and friendship have made this experience that much more meaningful, and it has been a blessing having him in my life and by my side. I also have to acknowledge our dogs Freckles and Emmett who have had to endure countless hugs from me during the most stressful of times.
Table of Contents

Abstract ..............................................................................................................i, ii
Copyright Notice ..................................................................................................iii
Acknowledgements .............................................................................................iv, v
Table of Contents ...............................................................................................vi-viii
List of Figures ....................................................................................................ix-x
List of Tables .....................................................................................................xi

Chapter 1: The Ron Receptor and Cancer

A. Definition ........................................................................................................2
B. Characteristics .................................................................................................3, 4
C. Expression and Function ...................................................................................5
D. Ligand ................................................................................................................5, 6
E. Signaling Through Ron .....................................................................................6, 7
F. Ron Overexpression in Multiple Tumor Types .............................................7
G. Ron Variants .....................................................................................................7, 8
H. Ron-Dependent Transformation .....................................................................8, 9
I. Transgenic Mouse Models Overexpressing Ron ..........................................9
J. Ron Loss in Murine Tumor Models .................................................................10
K. Oncogenic Ron Signaling ...............................................................................11, 12
L. Ron as a Therapeutic Target in Human Cancer ........................................12-14
M. Acknowledgements .......................................................................................14
Chapter 2: Introduction

A. Prostate Cancer ................................................................. 17, 18
B. Growth Factor Receptors in Prostate Cancer ................. 18, 19
C. Angiogenic Chemokines and the CXCR2 Receptor ......... 19, 20
D. Angiostatic Chemokines .................................................. 21
E. Prostate Tumor Angiogenesis .......................................... 22-24
F. Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model ............................................................... 24
G. Receptor Tyrosine Kinases in the TRAMP Mouse Model .... 24, 25
H. Ron Receptor Tyrosine Kinase in Prostate Cancer .......... 25-27
I. References ................................................................. 27-33

Chapter 3: The Ron Receptor Tyrosine Kinase Positively Regulates Angiogenic Chemokine Production in Prostate Cancer Cells

A. Abstract ................................................................. 35
B. Introduction ............................................................. 36-39
C. Materials and Methods .................................................. 40-45
D. Results ................................................................. 46-52
E. Discussion ............................................................. 53-57
F. Acknowledgements ......................................................... 58
G. References ............................................................. 59-62
H. Figures, Tables, Legends ............................................... 63-77
Chapter 4: The Ron Receptor is Required for Prostate Tumor Growth in the TRAMP Mouse Model

A. Abstract........................................................................................................79
B. Introduction...............................................................................................80-82
C. Materials and Methods...........................................................................83-86
D. Results.........................................................................................................87-90
E. Discussion....................................................................................................91-94
F. Acknowledgements...................................................................................94
G. References..................................................................................................95-99
H. Figures, Tables, Legends..........................................................................100-111

Chapter 5: Conclusions and Discussion

A. Summary.....................................................................................................113, 114
B. Ron Expression and Activation in Prostate Cancer...............................114-116
C. The Ron Receptor: Prostate Tumor Initiation or Tumor Progression?.................................................................116-117
D. Ron and Prostate Tumor Angiogenesis....................................................117-119
E. Ron Signaling in Prostate Cancer...............................................................119-121
F. Ron and the Androgen Receptor: Potential Combination Therapy?...............................................................................121-123
G. Conclusions and Future Directions..........................................................124, 125
H. References..................................................................................................126-134
List of Figures

Chapter 1: The Ron Receptor and Cancer

Figure 1: The human and mouse Ron gene structure.............................................2
Figure 2: Ron protein structure and signaling pathways activated by Ron...............4

Chapter 3: The Ron Receptor Tyrosine Kinase Positively Regulates Angiogenic Chemokine Production in Prostate Cancer Cells

Figure 1: Ron expression in human prostate specimens.................................63
Figure 2: Ron expression and activation in human prostate cells....................64
Figure 3: Levels of angiogenic chemokines produced by prostate cancer cells.....65
Figure 4: Decreased angiogenic chemokine production in Ron-knockdown PC-3 cells.................................................................67
Figure 5: Impact of Ron knockdown in DU145 cells..................................69
Figure 6: Ron overexpression in LNCaP cells..............................................71
Figure 7: Orthotopic transplantation of PC-3 cells containing a Ron knockdown...72
Supplemental Figure S1: Ron expression in prostate cells..............................74
Supplemental Figure S2: NF-kappaB activation following Ron knockdown........75
Supplemental Figure S3: Met expression in prostate cancer cells.....................76

Chapter 4: The Ron Receptor is Required for Prostate Tumor Growth in the TRAMP Mouse Model

Figure 1: Ron expression in wild-type and TRAMP mouse prostates.................100
Figure 2: Prostate weights in TRAMP mice +/- Ron.........................................102
Figure 3: Vascularization of TRAMP prostate tumors...................................103
Figure 4: BrdU and TUNEL immunohistochemistry on TRAMP prostates.............105

Figure 5: Cell viability in prostate cancer cells.............................................107

Supplemental Figure S1: Cleaved caspase-3 expression in TRAMP tumors.........109

Supplemental Figure S2: Annexin V/PI FLOW cytometry histograms...............110

Chapter 5: Conclusions and Discussion

Figure 1: Impact of PI3K inhibition on CXCL8 production............................120

Figure 2: Working model-Ron in prostate cancer cells...............................123
List of Tables

Chapter 2: Introduction
Table 1: Common angiogenic and angiostatic chemokines…………………………..22

Chapter 3: The Ron Receptor Tyrosine Kinase Positively Regulates Angiogenic Chemokine Production in Prostate Cancer Cells
Table 1: Ron expression in human prostate specimens………………………………77
Table 2: Ron expression in human prostate cell lines………………………………..77

Chapter 4: The Ron Receptor is Required for Prostate Tumor Growth in the TRAMP Mouse Model
Table 1: Lung metastases in TRAMP mice………………………………………….111
CHAPTER 1

THE RON RECEPTOR AND CANCER

**Synonym**

Macrophage Stimulating 1 Receptor (MST1R)

**Definition**

Cell surface receptor tyrosine kinases regulate critical signaling pathways and elicit a variety of important biological responses that contribute to tumorigenesis. The Ron receptor tyrosine kinase has recently been implicated in a number of human cancers. Ron belongs to a family of receptor tyrosine kinases that includes the Met proto-oncogene. The Ron ortholog in the mouse is also referred to as *stem cell derived kinase* (*Stk*) while the chicken counterpart encodes *c-sea*. The human Ron gene is located on chromosome 3p21.3 and contains 20 exons with a transcript length of 4,531 basepairs (bps) and a translation length of 1,400 residues. The murine counterpart contains 75% identity to the human protein and is located on chromosome 9. The mouse Ron gene contains 19 exons with a transcript length 4,710 bps and 1,378 residues. A comparison of the genomic organization of the human and mouse Ron is depicted in Figure 1 (1).

![Comparison of genomic organization of human and mouse Ron](image)
Figure 1. The human and mouse Ron gene structure. Exons are depicted by numbered boxes and introns by black lines. Red boxes code for the SEMA domain, aqua box for the signal peptide, green box for the plexin domain, yellow boxes for the immunoglobulin-like, plexins, transcription factor (IPT) domains, pink box for the transmembrane domain, and blue boxes for the tyrosine kinase domain. The Ys refer to the critical autophosphorylation sites in the kinase domain and for the docking site tyrosine residues in the C-terminal region of Ron. Note that exon 13 in human Ron is missing in the mouse gene.

Characteristics

The Ron protein is a heterodimeric glycoprotein with disulfide linked alpha (35 kDa) and beta (150 kDa) chains. The protein is synthesized as a single chain precursor of 185 kDa that is cleaved into its heterodimeric form before exposure to the cell surface. A diagram of the domain structure of Ron is presented in Figure 2. The protein includes a 24 amino acid signal peptide, an extracellular domain of 933 amino acids, a 25 amino acid transmembrane domain and an intracellular tyrosine kinase domain of 418 amino acids. The tyrosine kinase domain of Ron is 63% identical to the same domain in the Met receptor, while the extracellular domain is only 25% identical. The Ron alpha chain contains regions important for ligand binding while the beta chain includes an extracellular domain, a transmembrane domain and an intracellular region including the tyrosine kinase domain. Based on functional annotation of the Ron protein sequence, the extracellular domain contains a SEMA domain characterized by a conserved set of cysteine residues that form disulfide bonds to stabilize the protein structure. The
extracellular portion of Ron also contains a plexin domain, a cysteine rich motif found in many extracellular receptors, and several IPT domains, which contain an immunoglobulin-like fold and interestingly is found in intracellular transcription factors involved in DNA binding.

Figure 2. Ron protein structure and signaling pathways activated by Ron. The Ron receptor is a single pass membrane spanning, disulfide-linked heterodimeric protein. The
alpha chain is an extracellular glycoprotein and the beta chain spans the membrane and contains the intracellular tyrosine kinase domain. Activation of Ron leads to the phosphorylation of tyrosine residues within the kinase domain and subsequent phosphorylation of tyrosine residues that form docking sites for a number of downstream signaling proteins.

**Expression and Function**

Ron is preferentially expressed on epithelial cells and macrophages. Ron expression has been detected in the central nervous system, liver, kidney, testes, bone, lung, breast, and epithelia of the digestive tract. In macrophages, Ron signaling regulates select cytokine and chemokine production in response to injury. In epithelial cells, the normal functions of Ron include a wide array of biological activities such as inducing DNA synthesis, triggering cell scattering, branching morphogenesis, and the regulation of apoptosis. Based on the signaling pathways elicited by Met and Ron, the activities elicited by this receptor family have been termed “invasive growth” (2).

**Ligand**

The ligand for Ron is a protein termed hepatocyte growth factor-like protein (HGFL), and is also called macrophage-stimulating protein or macrophage stimulating 1 (MST1). HGFL is part of a larger family of plasminogen-related growth factors, which also includes hepatocyte growth factor (HGF)/scatter factor (the ligand that activates c-Met), plasminogen (ligand for several receptor) and urokinase (ligand for uPAR receptor). The HGFL gene, similar to Ron, is located at chromosome 3p21.3. HGFL is predominantly
produced in hepatocytes. Following synthesis, HGFL is constitutively secreted into the bloodstream, at a concentration of approximately 400ng/ml, as a biologically inactive single chain precursor of 80 kDa. Pro-HGFL is proteolytically cleaved by membrane-associated proteins on the cell surface or by members of the kallikrein family. Proteolytic cleavage results in the formation of a disulfide-linked heterodimer containing an alpha chain of approximately 50 kDa and a beta chain of approximately 35 kDa. The beta chain of HGFL contains a serine protease-like domain and is primarily responsible for binding to Ron, while the alpha chain contains four kringle domains and appears to regulate the functional consequences of this binding (3).

Interestingly, HGFL was originally identified as a chemoattractant for macrophages, but it can also act as a motogen or mitogen for several other cell types. Depending upon cell type and context, HGFL can also induce a number of pleiotropic effects. However, gene deletion studies in mice have shown that HGFL is not an essential gene product as HGFL deficient mice have limited phenotypic abnormalities.

**Signaling Through Ron**

A schematic representation of the downstream signaling molecules associated with Ron activation is presented in Figure 2. Binding of HGFL to Ron leads to activation of the receptor’s intrinsic tyrosine kinase activity and trans-autophosphorylation of two C-terminal tyrosine residues, creating high affinity binding sites for proteins containing Src homology 2 and phosphotyrosine-binding domains. These phosphorylated tyrosine residues can bind phospholipase C-gamma (PLC-γ), phosphoinositide kinase-3 (PI3K),
growth factor receptor-bound protein 2 (Grb2), and Shc. Ras, mitogen-activated protein kinase (MAPK), β-catenin, nuclear factor kappa B (NF-κB), and Akt mediate HGFL induced activities. Focal adhesion kinase (FAK), Src, c-Jun N-terminal protein kinase (JNK), and signal transducers and activators of transcription (STAT)3 activation result from HGFL/Ron signaling. Thus, based on cell type and context, Ron activation induces pleiotropic responses through the recruitment of numerous signaling molecules (4).

**Ron Overexpression in Multiple Tumor Types**

Increased Ron expression has been documented in several tumor types including breast, colon, lung, liver, kidney, ovary, stomach, pancreas, bladder and prostate. Ron expression is also elevated in cells derived from these tumors (5). Clinically, high expression of Ron is positively correlated with poor prognosis in several types of human cancers including breast, prostate, colon, and pancreas. Based on gene expression analyses, in the breast, increasing Ron expression is associated with metastatic disease, and in the prostate, Ron expression is correlated with advanced, androgen-independent cancers, suggesting that Ron is important in tumor progression. Overexpression of both Ron and Met in breast and bladder cancers is associated with overall decreased patient survival.

**Ron Variants**

Several variants of Ron result from alternative splicing or alternative initiation. These variants, designated by their molecular weight, have been found in lung, colon, and breast cancers, and many are associated with increased Ron expression. Four of these variants
(RonΔ165, RonΔ160, RonΔ155, RonΔ55) result in constitutive activation of the receptor and three (RonΔ160, RonΔ155, RonΔ55) have oncogenic properties. Less is known about RonΔ170 and RonΔ110 (6).

RonΔ55, also known as Short Form (SF)-Ron, is generated from an alternative start site in intron 10 of the Ron gene that eliminates most of the extracellular portion of the receptor. SF-Ron is expressed in ovarian and breast cancers, and in cell lines derived from colorectal, lung and pancreatic carcinomas and several leukemias. This variant is constitutively phosphorylated, has strong kinase activity, and alters morphology, growth, motility, and anchorage-dependent growth when transduced into epithelial cells. Expression of SF-Ron can also negatively regulate E-cadherin transcription, resulting in a loss of cellular adhesion (7).

Mouse strains that express SF-Ron in adult bone marrow tissue are susceptible to Friend virus-induced erythroleukemia, and SF-Ron kinase activity is necessary for erythropoietin-independent expansion of erythroid progenitors in response to Friend virus. Moreover, recent studies have also suggested that SF-Ron has distinct nonredundant biological functions relative to full-length Ron in the progression of inflammatory immune responses in vivo (8).

**Ron-Dependent Transformation**

Overexpression of the full-length Ron receptor has transforming capabilities in vitro and in vivo. Mouse fibroblasts overexpressing wild-type Ron exhibit loss of contact
inhibition, elongated cellular processes, and increased cellular proliferation and motility compared to fibroblasts with endogenous Ron expression. These phenotypes are augmented with HGFL. Overexpression results in tyrosine phosphorylation of the receptor, suggesting that overexpression is sufficient to elicit constitutive activation. Ron overexpression in colon epithelial cells induces cellular migration and invasion and is able to protect cells against apoptosis. Ron overexpression in fibroblasts and epithelial cell lines is sufficient for tumor formation in xenograft models.

**Transgenic Mouse Models Overexpressing Ron**

Important mouse models have been created to examine tissue-specific Ron overexpression. The mouse mammary tumor virus (MMTV) promoter was utilized to drive Ron overexpression in mammary epithelium. 100% of the female mice expressing the transgene developed mammary tumors. The tumors were highly metastatic, with metastases observed in about 90% of the lungs and livers of all tumor-bearing mice. This study demonstrates that Ron overexpression may be important in metastatic breast cancer. This data also supports the observation that Ron overexpression in human breast cancer is associated with an aggressive cancer phenotype with decreased disease free survival time and an increase in metastasis. In another mouse model, the surfactant protein C promoter was utilized to drive Ron overexpression in distal lung epithelial cells. Between 6 and 14 months of age, about 90-95% of the transgenic mice developed lung adenomas. These mouse models suggest that Ron is important to tumor formation, progression, and metastasis.
Ron Loss in Murine Tumor Models

The development of a mouse model containing a targeted deletion of the Ron tyrosine kinase domain has allowed the functional significance of Ron signaling in vivo to be examined. These mice have been crossed with mice engineered to develop tumors. In a well-characterized mouse model of skin cancer, v-Ha-Ras transgenic mice were utilized in which 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment results in the formation of benign skin papillomas that progress to frank tumors. TPA-treated v-Ha-Ras mice lacking Ron signaling have a greater number of benign skin papillomas, although the papillomas are smaller in size, and have decreased cell proliferation. Importantly, the loss of Ron in this model leads to significantly reduced progression of the benign papillomas toward malignancy (11).

Another transgenic mouse model lacking functional Ron has been created using mice that have expression of the polyoma virus middle T antigen (pMT) under the control of the MMTV promoter. In the pMT-induced model, the mice develop mammary tumors that metastasize to the lung. The loss of Ron in this model results in a significant decrease in the rate of mammary tumor initiation, fewer tumors, a decrease in tumor size, and is associated with decreased tumor cell proliferation and increased apoptosis. Interestingly, tumors lacking functional Ron also exhibit a decrease in microvessel density, suggesting that Ron may be important in regulating events impacting tumor angiogenesis (12).
Oncogenic Ron Signaling

Several signaling pathways (β-catenin, MAPK, PI3K) are activated by Ron overexpression as well as ligand binding (13). β-catenin is part of the Wnt signaling pathway and β-catenin expression has been associated with several human cancers. Mammary tumors derived from mice overexpressing Ron are associated with an increase in β-catenin and in the β-catenin target genes c-myc and cyclin D1 (9). Human pancreatic cancer cells treated with HGFL also have increased nuclear β-catenin (14), and human colorectal carcinoma cells lacking Ron expression have decreased β-catenin expression (15).

The MAPK pathway is also upregulated in a variety of cancers, and this pathway is important for many cellular processes including cellular differentiation, survival, proliferation and gene expression. In several human cancer cell lines with high levels of Ron expression, such as those derived from colon, prostate, lung, pancreas and stomach, MAPK activation can be abrogated using a neutralizing antibody against Ron (5). Mammary tumors from mice lacking the tyrosine kinase domain of Ron also have decreased MAPK activation (12). Conversely, the addition of HGFL to several cell types results in increased MAPK activation.

Similar results have been found for the regulation of the PI3K/Akt signaling pathway by Ron. Like β-catenin and MAPK, this pathway is often upregulated in various cancers. PI3K can bind directly to Ron. Akt, a serine/threonine kinase downstream of PI3K, is then activated and elicits responses decreasing apoptosis and increasing cellular survival.
In colon, prostate and stomach cancer cell lines, there is a decrease in activated Akt when Ron is neutralized (5). *In vivo*, the amount of phosphorylated Akt is decreased in mammary tumors from mice lacking functional Ron (12). Taken together, these data suggest that Ron expression is important to the regulation of signaling pathways that are often highly associated with many human cancers.

**Ron as a Therapeutic Target in Human Cancer**

The mounting evidence that Ron is important in tumor initiation, progression, and metastasis suggests that Ron may be a therapeutic target for cancer treatment. Several approaches have been initiated to target Ron. Antibodies have been developed to neutralize Ron activity by preventing HGFL binding, and may be effective in treating tumors containing high Ron expression. These antibodies appear to be specific to the Ron receptor, and do not inhibit HGF binding to Met (5). Antibodies directed against Ron are able to inhibit MAPK and Akt activation as well as cellular migration. Moreover, in xenograft models using cancer cells that overexpress Ron, antibody neutralization is able to reduce subcutaneous tumor growth. Increased apoptosis of Ron-expressing pancreatic cancer cells was demonstrated by concurrent Ron receptor antibody blockade and gemcitabine treatment (16). In addition to neutralizing antibodies to target Ron, small molecule inhibitors have also been developed. A small peptide inhibitor containing five amino acids can inhibit HGFL- and HGF-induced cellular scattering and migration. A soluble molecule homologous to the SEMA domain of Ron has been shown to exhibit a dominant-negative effect. This molecule is able to inhibit HGFL-induced phosphorylation of Ron, and has an inhibitory effect on the growth of colon cancer cells.
Geldanamycins, a class of antitumor drugs that inhibit tumor cell growth by preventing proper folding and increase the degradation of oncogenic proteins, have also been identified as possible treatment modalities leading to receptor tyrosine kinase degradation, including Ron, in human cancers.

The Ron receptor participates in cross-talk with other receptor tyrosine kinases and integrins, which may be important in regulating the therapeutic response of human tumors. Several studies have shown that Ron can interact with the epidermal growth factor receptor (EGFR). Moreover, treatment of one receptor can modulate the response of an interacting receptor. Targeting this interaction in cell lines results in the modulation of cellular responses including cell scattering and tumor regression. In addition to EGFR, Ron is also co-expressed with the Met receptor in bladder, breast and liver cancers, and this co-expression has been linked with poor prognosis. Combined, these studies suggest that targeting multiple receptor tyrosine kinases in these cancers might prove more effective than targeting one alone.

Although there are currently no clinical trials being done selectively targeting the Ron receptor, there are several ongoing clinical trials with compounds that target Met, and which cross-react with Ron. Initial data from these trials suggests that the compounds are clinically well tolerated, and show an impressive anti-tumor activity in patients with a broad range of metastatic tumor types who have failed prior treatment. In total, the data outlined suggests that Ron may be a critical factor in tumorigenesis and that inhibition of
Ron, either alone or in combination therapy, may prove to be beneficial in the treatment of high-risk cancer patients.

**Acknowledgements**

The authors would like to thank Claudia Hinzman for the artwork and Drs. Leonis, Peace and Zinser for their valuable comments and editing.

**References**


CHAPTER 2

INTRODUCTION: Prostate Cancer and the Ron Receptor
Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in men, excluding basal and squamous cell skin cancers. Approximately one out of every six men will develop prostate cancer over his lifetime. While the incidence and mortality rates for prostate cancer have been on the decline since the early 1990’s, it remains the second leading cause of cancer-related deaths among men in the United States (1). Since the approval by the U.S. Food and Drug Administration in 1994 for using prostate specific antigen (PSA) to help detect prostate cancer, the five-year survival rate for prostate cancer patients has significantly increased, at least partially due to using PSA as an early-detection marker (2).

First-line prostate cancer treatments include watchful waiting, radical prostatectomy, radiation therapy and hormone therapy (3, 4), however recurrence is common and most cancers progress to androgen-independent disease (5, 6). While androgen-deprivation therapy is a common treatment for advanced prostate cancer patients, recent evidence suggests that the overall survival rate is only minimally improved (7). Other treatments for advanced, hormone-refractory disease, including docetaxel-based chemotherapy, also resulted in only a modest increase in overall patient survival (8), supporting the need for new therapeutic targets for hormone-refractory prostate cancer.

As reviewed in (6), there are at least five potential mechanisms of cancer cells gaining androgen-independence. These include 1.) overproduction of the androgen receptor and/or increased sensitivity of the receptor, 2.) binding of the androgen receptor by non-androgens, resulting in activation of the androgen receptor, 3.) activation of
receptor tyrosine kinases that subsequently phosphorylate and activate the androgen receptor, 4.) activation of other signaling pathways that are pro-survival and anti-apoptotic, and 5.) selection of androgen-independent cells, possibly stem cells, during androgen-ablation therapy. While all of these potential mechanisms of gaining androgen-independence are plausible, there is strong evidence to support the notion that the androgen receptor becomes activated in the absence of androgens.

**Growth Factor Receptors in Prostate Cancer**

Recent evidence suggests that growth factor receptors are important for prostate tumor progression. While these receptors can impact a variety of downstream signaling pathways involved in prostate cancer, it is interesting that several growth factor receptors have been identified as being able to regulate androgen receptor signaling. Examples include the EGFR-related receptor tyrosine kinase, HER-2/neu (9-11), and the IL-6/STAT3 pathway (12). Additionally, PI3K signaling in high-passage LNCaP prostate cancer cells enhances androgen receptor activity (13). Interestingly, it has been postulated that the epidermal growth factor receptor (EGFR) can “crosstalk” with the androgen receptor and is an important mediator in androgen-independent cancer. In normal prostate cells, EGFR is negatively regulated by the androgen receptor, most likely to keep cells from proliferating unnecessarily. However in prostate cancer cells, there is a significant increase in EGFR levels following androgen ablation (14). Moreover, the androgen receptor has been shown to negatively regulate the Met receptor tyrosine kinase in LNCaP prostate cancer cells by interfering with Sp1-induced transcription of the receptor (15). These studies suggest that while targeting the androgen receptor alone is
benificial, more optimal results might be obtained for advanced prostate disease by further examining the role of other growth factors and receptors on either i.) activating the androgen receptor, ii.) becoming upregulated following androgen deprivation, or by iii.) impacting cell growth and survival by bypassing the androgen receptor completely.

The epidermal growth factor receptor (EGFR) has been the center of several investigations both in the laboratory as well as in the clinic as a potential therapeutic target in prostate cancer. The compound ZD1839 (‘Iressa’) binds to and inhibits EGFR, and has been shown to inhibit prostate cancer cell proliferation in both established prostate cancer cell lines and primary prostate cancer cells (16). Furthermore, the oral EGFR inhibitor, erlotinib, showed promising results in a clinical phase II trial in chemotherapy-naive castration-resistant prostate cancer patients (17). Additionally, the inhibition of Insulin-Like Growth Factor I Receptor by a human monoclonal antibody CP-751,871 (18, 19), or inhibition of the Her2/neu receptor by use of Herceptin resulted in decreased tumor growth in mouse xenografts (20), although whether these compounds are clinically relevant is still under investigation.

**Angiogenic Chemokines and the CXCR2 Receptor**

Chemokines are a family of molecules ranging in size from 8 to 12kDa and were first identified based on their chemotactic abilities during the inflammatory response through stimulating leukocyte movement. Their classification is based on the position of the first two cysteine residues (21). There are two main families of chemokines, one in which the two cysteine residues are next to each other, (CC chemokines) and the other in which the two cysteine residues are separated by a non-conserved amino acid (CXC
chemokines). Angiogenic chemokines are a group of CXC chemokines that share a common ELR (glutamic acid-leucine-arginine) motif immediately proximal to the CXC sequence in the amino terminus (Table 1). Originally discovered for their ability to attract neutrophils, angiogenic chemokines are also potent regulators of angiogenesis (22, 23). CXCL8 (formerly known as IL-8) was one of the earliest angiogenic chemokines identified, and two other common angiogenic chemokines CXCL1 (GROα) and CXCL5 (ENA-78) were subsequently identified.

All angiogenic chemokines bind to a common receptor, called CXCR2. CXCR2 is a rhodopsin-like seven-transmembrane G protein-coupled receptor (24), which is able to activate signaling pathways including PI3K/Akt, MAPK, PLCβ, Ras and Rho GTPases, p21 and NF-kappaB (25). CXCR2 expression on endothelial cells is required for endothelial cell migration (26). Using human intestinal microvascular endothelial cells (HIMEC), Heidemann et al demonstrated that pre-treatment with a CXCR2 neutralizing antibody significantly reduced endothelial cell migration (27). Moreover, mice deficient in CXCR2 exhibit decreased cornea vascularization in response to angiogenic chemokines (24). In an orthotopic mouse model of lung cancer, tumors formed in CXCR2-deficient mice were smaller in size, less vascularized, and were less metastatic compared to CXCR2-expressing tumors (28). These observations suggest that CXCR2 is necessary for tumor growth and metastasis. Angiogenic chemokines in prostate cancer is discussed on page 22.
Angiostatic Chemokines

Similar to angiogenic chemokines, angiostatic chemokines are part of the CXC family of chemokines, although they lack the ELR motif and are anti-angiogenic (Table 1). For example, tumors formed from human Burkitt lymphoma cells injected orthotopically in nude mice showed increased tumor necrosis when treated with the angiostatic chemokine CXCL10. Furthermore, Burkitt cells constitutively over-expressing CXCL10 had reduced tumor formation correlating with increased necrosis (29). Similarly, in a mouse model of lung cancer, lung squamous cell carcinomas treated intratumorally with CXCL10 had reduced growth, neovascularization and metastasis (30). While the common angiostatic chemokine receptor CXCR3 has also been implicated in prostate cancer, the signaling pathways activated by CXC ELR-chemokines are not fully understood. There is evidence however, that activation of STAT1, and secretion of interferon-α and interferon-γ play roles in inhibiting vascularization (31-33).
Table 1. Common angiogenic (CXC ELR+) and angiostatic (CXC ELR-) chemokines (adapted from (21))

<table>
<thead>
<tr>
<th>New Nomenclature</th>
<th>Human Ligand</th>
<th>Mouse Ligand</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CXCR ELR+</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL1</td>
<td>GROα</td>
<td>GRO/KC</td>
<td>CXCL2, CXCR1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>GROβ</td>
<td>MIP-2</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL3</td>
<td>GROγ</td>
<td>Dcip</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>LIX</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL8</td>
<td>IL-8</td>
<td></td>
<td>CXCR2, CXCR1</td>
</tr>
<tr>
<td><strong>CXCL ELR-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL9</td>
<td>MIG</td>
<td>MIG</td>
<td>CXCR3, CXCR3B</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td>IP-10</td>
<td>CXCR3, CXCR3B</td>
</tr>
<tr>
<td>CXCL11</td>
<td>I-TAC</td>
<td>I-TAC</td>
<td>CXCR3, CXCR3B, CXCR7</td>
</tr>
</tbody>
</table>

Prostate Tumor Angiogenesis

In normal, non-tumorigenic prostate cells, there is a balance between angiogenic and angiostatic chemokines that becomes deregulated in cancer cells, resulting in increased angiogenic chemokine levels and reduced angiostatic chemokine levels. This ultimately results in increased vascularization of the tumor, and is critical for prostate tumor growth (34). Without tumor angiogenesis, the tumor cannot grow and metastasize, and angiogenic chemokine production has been correlated with prostate cancer progression (32, 35-37).

In human prostate cancer specimens, CXCL8 levels were increased compared to benign hyperplastic or normal prostate patient specimens (38). In vitro, there is a progressive increase in angiogenic chemokines, and a progressive decrease in angiostatic chemokines, from the non-transformed prostate cell line PZ-HPV-7, to cells derived from a primary prostate tumor (CA-HPV-10), to the metastatic prostate cancer cell line PC-3. Interestingly, levels of active NF-kappaB correlate with levels of angiogenic chemokines,
and levels of STAT1 correlate with levels of angiostatic chemokines. Moreover, experiments utilizing inhibitors of either NF-kappaB or of STAT1 demonstrate that levels of angiogenic chemokines are at least partially dependent on NF-kappaB activation, and that STAT1 is an important regulator angiostatic chemokines (32).

PC-3 and DU145 prostate cancer cells have elevated production of the angiogenic chemokines CXCL1 and CXCL8 compared to LNCaP cells. When PC-3 or DU145 cells were injected subcutaneously into SCID mice, prostate tumor growth could be inhibited by using neutralizing antibodies against CXCL1 (DU145 cells) or against CXCL8 (PC-3 cells) (39). In another study, PC-3 and DU145 cells were stimulated with recombinant CXCL8, which resulted in increased Cyclin D1 expression and increased Akt and MAPK signaling (40). Furthermore, Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice lacking the CXCR2 receptor had decreased prostate tumor size and tumor vascularization while TRAMP mice lacking the CXCR3 receptor (receptor for angiostatic chemokines) had increased prostate tumor size and tumor angiogenesis (41).

The Duffy antigen receptor for chemokines (DARC) binds angiogenic chemokines CXCL1, CXCL5 and CXCL8, among others. Interestingly, evidence suggests that ligand binding to DARC does not initiate signal transduction pathways, however the receptor becomes internalized (42). Therefore it is hypothesized that DARC acts like a sponge to sequester circulating chemokines and therefore is an inhibitor of tumor vascularization. In prostate cancer, DARC-deficient mice crossed with mice that are predisposed to prostate cancer (TRAMP mice) exhibit increased prostate tumor size, increased tumor vasculature, and increased levels of angiogenic chemokines relative to
TRAMP mice expressing DARC (43). This is further evidence demonstrating the importance of angiogenic chemokines in prostate tumor growth.

**Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model**

The TRAMP mouse model was first described in 1995. TRAMP mice have expression of the SV40 early genes (large and small T antigen) driven by the rat probasin promoter (PB-SV40 Tag), and expression is specific to the dorsolateral and ventral lobes of the prostate. This is a useful model for studying prostate cancer because the age of tumor formation and metastases is relatively young. By as early as 10 weeks of age, some mice have mild to severe prostatic epithelial hyperplasia, and as early as 18 weeks of age invasive adenocarcinoma can be detected (44). Moreover, metastases (primarily to the periaortic lymph nodes and lungs) can occur as early as 18 weeks (45).

Interestingly, TRAMP mice that are castrated at age 12 weeks initially have a reduction in tumor size, although if the tumor is allowed to continue to grow, the majority of these mice exhibit prostate tumors of similar size to their non-castrated counterparts. However, the tumors formed in castrated mice are more poorly differentiated and have approximately twice the incidence of lymph node metastases compared to intact mice (46).

**Receptor Tyrosine Kinases in the TRAMP Mouse Model**

Several studies have examined the role of receptor tyrosine kinases in the TRAMP mouse model. For example, utilizing cells established from TRAMP prostate tumors (termed TRAMP C1 cells), it has been shown that EGFR inhibition does not
effect cellular proliferation, but it does result in decreased invasion through matrigel (47). Furthermore, adenoviral delivery of a soluble VEGFR-2 domain in the prostates of TRAMP mice resulted in reduced prostate tumor size, decreased tumor vasculature and increased survival of the animals (48). Additional evidence demonstrates the importance of VEGFR in TRAMP prostate tumor progression, in studies that utilized a small molecule inhibitor of VEGFR, referred to as SU5416. SU5416, when administered to TRAMP mice between 10 and 16 weeks of age (when VEGFR1 levels are high), resulted in no impact on tumor growth. However, when SU5416 was given between ages 16 and 22 weeks (when VEGFR2 levels are high), there was a dramatic reduction in prostate vessel density and an increase in the number of apoptotic cells and areas of cell death compared to control mice (49). While these data suggest receptor tyrosine kinase signaling is important in promoting prostate tumor growth and angiogenesis in the TRAMP mouse model, the role of the Ron receptor tyrosine kinase has not been established.

**Ron Receptor Tyrosine Kinase in Prostate Cancer**

Our studies, as outlined in Chapters 3 and 4, involve studies to elucidate the functions of the Ron receptor tyrosine kinase i.) in the regulation of angiogenic chemokine production in vitro, and ii.) in the regulation of prostate tumor growth and vascularization in vivo. Based on recent evidence demonstrating that i.) non-transformed prostate cells have low levels of angiogenic chemokines and high levels of angiostatic chemokines, ii.) prostate cancer cells have high levels of angiogenic chemokines and low levels of angiostatic chemokines (32), and iii.) Ron is overexpressed in prostate cancer
specimens (50), we sought to determine whether Ron was indeed a positive regulator of angiogenic chemokine production. Our data confirm that Ron is overexpressed in human prostate cancer specimens, and that prostate cancer cells expressing high levels of Ron similarly have high levels of angiogenic chemokines produced. Moreover, Ron inhibition in these cells dramatically reduced angiogenic chemokine production. Conversely, Ron overexpression in non-transformed prostate cells induced production of angiogenic chemokines. Further investigation demonstrated that the NF-kappaB transcription factor is a signaling molecule through which Ron is able to regulate angiogenic chemokine production.

In vivo, our data demonstrate that high Ron-expressing prostate cancer cells (PC-3 cells) form tumors in nude mice when orthotopically transplanted into the prostate. However, prostate tumor formation and prostate tumor vascularization is significantly reduced when Ron-knockdown PC-3 cells are transplanted into the mouse prostate. These results are similar to a mouse model of breast cancer, in which Ron loss resulted in decreased mammary tumor size and reduced mammary vascularization (51). We expanded upon the in vivo studies and utilized the TRAMP mouse model crossed with mice containing a targeted deletion of the Ron receptor tyrosine kinase domain (Ron TK-/-). These mice are phenotypically normal although they have some alterations in inflammation in response to stress (52). We have shown that TRAMP TK-/- mice have significantly smaller prostate tumors, decreased prostate tumor microvessel density, and an increase in TUNEL-positive cells compared to TRAMP TK+/+ mice. Additionally, prostate cancer cells lacking functional Ron have decreased viability compared to Ron-
expressing prostate cancer cells, and this correlates with altered signaling through pAkt and pMAPK.

Altogether, our data show that Ron is not only important in regulating prostate tumor angiogenesis by modulation of angiogenic chemokine production, but this regulation has dramatic consequences as determined by decreased prostate tumor size in Ron-knockdown PC-3 tumors and in TRAMP TK-/- mice. As Ron is highly expressed in prostate cancer compared to normal prostate or benign prostate hyperplastic tissue, it is interesting to speculate that targeting the Ron receptor in human prostate cancer patients might reduce prostate tumor growth. Our data in whole, suggest that Ron inhibition might impact prostate tumor growth by regulating tumor vasculature as well as prostate cancer cell viability.

References


CHAPTER 3

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

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 (1). Therefore, to 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 (2). 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 (3-4).

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 (5-8). The Ron receptor is expressed preferentially in macrophages and epithelial cells (9-10). 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 (11-12). 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 (9-10). 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 (13). 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 (14). 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 (15). 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 (16). 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 (17). This process results in the formation of new blood vessels from pre-existing vessels, that integrate into the tumor (18). CXCL8 in particular, has
been shown to be a strong inducer of endothelial cell migration through binding to the CXCR2 receptor (18-19).

Increased angiogenic chemokine production has been implicated in the pathogenesis of prostate cancer (20-22). 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 (23). 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 (19). Interestingly, inhibiting the NF-kappa B (NF-κB) transcription factor in PC-3 prostate cancer cells results in decreased angiogenic chemokine production (17), 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
\textit{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 (13). 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 (7) 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: 1X10⁶ 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 (24), VEGF<sub>165</sub> (25), and CXCL5 (26). 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 (27). 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 (27) 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. 1x10⁵ 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 (17, 28).

**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, $1 \times 10^5$ 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 $3 \times 10^4$ 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 (5x10^5, 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 (29). 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 (13).

**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 have been no published reports analyzing relative Ron expression during prostate tumorigenesis (7, 30). 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 (5, 30-32). 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 (17, 33). 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 NF-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 (17, 28). 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 (34). 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 (19). 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 (7). 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 (18), 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 (8). 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 (35-36). In addition, multiple studies have indicated that Ron overexpression examined by microarray analyses also correlates with hormone-refractory/androgen-independent prostate cancers (37-39). 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 (40). 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 (40). 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 (5, 32). 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 (31). 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 (11-12). 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 (41).

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 (21-22, 42-44). 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 (17).

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 (45). Similarly, Met activation by its ligand, hepatocyte growth factor (11), 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 (46). 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.
ACKNOWLEDGEMENTS

The authors would like to thank Sandy Schwemberger for her assistance with the flow cytometry experiments as well as Sarah Kader for her technical contributions. This work was supported by Public Health Services Grants CA-125379 (S.E.W.) from the National Institutes of Health, and by grant project # PC060821 (M.N.T.) from the Department of Defense Congressionally Directed Medical Research Programs.
REFERENCES


**FIGURES**

**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 IkBα 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. 
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 200x). Data are expressed as means ± SE. *p<0.05 compared to PC-3 Control group.
Supplemental Figure S1. PZ-HPV-7 and CA-HPV-10 cells have low Ron expression. A, There is increased Ron expression in CA-HPV-10 cells compared to PZ-HPV-10 cells, and a further increase in Ron expression in PC-3 cells as determined by Western analysis. B, Representative histograms from flow cytometry data depicted in Table 2. Shown are DU145 and 22RV1 isotype controls or samples analyzed with a Ron-specific antibody.
Supplemental Figure S2. Inhibition of NF-κB in DU145 cells containing a Ron knockdown, and decreased endothelial cell migration with CXCR2 inhibition. A and B, Ron knockdown in DU145 cells results in decreased NF-κB activity as determined by electrophoretic mobility shift assays with NF-κB binding composed primarily of the p65 and p50 NF-κB subunits (arrow indicates mobility shift). Binding specificity was also examined in the presence of a 10-fold excess of cold probe. C, Treatment of HUVEC cells with the CXCR2-specific inhibitor, SB225002 (300uM), leads to a significant decrease in HUVEC migration towards conditioned media from DU145 cells. Data are expressed as means ± SE. *p<0.05 compared to control DU145 group.
Supplemental Figure S3. Met is highly expressed in PC-3 cells, and Met expression is unaltered by Ron loss in DU145 cells. A, As detected by quantitative real-time PCR, PC-3 cells have high levels of Met expression with LNCaP cells having no detectable levels of Met. DU145 and 22RV1 cells have intermediate levels of Met. B and C, Met expression is unchanged in Ron knockdown DU145 cells compared with control DU145 cells as determined by real-time PCR (B) and by Western analysis (C).
Table 1. Ron expression in human prostate specimens. 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>
<thead>
<tr>
<th>Prostate tissue</th>
<th>% Positive</th>
<th>Mean intensity</th>
<th>Intensity range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal prostate</td>
<td>12.5% (1/8)</td>
<td>50</td>
<td>0–160</td>
</tr>
<tr>
<td>Benign prostatic hyperplasia</td>
<td>74% (14/19)</td>
<td>143</td>
<td>0–180</td>
</tr>
<tr>
<td>Prostate adenocarcinoma</td>
<td>86% (38/44)</td>
<td>156</td>
<td>20–300</td>
</tr>
<tr>
<td>Metastatic adenocarcinoma</td>
<td>100% (3/3)</td>
<td>285</td>
<td>270–300</td>
</tr>
</tbody>
</table>

Table 2. Ron expression in human prostate cell lines. 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.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ron expression</th>
<th>Mean fluorescent intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>+++</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>PC-3</td>
<td>++</td>
<td>60–80</td>
</tr>
<tr>
<td>CA-HPV-10</td>
<td>+</td>
<td>40–60</td>
</tr>
<tr>
<td>LNCaP</td>
<td>+</td>
<td>40–60</td>
</tr>
<tr>
<td>22RV1</td>
<td>+/-</td>
<td>20–40</td>
</tr>
<tr>
<td>PZ-HPV-7</td>
<td>+/-</td>
<td>20–40</td>
</tr>
</tbody>
</table>
CHAPTER 4

THE RON RECEPTOR IS REQUIRED FOR PROSTATE TUMOR GROWTH IN THE TRAMP MOUSE MODEL

Megan N. Thobe¹, Jerilyn K. Gray¹, Devikala Gurusamy¹, Peterson Pathrose¹, Alex B. Lentsch², and Susan E. Waltz¹*

Departments of Cancer and Cell Biology¹ and Surgery², University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521.
ABSTRACT

The Ron receptor tyrosine kinase is overexpressed in many cancers, including prostate cancer. In order to examine the significance of Ron in prostate cancer in vivo, we utilized a genetically engineered mouse model, referred to as TRAMP mice, that is predisposed to develop prostate tumors. In this model, we demonstrate that prostate tumors from 30-week old TRAMP mice have increased Ron expression compared to age-matched wild-type prostates. Based on the upregulation of Ron in human prostate cancers and in this murine model of prostate tumorigenesis, we hypothesized that this receptor plays a functional role in the development of prostate tumors. To test this hypothesis, we crossed TRAMP mice with mice that are deficient in Ron signaling (TK-/-). Interestingly, TRAMP TK-/- mice show a significant decrease in prostate tumor mass relative to TRAMP mice containing functional Ron. Moreover, Ron TK-/- TRAMP prostate tumors exhibited decreased tumor vascularization relative to wild-type TRAMP prostate tumors, which correlated with reduced levels of the angiogenic molecule VEGF. While Ron loss did not alter tumor cell proliferation, a significant decrease in cell survival was observed. Furthermore, human prostate cancer cell lines containing a Ron deficiency exhibited decreased levels of phosphorylated MAPK and Akt, suggesting that Ron may be important in regulating prostate cell survival at least partly through these pathways. In total, our data show for the first time that Ron is required for prostate tumor growth, prostate tumor angiogenesis, and prostate cancer cell survival in vivo.
INTRODUCTION

Prostate cancer remains one of the most commonly diagnosed cancers among men in the United States. Unfortunately, following treatment, most prostate cancers will recur and become increasingly aggressive and resistant to therapy. Receptor tyrosine kinases are emerging as potential therapeutic targets in prostate cancer. For example, inhibition of the epidermal growth factor receptor results in decreased prostate cell proliferation (1), and inhibition of the insulin-like growth factor 1 receptor (2, 3) or Her2/neu receptor (4) reduces tumor growth in mouse prostate cancer cell xenograft models. These data suggest that receptor tyrosine kinases play an important role in prostate tumor growth and should be considered in the development of new treatments in prostate cancer.

One such potential receptor, the Ron receptor, is a member of the Met family of receptor tyrosine kinases. Ron is a heterodimeric glycoprotein consisting of an extracellular alpha-chain and a transmembrane beta-chain (5). Binding of ligand, hepatocyte growth factor-like protein (HGFL), to Ron results in receptor dimerization and phosphorylation on key tyrosine residues within the tyrosine kinase domain (6). Trans-autophosphorylation also occurs on two tyrosine residues within the carboxyl-domain, resulting in the formation of docking sites for adaptor molecules, including PI3K, PLC-γ, Grb2 and Shc. Therefore, Ron is able to signal through multiple signaling pathways to elicit a multitude of biological responses, including cellular proliferation, migration, scattering, survival, and branching morphogenesis (7-9).

The Ron receptor tyrosine kinase has been implicated in the initiation and progression of several human cancers, including those of the breast, colon, skin, and prostate (10-13). In prostate cancer cells, Ron is an important regulator of angiogenic chemokine production. Furthermore, Ron-knockdown PC-3 cells orthotopically transplanted into prostates of nude mice
resulted in decreased prostate tumor size and vascularization compared to PC-3 control cells (14). While these studies suggest that Ron is important in prostate tumor growth in vivo, they are limited in scope because the natural context of the prostate is disrupted by the introduction of human prostate cancer cells into the mouse prostate.

Mice harboring a deletion of the Ron tyrosine kinase domain, termed Ron TK-/- mice, are overtly normal, although they exhibit alterations in inflammatory responses following stress (19). We have previously published in a skin model of chemically-induced carcinogenesis that loss of Ron results in decreased growth of benign skin papillomas, as well as the percent of papillomas that progress to malignancy (20). In addition, Ron TK-/- mice crossed with mice that are predisposed to develop mammary tumors (MMTVpMT mice), display decreased mammary tumor initiation and growth, and have reduced levels of phosphorylated Akt and MAPK (21). In order to elucidate the role of the Ron receptor in prostate tumorigenesis in vivo, we generated TRAMP mice that are deficient in Ron signaling (TRAMP TK-/- mice).

TRAMP mice have expression of the simian virus 40 early genes driven by a prostate-specific rat probasin promoter, and is specifically expressed in the dorsolateral and ventral lobes of the prostate (15). Male TRAMP mice develop prostate hyperplasia as early as 10 weeks of age, and as young as 18 weeks of age can have invasive adenocarcinoma of the prostate. Interestingly, all mice eventually develop metastasis, mainly to the lymph nodes and lung (16).

Utilizing cell lines derived from TRAMP tumors, it has been shown that inhibition of the receptor tyrosine kinase EGFR, results in decreased cell migration, although there is no impact on cellular proliferation (17). Adult TRAMP males treated with Genistein, a phytoestrogen found in soy food known to have tyrosine kinase inhibitory effects, have more well-differentiated prostate tumors compared to control mice (18). While these data suggest receptor tyrosine
kinases are important in prostate tumorigenesis in the TRAMP mouse model, the role of the Ron receptor has not been established.

Our data show for the first time that Ron receptor signaling is required for prostate tumor growth \textit{in vivo}. Thirty-week old TRAMP TK-/- mice have a significant reduction in prostate tumor weight and decreased lung metastases compared to TRAMP TK+/+ mice. Moreover, prostate tumors taken from Ron-deficient TRAMP mice show decreased vascularization, and decreased levels of VEGF. Surprisingly, TRAMP TK-/- tumors demonstrate no difference in tumor cell proliferation compared to TRAMP TK+/+ tumors. While proliferation in the tumors does not appear to differ, a significant reduction in the number of viable cells was observed in both the TRAMP TK-/- prostate tumors and in TRAMP TK-/- primary prostate tumor cell cultured \textit{ex vivo} compared to controls, suggesting that Ron may modulate signaling pathways involved in prostate cancer cell survival. Prostate cancer cells with a Ron loss exhibited dramatically reduced levels of phosphorylated Akt and MAPK compared to control cells, further supporting our findings that Ron confers a survival advantage in prostate cancer cells. Taken together, our studies provide insight into the functional significance of the Ron receptor in promoting prostate cancer cell viability and increased prostate tumor size \textit{in vivo}.
MATERIALS AND METHODS

**Mice.** C57Bl/6 Transgenic Adenocarcinoma of the Mouse Prostate mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice containing a germline deletion of the Ron tyrosine kinase domain (Ron TK-/-) have been previously characterized (19). TRAMP+/- mice were crossed with Ron TK-/- mice to generate Ron TK+/- mice with TRAMP. These latter mice were crossed with TK+/- mice to obtain wild-type Ron TRAMP mice (TRAMP+/-, TK+/+) and Ron-deficient TRAMP mice (TRAMP+/-, TK-/-). Only hemizygous TRAMP males were used for experimental analyses. The use and maintenance of animals was performed under protocols approved by the Institutional Animals and Use Committee of the University of Cincinnati.

**Prostate Histology and Immunohistochemistry.** Immunohistochemistry of prostate sections from paraffin embedded 30-week old wild-type or TRAMP prostates were processed as previously described (21, 22). For Ron staining, the Ron C-20 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) was applied to tissues overnight at 4 degrees. The next day, goat anti-rabbit secondary antibody (Vector Laboratories, Burlington, CA) was added to the tissues, and following amplification with a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA), positive cells were visualized with DAB substrate (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and mounted with permount (Fisher Scientific, Pittsburgh, PA). CD31 staining was performed and quantified as previously described (14), and cleaved caspase-3 staining was performed according to the manufacturer’s instructions (1:100 Catalog #9664, Cell Signaling Technology, Danvers, MA) For the analysis of proliferation, two hours before sacrifice, 30-week old TRAMP TK+/+ or TRAMP TK-/- mice were injected into the intraperitoneum with bromodeoxyuridine (BrdU) and sections from
paraffin embedded prostate tissue were stained utilizing a BrdU staining kit (Amersham, Piscataway, NJ) according to the manufacturer’s instructions. Image J software was used to determine the number of positive cells normalized to area of prostate tissue. For TUNEL analyses, prostate sections from 30-week old mice were stained using an In Situ Cell Death Detection Kit, AP (Roche Applied Science, Indianapolis, IN) according to the manufacturer's directions, with a 1:2 dilution of TdT enzyme. The number of TUNEL-positive cells was determined by counting as previously described (21).

Lung Metastasis
For analysis of lung metastases, lungs from 30-week old TRAMP TK+/+ or TRAMP TK-/- mice were harvested, fixed in 10% neutral buffered formalin overnight, processed and were paraffin-embedded. 4-μm sections were cut at 50-μm intervals, and were examined by hematoxylin and eosin staining for metastases. Immunohistochemistry with a Cytokeratin 18 antibody (Epitomics, Burlingame, CA) was used to confirm metastatic foci. Mice were counted as positive for lung metastasis if at least one metastatic foci was observed.

Quantitative Real-Time PCR. RNA was isolated from frozen prostate tissue from 30-week old mice using the Trizol method (Invitrogen, Carlsbad, CA). The High Capacity complementary DNA kit (Applied Biosystems, Foster City, CA, USA) was used to convert RNA to cDNA as per the manufacturer's instructions, and SYBR green incorporation (Applied Biosystems, Foster City, CA) was used to measure gene expression. Primer sequences used were as follows: VEGFa forward: GCA GAA GTC CCA TGA AGT GA VEGFa, reverse: TCC AGG GCT TCA TCG TTA; CXCL2 forward: AGTGAACTGCGCTGTCATGC, reverse:
AGGCAAAACTTTTTGACCGCC. Primers for Ron were previously described (8). Gene expression values were normalized to Gus (forward: TTGAGAAGCTTATAAGACGCATCAG, reverse: TCTGGTACTCCTCAGACATGC). Relative gene expression values are shown.

Western Analyses. Frozen prostate tissues were homogenized in 1x Laemmeli buffer and were briefly sonicated, and DU145 cells were lysed in 1x Laemmeli buffer. Protein concentrations were determined using the MicroBCA kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. 200ug of protein (for Ron) or 50ug of protein (for Akt and MAPK) was loaded into a 6% polyacrylamide gel as previously described (14). Primary antibodies used were: Ron C-20 (1:200 Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Akt (1:1,000 Cell Signaling Technology, Danvers, MA), Total Akt (1:1,000 Cell Signaling Technology, Danvers, MA), phospho-p42/44 (1:1,000 Cell Signaling Technology, Danvers, MA), Total MAPK (1:1,000 Millipore Billerica, MA), and Actin (1:40,000 a gift from Dr. James Lessard, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). Membranes were then incubated with a peroxidase-conjugated anti-rabbit or an anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by detection of the antibody with the ECL Plus reagent (Amersham Biosciences, Piscataway, NJ). Protein bands were detected using autoradiography.

Flow Cytometry. 48 hours after plating, media was collected and spun down to obtain cells, and adherent cells were collected by brief trypsinization. The cell pellets were washed with phosphate buffered saline and binding buffer. Annexin V and Propidium Iodide were added to
the cells according to the manufacturers’ instructions (ApoAlert Annexin V-FITC Apoptosis Kit, Clontech, Mountain View, CA). The samples were analyzed using a Coulter Epics XL instrument (Beckman Coulter, Miami, FL).

**Statistical Analyses.** Data are expressed as mean ± standard error. Student’s t-test was used for pair-wise comparisons and Mann-Whitney Rank Sum Tests were applied when applicable using SigmaPlot 11.0 software (Systat Software, Inc., San Jose, CA). Differences between groups were accepted as significant when p<0.05.
RESULTS

Wild-type mouse prostates are similar to Ron TK-/- prostates. Prostates taken from 30-week old wild-type mice or age-matched Ron TK-/- mice were analyzed by H&E staining to determine if loss of Ron results in any abnormalities in the prostate. No appreciable differences were observed in the prostates of Ron TK-/- mice compared to controls. Representative dorsolateral prostate lobes from Wild-type and TK-/- mice are depicted in Figure 1A.

Ron is highly expressed in TRAMP prostate tumors. To determine if similar to human prostate disease (10) (14) Ron expression is increased in murine prostate tumors, prostates taken from either 30-week old wild-type or age-matched TRAMP mice were stained by immunohistochemistry for Ron expression (Figure 1B). Compared with the minimal expression of Ron observed in the wild-type mouse prostate, TRAMP prostate tumors have significantly elevated levels of Ron. In addition, we analyzed Ron mRNA levels by quantitative real-time PCR and similarly observed increased Ron transcript levels in TRAMP prostates compared to wild-type prostate (Figure 1C). Further examination of Ron expression by Western analyses demonstrates elevated Ron protein expression in TRAMP prostates relative to wild-type prostates (two representative samples are shown, Figure 1D). Relative levels of Ron are displayed in Figure 1E.

Ron TK-/- TRAMP mice have decreased genitourinary (GU) complex and prostate tumor size. Thirty-week old TRAMP mice crossed with mice harboring a targeted deletion of the tyrosine kinase (TK) domain of Ron showed a significant reduction in GU complex and prostate size compared to TRAMP prostates expressing wild-type Ron (Figure 2A). While the majority
of the TRAMP TK-/- mice had enlarged prostates and/or seminal vesicles, none had prostate tumors to the extent of the TRAMP TK+/- mice (Figure 2B). Histological evaluation confirmed that compared to the tumor-bearing TRAMP TK+/- prostate, TRAMP prostates lacking the TK domain of the Ron receptor have a more well-defined architecture, although hyperplasia was observed (Figure 2C). Lungs from these mice were also analyzed for metastasis by histological analysis. Three out of seven TRAMP+ TK+/- lungs had detectable metastases, while none of the five TRAMP TK-/- lungs examined contained metastasis (Table 1).

**Murine TRAMP prostates lacking functional Ron display decreased microvessel density.** To determine the extent of prostate vascularization, prostates taken from TRAMP mice containing wild-type Ron or prostates taken from TRAMP TK-/- mice were stained with a CD31-specific antibody (marker of endothelial cells). TRAMP TK-/- prostates exhibited a decrease in the number of vessels per unit area compared to TRAMP TK+/- prostates (Figure 3A, B). This decrease in microvessel density was correlated with a significant decrease in the levels of VEGF (Figure 3C).

**TRAMP prostate tumors from TK-/- mice have similar rates of cellular proliferation, but decreased cellular survival.** Because of the significant difference in tumor size between TRAMP TK+/- and TRAMP TK-/- prostate tumors, we sought to determine if there were any differences in cell proliferation or cell death. Interestingly, both TRAMP TK+/- and TRAMP TK-/- tumors had similar amount of proliferation as determined by BrdU incorporation (Figure 4A and 4C). There was however, a significant increase in the number of TUNEL-positive cells
in the TRAMP TK-/- tumors, suggesting a survival advantage may exist in the TRAMP TK+/+ prostate cancer cells (Figure 4B and 4C).

**Cells derived from TRAMP prostate tumors lacking functional Ron have decreased viability.** The increased number of TUNEL-positive cells in Ron-deficient TRAMP tumors suggests that Ron signaling may provide a survival advantage. To further examine if cells expressing Ron have increased viability *ex vivo*, we isolated primary prostate cells from 30-week old TRAMP TK+/+ and TRAMP TK-/- mice. These epithelial cells were then analyzed for plasma membrane integrity by propidium iodide (PI) staining and for apoptosis by Annexin V staining by flow cytometry. In examining the primary tumor cells, no appreciable Annexin V staining was observed under our experimental conditions (data not shown). Similarly, although the TRAMP TK-/- tumors trended towards increased cleaved caspase-3 staining, there was no significant difference compared with TRAMP TK+/+ tumors (Supplemental Figure S1). However, consistent with our TUNEL staining, we did observed a consistent and significant increase in the number of dead cells, which lost plasma membrane integrity and were permeable to PI staining, in the TRAMP TK-/- cells compared to TRAMP TK+/+ cells. The average number of dead TRAMP TK+/+ cells +/-SEM was 6.6+/−1.36 while the average number of dead TRAMP TK-/- cells was 13.7+/−1.32 (Figure 5A, Supplemental Figure S2).

**Ron activates Akt and MAPK phosphorylation in prostate cancer cells.** Ron stimulation through ligand binding has been shown to be a strong inducer of both MAPK and Akt activation and activation of both of these pathways by Ron has been shown to promote survival of RE7 epithelial cells (Madin-Darby canine kidney cells transfected with a Ron cDNA) cultured under
serum-free conditions (23). To elucidate a potential mechanism by which Ron may be regulating the viability of the TRAMP prostate epithelial cells, we analyzed levels of phosphorylated Akt and MAPK. **Figure 5B** demonstrates that in DU145 prostate cancer cells, a Ron knockdown is able to abrogate ligand-induced phosphorylation of both Akt and MAPK compared to control DU145 cells.
DISCUSSION

An analysis of Ron expression in prostate tissue from either wild-type or age-matched TRAMP mice showed that the Ron receptor is highly expressed in prostate tumors relative to normal murine prostate tissue (Figure 1). This data is consistent with several reports documenting increased Ron expression in prostate cancer compared with normal prostate tissue (14) (10) (24-25). The overexpression of Ron is an important finding as prior studies have shown that exogenous Ron overexpression is sufficient to induce the formation of both lung and mammary tumors in mice (22, 26), and Ron activation has been shown to induce oncogenic phenotypes in a variety of cell lines including mouse and human pancreatic cells with associated increases observed in the activation of Akt and MAPK signaling cascades (11).

Our data show for the first time that TRAMP mice deficient in Ron receptor signaling have decreased prostate tumor growth and metastasis to the lung (Figure 2 and Table 1) compared to Ron-expressing mice. Similar studies from our laboratory have documented that the Ron receptor is necessary for mammary tumor growth in a polyoma virus middle T (pMT) antigen model of breast cancer. pMT mice deficient in functional Ron were shown to have a longer tumor latency, decreased mammary tumor size and decreased metastasis. However, in the pMT mouse model, a significant decrease in cellular proliferation in Ron-deficient pMT tumors compared to Ron-expressing pMT tumors was observed (21). In contrast, no appreciable differences in cellular proliferation were observed between 30-week old TRAMP TK-/- and TRAMP TK+/+ prostate tumors (Figure 4A). These differences may reflect the aggressiveness of the respective tumors, with significant tumor burden observed in the pMT-induced mammary tumor model occurring in less than 2 months while the TRAMP model was evaluated at 8 months of age.
While the difference in prostate tumor size in the TRAMP TK+/+ and TK-/- mice may not be due to differences in tumor cell proliferation (Figure 4A), a significant difference in tumor cell viability was observed between groups. Marked increases in the number of TUNEL-positive cells were observed in prostates from TRAMP TK-/- mice compared controls (Figure 4B), suggesting that Ron may be acting as a pro-survival factor in this model. In addition, primary prostate cells derived from TRAMP TK-/- prostates were less viable \textit{ex vivo} than cells derived from TRAMP TK+/+ prostates (Figure 5A), supporting Ron as a regulator of cellular survival, although at present the exact mechanism for these differences remains unclear.

Several reports have documented the regulation of PI3K signaling by the Ron receptor. Breast cancer cells stimulated with HGFL have increased phosphorylation of Akt and MAPK (27-28). Furthermore, colon cancer cells containing a Ron-knockdown have decreased levels of pAkt following growth factor reduced conditions compared to Ron-expressing cells, potentially due to decreased binding of the Ron receptor to the p85 subunit of PI3K (29).

We observed that DU145 prostate cancer cells with a Ron knockdown have decreased levels of phosphorylated Akt and MAPK compared to Ron-expressing control cells following ligand stimulation (Figure 5B), suggesting that Ron may be acting as a pro-survival factor through the regulation of these pathways. It is important to note that the Ron ligand, HGFL, is a systemic hormone present and available in the prostate microenvironment to active Ron signaling. In the case of the Met receptor, a closely related receptor tyrosine kinase to Ron, there is production of ligand (HGF) by the stromal cells, and most likely specifically by myofibroblasts. Moreover, conditioned media taken from prostate stromal cells is sufficient to induce Met activation in DU145 cells (30), suggesting that while the prostate epithelial cells do not produce HGFL, an ample source of ligand is available systemically. Future studies are
needed to determine the precise requirement of ligand for Ron receptor activation in the mouse prostate.

TRAMP TK-/- tumors also demonstrate a reduction in tumor vascularization compared to TRAMP TK+/+ tumors, suggesting prostate tumor neovascularization is not only regulated by Ron, but is also important for tumor growth (Figure 3). Prostate tumor angiogenesis is critical in the TRAMP mouse model, and TRAMP mice crossed with mice containing a knockout of the common angiogenic chemokine receptor, CXCR2, have decreased prostate tumor growth and time to palpable tumor (31). In addition, TRAMP mice treated with soluble VEGFR-2 demonstrated decreased prostate tumor volume correlating with decreased vasculature (32). We have previously reported that prostate cancer cells with high Ron expression have increased production of angiogenic chemokines, and Ron-knockdown in these cells leads to diminished levels (14).

Consistent with these data, we also observed a slight decrease in levels of the angiogenic chemokine, CXCL2, in TRAMP TK-/- prostates relative to TRAMP TK+/+ prostates (Figure 3D). While in our prior analyses we observed minimal levels of VEGF in prostate cancer epithelial cells in vitro (14), our current in vivo studies show decreased VEGF in TRAMP TK-/- prostates in vivo. Tumors taken from 24-week-old TRAMP mice have elevated levels of VEGF relative to wild-type prostate (33), suggesting there may be factors involved in the upregulation of VEGF in vivo found in the prostate microenvironment that are not present in cell culture media.

Our studies strongly support the Ron receptor tyrosine kinase as being critical for prostate tumor growth in vivo. Further studies are needed to elucidate the potential role of Ron in the growth of prostate cancers, and to determine if advanced prostate cancers upregulate Ron
expression in order to bypass androgen-dependence. Based on our data, targeting the Ron receptor in prostate cancer might result in decreased prostate tumor angiogenesis and an increase in prostate cancer cell death, and therefore potentially leading to a reduction in prostate tumor size. These new findings provide insight into a potential therapeutic target that might aide in the advancement of prostate cancer therapies.

Acknowledgements

The authors would like to thank Sandy Schwemberger for her assistance with the flow cytometry experiments as well as Gina Ciovacco for her technical contributions. This work was supported by Public Health Services Grant CA-125379 (S.E.W.) from the National Institutes of Health, and by grant project #PC060821 (M.N.T.) from the Department of Defense Congressionally Directed Medical Research Program.
REFERENCES


Figure 1. Ron expression in wild type and TRAMP mouse prostates. A, Prostates from 30-week old wild-type or Ron TK-/- mice are structurally similar. Representative sections of wild-
type and Ron TK-/- prostates are shown.  B, Ron is highly expressed in 30-week old TRAMP prostates compared to normal prostate tissue. Representative tissue sections were stained with a Ron-specific antibody or with an isotype control IgG. Wild-type prostates have minimal Ron expression whereas TRAMP prostate tumors have significantly elevated Ron expression.  C, Quantitative real-time PCR analysis demonstrates increased Ron mRNA levels in TRAMP prostate tumors (n=5) relative to wild-type mouse prostates (n=6). Data are expressed as means ± SE.  D, Western analysis of Ron protein levels in wild-type prostate and in prostate tumors from TRAMP mice.  E, Densitometry analysis of the relative Ron protein levels in wild-type (n=4) and TRAMP (n=5) prostates. Data are expressed as means ± SE. *p<0.05 compared to wild-type group.
Figure 2. TRAMP mice deficient in functional Ron have decreased genitourinary tract and prostate tumor size. **A**, 30-week old TRAMP TK-/- mice (n=12) exhibit decreased genitourinary (GU) tract and prostate tumor mass (*p<0.05 compared to TK+/+ group) relative to TRAMP TK+/+ mice (n=16). Data are expressed as means ± SE. **B**, Gross examination of genitourinary tracts from TRAMP TK+/+ mice and TRAMP TK-/- mice demonstrates decreased GU tract size in the TRAMP TK-/- mice. **C**, Representative histological analysis of TK+/+ and TK-/- prostates from TRAMP mice.
Figure 3. Prostate tumors from TRAMP TK−/− mice are less vascularized than prostates from TRAMP mice expressing wild-type Ron.  

A, TRAMP mice lacking functional Ron have decreased vascularization as determined by immunohistochemistry with a CD31-specific antibody.  

B, Mean vessel density per area was determined as described in Materials and Methods with n=4 mice for the TRAMP TK+/+ group and n=3 mice for the TRAMP TK−/− group.  Data are expressed as means ± SE.  *p<0.05 compared to TK+/+ group.  

C&D, Quantitative real time PCR was performed on RNA isolated from TRAMP prostate tumors from
TRAMP TK+/+ and TRAMP TK-/− mice. Expression of the angiogenic factors VEGF (C) and CXCL2 (D) are shown. Data are expressed as means ± SE with n=4 prostates per group. *p<0.05 compared to TK+/+ group.
Figure 4. BrdU and TUNEL analyses on TRAMP+ TK+/+ and TRAMP+ TK-/- prostates.

A, BrdU immunostaining was performed on TRAMP prostates from 30-week TK+/+ and TK-/- mice. B, Detection of TUNEL positive cells in TRAMP prostate tissue of TK+/+ and TK-/- mice at 30-weeks of age. C, Prostates from 30-week old TRAMP+ TK+/+ and TK-/- mice did not exhibit differences in BrdU (left) staining but did contain significant differences in the extent
of TUNEL-positive cell staining (right). Data are expressed as means ± SE. Five separate areas were counted from four independent specimens per group, and representative images are shown. *p<0.05 compared to TK+/+ group.
Figure 5. Prostate cancer cells expressing Ron have increased survival compared to Ron-deficient prostate cancer cells. A, Primary prostate cancer cells from TRAMP TK-/- prostates are less viable than TRAMP TK+/+ cells, as determined by the increase in propidium iodide-positive cells (used as a measurement of membrane permeability). Data are expressed as means ± SE. Experiments were performed three times in triplicate with values from a representative
experiment shown. *p<0.05 compared to TK+/+ group. B, Western analysis of DU145 prostate cancer cells. A representative Western analysis of Ron protein expression in DU145 cells with and without a knockdown of Ron is shown. Following serum-starvation and stimulation with HGFL, Ron-expressing DU145 cells exhibit higher levels of phosphorylated Akt and phosphorylated p44/42 MAPK compared to Ron knockdown cells.
Supplemental Figure S1. No difference between TRAMP TK+/+ and TRAMP TK-/ prostates in levels of cleaved caspase-3.  A, Representative images of prostates stained with active caspase-3-specific antibody (image taken at 40x magnification).  B, Average number of cleaved caspase-3 positive cells (TRAMP TK+/+ n=3, TRAMP TK-/ n=4, four areas/section were counted)
Supplemental Figure S2. Increased number of PI-positive TRAMP TK-/- cells.

Representative FLOW cytometry histograms for Figure 5A depicting either TRAMP TK+/+ or TRAMP TK-/- cells analyzed for propidium iodide (PI, y-axis) and Annexin V (x-axis). There is increased number of PI-positive cells (top left quadrant) in TRAMP TK-/- cells compared with TRAMP TK+/+ cells. Numbers shown represent % of cells that were positive for PI in this particular experiment. Minimal detection of Annexin V staining (bottom and top right-hand quadrants) was observed in either cell line.
Table 1. Lung metastases in TRAMP mice. 30-week old TRAMP TK+/+ and TRAMP TK-/- mice were evaluated for lung metastases.

<table>
<thead>
<tr>
<th>% Mice with Lung Metastases (# mice with lung metastases/total mice analyzed)</th>
<th>TRAMP+ TK+/+</th>
<th>TRAMP+ TK-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>43% (3/7)</td>
<td>0% (0/5)</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 5

CONCLUSIONS AND DISCUSSION
Summary

The Ron receptor tyrosine kinase has been implicated in a wide variety of human cancers, and overexpression of Ron has been previously documented in human prostate cancer specimens (1, 2). Our studies, as outlined in Chapters 3 and 4, are the first to elucidate a functional role of the Ron receptor in regulating prostate tumor growth, by impacting tumor angiogenesis and cellular survival. Specifically, in DU145 and PC-3 prostate cancer cells Ron is highly expressed and is constitutively active. Moreover, Ron expression correlates with production of angiogenic chemokine production, and Ron inhibition results in decreased levels of angiogenic chemokines. Conversely, Ron overexpression in LNCaP cells is sufficient to induce CXCL8 production, and the regulation of angiogenic chemokine levels by the Ron receptor is at least partially dependent on the NF-κB transcription factor. The modulation of angiogenic chemokines in prostate cancer cells by Ron is biologically relevant, as we have demonstrated supernatant taken from Ron-knockdown cells is a poor chemoattractant for endothelial cells. Taken together, these data suggest that Ron might be playing a role in prostate tumor angiogenesis, and hence impacting tumor growth and metastasis.

Further analysis of the impact of Ron in prostate cancer demonstrated a significant impact of Ron knockdown on prostate tumor growth in vivo. PC-3 cells containing a Ron-knockdown orthotopically transplanted into prostates of nude mice formed significantly smaller tumors than control PC-3 cells, and these tumors were also less vascularized. Moreover, prostates taken from 30 week-old TRAMP mice lacking functional Ron (TRAMP TK-/- mice) have a significant reduction in tumor mass and decreased tumor vascularization. Interestingly, there was not a significant difference in
cellular proliferation between TRAMP TK+/+ and TRAMP TK-/- prostates, although TRAMP TK+/+ tumors might have a survival advantage which correlates with alterations in Akt and MAPK signaling. Together, these data suggest that Ron signaling has multiple roles in prostate cancer, including regulating tumor angiogenesis and tumor cell survival, and are described in further detail below.

**Ron Expression and Activation in Prostate Cancer**

Our data demonstrate that Ron is highly expressed in prostate cancer cells compared to non-transformed prostate cells. Moreover, Ron expression is significantly increased in prostate tumors taken from TRAMP mice, whereas Ron expression is minimally detected in normal mouse prostate. Likewise, Ron expression was increased in colorectal carcinoma cells (3), pancreatic cancer cells (4), breast cancer cells (5), and ovarian carcinomas (6), among several other types of human cancer. Moreover, normal mammary gland and benign mammary lesions had minimal expression of Ron, whereas primary mammary carcinomas have a significant increase in Ron expression (5). Additionally, the majority of pancreatic tumors and metastatic lesions have overexpression of Ron, while normal ducts and low-grade PanIN have minimal Ron expression (4), demonstrating that Ron expression is increased in cancer specimens relative to normal tissue, similar to our analyses in the prostate.

We have also shown that in DU145 and PC-3 cells lines, the Ron receptor is constitutively active, although the mechanism of activation remains unclear. It has been demonstrated however, that murine Ron overexpression in NIH3T3 fibroblasts leads to activation of oncogenic phenotypes. These Ron-overexpressing fibroblasts exhibit
increased rates of proliferation and are able to form tumors in vivo. These data suggest that high Ron expression is sufficient to induce and maintain activation. Interestingly, introducing single amino acid point mutations in the Ron gene (natural occurring point mutations found in the Met receptor in human hereditary papillary renal carcinoma) also results in increased proliferation, tumor growth, as well as metastasis to the lung (7). Additionally, Ron overexpression in mouse mammary gland is sufficient to induce mammary tumorigenesis (8).

What remains unclear is the contribution of HGFL in prostate cancer. HGFL is predominantly produced by hepatocytes in the liver, and is secreted into the bloodstream where it circulates (9). In addition to becoming cleaved and activated by the membrane-bound protease MT-SP1, HGFL can also be activated by proteases of the coagulation cascade, including kallikrein, factor XIIa, and factor XIa (10).

It remains unclear if HGFL levels are altered during prostate tumor progression or in androgen insensitive prostate cancer (11, 12). Our preliminary data suggests that PC-3 prostate cancer cells do not express HGFL (data not shown). However, there is an abundance of evidence to suggest that epithelial cells and prostate stromal cells crosstalk (13-16). In the case of the Met receptor, stromal myofibroblasts are a source of the Met ligand HGF. Secretion of HGF by myofibroblasts is able to activate Met on DU145 prostate cancer cells, and is sufficient to induce migration (17). Therefore, further analyses are needed to determine which, if any, cell type in the prostate microenvironment produces and secretes HGFL. This would help further elucidate the activation of the Ron receptor during tumor progression and could also act as a prognostic factor for prostate cancer patients.
In addition to examining expression of HGFL within the tumor microenvironment, activation of HGFL should also be considered. Coordinate expression of MT-SP1 (a serine protease that cleaves pro-HGFL to active HGFL), HGFL, and Ron in breast cancer patients correlates with increased metastasis and poor patient prognosis. Additionally, HGFL overexpression in polyomavirus middle T antigen-driven mammary tumor cells accelerates tumor growth and increases the spectrum of metastasis compared to control mice expressing wild-type HGFL (18). Furthermore, there is evidence that prostate cancer cells have higher levels of MT-SP1 mRNA compared to non-tumorigenic cells, and that prostate tumor tissue has increased MT-SP1 expression compared to normal prostate tissue (19). These data suggest that HGFL might become activated in prostate cancer whereas there are low levels of active HGFL present in normal prostate. Moreover, it would be important to determine if similar to breast cancer cells, there is coordinate expression of Ron, HGFL and MT-SP1 (or any protease able to cleave pro-HGFL to active HGFL) in prostate tumor cancer cells or in the tumor microenvironment. Coordinate upregulation would suggest that HGFL becomes activated within the tumor, which activates the Ron receptor, and ultimately promotes prostate tumor growth.

**The Ron Receptor: Prostate Tumor Initiation or Tumor Progression?**

Based on our data wherein there is a significant proportion of human benign prostate hyperplastic specimens exhibiting increased Ron expression (Chapter 3, Figure 1), it suggests that Ron may be important in early tumor initiation. Similarly, in an analysis of non-transformed prostate cells (PZ-HPV-7), prostate cells derived from a
primary prostate tumor (CA-HPV-10), and metastatic prostate cells (PC-3), for Ron expression, we found increasing Ron expression in CA-HPV-10 and PC-3 cells compared to PZ-HPV-7 cells, with PC-3 cells expressing the highest amount of Ron (data not shown). Furthermore, our studies wherein TRAMP TK-/- mice displayed decreased prostate tumor mass and exhibited reduced lung metastasis compared to TRAMP TK+/+ mice, suggest that Ron is also playing a role in prostate tumor progression. However, because we analyzed tumor burden and metastases at a later timepoint (30 weeks), it remains unclear if Ron is critical for tumor initiation in this model.

An interesting experiment would be to conditionally knock out Ron in the TRAMP mouse model after palpable tumors are observed. This would determine the extent of the impact of Ron on tumor progression in this model. Another critical experiment that is currently being done in our laboratory is to genetically engineer mice that have overexpression of Ron specifically in the prostate in order to elucidate if Ron is sufficient for prostate tumor initiation. To do this, we have utilized a Ron mini-gene driven by the rat probasin promoter. In prostates harvested from mice at one year of age, preliminary results suggest that Ron is sufficient to induce PIN lesions in the prostate. Current studies are in progress to examine if these PIN lesion progress to hyperplasia at later timepoints, and any altered signaling pathways in the prostate as a result of Ron overexpression.

**Ron and Prostate Tumor Angiogenesis**

One of the ways in which the Ron receptor might impact prostate tumor growth is by regulating production of angiogenic chemokines. Therefore, this regulation most
likely influences prostate tumor angiogenesis, which is critical for tumor growth and metastasis. CXCL8 levels progressively increase during prostate cancer progression (20), and prostate epithelial cancer cells overexpressing CXCL8 are more tumorigenic and metastatic compared to control cells (21). Moreover, our data demonstrate that Ron knockdown in DU145 or PC-3 prostate cancer cells resulted in a dramatic reduction in angiogenic chemokine production. Furthermore, media taken from Ron-knockdown cells is a poor chemoattractant for endothelial cells compared to media taken from control cells. Interestingly however, in vivo, there was no change in the angiogenic chemokine CXCL2 in the TRAMP prostate tumors with or without Ron. While these studies do not directly support each other, this might be explained by influences provided by the tumor microenvironment. Utilizing DU145 and PC-3 cells, serum-starvation was performed in order to assay chemokine levels. However in vivo, factors found in serum are abundant, and may potentially influence signaling pathways in other cell types that might be important regulators of angiogenic chemokine production, and hence impacting prostate tumor angiogenesis.

Interestingly, the prostate cancer cell line PC-3 did not produce and secret measurable levels of the pro-angiogenic molecule VEGF, although VEGF upregulation most often accompanies prostate tumor growth in human patients (22-24). In the TRAMP mouse model of prostate cancer, VEGF is also upregulated in prostate tumors (25-27). In our studies, we found VEGF to be significantly reduced in TRAMP TK-/- prostates compared with TRAMP TK+/- prostates (Chapter 4, Figure 3). While in vitro, prostate cancer cell lines likely do not produce significant levels of VEGF, it is probable
that in vivo, VEGF production by prostate tumor cells or by cell types in the stroma may be important for tumor growth (28-30).

Based on our studies utilizing human prostate cancer cells, it is likely that angiogenic chemokines are also regulated by Ron in vivo, however because of the contribution of other factors, such as VEGF, the regulation is not as apparent. There is evidence however that angiogenic chemokines are also important in prostate tumor growth in TRAMP mice. Mice crossed with CXCR2 knockout mice exhibit much smaller tumors and are less vascularized. Interestingly, TRAMP mice crossed with mice containing a knockout of the common angiostatic chemokine receptor, CXCR3, have tumors palpable at an earlier age (31). Therefore, it is likely that Ron is able to regulate a combination of angiogenic chemokines and other pro-angiogenic factors such as VEGF, that contribute to prostate tumor growth.

**Ron Signaling in Prostate Cancer**

The transcriptional regulation of angiogenic chemokines by NF-κB has been documented in prostate cancer cells (32). While our data have demonstrated that the regulation of angiogenic chemokines by the Ron receptor is at least partly dependent on NF-κB, it remains unclear how Ron signals to activate NF-κB. Ron has been shown in other cell types to be able to activate PI3K/Akt signaling (33-35), and PI3K/Akt is able to activate NF-κB (36-38). Therefore it is plausible that Ron signals through PI3K/Akt to activate NF-κB, resulting in transcriptional upregulation of angiogenic chemokines. Our preliminary data show that inhibition of PI3K in prostate cancer cells leads to diminished levels of CXCL8 (Figure 1). Further studies are needed to determine if Ron is upstream
of the PI3K pathway and if PI3K/Akt signaling activates NF-κB in prostate cancer cells. More recently, utilizing an NF-κB luciferase assay, it was demonstrated that inhibition of PI3K signaling in C4-2B (androgen-independent LNCaP subline) and LNCaP prostate cancer cells leads to a reduction in NF-κB target genes (39), suggesting that PI3K signaling can activate NF-kB in prostate cancer cells.

Ron is a known upstream regulator of PI3K/Akt and MAPK signaling. These two signaling pathways are involved in regulating cellular survival and proliferation, and are often deregulated in prostate cancer (40). We found that Ron-knockdown DU145 cells do not respond to HGFL stimulation, whereas control DU145 cells have increased levels of phosphorylated Akt and phosphorylated MAPK in response to HGFL treatment (Chapter 4, Figure 5). These data are consistent with studies performed in other cancer types including breast and pancreatic cancer as well as in skin papillomas, where HGFL treatment resulted in Ron activation and increased levels of pAkt and pMAPK (41-43). Currently, we are analyzing pAkt and pMAPK levels in cells derived from TRAMP TK+/+ and TRAMP TK-/- prostates following treatment with HGFL.

Based on our data and previously published data, inhibiting the Ron receptor in prostate cancer might result in decreased cellular survival and would therefore inhibit tumor growth. Interestingly, our data demonstrate no significant difference in the number
of Annexin V positive cells between TRAMP TK+/- cells and TRAMP TK-/- cells, and no difference in the number of cleaved caspase-3 cells in prostate tumor sections from these mice.

While these studies suggest that there is no difference in prostate tumor cell apoptosis, a more thorough examination by analyzing earlier timepoints in TRAMP prostate tissue and using other markers of apoptosis such as cleaved PARP could be undertaken to substantiate this claim. Furthermore, our PI/Annexin V analyses were performed with cycling cells maintained in serum-containing media. It would be interesting to determine if there are any differences in viability in serum-starved cells or in cells undergoing another type of stress such as treatment with chemotherapeutic drugs. Rapamycin is a drug that inhibits Akt/mTOR signaling and when combined with the chemotherapeutic agent docetaxel, results in decreased cell viability in LNCaP cells and androgen-independent LNCaP cells compared to rapamycin treatment or docetaxel treatment alone (44). Additionally, Akt has been implicated in lung cancer cells in the resistance to docetaxel (45). These studies suggest that increased PI3K/Akt signaling in prostate cancer cells gives a survival advantage. We observed increased levels of pAkt in Ron-expressing DU145 cells treated with HGFL compared to Ron-knockdown DU145 cells (Chapter 4, Figure 5), and therefore combining chemotherapy with Ron inhibition might prove more effective than either treatment alone.

**Ron and the Androgen Receptor: Potential Combination Therapy?**

While current treatment modalities and using PSA as a screening tool for early detection have greatly reduced the mortality rate from this disease, there are still long
strides to go before successfully and completely treating prostate cancer. While most treatment options include hormone therapy and initially the tumors regress, tumor recurrence is common, and the prognosis is poor due to advanced metastatic disease. Interestingly, our data demonstrate that two androgen-independent prostate cancer cell lines DU145 and PC-3 have increased Ron expression while two androgen-dependent cell lines 22RV1 and LNCaP have undetectable levels of Ron. While there are other differences between these four cell lines such as PTEN mutations, Rb and p53 status, the striking demarcation between cells expressing or lacking Ron seems to be that of dependence on androgen.

While Ron expression in prostate cancer cells suggests there is some correlation or interaction between the Ron receptor and the androgen receptor, extensive studies are needed to decisively determine the extent, if any, of this interaction. Similar to Ron expression, the Met receptor is also highly expressed in DU145 and PC-3 cells and is minimally detected in LNCaP cells (46). Furthermore, in LNCaP cells, an androgen-dependent cell lines, the removal of androgen resulted in increased expression of the Met receptor (47). Further studies have determined that the androgen receptor has a repressive role for the Met receptor. The mechanism of repression by the androgen receptor is at least partially due to the androgen receptor interfering with SP1 binding on the Met promoter (48). Given that the Ron promoter also contains an SP1 site (49), and the expression pattern of Ron in prostate cancer cells, it is worth determining if the androgen receptor can also negatively regulate Ron.

In addition to the potential direct interaction between the androgen receptor and Ron, it is also possible that Ron is able to modulate signaling pathways such as PI3K/Akt
and MAPK that are important for cellular proliferation and survival, and that this is sufficient for prostate cancer cells to survive without the presence of androgens. Growth factor receptors such as Her2/Neu (50-52) and EGFR (53) have been shown to not only be overexpressed during prostate cancer progression, but have been implicated in the advancement of prostate cancer cells towards androgen-independence. It would prove to be a significant milestone, if by targeting the androgen receptor and the Ron receptor, prostate tumor progression could be delayed or stopped completely.

**Figure 2. Working model: Ron in prostate cancer cells.** Shown is a schematic of a prostate cancer cell with high Ron expression. Activation of Ron results in activation of at least two pathways: 1.) activation of PI3K/Akt and MAPK signaling promotes tumor cell growth and survival, and 2.) activation of NF-κB through an unknown mechanism results in increased production of angiogenic chemokines, impacting prostate tumor growth and tumor angiogenesis.
Conclusions and Future Directions

As described in Chapter 1, there are ongoing clinical trials targeting the Met receptor, such as ARQ 650-RP (ArQule), GSK1363089 (Exelisis), and MP470 (SuperGen), which look promising for inhibiting tumor growth. There are currently no trials targeting the Ron receptor in cancer, although neutralizing antibodies and small molecule inhibitors have proven efficient in reducing oncogenic phenotypes and should be further considered as therapeutic agents (1, 54, 55). Furthermore, drug trials targeting the VEGF receptor with inhibitors such as sunitinib (56) are promising, and combined with chemotherapeutic agents might be an effective treatment for prostate cancer.

Tumor angiogenesis is not only critical for tumor growth, but also for tumor metastasis, and therefore targeting angiogenic processes might prove beneficial in inhibiting prostate tumor growth (57, 58). While there are likely several signaling pathways regulated by Ron in prostate cancer cells, Figure 2 depicts the regulation of the NF-κB, PI3K/Akt and MAPK pathways. Furthermore, the working model as shown is not limiting, and it should be noted that there is crosstalk between these pathways, especially between NF-κB and Akt (59). Moreover, NF-κB is also well-known as a pro-survival factor and in addition to regulating angiogenic chemokine production, should also be considered as a pro-survival signal downstream of Ron (60).

It should also be considered that Ron may also impact prostate cancer by interacting with other transmembrane receptors such as integrins, which are important mediators of cell-matrix adhesions. In prostate cancer, deregulation of integrins occurs during prostate cancer progression, and this deregulation may impact prostate tumor
angiogenesis and metastasis (61). Interestingly, several reports have described an interaction between the Ron receptor and integrins. This interaction has been shown to be critical for keratinocyte migration and wound healing (62), and cellular survival (63), although the potential interaction between Ron and integrins in prostate cancer, and any resulting consequence of this interaction has not been elucidated. It is possible however, that Ron-integrin interactions in prostate cancer promotes vascularization, cell survival and metastasis, and should therefore be carefully evaluated.

Ron inhibition in prostate cancer not only impacts prostate tumor angiogenesis, but also cellular survival, and therefore targeting the Ron receptor in prostate cancer would likely result in decreased tumor vascularization as well as decreased tumor cell survival. This combination could prove very effective by impacting multiple key components in prostate tumor growth, and the Ron receptor tyrosine kinase should be considered as a potential therapeutic target in prostate cancer.
References


