University of Cincinnati

Date: 1/25/2013

I, Devikala Gurusamy , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Cancer and Cell Biology.

It is entitled:

EPITHELIAL AND STROMAL RON RECEPTOR EXPRESSION PROMOTES TUMOR GROWTH IN A MURINE MODEL OF PROSTATE CANCER

Student's name: **Devikala Gurusamy**

This work and its defense approved by:

Committee chair: Susan Waltz, PhD

Committee member: Vladimir Kalinichenko, MD, PhD

Committee member: David Plas, PhD

Committee member: Shao-Chun Wang, PhD

Committee member: Susanne Wells, PhD

Committee member: Aaron Zorn, PhD

3181

Epithelial and stromal Ron receptor expression promotes tumor growth in a murine model of prostate cancer

A dissertation submitted to the Graduate School

at 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

Devikala Gurusamy

Bachelor of Technology - Industrial Biotechnology
Anna University, India.

January 25 2013

Dissertation Committee

Susan Waltz, PhD, Chair
Shao-Chun Wang, PhD
Susanne Wells, PhD
Vladimir Kalinichenko, MD, PhD
David Plas, PhD
Aaron Zorn, PhD

Abstract

The Ron receptor tyrosine kinase is an oncogene expressed on epithelial cells and on several tissue resident macrophage populations. Over-expression and/or constitutive activation of Ron have been reported in several epithelial cancers, including prostate cancer. To investigate the significance of Ron expression in prostate cancer our laboratory had examined the loss of function of Ron in the Transgenic Adenocarcinoma of the Mouse Prostate [TRAMP] mouse model. In this model, TRAMP mice crossed with mice that have a germline loss of the tyrosine kinase signaling domain of Ron, referred to as TK-/-, had decreased prostate tumor size when compared to wild type, TK+/+ mice. This study demonstrated the functional importance of Ron signaling in promoting prostate tumor growth; however, the data did not address the selective contributions of Ron signaling in epithelial versus stromal cells during tumorigenesis. Analysis for Ron expression by qRT-PCR showed Ron expression in normal prostate epithelial cells and stromal cells including macrophages. Utilizing human epithelial cell line PC-3, we demonstrated that knockdown of Ron in PC-3 cells leads to decreased prostate tumor growth and metastasis following orthotopic prostate transplantation compared to cells with high Ron expression suggesting that the Ron receptor expressed in epithelial cells is an important factor in prostate tumorigenesis. To test the significance of stromal Ron expression, prostate cancer cells were orthotopically implanted into the prostates of either TK+/+ or TK-/- hosts. In TK-/- hosts, prostate cancer cell growth was significantly reduced compared to controls, suggesting that Ron signaling in the tumor microenvironment is critical to prostate tumor development. Prostate tumors in TK-/- hosts exhibited an increase in tumor cell apoptosis, macrophage infiltration and altered cytokine expression. Reciprocal bone marrow transplantation studies and studies utilizing mice with myeloid cell specific ablation of Ron, demonstrated that loss of Ron in myeloid cells is sufficient to inhibit prostate cancer cell growth. Interestingly, depletion of CD8+ T-cells, but not CD4+ T-cells, was able to restore prostate tumor growth in hosts devoid of

myeloid-specific Ron expression. These studies demonstrated a critical role for the Ron receptor in the tumor microenvironment, whereby Ron loss in tumor-associated macrophages inhibits prostate cancer cell growth, at least in part, through the regulation of CD8+ T lymphocytic activity. We also demonstrated that Ron deficient macrophages function dominant negatively and its potential use as an immunotherapeutic tool for therapeutic intervention. Furthermore, bone marrow transplantation experiments hinted the importance of Ron expression on other non-hematopoietic stromal cells, as TK+/+ myeloid cells were not able to promote tumor growth in the TK-/- animals. Our data shows that Ron is expressed on fibroblasts isolated from normal and tumor bearing prostates and that Ron expression in the fibroblasts increases TRAMP-C2Re3 tumor growth in an *in vivo* co-culture model system. Taken together, we propose a model that Ron expression in epithelial cells, tumor associated macrophages and tumor associated fibroblasts is important for supporting prostate tumorigenesis. Targeting Ron in the clinic would be thus beneficial for the treatment of prostate cancer through its impact on multitude of cell types.

Copyright Notice

Chapter 2 contains part of published original research; citation:

The Ron receptor tyrosine kinase positively regulates angiogenic chemokine production in prostate cancer cells. Thobe MN, Gurusamy D, Pathrose P and Waltz SE. Oncogene. 2010 Jan 14; 29 (2): 214-26.

doi: 10.1038/onc.2009.331.

PMID: 19838218

Chapter 3 contains published original research; citation:

Myeloid-specific expression of Ron receptor kinase promotes prostate tumor growth. Gurusamy D, Gray JK, Pathrose P, Kulkarni RM, Finkleman FD and Waltz SE. Cancer Res. 2013 Mar 15; 73(6): 1752-63.

doi: 10.1158/0008-5472.CAN-12-2474.

PMID: 23328584 [PubMed - in process]

Acknowledgements

This work would not have been possible without the help and guidance of my advisor and mentor, Dr. Susan Waltz. I would like to express my sincere gratitude for her continued support and encouragement during the past five years of my graduate study. Her guidance and mentoring of experimental design, grant and manuscript writing, oral and poster presentation and helpful discussions have really been instrumental in shaping my progress as a scientist. I also thank her for being my role model and for her continuous support of my goals and aspirations as a young researcher. I look forward to many years of collaboration and continued interaction.

I would like to thank my thesis committee Dr. Shao-Chun Wang, Dr. Susanne Wells, Dr. Aaron Zorn, Dr. Vladimir Kalinichenko and Dr. David Plas for their insightful ideas, comments and advice throughout the development of my thesis project that had not only helped my research evolve into a more comprehensive body of work, but also played an important role in my development as a scientist.

I would like to acknowledge the Department of Defense Predoctoral fellowship [Project # PC101840] for funding me for three years. I would like to thank all the present and past members of the Waltz laboratory, as they have not only offered me good critiques and advice on a scientific level but have become some of my good friends who have been wonderful to work with. Also, I would like to acknowledge past and present members of the Cancer and Cell biology graduate program for their support.

I would like to thank my friends and family for believing in me and for providing me their unwavering support. Without my friends these past five years wouldn't have been successful. I

would like to thank my sister for being my life long friend and my parents Mr. Gurusamy and Mrs. Chandra Gurusamy who have always been by my side and have undoubtedly been my “rock” throughout my life. They always have gone far and beyond to ensure that I succeed in anything that I venture and this would not have been possible without their love and support. Your love and support, your determination and faith have made me the person I am today. Love you always!

Finally, I would love to dedicate this thesis to the memory of my beloved grandmother **Perundevi Govindaraj,** whose role in my life was and remains immense.

Table Of Contents

List of figures

Chapter 2

Chapter 3

Chapter 3 - Clinical Implication

Chapter 4

Chapter 5

List of tables

Chapter 1

Chapter 1

RON RECEPTOR TYROSINE KINASE IN INFLAMMATION AND TUMORIGENESIS

Abstract

The Ron receptor tyrosine kinase and its ligand Hepatocyte Growth Factor Like protein, are involved in the regulation of multiple biological processes including macrophage activity, wound healing and tumor progression. Numerous studies have investigated the functional role of Ron and HGFL signaling in normal and cancer epithelial cells and have outlined the importance of this receptor pathway in the regulation of epithelial cell proliferation, survival, migration and epithelial to mesenchymal transition. Several studies have also testified that Ron expression in macrophages serves to be a critical negative regulator of inflammation, influencing inflammation and wound healing responses. Recent work from our laboratory has identified the important role for Ron expression in the stromal cells of the tumor microenvironment in supporting tumor growth through the regulation of both innate and adaptive anti-tumor immune responses. This chapter summarizes the tumor-intrinsic and -extrinsic functional roles of Ron, emphasizing the potential for Ron to be a novel and efficacious therapeutic target in the treatment of cancers through the targeting of multiple cell types, with special emphasis on macrophages as an exemplary Ron dependent cell type that supports tumor progression.

Introduction

The receptor tyrosine kinase Receptor d' Origine Nantaise [Ron], also known as human Macrophage Stimulating 1 Receptor [MST1R] or murine stem cell derived tyrosine kinase [STK] is a cell surface receptor belonging to the Hepatocyte growth factor [HGF]/ cMet receptor family (1). The receptor was first identified in 1993 from a human foreskin keratinocyte cDNA library (2) and the murine ortholog was later cloned in 1994 from hematopoietic stem cells (3). Several orthologs for human Ron receptor have been identified and numerous confirmed since including murine (3), xenopus (4), rat (3), chicken (5), zebrafish (6) and feline (7), suggesting its essential importance and conservation through evolution.

The ligand for Ron was initially identified in 1976 as a serum chemotactic protein capable of inducing macrophage shape change, chemotactic migration and phagocytosis (8). This protein was subsequently isolated from human serum in 1978 and was named the Macrophage Stimulating Protein [MSP] (9). The gene for MSP was cloned in 1991 from a human genome library after screening for proteins that contained the characteristic kringle domains found in prothrombin and in other proteins of the blood coagulation system. The protein was termed Hepatocyte Growth Factor like protein [HGFL] given its 50% homology to the cMet ligand Hepatocyte Growth Factor [HGF] (10). These proteins are believed to have diverged from the family of serine proteases involved in blood coagulation and fibrinolysis and lack protease activity (10, 11). HGF and HGFL do not cross react with Ron or cMet respectively and hence are specific for their receptors (12). HGFL, similar to Ron, is also evolutionally conserved (4, 11, 13).

Biology of the RON/HGFL axis

Ron structure and expression

The human Ron gene is located on chromosome 3p21.3 and contains 20 exons and 19 introns (14). The protein is translated as an 185kD glycosylated single chain polypeptide precursor that is proteolytically processed by furin-like proteases before being delivered to the cell surface (12). At the cell surface, Ron exists as a disulphide linked heterodimer comprising of a 35kD alpha chain and a 150kD beta chain. The beta chain spans the membrane and entails an extracellular domain, transmembrane domain and an intracellular tyrosine kinase-signaling domain. The alpha chain is completely extracellular and is linked by disulphide bonds to the beta chain's extracellular domain. The murine Ron gene is located on chromosome 9 and contains 19 exons. Human and murine protein sequences for Ron share about 73.6% overall sequence homology with about 88.6% similarity detected in the kinase domain (3, 15). Altered splicing events of the murine Ron regions results in the deletion of a small juxtamembrane

region that is present in human Ron (16). Amino acid sequence of the intracellular tyrosine kinase domain of the Ron receptor is about 63% identical to the cMet tyrosine kinase domain exemplifying similarities between these receptors (3). Ron and cMet have similar c-terminal multifunctional docking sites with two-tyrosine kinase signaling residues, a characteristic feature of the cMet family of tyrosine kinases.

The expression of Ron specific transcripts has been observed during early stages of developing embryo with expression observed in the liver and neural structures as early as e12.5. Ron expression also extended to other organs during development to e16.5, with Ron mRNA being essentially restricted to the central and peripheral nervous system, adrenal glands, the digestive tract epithelia, and the ossification centers in the developing bones (17, 18). In humans and mice, Ron expression has been detected in the epithelium of normal esophagus, stomach, duodenum, small intestine, colon, rectum, gallbladder, pancreas, spleen, testes, skin, lung, liver, brain and bone marrow tissues (17, 19). The expression of the Ron receptor in the liver during early development has been correlated with its involvement in hematopoiesis and consistent with this observation a truncated variant of the Ron receptor, conferred susceptibility to Friend virus induced erythroleukemia, indicating a functional link between Ron and blood cell development (20). Ron was also showed to be critical during pubertal mammary gland development controlling branching morphogenesis and ductal elongation (21).

Ron is expressed during macrophage terminal differentiation (22) and expression of the receptor has been reported on several tissue resident macrophage populations including peritoneal macrophages (22), human alveolar macrophages (23), osteoclasts (24) and dermal macrophages (25), Kupffer cells of the liver (19, 26) and microglial cells of the brain (27, 28). However, its expression was not detected on circulating monocytes (22). A significant fraction of epithelial tumors, including prostate, breast, pancreas, kidney, colon, lung, bladder, liver,

thyroid, uterus, brain and stomach overexpress Ron. There have been contradicting reports on the expression levels of Ron observed in adult normal tissues, and this can be attributed to the very low expression levels and the quality of the detecting antibody (29, 30).

The Ron Ligand HGFL - Structure And Expression

HGFL is also located on chromosome 3p21.31 and is secreted into the blood stream as an 80kDa single chain inactive precursor, pro-HGFL, and is present in the blood stream in an inactive form at a concentration of about 5nM (10, 25). Pro-HGFL then works in an endocrine fashion, when locally cleaved by proteases of the coagulation cascade [kallikrein, factor XIa, and factor XIIa] (31), nerve growth factor, epidermal growth factor binding protein (32), hepatocyte growth factor activator-1(33), and by matriptase-1 expressed on macrophages (34) to form an active heterodimer comprising of a disulfide linked alpha [53kDa] and beta chains [25kDa]. The two chains of the activated ligand have distinct functional roles; the alpha chain containing the kringle domain influences the Ron signaling activities while the serine protease like domain encoded by the beta chain regulates ligand receptor binding (35, 36). Pro-HGFL is primarily produced by the hepatocytes with low levels of messenger RNA detected in lung, adrenal gland and placenta (11). Recent published papers have documented the expression of HGFL in tumors including both the epithelial and stromal cells (13, 28, 37-39). HGFL can induce a number of pleiotropic effects in a cell type and context specific manner and is discussed in detailed in the subsequent sections.

Mouse models of Ron and HGFL

The development and use of transgenic mice that harbor Ron deletion have greatly aided in the understanding of the physiological roles of the Ron receptor and its ligand HGFL during inflammation and tumorigenesis. The various transgenic animal models and the phenotypic properties are discussed below and summarized in Table 1.

To understand the functional importance of the Ron receptor *in vivo*, two groups produced mouse models in which the Ron gene was disrupted by different methods. One of the models had total loss of the Ron protein, referred to as Ron-/-, and was produced by the global deletion of exon 1-14 of the mouse Ron gene that eliminated the extracellular domain, the transmembrane domain, and a portion of the intracellular domain. Genetic knockout of the entire receptor resulted in embryonic lethality by e7.5. Ron signaling has been suggested to affect the implantation of the placenta through its expression in the ectoplacental cone and trophoblastic giant cell regions of the implanting embryo (40). The hemizygous Ron+/- mice were viable and fertile, however exhibited enhanced susceptibility to endotoxic shock and the macrophages displayed an impaired ability to regulate nitric oxide.

The second model that was generated to study the biological significance of the Ron receptor was a knock-in mouse model referred to as the stk-/-. In this model, Correll et al. replaced approximately 850bp of exon 1 of Ron with a β-*galactosidase* expression cassette, leaving the promoter region intact as well as the gene sequences encoding a large portion of the ligand binding domain, the transmembrane domain, and the intracellular tyrosine kinase domain. The homozygous stk-/- mice were viable and phenotypically normal in contrast to the Ron-/- mice that were embryonic lethal. Nevertheless, consistent to the Ron+/- mice the macrophages from the stk-/- mice showed reduced activity and survival upon LPS challenge confirming that Ron is important for macrophage activity. The apparent discrepancy between the survival of the stk-/- mice and Ron-/- mice in these studies may be probably due to the expression of alternate splicing variants from the mutant Ron gene in the stk-/- model that would be sufficient to support embryonic development and growth (41).

Since the germline deletion of a larger region of the Ron receptor was embryonic lethal, another mouse model harboring a deletion of the tyrosine kinase signaling domain was developed to

study its physiological role. In this model Cre-mediated excision of the intracellular domain, spanning exons 13-18 of mouse Ron gene was generated (42). The TK-/- animals were viable and the macrophages isolated from these animals expressed a 105kDa extracellular β chain. All organs examined from Ron TK-/- mice appeared overtly normal with the exception of the ovaries, which were smaller and had fewer corpora lutea (CL). In agreement with the Stk-/- and Ron+/- models, TK-/- macrophages exhibited reduced activation, decreased survival, and were unable to block NO production when challenged with LPS and INFγ (42). Ron tyrosine kinase knockout [TK-/-] mice have been valuable tools for the study of Ron biology during development, inflammation, injury and tumorigenesis. Importantly the TK-/- animals were used in this thesis to test the hypothesis if Ron expression in the epithelial cells and stromal cells is important for prostate tumorigenesis.

Total body HGFL knockouts in mice have shown that HGFL is not essential for embryogenesis, fertility or wound healing, however, these mice do exhibit lipid filled vacuoles; the significance has been unclear (43). The HGFL null mice phenotype is in contrast to the deletion of the HGF null mice, the HGFL homolog, wherein HGF deletion results in embryonic lethality and impaired liver development highlighting the fundamental differences in signaling between these two ligands (44).

Studies on the functional roles of Ron/HGFL signaling have been arrived from a variety of cell types, including macrophages and epithelial cells, in a context and cell type specific manner. This chapter reviews both the context and cell type specific roles of Ron/HGFL signaling during inflammation, wound healing and tumorigenesis, with a special emphasis on the current strategies that are employed for the therapeutic targeting of this pathway for cancer treatments.

Table 1. *In vivo* model of Ron receptor tyrosine kinase

Model name	Details	Phenotype	Ref.
Stk -/-	Germline deletion; Knock in mutant Beta-galactosidase introduced in exon1	Mice viable; Increased macrophage NO Production and increased inflammation *in vivo* upon IFNγ /LPS treatment	(45)
Ron -/-	Germline deletion of mouse Ron exon 1-14	Ron-/- mice embryonic lethal day E6.5-E7.5; Ron+/- mice viable; have reduced macrophage activation, increased NO production, and increased death of mice after LPS treatment.	(40)
TK -/-	Germline deletion of tyrosine kinase domain [exon 13-18]	Mice viable; macrophages exhibit reduced activation and survival after LPS challenge.	(46)
HGFL -/-	Germline deletion [exon 3-8]	Mice viable; Hepatocytes have lipid filed vacuoles	(43)

Cell and context specific function roles of RON receptor

HGFL/RON signaling in macrophages

Since the identification of HGFL as a serum factor capable of regulating macrophage chemotaxis and phagocytosis (8), numerous studies have highlighted essential roles for Ron/HGFL signaling pathway in regulating macrophage effector functions. Ron/HGFL signaling in macrophages regulates both the extent of macrophage activation and the inflammatory responses and is considered to be critical negative regulator of inflammation. The various studies and the signaling pathways altered by Ron/HGFL signaling in macrophages are discussed below with emphasis on their wound healing and tumorigenic functions.

The Ron receptor is expressed on a variety of tissue resident macrophages and is upregulated during macrophage terminal differentiation (22). Mice that lacked Ron activity, both stk-/- and TK-/- mice, were viable and fertile, but had severe defects in macrophage activation during an inflammatory insult (41, 42). Ron activation in macrophages diminished lipopolysaccharide [LPS] induced NF-κB activation and subsequent expression of pro-inflammatory chemokines/cytokines (42, 47, 48). The studies that outline Ron/HGFL mediated regulation of inflammatory responses in macrophages are discussed here.

Acute lung injury [ALI] is a severe respiratory syndrome that develops in response to numerous insults and has poor response to therapeutic interventions. Studies in mice that lacked functional Ron expression, both TK-/- and stk-/-, showed that macrophages from these deficient mice produced elevated levels of nitric oxide [NO], both *in vitro* and *in vivo* (41, 42, 49). The Ron tyrosine kinase knockout mice, TK-/-, are more susceptible to endotoxic shock even when challenged at sub-lethal LPS doses demonstrating the vital role of the Ron/HGFL pathway in limiting inflammatory response and protection from infections. Acute lung injury induced by intrapulmonary administration of LPS, in wild type and Ron tyrosine kinase deficient [TK-/-]

mice, demontrated that mice lacking Ron tyrosine kinase [TK-/-] had substantial increase in lung injury compared to controls that was associated with an increase in NF-κB activation as well as enhanced production of TNF-α and NO (50). Further studies on isolated alveolar macrophages, pretreated with HGFL, showed significant decrease in TNF-α production mediated by reduced NF-κB activity levels and an increase in IκB levels. In this study, Ron was also shown to negatively regulate Adam17 levels, the metalloproteinase responsible for releasing TNF-α from membrane surfaces at the level of both mRNA and protein (51). Additionally, studies in mice that harbored a conditional deletion of Ron receptor in the myeloid cells confirmed that myeloid specific knockout of Ron tyrosine kinase signaling domain was sufficient to increase TNF-α production, and lung injury upon LPS administration. An increase in neutrophil numbers was also observed in these mice following LPS challenge (52).

In a similar acute lung injury model, Wilson *et al.,* showed that stk-/- mice under endotoxin insult had increases in systemic levels of IL-12p40 and IFN-γ. The elevated levels of IL-12p40 after endotoxin challenge resulted in enhanced IFN-γ production by Natural Killer cells, thereby increasing susceptibility to septic shock (53). Furthermore, this study showed that the susceptibility of stk-/- mice to septic shock was dependent on the signaling through the IFN-γ receptor (53). HGFL dependent Ron activation reduced the susceptibility to infection by negatively regulating signaling through the IFN-γ receptor and by down regulating tyrosine phosphorylation of STAT1 via the up regulation of SOCS1. This study pointed Ron to be a critical checkpoint in the regulation of endotoxin-induced injury through regulating the production and response to IFN-γ (53). Ray *et al*. demonstrated that the Ron receptor plays an important role in shaping the repertoire of LPS-dependent responses primarily by targeting IKK activation and its downstream targets by LPS, and that Ron does not inhibit proximal signaling events initiated by LPS stimulation of TLR4 (54).

Although Ron expressing was not detected on monocytes (22), recent studies using rat and murine monocytes showed that the expression can be induced by LPS stimulation (54, 55). Study by Ray *et al.,* showed that LPS mediated activation of macrophages induces the expression of both the full length and the short form [SF-Ron] (54). The SF-Ron is generated from an alternative start site in intron 10 of the Ron gene and lacks extracellular portion of the receptor (56). Based on previous studies on SF-Ron in concanavalin-A induced murine model of acute liver injury, mice that solely express the full-length version of the receptor and lack SF-Ron expression, showed increases in levels of serum INF-γ and alanine aminotransferase along with worsened liver histology and overall survival, compared with wild-type mice that expressed both isoforms (57). This study identified SF-Ron to be important for limiting the inflammatory response to concanavalin-A induced lung injury suggestive of its negative inflammatory role (57). Overall, the induction of Ron expression in monocytes and macrophages upon LPS stimulation suggests the potential negative feedback loop for regulating LPS induced TLR4 signals (54, 55).

In another model of ALI induced by nickel exposure, Ron TK-/- knockout mice showed increased sensitivity and decreased survival and showed earlier onset of pulmonary pathology. The Ron TK-/- mice in these studies had earlier increases in levels of cytokines like IL-6, MCP-1, MIP-2 and NO compared to controls. Furthermore, untreated Ron TK-/- mice had increased clustering of a variety of immune cells, including macrophages, T-cells and neutrophils, within the perivascular and peribronchial within the lung. The lymphocytes stained positive for granzyme D, a marker for cytotoxic T-cell function. These results suggest that Ron signaling may be involved in the regulation of macrophage and T-lymphocyte activation *in vivo* during injury (58, 59).

Consistent with the functions regulated by Ron in macrophages during an endotoxin insult, *in*

vivo studies using stk-/- animals challenged with *Listeria monocytogenes* demonstrated that Ron activation in macrophages is important for the protection of the animal from gram positive bacterial infections. Stk-/- mice challenged with *Listeria monocytogenes* showed increased susceptibility to infection with an increase in bacterial burden (60). The susceptibility of the Ron knockouts to *Listeria* infection is similar to that of the TNF-α receptor p55 knockout mice (60). In this study, Ron activation promotes CR3- mediated phagocytosis and ICAM-1 dependent adhesion of macrophages (61). The ability of Ron to promote the rapid phagocytosis through complement receptor 3 is therefore a potential mechanism through which Ron/HGFL pathway might function to reduce bacterial burden upon *Listeria* infection (61).

Ligand stimulation of Ron prompts the rapid phagocytosis of C3bi coated erythrocytes via complement receptor 3; the phagocytosis was not apparent in the absence of the HGFL suggestive of a multifactorial based regulation of Ron activity (61, 62). Recent work from Bhatt and colleagues had shown that Matriptase-1, a protease capable of cleaving pro-HGFL to HGFL, was expressed on macrophages and could potentially explain the local influx of the active ligand *in vivo* (63). HGFL in itself was also shown to be a monocyte chemoattractant in an *in vivo* rat model of anti-Thy1 glomerulonephritis [complement induced immune mesangial injury], wherein neutralization of HGFL using a HGFL neutralizing antibody reduced the infiltration of the monocytes to the kidneys (55). The expression of HGFL although not important for macrophage differentiation, the absence of the ligand delays the activation of the macrophages suggestive of the importance of ligand activation and the possibility for other serum factors that can activate Ron receptor signaling (43). Overall, studies on HGFL/Ron signaling in macrophages through both endotoxin and gram positive bacterial infections indicate that the Ron pathway is involved in macrophage activation and protection from particular microorganisms and is critical for resolution of inflammation.

Paradoxical to all these studies, in a model of LPS- induced acute liver failure in galactosamine -sensitized mice [LPS-GalN], Ron TK-/- displayed marked protection compared to the control wild type mice. TK-/- mice had reduced liver injury determined by histopathology as well as a decrease in serum alanine amino transferase expression, which was associated with decreased apoptosis of the liver cells (64). An elegant study by Stuart and Kulkarni *et al.* demonstrated the cell and context specific role of the Ron receptor signaling in the LPS-GalN model. Using mice with a conditional loss of Ron in hepatocytes or myeloid cells, the authors demonstrated that Ron expression in hepatocytes is protective while loss in the myeloid compartment was detrimental in response to inflammation. Using primary hepatocytes and Kupffer cells from Ron proficient [TK+/+] and Ron deficient [TK-/-] mice; the authors found that TK-/- Kupffer cells had increased production of TNF-α and other cytokines compared to the controls and that treatment of TK+/+ hepatocytes with either TK-/- Kupffer cell media or TNF-α significantly increased their death compared to treated TK-/- hepatocytes, suggesting that loss of Ron in the hepatocytes desensitizes hepatocytes to the excess of inflammatory mediators produced by TK-/- hepatocytes. These results provide support for the differential role of Ron regulation in epithelial and immune cells (26).

In addition to its role in suppressing inflammation, Ron also promotes macrophage M2 polarization. Macrophages are plastic cells whose functional activation state are determined by their environmental signals and can be classified into two broad polarization states. Classically activated [M1] macrophages are present during the initial phase of wound healing and facilitate stimulation of inflammatory responses and pathogen clearance. During the later phase of the wound healing process, macrophages become M2 polarized where they secrete extracellular matrix proteins and polyamines, which can influence the production of cytokines, inhibit the clonal expansion of T lymphocytes and stimulate re-epithelialization, a process that involves the migration and proliferation of epidermal keratinocytes [Reviewed in (65, 66)]. Ron promotes

hallmarks of an M2, anti-inflammatory phenotype in primary macrophages demonstrated by the expression of Arginase-1, Macrophage Scavenger Receptor-A [SR-A], IL-1 receptor antagonist expression (67, 68). Ron expression also suppresses the hallmarks of M1 polarization, including expression of iNOS, TNF-α, COX-2 and IL-12p40 in primary macrophages stimulated with IFN-γ and LPS (47, 68, 69). This inhibition was shown to be regulated by the ability of Ron to inhibit the phosphorylation of Stat1 in response to IFN-γ (53, 69) and the inhibition of NF-κB activation in response to LPS stimulation as discussed above (54, 70). Another potential mechanism through which the expression of proinflammatory genes is inhibited includes the activation of STAT3 by Ron (53). Activated STAT3 can drive the expression of anti-inflammatory genes that can subsequently inhibit NF-κB dependent gene expression. Moreover, a recent study in mouse peritoneal macrophages after polarizing the macrophages to an M1 or an M2 polarized state with either LPS or IL-4 treatment respectively showed that MSP stimulation decreased the expression of both M1 and M2 markers, but sustained the expression of Arg-1 even under M1 polarization signals, suggestive of the involvement of Ron in both Th1 and Th2 mediated inflammatory responses (71). In summary, ligand stimulation of Ron has been shown to be important in maintaining a controlled immune responses and expression of genes that will be involved in macrophage M2 polarization signals.

These elegant studies have provided significant understanding on the complexity of the Ron/HGFL signaling processes and demands for further research into identifying the cell type specific roles of Ron in the context of various diseases. The central role of the Ron receptor in protecting the animal from septic shock, limiting Th1-mediated inflammation, promoting macrophage M2 polarization and clearance of *Listeria monocytogenes* infections suggests that the reshaping of macrophage activation by Ron is important in the maintenance of a critical balance to infection, at the same time protecting the host from tissue damaging inflammation. Targeting Ron in macrophages or its downstream components could be therapeutic in a variety

of chronic inflammatory diseases including tumors.

RON /HGFL signaling in epithelial cells

Numerous studies have outlined the functional importance of Ron/HGFL signaling in normal and cancer epithelial cells in regulating epithelial cell proliferation, survival, migration, and epithelial to mesenchymal transition (72). The signal transduction processes regulated by Ron in the epithelial cells as well as the various mouse models that helped elucidate the functional role of Ron in tumor epithelial cells are discussed below.

Signal transduction through the multifunctional docking sites of Ron can be mediated by both ligand dependent and independent mechanisms. Ron activation by HGFL induces conformational change and receptor homodimerization and the activation of intrinsic tyrosine kinase activity leading to trans autophosphorylation of key tyrosine kinase residues in the intracellular C-terminal domain. These phosphorylated residues serve as high affinity docking sites for signal transduction molecules through binding of effector proteins containing Src homology 2 and phosphotyrosine binding domains including phospholipase C gamma [PLC-γ], phosphoinositide kinase-3 [PI3K], growth factor receptor bound protein 2 [Grb2] (73, 74). Grb2 and Son of sevenless receptor recruitment to the Ron receptor leads to the activation of PI3K/Akt and/or MAPK signaling through the conversion of Ras GDP to its active form. Other downstream signaling molecules activated by HGFL/Ron signaling include FAK, Src, Jun kinase, STAT3, NF-κB and β-catenin [reviewed in (75) ,(76) (72, 77)].

Ron can also be activated in epithelial cells through receptor herterodimerization with other membrane bound receptor tyrosine kinases. Ron has been shown to interact with other cell surface receptors including c-Met (78), Epidermal Growth Factor Receptor [EGFR] (79), Insulin

like growth factor 1 receptor [IGF1R] (80), Integrin's (81) and B-type plexins (82). The interactions with these membrane bound receptors can occur through both the tyrosine kinase domains as well as their multifunctional docking sites. In addition to transactivation of Ron kinase domain by these receptors in a HGFL independent manner, HGFL mediated Ron activity has been shown to be capable of transactivating Met and EGFR in the absence of their respective ligands (79, 83). Plexin-B can also interact with Ron in the absence of the ligand HGFL and confers invasive growth properties (82). Interestingly, the interactions between IGF1R and Ron were important for the induction of migration induced by IGF1 stimulation. The signaling was described unidirectional wherein binding of IGF1 to IGF1R is required to initiate the formation of the heterodimer with Ron (80). The interaction of Ron and EGFR or cMET is bidirectional (79, 83).

Ron is overexpressed in a variety of human cancers (29, 30) and the coordinated overexpression of Ron/HGFL and Matriptase-1 in human breast tumor patients has been shown to be associated with increased metastasis and death (84). The significance for Ron overexpression and activation in the epithelial cells for tumor progression was arrived from transgenic mice that expressed both the wild type and constitutively active form of the Ron receptor in the mammary epithelium (85). In this mouse model, Ron overexpression was sufficient to induce mammary transformation in animals with 100% penetrance. Furthermore, these animals had a high degree of metastasis, with metastatic foci detected in the liver and the lungs of greater than 86% of the transgenic animals. This was the first study to demonstrate that Ron overexpression in the breast tumor epithelium was sufficient to metastatic tumor progression (85). Transgenic overexpression of human Ron has also been performed in murine pulmonary epithelium with about 95% of the transgenic animals developing adenocarcinomas between 6-14 weeks of age (86). Additionally, transgenic Ron receptor overexpression in the murine prostate using a prostate specific probasin promoter, was shown to lead to the

development of prostate intraepithelial neoplasia in these mice compared to the control wild type mice and was associated with an accumulation of β-catenin and increased pErk expression (87).

In addition to the mouse models that overexpressed Ron the functional importance of Ron in supporting tumorigenesis was also assessed using loss of function models. Mice containing a germline targeted deletion of the Ron tyrosine kinase signaling domain showed decreased mammary tumor initiation and growth when crossed with the polyoma virus middle T antigen [pMT], murine model of breast cancer. A significant decrease in microvessel density and proliferation, and increased cell apoptosis were hallmark features of these tumors lacking the Ron tyrosine kinase domain (88). Our lab had also shown a significant decrease in tumor size and angiogenesis in the Transgenic Adenocarcinoma of the Mouse Prostate [TRAMP], prostate cancer mouse model, crossed with mice with germline deletion of the Ron tyrosine kinase domain (89). The TRAMP mouse model has the SV-40 large T antigen specifically expressed in the prostate using a prostate specific probasin promoter resulting in prostate tumors by 30 weeks of age. These studies have demonstrated the functional importance of Ron signaling in promoting tumor growth and angiogenesis, however, it has to be considered that Ron signaling was lost in both the epithelial and stromal cellular compartments. Ron expression in prostate tumor epithelial cells has been shown to be important for angiogenic chemokine production CXCL-1 and CXCL-8 in a NF-κB dependent pathway (90). Although several studies have outlined the importance for Ron expression in the tumor epithelial cells, the contributions of stromal Ron expression in regulating tumor growth and angiogenesis needs to be determined and is the subject of this thesis.

Intersection of the cell type specific functions of RON/HGFL

Ron in wound healing responses: Wound healing is a dynamic process involving the

continuous overlap of precisely programmed events. Optimal wound healing involves the establishment of tissue homeostasis, inflammation and tissue remodeling, which includes mesenchymal cell differentiation, angiogenesis and re-epithelialization. Once tissue homeostasis is established after vascular constriction and fibrin clot formation, inflammatory cells migrate into the wound and promote inflammation to facilitate clearance of microbial and cellular debris. As the immune cells clear the infection and cellular debris, they undergo a phenotypic transition to a reparative state that stimulates the keratinocytes and fibroblasts to promote tissue regeneration and wound healing [Reviewed in **(65, 66)**].

Macrophages are major immune cell types that are involved during the various phases of the wound healing response. Importantly as discussed previously the Ron/HGFL signaling is important for skewing the macrophages towards a M2 polarized state and the expression of Ron, HGFL and the HGFL converting enzyme has been found at wound sites in human wound fluids (25). In a rat experimental model of excisional wounds, the expression of both HGFL and Ron occurred between days 7 and 21 post-wounding suggestive of specific roles during the tissue regeneration phase of the wound healing process. The expression of HGFL co-localized to a specific subset of macrophages [ED1] suggestive of the potential role in the specific activation of macrophage populations that can support tissue repair (91). Notably mice with whole body HGFL knockout exhibited no defects in wound repair demonstrating the functional redundancies that exist amongst other signal mediators during the wound healing process (43). Ron/HGFL signaling has been shown to be important for facilitating both inflammation resolution and tissue repair.

The expression of Ron was also increased in migrating keratinocytes along the leading wound edge and in dermal macrophages (25). An autocrine loop for HGFL was observed in migrating kertatinocytes during the reepithelialization process in both *in vivo* and *in vitro* model systems

(81). Santoro *et al.* had shown that Ron promoted keratinocyte reepithelialization through the specific activation of gene expression programs necessary to support wound closure. They showed that HGFL-mediated activation of the PI3K pathway resulted in serine phosphorylation of both Ron and α6β4 phosphorylation to generate 14-3-3 binding sites on both molecules. Ron activation and 14-3-3 binding are responsible for shift in integrin dominance, therefore modulating keratinocyte spreading/migration on laminin-5 extracellular matrix thereby promoting wound closure (81). Taken together, Ron function and expression in both the epithelial cells as well as the macrophages is important during wound healing. Given that tumors are considered as wounds that never heal (92), our study presented in this thesis assesses the cell type specific roles for Ron expression in the tumor epithelial cells and associated stromal cell types including tumor associated macrophages and tumor associated fibroblasts.

RON/HGFL pathway as a therapeutic target

Targeted therapies are aimed at oncogenic signaling molecules that dictate the survival and growth of tumor cells, a process defined as oncogenic addiction (93). Data from clinical trials have indicated that most tumors acquire drug resistance with the help of the stromal cells present in the tumor microenvironment. Targeting both the tumor and the stromal cells at the same time would provide better therapeutic efficacy through the inhibition of multiple cell types and factors that support tumorigenesis. This suggests the potential use of multiple kinase inhibitors that can simultaneously target both tumor and stromal cells (94). As reviewed above, the Ron/HGFL pathway is overexpressed in a variety of cancers and regulates a plethora of oncogenic functions including proliferation, survival, migration and invasion when expressed in the tumor cells as well as regulates the cellular functions of the cells present in the stromal microenvironment include the cells of the immune system. Drugs that target the Ron receptor will be valuable for promoting tumor regression through the targeting of both the intrinsic and

extrinsic tumor cell functions of Ron. The various strategies for targeting Ron that are currently under preclinical and clinical studies are discussed below.

Monoclonal Antibodies: A fully humanized monoclonal antibody that binds with high affinity to Ron and effectively blocks its interaction with its ligand HGFL was developed by Imclone systems, IMC 41A10 [ImClone systems, New York, NJ]. IMC 41A10 was shown to inhibit both the phosphorylation of the receptor as well as the downstream signaling, including cell migration and growth. The IMC 41A10 antibody was also capable of significantly decreasing tumor growth of colon, lung and pancreatic cells in murine xenograft models (29). Furthermore, in the same study the antitumor efficacy of IMC-41A10 on BXPC-3 pancreatic cancer cell xenograft was enhanced when used in combination with Erbitux, an EGFR inhibitor. The combination of both these tyrosine kinase inhibitors decreased tumor growth in a synergistic manner compared to the growth observed in the presence of the individual inhibitors for Ron or EGFR. This study sheds importance to the efficacy achieved with combinatorial drug therapies against tyrosine kinases, and also emphasizes the involvement of Ron and EGFR interactions to the pathogenesis of pancreatic tumors in this model (79, 95). It is noteworthy that the IMC-41A10 antibody showed strong antitumor activity in HT-29 cells that expressed both the full length Ron receptor as well as the multiple splice variants. (29, 96). The IMC 41A10, also called Narnatumab/IMC-Ron8, is currently under phase1 of the clinical trials, which are in the process of enrolling patients [2012; http://clinicaltrials.gov/show/NCT01119456]. A recent study published in 2011 by Gunes et al., suggested that the anti-Ron monoclonal antibodies must have distinct properties besides ligand antagonism and inhibition of downstream signaling to achieve *in vivo* efficacy. They showed that the neutralizing antibodies developed in their laboratory, similar to the IMC 41A10, exhibited effective inhibition of tumor cells *in vitro* with no effect detected *in vivo* (97). Additional antibodies have been developed to block ligand binding to Ron are ID-1 and ID-2. These antibodies diminished HGFL induced HT-29-D4 human

intestinal cell migration in preclinical studies, suggesting the capability of this antibody in blocking Ron mediated oncogenic signaling (98).

Apart from the antibodies described above that specifically block ligand binding and the inhibition of downstream signaling; M.H Wang and colleagues developed a series of antibodies that bind to exon 11 of the extracellular domain of the Ron receptor [Zt/g4, Zt/f2, Zt/c9]. The sequence encoded by this region is referred to as the MRS, Maturation Required Sequence, which regulates Ron maturation and phosphorylation (99-101). Binding of the antibody to the MRS epitope prompts receptor internalization and subsequent degradation by proteasome machinery (100). Zt/f2 antibody was capable of inhibiting the *in vivo* growth of colon cancer cells (100). Furthermore, Zt/f2 antibodies coupled to immunoliposomes formulated with chemotherapeutic drug 5-flourouracil decreased the growth of human HT-29 colon cancer in athymic nude mice. An increase in incubation of tumor growth was observed in the presence of the Zt/f2 antibody coupled to 5-flourouracil immunoliposomes as opposed to the treatment with the Zt/f2 antibody alone (99, 101-103). Similar inhibition was also observed on breast cancer cells *in vitro* using Zt/f2 antibody coupled to doxorubicin or 5-FU targeted immunoliposomes (99, 103), and on purified pancreatic cancer stem cells using doxorubicin targeted immunoliposomes (79). The delivery of doxorubicin to the tumors was shown to be dependent on the dosage and the time of delivery; the antibody had no impact on the expression of Met and EGFR (103). Targeting Ron driven cancer cells using antibodies coupled to immunoliposomes that carry the chemotherapeutic drugs, presents proof of principle that antibodies targeted to the MRS sequence of Ron can be used to specifically deliver chemotherapeutic drugs to Ron expressing tumors, reducing off-target effects and facilitating efficient delivery of cytotoxic drug to the tumors (100). These series of antibodies represent a novel approach by which Ron can be used for cancer therapy and has proven to be promising in these pre-clinical studies.

Several studies on the use of Ron inhibitors for tumor targeting have shed insights into the role of Ron receptor in imposing resistance to therapies. The treatment of pancreatic cancer cells BxPC3 cells in vitro with a combination of non-humanized monoclonal Ron blocking antibody and gemcitabine increased the tumor cell apoptosis by 32% when compared to the treatment of these cells with gemcitabine alone (104). Studies from the same group also found out that knockdown of Ron using shRNA transfection in pancreatic xenografts delayed acquired gemcitabine resistance compared to Ron expressing xenografts (105). In the same study, more than 50% of the acquired resistance in the Ron knockdown group was attributed to the re-expression of Ron as well as the activation of other kinases including Met and EGFR, suggestive of kinase switching and hence acquired resistance to treatments, suggestive of the mechanisms by which pancreatic cancer cells may circumvent Ron-directed therapies (105). The sensitization of SW620 colon cancer cells to gemcitabine treatment was also increased in the presence of the Ron binding antibody Zt/g4. The death of SW620 cells was increased when Zt/g4 was used in combination with gemcitabine, suggestive of the use of Ron inhibitors in combination with chemotherapy (101).

A recent published study from our laboratory had also shown an important connection between Ron activation and tamoxifen cytotoxicity. McClaine et al. found that HGFL induced Ron activation in both human and murine breast cancer cell lines is sufficient to confer resistance to tamoxifen induced cytotoxicity (106). This study also showed increases in cyclin D1 expression and an increase in ERE luciferase activity after Ron activation, suggestive that Ron augmented ER signaling drives tamoxifen resistance in breast cancer cells. Taken together, the mechanisms of acquired resistance to chemotherapies provided by Ron expression as well as the mechanisms of kinase switching during Ron inhibition needs further evaluation for the better design of combinatorial therapies and for improving treatment prognosis.

Small molecule inhibitors [SMI]: Most of the SMI that can target Ron can also target cMet given the similarities in the tyrosine kinase domain of these receptors. The Ron/c-Met dual kinase inhibitors that are currently under clinical trials are is summarized in Table 2. Of particular note is the SMI called Foretinib/EXEL-2880 that has currently completed phase-1 of the clinical trials and is under phase 2 of the clinical trials. Foretinib is a broad-spectrum tyrosine kinase inhibitor capable of binding to and inhibiting the functions of Met, Ron and VEGFR, leading to the inhibition of both proliferation and angiogenesis (107). Foretinib was developed to target Met and VEGFR, however, initial studies also showed potency against Ron receptor similar to the properties exhibited by other tyrosine kinase inhibitors (108). Phase 1 clinical trial results have confirmed both the dosage as well as the delivery schedule for the treatment of patients with metastatic or end terminal solid tumors (107). Most of side effects observed during the phase-1 clinical trials were attributed VEGFR inhibition making Met and Ron a valid drug target. Furthermore, analysis of the patient samples before and after treatment indicated significant reduction in the phosphorylation of Ron and Akt along with decreases in proliferation and increases in tumor survival (107). Currently, Foretinib is under phase 2 of clinical trials used alone or in combination with chemotherapeutic drugs for the treatment of several solid tumors [www.clinicaltrials.gov].

Table 2. Small molecule inhibitors for cMet/Ron currently under clinical trials

Company	Compound	Targeted RTK	Clinical Development
Amgen	AMG 208	RON, c-MET	Phase I
Angion	ANG 707	c-MET,RON,TIE2	Phase I
Bristol-Myers Squibb	BMS-796302	c-MET,RON,AXL, VEGFR2,FLT3, TYRO3, MER	Phase I
EMD Serono	EMD 1214063/ EMD 1204831	c-MET	Advanced, refractory solid tumors
GlaxoSmithKline	Foretinib	c-MET, RON, VEGFR2, AXL, PDGFR, KIT, FLT3, TIE2	Phase II: breast, NSCLC, papillary renal, gastric and head and neck cancers
Methylgene	MGCD-265	c-MET,RON, VEGFR1, VEGFR2, VEGFR3, TIE2	Phase II: NSCLC
Merck	MK-2461	cMET, RON, VEGFR1, VEGFR3, FLT1, FLT3, FLT4, FGFR1, FGFR2, FGFR3	Phase II: solid tumors
Pfizer	Crizotinib	cMET, ALK, RON, AXL, TIE2	Phase III: ALK-altered NSCLC Phase I–II: lung cancer, ALCL and other tumors
Pfizer	PHA665752	c-MET/RON	Preclinical

Antagonists: The extracellular region of Ron ß chain is characterized by the presence of a SEMA domain [a stretch of approximately 500 amino acids with multiple highly conserved cysteine residues], a PSI domain [plexin, semaphorin and integrin domain] and four immunoglobin like folds (109). A study in which two soluble molecules designed over the sema domain [Ron sema] and sema + PSI domain [Ron PSI], when expressed on mammalian cells underwent post-translational processing and were secreted from the cells. Both these molecules were capable of inhibiting the ligand binding to Ron receptor and had a highly specific dominant negative effect on Ron kinase activation when tested on HCT116 colon cancer cells. The forced overexpression of either of these molecules can be used for targeted gene therapy in HGFL responsive tumor cells (110).

Global Tyrosine kinase inhibitors: In addition to neutralizing antibodies, small molecule inhibitors or Ron antagonists several other indirect strategies have been developed that can be used to treat both Ron and other tyrosine kinase dependent tumors. An example for such treatment is the chemotherapeutic agent geldanamycins that impairs Heat shock protein [Hsp] functions. Hsp are chaperone proteins that facilitate correct protein folding and assembly and several receptor tyrosine kinases including Ron are sensitive to these drugs (111). Targeting Ron in cancer therapy is likely to provide benefit to patients, especially when used in conjunction with other approved therapies.

Given the role of Ron in conferring therapeutic resistance to chemotherapeutic drugs like tamoxifen and gemcitabine as well as the prospects of kinase switching involved during Ron inhibition, drugs that can simultaneously target multiple pathways and cell-types are necessary in order to completely abolish the growth of tumor cells. Future studies directed towards understanding the importance of Ron receptor signaling during chronic inflammation and tumorigenesis is important to fully understand the impact that targeting Ron would have in these

various disease states. The work presented in this thesis identifies the cell type specific contribution of the Ron receptor tyrosine kinase to prostate tumor growth. Specifically, our work has identified that Ron receptor expression in both the epithelial cells and stromal cells is important for supporting the various aspects of prostate tumorigenesis.

Chapter 2

RON RECEPTOR TYROSINE KINASE EXPRESSION IN EPITHELIAL CELLS PROMOTES PROSTATE TUMOR GROWTH AND IS ASSOCIATED WITH AN INCREASE IN PELVIC BONE METASTASIS

Part of this work is published in Oncogene. 2010 Jan 14; 29 (2): 214-26.

Abstract:

The Ron receptor tyrosine kinase is overexpressed in numerous epithelial cancers including prostate cancer. In this chapter we investigate the importance for Ron expression in the epithelial compartment for supporting prostate cancer growth and metastasis. We have characterized the expression levels of Ron in a variety of murine PCa cell lines with differing tumorigenic properties derived from the Transgenic Adenocarcinoma of the Mouse Prostate Model [TRAMP]. Our data demonstrates that Ron is overexpressed in TRAMPC1 and TRAMPC2 cells with very little expression detected in TRAMPC3 cells by RNA and protein analyses. This expression pattern is consistent with the ability of these cells to form tumors *in vivo*. Ron is functionally active in TRAMPC1 and TRAMPC2 cells as stimulation with the Ron ligand, hepatocyte growth factor-like protein [HGFL] induced MAPK activity. Furthermore, HGFL signaling was sufficient to significantly increase the growth of the TRAMP-C1 cells. The importance for Ron expression in tumor growth *in vivo* was examined using orthotopic PC-3 xenografts in nude mice. A significant decrease in prostate tumor mass was observed in mice injected with prostate cancer cells that have a stable knockdown of the Ron receptor. We also observed a decrease in the incidence of pelvic bone metastasis in animals that were injected with Ron knockdown PCa cells.

Introduction

The Ron receptor tyrosine kinase is expressed on epithelial cells and is important for promoting proliferation, survival, cell migration, EMT, invasion and metastasis (72, 75, 76). Over-expression and/or constitutive activation of the Ron receptor have been reported in several epithelial cancers, including prostate cancer (29, 72, 90). Analysis for Ron expression by immunohistochemistry in human prostate tissues showed that Ron was overexpressed in >92% of the prostate tumor tissues examined (29). The expression of the receptor is limited in normal prostate epithelium and increases proportionally with disease progression from benign prostate

hyperplasia to prostate adenocarcinoma samples (90). To investigate the significance of Ron receptor expression in prostate cancer, our laboratory has examined the loss of function of Ron in the Transgenic Adenocarcinoma of the Mouse Prostate [TRAMP] mouse model. In this model, TRAMP mice crossed with mice that have a germline loss of the tyrosine kinase signaling domain of Ron [TK-/-] had decreased prostate tumor size when compared to the TRAMP mice with Ron expression [TK +/+] (89). This study demonstrated the functional importance of Ron signaling in promoting prostate tumor growth; however, the data did not address the selective contributions of Ron signaling in epithelial versus stromal cells in tumor formation. This is important as Ron expression is also detected on several tissue resident macrophage populations and activation of Ron plays a vital role in regulating the threshold of macrophage activation, promoting from tissue from inflammation-induced tissue damage (68). Given that Ron receptor is important for tumor progression and in macrophage activation, the functional importance of Ron in epithelial and tumor associated stromal cells, such as macrophages, in promoting tumorigenesis needs to be elucidated. Understanding the compartment specific roles of Ron receptor will aid in the development of efficient therapeutic targets and strategies. In this chapter, we have addressed the importance for Ron expression in prostate tumor epithelial cells using orthotopic xenograft of human prostate cancer cell line PC-3M in nude mice. We have also identified a novel assay for the study of spontaneous bone metastasis of the prostate cancer cells.

Materials and Methods

Cell Lines: Murine Prostate cancer cells TRAMP-C1, TRAMP-C2 and TRAMP-C3 obtained from ATCC [Manassas, VA] was cultured in DMEM media supplemented with 5% Nu Serum IV and 5% FBS. TR-9 epithelial cells were primary cells established from TRAMP+ TK-/- prostate tumors. These cells were also maintained in DMEM media supplemented with 5% Nu Serum IV and 5% FBS. Human Prostate cancer cell line PC-3M-luc2 cells obtained from Caliper Life

Sciences [Hopkinton, MA, USA], was cultured in 5% MEM media supplemented with 5% FBS. All cell line were maintained at 37^{o}C, 5% CO2 and harvested at 90% seeding density for further experiments.

Western and Real Time PCR: TRAMP-C1, TRAMP-C2, TRAMP-C3 and TR-9 cells were analyzed for Ron expression by protein or RNA analysis as previously described (89, 90). The mRNA levels of Ron receptor in the cell lines were measured by qRT-PCR analysis using the following primer pair for Ron 5'TCCCATTGCAGGTCTGTGTAGA3' and 5'CGGAAGCTGTATCGTTGATGTC3'. The expression levels for Ron were normalized to the levels of 18S mRNA and the data is represented as relative mRNA levels. Levels for Ron expression by protein was detected after western analysis using RON-C20 antibody [Santa Cruz Biotechnologies, Santa Cruz, CA] and C4 Actin [CCHMC, Cincinnati, OH]. For activation assays, the cells were serum starved overnight and stimulated with either vehicle [0.1% BSA in PBS] or human recombinant [100ng/ml] HGFL [R&D Systems, Minneapolis, MN] for 20min. The cells were washed twice after treatment and the lysates were subjected to western analysis for pMAPK [p p42/p44] and total MAPK [p42/44] [Cell Signaling Technologies, Danvers, MA].

Growth assay: TRAMP-C1 cells were used to determine the growth rate of these cells in the presence of HGFL. Cells were seeded in 24 well plates and serum starved overnight. The cells were then pulsed with serum free media containing 100ng/ml of recombinant human HGFL and the growth rate of these cells was estimated using MTT assay at 48hrs. Control cells were given 0.1% BSA in PBS [vehicle] as a negative control.

Orthotopic prostate injections and imaging: For orthotopic injections, human prostate cancer cell line PC-3M-luc2 cells, a PC-3 cell line containing stable luciferase expression were used. We also created PC-3M-luc2 cells with a stable Ron knockdown after stable infection of cells

with human Ron shRNA [cat# RMM3979-9571732] from Open Biosystems [Huntsville, AL]. These cells [5 x 10^5] 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 growth of the tumors was monitored weekly using the [Xenogen™, IVIS] after *in vivo* injection of 200µl of D-luciferin per mouse for 15min. Imaging and quantification of signals were controlled by acquisition and analysis software Living Image® [Xenogen]. *Ex vivo* imaging of the tissues was carried out at the time of sacrifice by imaging the tissues, isolated from mice injected with D-luciferin prior to harvest, in 1X PBS, using the Xenogen IVIS.

Immunohistochemical staining for CD-31: Formalin-fixed paraffin embedded tissue sections was used for histological analysis for microvessel density after staining for CD31. Tissues were incubated with an anti-CD31 antibody [1:500,Dako, CA] and the sections were developed using the 3,3'-diaminobenzidine tetrahydrocloride enhanced liquid substrate system [Sigma, MO, USA] after incubation with biotinylated secondary antibody and Avidin-Biotin kit [PK-6100, Vector labs]. Microvessel density was calculated as previously described (88).

Statistical Analysis: Data are represented as mean ± standard error. Statistical significance was determined by students t test using the graph pad prism software [Graph Pad Software, Inc. La Jolla, CA]. Data was considered significant when $p<0.05$.

Results

Ron is expressed and functional in murine prostate cancer cell lines

The expression and functional characterization of Ron in murine prostate cancer cell lines are shown in Figure 1. To investigate the function of Ron in prostate epithelial cells, we analyzed Ron expression in a variety of murine prostate cancer cell lines with differing tumorigenic

properties derived from TRAMP prostate tumor (112). Our data shows that Ron is overexpressed in TRAMPC1 and TRAMPC2 cells with very little expression detected in TRAMPC3 cells by RNA and protein analyses [**Figure 1A -1C**]. This expression pattern is correlates with the ability of these cells to form tumors *in vivo* [**Figure 1B** and (112)]. Ligand dependent activation of Ron was also examined in these cells after serum starvation and HGFL stimulation for 20 min. Ron was ligand responsive in TRAMPC1 and TRAMPC2 cells, where stimulation by HGFL induced an increase in pMAPK, a key downstream target for the Ron receptor tyrosine kinase (64) [**Figure 1D**]. Furthermore, the growth of the TRAMP-C1 cells in the presence of recombinant HGFL was significantly more compared to their growth in the presence of the vehicle control, suggesting the importance for the ligand dependent Ron activation in supporting the tumor cell growth [**Figure 1E**].

Ron expression in epithelial cells is important for prostate tumor growth and increases pelvic bone metastasis

We also examined the tumorigenic potential of the human prostate cancer cell line PC-3 *in vivo* using orthotopic xenografts in nude mice. Western analysis on PC-3M-luc2 cells and PC-3M-luc2 shRON cells shows greater than 95% efficiency in the reduction in the levels of Ron in PC-3M-luc2 shRON cells [**Figure 2A**]. Our experiments demonstrated that knockdown of Ron in PC-3M luc cells leads to decreased prostate tumor growth rate as measured by bioluminescence imaging [**Figure 2B-2C**]. A significant decrease in tumor mass was observed in mice with the Ron knockdown cells compared to control mice at the end terminal time point [**Figure 2D**]. Loss of Ron in the epithelial cells also resulted in a significant decrease in microvessel density in PC3-shRon tumors compared to controls [**Figure 2E**]. *Ex vivo* bioluminescence imaging of specific tissues and organs including the brain, lungs, liver, kidney, colon, intestine, lymph nodes, femur and pelvic bones revealed that Ron expression in the

prostate tumor cells specifically increases the frequency of metastasis to the pelvic bones but not to the femur and other tissues analyzed [**Figure 2F** and data not shown].

Discussion

This study characterizes the expression levels of Ron in murine cancer cell lines and shows that expression levels correlate well with the known *in vivo* tumorigenecity of these cell lines. Furthermore, our experiments in these murine prostate cancers cell lines show that Ron expressed in these cells is ligand responsive and exhibits a growth advantage when cultured in the present of the Ron ligand, HGFL. Understanding whether Ron retains ligand independent signaling in these cells that contributes to tumor growth and survival remains to be discovered.

In vivo experiments using human prostate cancer cell lines have shown that Ron is important for promoting prostate tumor growth and is involved in promoting the specific metastasis to the pelvic bones The use of luciferase tagged cells in an orthotopic tumor model along with *ex vivo* tissue imaging has proved to be a valuable tool for study of spontaneous bone metastasis. Bone is the most common metastatic site in patients with advanced stages of prostate cancer; importantly, the disease becomes practically incurable once there is metastasis to the bone resulting in significant morbidity prior to the death of the patient (113) . Metastasis to the bone is a multifactorial process, involving contributions by stromal cells as well as production of bone homing factors by the cancer cells (113). Understanding these processes will have important clinical implications in the treatment of advanced stages of prostate cancer. Our results show that Ron expression in epithelial cells is important for promoting bone metastasis. Consistent with our observation, patients with the coordinated overexpression of Ron, its ligand HGFL and ligand activating protease Matriptase-1 have been shown to have an increase in metastasis to the bone compared to those patients that did not overexpress Ron, HGFL and Matriptase-1

(84). Taken together, our data shows that Ron expression in the epithelial cells is important for prostate tumor growth and can potentially impact metastasis to the bone. Future studies are aimed at understanding the mechanism by which Ron expression in the tumor cells enable bone homing or osteolysis; this would provide significant insights in the treatment of the metastatic disease using Ron targeted therapies.

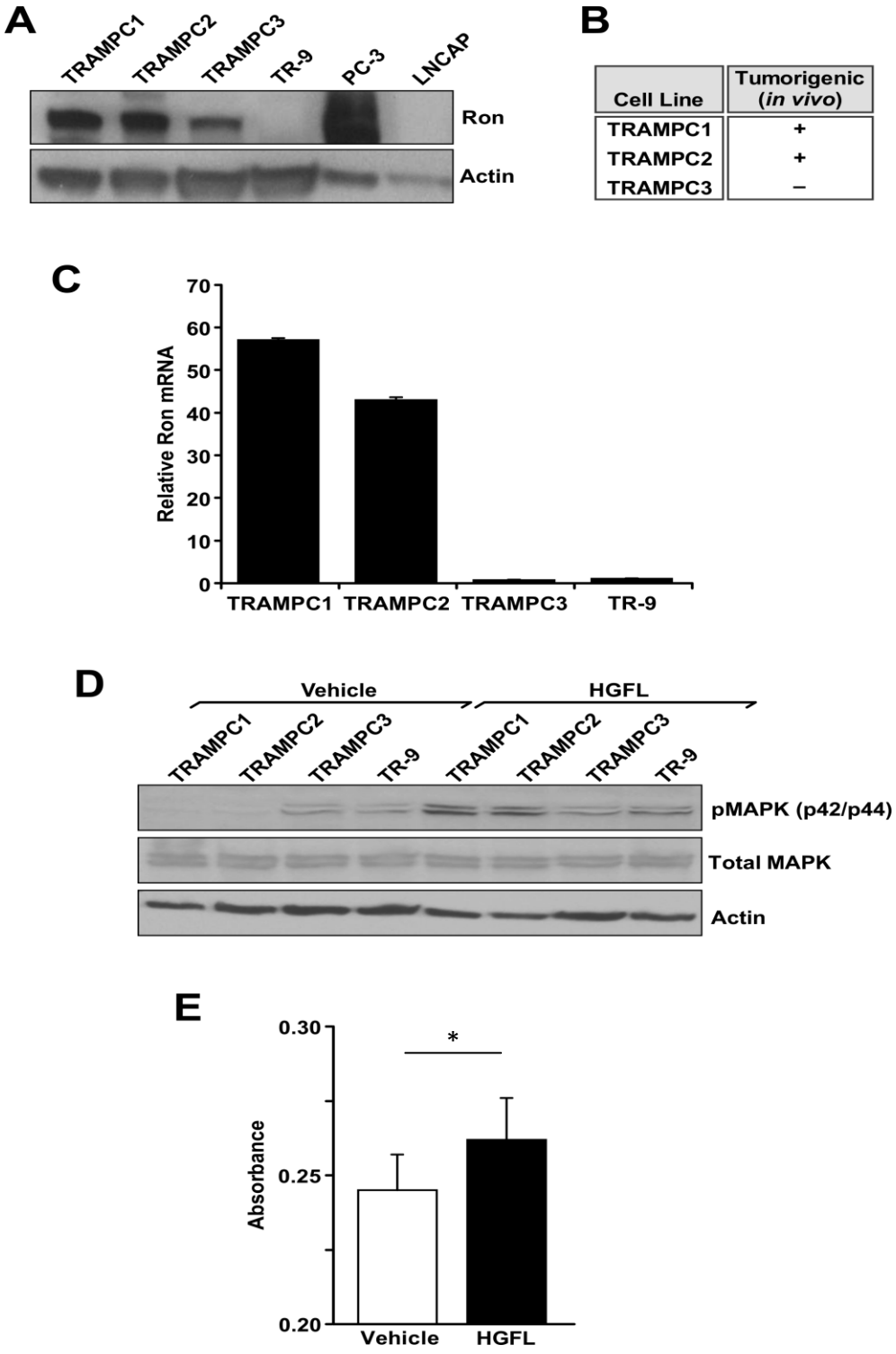


Figure 1: Ron expression and function in murine prostate cancer cell

A. Ron expression in murine prostate cancer cells derived from TRAMP+ TK+/+ tumors [TRAMP-C1, TRAMP-C2, TRAMP-C3] and TRAMP+ TK-/- tumors [TR-9] by western analysis. Human prostate cancer cells PC-3 and LnCaP were used as controls. **B.** Table showing the ability of TRAMP cells to form tumors *in vivo.* **C.** Quantitative Real Time PCR analysis for Ron expression in mouse prostate cancer cell lines. **D**. Functional readout for Ron activation in murine prostate cancer cells determined by western analysis for pMAPK [p42/p44] after stimulation with the Ron ligand, HGFL, or vehicle. **E.** Increase in TRAMPC1 cell number in response to HGFL treatment for 48hrs.

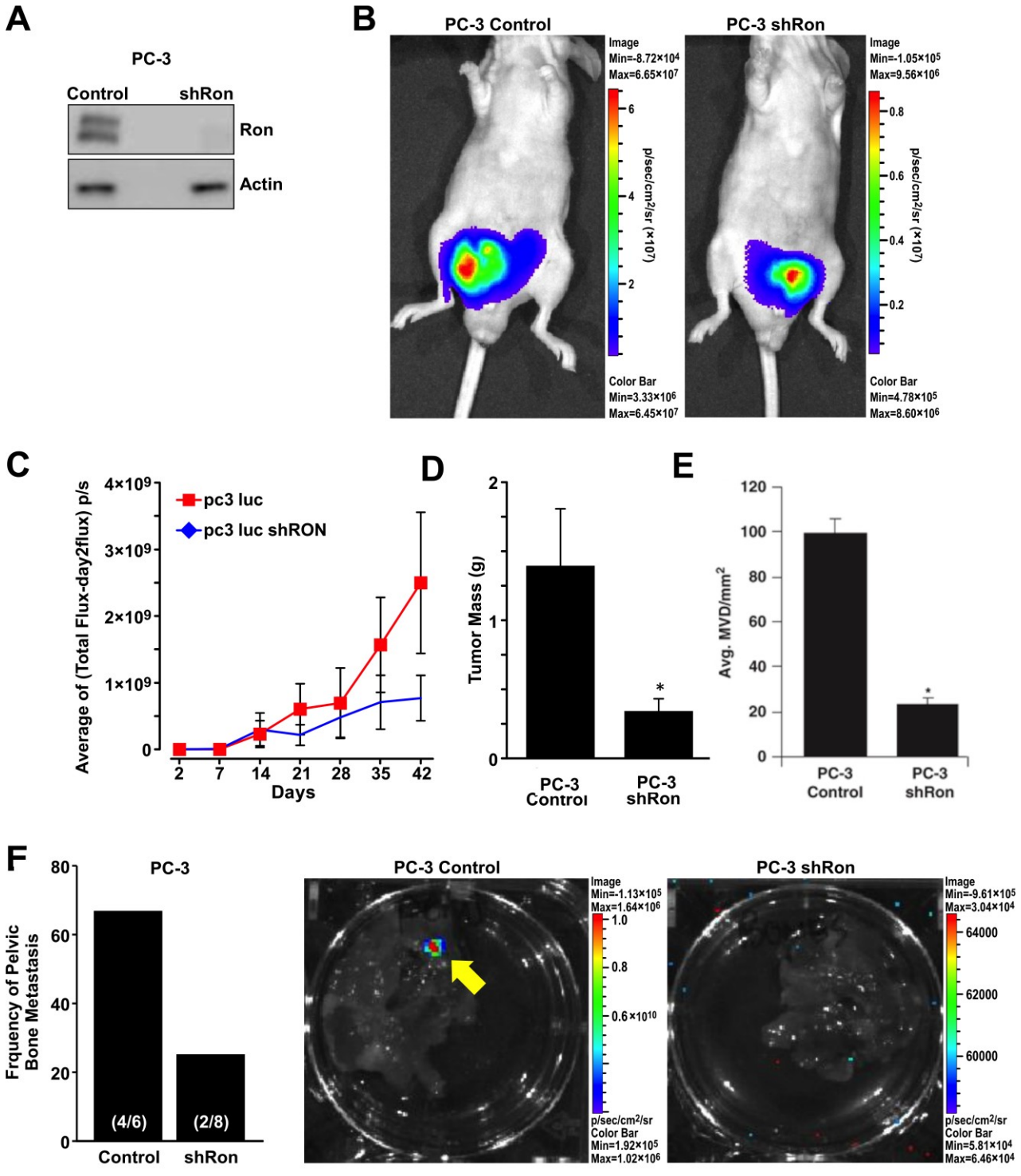


Figure 2: Ron expression in epithelial cells is important for promoting tumor growth *in vivo*

A. Western analysis showing stable knockdown of Ron in PC-3M luciferase cells. **B**. Representative bioluminescent images of mice harboring orthotopic PC-3M Control or PC-3M shRON cells 21 days post injection **C.** *In vivo* growth rate of PC-3M control and PC-3M shRON cells after orthotopic injection into the prostates of nude mice measured using bioluminescence imaging (PC-3M Control, n=6; PC-3M shRON, n=8) **D.** Tumor weights determined at the time of harvest showing significant reduction in tumor weight in mice that received PC-3 shRON cells. (PC-3M Control, n=6; PC-3M shRON, n=8; *P<0.05) **E.** Histogram showing the quantification of the microvessel density determined by CD-31 staining. (PC-3M Control, n=6; PC-3M shRON, n=8; *P<0.05) **F.** Histogram showing the frequency of bone metastasis observed in the animals after *ex vivo* bioluminescence imaging. Representative pictures showing bone metastasis marked by yellow arrow.

Chapter 3

MYELOID-SPECIFIC EXPRESSION OF RON RECEPTOR KINASE PROMOTES PROSTATE TUMOR GROWTH

Devikala Gurusamy[1], Jerilyn K. Gray[1], Peterson Pathrose[1], Rishikesh M. Kulkarni[1], Fred D. Finkleman[2, 3] and Susan E. Waltz[1, 3,*]

This work is published in Cancer Research 2013; 73: 1752-1763.

Published Online First January 17, 2013.

Abstract

Ron receptor kinase [MST1R] is important in promoting epithelial tumorigenesis, but the potential contributions of its specific expression in stromal cells have not been examined. Herein, we show that the Ron receptor is expressed in mouse and human stromal cells of the prostate tumor microenvironment. To test the significance of stromal Ron expression, prostate cancer cells were orthotopically implanted into the prostates of either wild type (WT) or Ron tyrosine kinase deficient [TK-/-; Mst1r-/-] hosts. In TK-/- hosts, prostate cancer cell growth was significantly reduced compared to tumor growth in TK+/+ hosts. Prostate tumors in TK-/- hosts exhibited an increase in tumor cell apoptosis, macrophage infiltration and altered cytokine expression. Reciprocal bone marrow transplantation studies and utilizing myeloid cell specific ablation of Ron demonstrated that loss of Ron in myeloid cells is sufficient to inhibit prostate cancer cell growth. Interestingly, depletion of CD8+ T-cells, but not CD4+ T-cells, was able to restore prostate tumor growth in hosts devoid of myeloid-specific Ron expression. These studies demonstrate a critical role for the Ron receptor in the tumor microenvironment, whereby Ron loss in tumor-associated macrophages inhibits prostate cancer cell growth, at least in part, by de-repressing the activity of CD8+ T cells.

Introduction

Coordinated signaling between different cell types of the stroma is required for the development and maintenance of an adult prostate secretory epithelium. The stroma also supports prostate tumor initiation and progression (114, 115). Inflammatory infiltrates are major cellular components of tumor stroma and include adaptive and innate immune cell types (116, 117). Tumor-associated immune cells support tumor growth by producing an immunosuppressive microenvironment capable of blocking productive antitumor immunity. Tumor-associated macrophages [TAMs] play pivotal roles in tumor progression and can promote or inhibit tumor growth depending on their activation state (118, 119).

The Ron receptor tyrosine kinase is expressed on several tissue-resident macrophage populations (22-25, 51). Ron activation in peritoneal macrophages suppresses inflammation and promotes alternative macrophage activation (54, 71). Following LPS stimulation in macrophages, Ron activation inhibits the expression of inducible nitric oxide synthase [iNOS] while promoting expression of arginase-1, an enzyme that competes with iNOS (68, 120). iNOS-mediated nitric oxide production is cytotoxic and tumor suppressive, whereas products of arginase-1 promote tissue repair and tumorigenesis (121).

Studies from our laboratory have shown Ron to be a negative regulator of inflammation in lung injury and bacterial peritonitis models (59, 122). Utilizing mice with myeloid cell-specific Ron deletion, macrophages were identified as the major cell type regulating inflammatory responses in lung and liver injury models (26, 52). Ron signaling in peritoneal macrophages increases the phosphorylation of STAT3, suggesting an interaction between these two pathways (53, 122). Moreover, STAT3-/- mice show similar phenotypic responses as that of Ron-deficient mice

(123). Phosphorylation of STAT3 in TAMs is important for attenuating antitumor immunity by suppressing macrophage-mediated antigen presentation to cytotoxic CD8+ T-lymphocytes (124, 125). Given that Ron regulates macrophage activation and inflammation, Ron signaling in TAMs may be important for maintenance of an immunosuppressive microenvironment.

Ron is overexpressed in > 90% of human prostate cancers (29, 72, 90). Ron activation in tumor cells elicits a range of cellular responses including growth, differentiation, migration, and survival through various signaling pathways (72, 75). Gain- and loss-of-function studies in tumor epithelial cells, including human prostate cells, have shown that Ron expression is important for promoting tumor growth *in vivo* (85, 88, 89, 104). Moreover, data from our laboratory has shown that mice containing a germline deletion of the Ron tyrosine kinase signaling domain exhibit significant reduction in tumor mass when bred to mice predisposed to develop prostate cancer (89). While numerous studies have outlined the importance of epithelial-expressed Ron in supporting tumorigenesis, the importance of Ron expression in the tumor stroma has not been extensively investigated. Although a recently published study outlined the importance of Ron expression in the tumor microenvironment, the Ron-expressing stromal cell lineage that supported tumor growth was not identified (71).

This study identifies Ron expression in the stroma, particularly in TAMs, to be a critical factor that supports tumor growth through the regulation of apoptosis in tumor epithelial cells. Additionally, we show that Ron expression in TAMs suppresses tumor immune surveillance through CD8+ T-cell regulation and that Ron-expressing TAMs have increased activation of STAT3. Our findings indicate that in addition to the well-established tumor-cell-autonomous role, Ron plays a novel role in TAMs by promoting tumor cell survival through cytotoxic CD8+ T-cell regulation.

Materials and Methods

Mice. Wild-type (TK+/+), Ron tyrosine kinase-deficient mice (TK-/-), homozygous Ron-floxed mice ($TK^{f/f}$), and LysMcre ($TK^{f/f}$ LysMcre+) mice were generated and maintained in a C57BL/6 background as described (26, 42). All experiments utilized 8-12 week old male mice. For bone marrow transplantation, donor bone marrow cells from TK+/+ and TK-/- mice were injected into the tail vein of irradiated mice (**Supplemental Methods**). All mice were maintained under specific pathogen-free conditions and were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Cell Lines and Orthotopic Injections. Murine TRAMP-C2Re3 cells were obtained from Dr. Zhongyun Dong (University of Cincinnati, OH) and grown as previously described (126). The cells are of murine origin and were tested through growth in syngeneic mice; no further validation was performed. Cells ($2.5x10^5$) were injected into the ventro-lateral prostates of mice and harvested after 30 days (90).

T-Cell Depletion. Mice were subcutaneously injected with $5x10^5$ TRAMP-C2Re3 cells. To deplete T-cell subsets, mice were injected on days 1, 7, 14, 21, and 24 with 1mg/ml of rat monoclonal antibody that depleted specific lymphocyte subsets: mAb-GK1.5 (anti-CD4, IgG2b); mAb-2.43 (anti-CD8, IgG2b); and Rat-IgG2b isotype control antibody (127) (Bio X Cell). Tumor volume was recorded biweekly and tumors were harvested on day 28.

Cell Isolations. A single-cell suspension of normal or tumor-bearing prostate tissue was obtained by mechanical dissociation and enzymatic digestion. Enrichment for epithelial cells, fibroblasts and immune cells was completed by differential centrifugation using standard procedures (**Supplemental Methods**). TAMs were enriched from the immune cell fraction by

magnetic beads coated with mouse CD11b according to manufacturer's instructions. (Miltenyi Biotech). The isolated cells were >95% pure. 121qq

Flow Analysis. Immune infiltrates were treated with Fc anti-CD16/CD32 antibody and stained with antibodies that detect macrophages (anti-mCD11b), granulocytes (anti-mGr-1) and CD8+ T-cells (anti-mCD8a) (eBiosciences). Epithelial cells were stained for AnnexinV/PI, per manufacturer's instructions (BD Biosciences). Cells were analyzed using the FACS Aria and FACS Diva software (BD Biosciences).

Luminex Array. Plasma was analyzed utilizing the Milliplex Map Mouse Cytokine/Chemokine Panel with Luminex Map detection per manufacturer's instructions (Millipore, #MPXMCYTO-70).

Immunohistochemistry. Formalin-fixed paraffin-embedded sections were stained for F4/80 (eBiociences), CD-31 (Dako), and α-SMA (Sigma Aldrich) using standard procedures.

Human Tissue Array and Scoring. Immunohistochemistry for Ron was performed on human prostate cancer tissue microarray specimens (IMH-303, Imgenex; TMA1202-4, Chemicon/Millipore; 75–4063, Zymed), using the Ron-α antibody (BD-Transduction Laboratories). The percentage of Ron-positive stromal cells was determined and staining intensity graded (0-3). The stromal staining index, a factor of the staining percentage and intensity, was obtained for each tissue section.

Quantitative Real-Time (qRT)-PCR and Immunoblot Analyses. qRT-PCR was performed utilizing primer pairs listed in the **Supplemental Methods** as previously described (26). Expression levels were normalized to β-glucuronidase and relative gene expression reported. Western analyses utilized the following antibodies: Ron-C20 (Santa-Cruz-biotechnologies)

STAT3 and pSTAT3-Y705 (Cell Signaling) and Arginase-1 (BD Biosciences).

Statistical Analysis. Data are expressed as mean ± standard error (SE). Statistical significance was determined by t-tests for pairwise comparisons or ANOVA for comparison of multiple groups with Graph Pad Prism software. Significance was set at $P<0.05$.

Results

Ron is expressed in stromal cells of normal and tumor-bearing prostates and is critical for promoting prostate tumor growth.

Ron mRNA expression was detected in primary cells isolated from the prostates of TK+/+ mice including resident prostate macrophages, epithelial cells, and fibroblasts (**Figure 1A)**. Ron expression in human stromal cells was examined by immunohistochemistry on a panel of human prostate tissues. As depicted in **Figure 1B**, Ron was detected in the stromal cells of normal human prostates, benign prostate hyperplasia and prostate adenocarcinoma samples at approximately similar levels. Interestingly, Ron expression in stromal cells did not parallel its expression in the prostate epithelium, which has been previously shown to increase with disease progression (90). While the levels of stromal Ron are not altered with prostate tumor progression, the functional role of this receptor may be regulated by the availability/processing of its ligand, hepatocyte growth factor-like protein, whose expression has been reported to increase with tumor progression (38, 39, 63, 128).

The role of Ron expression in the tumor microenvironment was assessed after orthotopic transplantation of TRAMP-C2Re3 prostate cancer cells into the prostates of TK+/+ and TK-/- mice. TRAMP-C2Re3 cells were able to grow in TK+/+ and TK-/- animals; however, growth was markedly inhibited in the TK-/- host microenvironment with an average prostate weight of 1.10±0.20g in TK+/+ mice compared to 0.27±0.05g in TK-/- mice (**Figure 1C**). Histological

analysis of the TRAMP-C2Re3 cells from the prostates of TK+/+ and TK-/- hosts revealed similarities in tissue architecture (**Figure 1D**). These findings demonstrate that Ron expression in the prostate tumor microenvironment is a critical mediator of prostate tumor growth.

Comparison of the stromal compartment in TK+/+ and TK-/- prostates following TRAMP-C2Re3 cell implantation.

To characterize the host environment following transplantation of TRAMP-C2Re3 cells into the prostates of TK+/+ and TK-/- mice, histological sections were stained for cell-surface markers. F4/80 staining showed significantly more macrophage infiltration in tumors from TK-/- hosts compared to tumors from TK+/+ hosts (**Figure 2A**). Interestingly, tumor macrophage distribution was distinct within each group, with macrophages observed around the tumor periphery in prostates from TK+/+ mice as opposed to diffuse intratumoral macrophages in tumors from TK-/- mice. Flow analyses confirmed increased macrophage infiltration in tumors from TK-/- hosts compared to controls (**Supplementary Figure S1A)**. To examine Ron-dependent macrophage migration *in vitro*, the murine macrophage cell line, MH-S cells, without (shNT) and with Ron knockdown (shRon) were utilized (51). Loss of Ron in these cells significantly increased their migration potential compared to the controls (**Supplementary Figure S1B)**.

The impact of stromal Ron on tumor vascularization was determined by microvessel staining which showed a significant increase in microvessel density in tumors from TK+/+ hosts compared to tumors in TK-/- mice (**Figure 2B**). Immunohistochemistry for tumor-associated myofibroblasts showed an increase in α-SMA staining in tumors from TK-/- prostates compared to controls (**Figure 2C**). Taken together, our data show that Ron signaling in the host regulates

key events linked with tumor malignancy including angiogenesis, α-SMA expression, and macrophage infiltration.

Lack of host Ron expression promotes prostate cancer cell apoptosis.

Given the significant difference in tumor size in TK+/+ versus TK-/- host environments, we examined tumor cell proliferation and death. There was no difference in proliferation of the transplanted TRAMP-C2Re3 cells (data not shown). However, a 2-fold increase in necrosis was noted in histological sections of tumors from TK-/- compared to TK+/+ hosts (**Figure 3A**). To evaluate differences in survival between groups, tumor cells from each host genotype were isolated and examined by AnnexinV/PI staining. A significant increase in the number of apoptotic ($AnnexinV^{+}PI^{-}$) and dead cells ($AnnexinV^{+}PI^{+}$ and $AnnexinV^{-}PI^{+}$) was detected in TRAMP-C2Re3 tumor cells isolated from TK-/- hosts compared to controls (**Figure 3B)**. These data are consistent with immunohistochemistry for cleaved caspase-3, which demonstrated an appreciable increase in the number of activated/cleaved caspase-3–positive tumor cells from TK-/- hosts compared to controls (data not shown). Furthermore, AnnexinV/PI staining on the F4/80 positive macrophages showed a trend towards increased cell death in the TK-/- macrophages compared to TK+/+. However, this latter observation may be attributed to the increased necrotic environment observed in tumors from TK-/- hosts (**Supplementary Figure S2** and **Figure 3A**).

Ron regulation of the inflammatory tumor microenvironment is associated with STAT3 activation in TAMs.

We next examined the inflammatory milieu in tumor-bearing TK+/+ and TK-/- hosts. A 3-fold increase in iNOS mRNA was observed in tumors isolated from TK-/- mice compared to controls (**Figure 4A**). Increased iNOS protein from tumors in TK-/- hosts was confirmed by Western

analysis (data not shown). Blood nitrite levels were also increased in the sera from tumor-bearing TK-/- animals (**Figure 4B**). Expression of IL-9, MIG, and IL-17 was significantly greater in tumor-bearing TK-/- sera while M-CSF was higher in TK+/+ sera (**Figure 4C)**. Intratumoral gene expression examined by qRT-PCR showed significant increases in MIG and TNF-α expression in tumors from TK-/- hosts as well as a significant increase in TGF-β in tumors from TK+/+ hosts (**Supplementary Figure S3A**). Significant increases in the intratumoral expression of STAT1, CXCR3, IL-12b, and ICOS were observed in tumor cells from TK-/- hosts compared to controls while CD80 and IL-27 levels did not change (**Figure 4D)**.

To examine signaling pathways in the TK+/+ versus TK-/- microenvironment that may account for altered cytokine expression, TAMs were isolated from TK+/+ and TK-/- tumors. Western analysis of TAMs from TK-/- hosts tumors showed a significant reduction in phosphorylated (p)STAT3 compared to controls (**Figure 4E)**. TAMs were isolated by CD11b coated magnetic bead pulldown assays and cells from this isolation included both CD11b+Gr1- (TAMs) and CD11b+Gr1+ (myeloid-derived-suppressor cells, MDSCs) cells. We refer to this population as TAMs hereafter as flow cytometry analysis demonstrated a 7.3-fold higher number of CD11b+Gr1- compared to CD11b+Gr1+ cells (data not shown). qRT-PCR analysis of flow-sorted immune cells demonstrated Ron mRNA expression in the various myeloid cell populations including CD11b+Gr1- (TAMs), CD11b+ Gr1+ (MDSCs) and to a lesser extent in CD11b-Gr1+ (granulocytes) (**Supplementary Figure S3B)**. Ron protein expression in TAMs isolated from TRAMP-C2Re3 tumors from TK+/+ mice is also depicted by Western analysis (**Supplementary Figure S3B)**.

TK-/- bone marrow-derived cells inhibit the growth of TRAMP-C2Re3 cells in chimeric TK+/+ mice.

Given the role of Ron in regulating macrophage activation, as well as the increase in proinflammatory cytokines observed in the TK-/- microenvironment, we next determined whether the hematopoietic compartment of TK-/- mice is sufficient to inhibit TRAMP-C2Re3 tumor growth *in vivo*. Utilizing reciprocal bone marrow transplantation experiments, TK+/+ and TK-/- mice were irradiated and reconstituted with either TK+/+ or TK-/- donor bone marrow to generate the following groups TK+/+→TK+/+ (irradiated TK+/+ mice transplanted with TK+/+ donor marrow), TK-/-→TK-/-, TK+/+→TK-/- and TK-/-→TK+/+. Following bone marrow reconstitution, the chimeras were intraprostatically injected with TRAMP-C2Re3 cells and tumor weight was measured after 30 days. TRAMP-C2Re3 cells injected into non-irradiated TK+/+ and TK-/- mice served as controls. qRT-PCR analysis for Ron expression on whole bone marrow-derived cells and on CD11b+ TAMs isolated from tumors indicated efficient reconstitution (82%) of donor-derived bone marrow cells into recipient animals (**Figure 5A-5B**). At baseline, no appreciable differences in the number of myeloid precursor cells were noted between genotypes (**Supplementary Figure S4).**

Irradiation had no effect on the growth of TRAMP-C2Re3 cells (**Figure 5C**). However, tumor growth was significantly delayed in TK+/+→TK-/- and TK-/-→TK+/+ chimeras relative to TK+/+→TK+/+ mice. Interestingly, tumor growth in TK+/+→TK-/- and TK-/-→TK+/+ mice was not significantly different from growth in TK-/-→TK-/- mice. These data indicate that tumor growth in Ron-deficient animals is due to contributions by hematopoietic and non-hematopoietic cells in the host microenvironment, as TK+/+ bone marrow-derived cells were not sufficient to restore tumor growth in TK-/- animals. In contrast, TK-/- bone marrow-derived cells significantly reduced tumor growth in TK+/+ animals. Analysis of tumor infiltrates from the chimeric mice demonstrated a significant infiltration of TK-/- donor-derived macrophages compared to TK+/+

donor macrophages (**Figure 5D**) similar to that observed with TK-/- macrophages from tumors of TK-/- mice (**Figure 2A, Supplementary Figure S1)**.

Myeloid-specific Ron loss is sufficient to decrease prostate tumor cell growth.

To delineate the Ron-expressing hematopoietic cell type that influences tumor growth, we crossed LysMcre+ mice with $TK^{f/f}$ to generate $TK^{f/f}$LysMcre+ mice. LysMcre+ mice specifically express Cre recombinase in myeloid cells and efficiently delete Ron in this cell population (26, 52). To test the efficiency of Ron deletion in TAMs, TRAMP-C2Re3 cells were intraprostatically injected into $TK^{f/f}$ control and $TK^{f/f}$LysMcre+ mice and TAMs were isolated 30 days post transplantation. A dramatic loss of Ron mRNA expression (>90%) was observed in TAMs isolated from $TK^{f/f}$LysMcre+ tumors compared to TAMs from $TK^{f/f}$ tumors (**Figure 6A**). To test the functional importance of Ron-expressing myeloid cells, prostate tumor weight was compared across genotypes. Growth of TRAMP-C2Re3 cells was significantly reduced in $TK^{f/f}$LysMcre+ animals compared to controls (**Figure 6B)**. Flow analysis for AnnexinV/PI on tumors from $TK^{f/f}$LysMcre+ mice demonstrated a significant increase in total apoptotic and dead cells compared to controls, signifying that lack of Ron in myeloid cells is sufficient to inhibit tumor growth through a mechanism that induces tumor-cell apoptosis (**Figure 6C**). Immunohistochemistry for macrophages on tumors from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice indicated a significant increase in macrophage numbers in $TK^{f/f}$LysMcre+ tumors compared to controls, thus reaffirming studies whereby Ron-deficient macrophages localize to the tumors in greater numbers (**Figure 6D)**. Moreover, iNOS expression was increased in tumors from $TK^{f/f}$LysMcre+ mice (**Figure 6E**). Western analysis on TAMs isolated from $TK^{f/f}$LysMcre+ tumors showed significantly less pSTAT3 and arginase-1 expression than $TK^{f/f}$ TAMs (**Figure 6F- 6G)**. Despite the similarities in tumor growth, no differences were observed in microvessel density of tumors transplanted into $TK^{f/f}$LysMcre+ mice and $TK^{f/f}$ compared to tumors from TK-/- mice, suggesting that myeloid-specific Ron deletion does not replicate a complete Ron deficiency

(**Supplementary Figure S5**). Combined, these studies demonstrate that Ron signaling in macrophages/myeloid cells promotes tumor growth and is associated with STAT3 activation, increased arginase-1 and decreased iNOS.

CD8+T-cells are essential for the antitumor immune response in TK-/- animals.

Flow cytometry and qRT-PCR analyses of tumors for T-cells showed an increase in CD8+ T-cells in tumors from TK-/- hosts compared to the controls, although this number was not significant (**Supplementary Figure S6A-S6B).** To determine if macrophage-mediated education of CD4+ and CD8+ T-cells was important for reduced tumor burden in TK-/- mice, mice were depleted of CD4+, CD8+, or both T-cell subsets by injection of cytotoxic monoclonal antibodies. To allow for accurate measurement of tumor growth, TRAMP-C2Re3 cells were injected subcutaneously and tumor growth was assessed biweekly. The efficiency with which T-cell subsets were depleted was determined by flow analysis (>99%) for T-cell subset markers on splenocytes obtained at the time of sacrifice (**Supplementary Figure S6C**). As shown in **Figure 7A**, TRAMP-C2Re3 cells grew similarly in the TK+/+ and TK-/- mice in the subcutaneous environment compared to the prostate microenvironment (**Figure 1C**), wherein a 3-fold increase in tumor growth was observed in the TK+/+ mice as compared to TK-/- mice; however, TRAMP-C2Re3 cells implanted orthotopically grew approximately 2-fold faster than cells implanted subcutaneously, in spite of the reduced inoculum cell numbers (129).

Depletion of CD4+ T-cells had a limited effect on tumor growth in both genotypes, as tumor growth in TK+/+ versus TK-/- mice was similar to that of isotype treated controls (**Figure 7A-7B**). In contrast, depletion of CD8+ T-cells restored tumor growth in TK-/- animals to that of controls. Depletion of CD4+ and CD8+ T-cell subsets resulted in similar growth rates in TK+/+ and TK-/- mice; however, a dramatic increase in tumor growth was observed in both genotypes.

Depletion of CD8+ T-cells in TKf/fLysMcre+ mice restores TRAMP-C2Re3 tumor growth.

To determine if Ron loss in macrophages was sufficient to engage an antitumor immune response, CD8+ T-cell depletion studies were performed in $TK^{f/f}$ and $TK^{f/f}$LysMcre+ tumor-bearing mice. Depletion of CD8+ T-cells in $TK^{f/f}$LysMcre mice abolished the antitumor immune response (**Figure 7C-7D**). Taken together, our results indicate that Ron expression in the tumor microenvironment, specifically in macrophages, aids in evasion of tumor immune surveillance by impacting CD8+ T-cell function.

Discussion

Tumors are regulated by complex signaling networks involving interactions between malignant epithelial cells and the adjacent stroma. While many publications have reported the importance of tumor-stromal interactions for supporting tumor growth, further investigations are required for the identification of the stromal cellular mediators and their associated signaling networks that promote tumorigenesis. In this report, we provide compelling evidence supporting the novel role for the Ron receptor in regulating tumor growth by virtue of its signaling in host bone marrow-derived cells, particularly myeloid cells, of the tumor microenvironment.

Myeloid cells and bone marrow-derived precursors are recruited to tumors and promote tumor growth, neovascularization, and aid in antitumor immune suppression (116, 130). Our experiments indicate that Ron loss in macrophages increases their localization to tumors and is associated with decreased tumor growth. Interestingly, this influx of macrophages into tumors was only observed in the presence of TK-/- bone marrow-derived cells, even in the context of bone marrow transplantation experiments (**Figure 3A**, **6D**). Furthermore, TK-/- mice were on par with wild types as they exhibited similar numbers of bone marrow-derived cells and hematological values [**Supplementary Figure S4** and (42)]. These results suggest that Ron deficiency does not affect the number and development of the hematopoietic cell lineages, but

instead improves localization of macrophages to tumors. Loss of Ron expression in the host environment also resulted in an increase in the expression of inflammatory cytokines including IL-9, MIG, IL-17, and TNF-α, which are known to promote macrophage infiltration and inhibit tumor growth through mechanisms that regulate phagocytic activity or support the activation of effector cells of the innate and adaptive immune responses. Alternatively, the increase in TK-/- TAMs in either host genotype may be due to the need to clear cellular debris. The underlying mechanism that leads to the increased localization of macrophages to tumors in the presence of TK-/- bone marrow-derived cells as well as the functional significances of increased macrophage numbers on tumor growth warrants further investigation.

In addition to the increased influx of Ron-deficient macrophages to tumors, our data show that a conditional loss of Ron in myeloid cells is sufficient to inhibit tumor growth *in vivo*. These studies are analogous to several previous studies which demonstrated decreased tumor growth in mice harboring myeloid-specific deletions of IKKβ (131), STAT3 (132, 133), HIF-1α (134) and HIF-2α (135). A previous study on IKKβ has shown that deletion of this gene in either epithelial cells (enterocytes) or myeloid cells reduces colitis-associated cancer. In this study, enterocyte IKKβ expression promoted tumorigenesis by suppressing apoptosis while myeloid expression enhanced tumor growth by controlling the production of inflammatory mediators, showing distinct roles for the protein in different cell types (131). The cell-type specific regulation of tumor growth by Ron is similar to that observed for IKKβ. Published studies have shown that loss of Ron in prostate cancer epithelial cells leads to a reduction in tumor growth by promoting epithelial cell apoptosis and limiting tumor vascularization (89, 90). In myeloid cells, Ron loss leads to increased tumor cell apoptosis associated with alterations in the inflammatory microenvironment and is independent of an effect on blood vessel formation Of note, our data utilizing bone marrow transplantation in chimeric mice suggested that in addition to hematopoietic cells, other non-hematopoietic Ron expressing stromal cell types contribute to

tumor progression. These data reinforce the notion of multiple cell-type specific roles for Ron in promoting tumor growth.

Previous studies from our laboratory in peritoneal macrophages have shown that Ron activation enhances macrophage STAT3 phosphorylation, while macrophages from TK-/- mice exhibit decreased STAT3 activation (122). Consistent with this data, our current study shows that Ron loss is associated with lower STAT3 activity in TAMs. STAT3 signaling in macrophages attenuates antitumor immunity in a variety of *in vivo* tumor models (124, 136). Similar to the studies in this report, disruption of STAT3 in myeloid cells through the use of LysMcre mice enhanced the ability of macrophages to stimulate CD8+ T-cell activity in response to tumor antigens (124). Ron signaling may also regulate T-cell activity through the induction of T-cell anergy by downregulating the expression of costimulatory molecules or regulating cytokine/chemokine production. A significant induction in costimulatory molecules CXCR3 and ICOS as well as an induction of IL-9, iNOS, MIG were observed in tumors taken from TK-/- compared to wild-type hosts, suggesting multiple components downstream of Ron that may regulate T-cell activity. While further studies are needed to determine the mechanism(s) by which myeloid-specific Ron signaling regulates tumor growth, our experiments with depletion of CD8+ T-cells in $TK^{f/f}$LysMcre+ mice suggest that Ron loss in myeloid cells leads to enhanced tumor cell death through a mechanism that may depend upon STAT3 signaling and the activation of the immune response. Moreover, given the lack of current technologies to effectively target transcription factors such as STAT3, targeting Ron may serve as an important surrogate to activate the immune response for cancer therapy.

In addition to alterations in the immune response, Ron signaling has been shown to regulate key metabolites that support an immunosuppressive tumor microenvironment. Ron activation in macrophages results in the inhibition of iNOS and increased arginase-1 expression (42, 120).

iNOS expression in tumor tissues has been shown to be either pro- or anti-tumorigenic, depending on the level of expression and the duration of NO (137). Consistent with published observations, Ron loss in the tumor microenvironment increased local expression of iNOS. This increase in iNOS expression may be partly attributed to the expression of Ron in myeloid cells, as tumors from $TK^{f/f}LysMcre^{+}$ mice also displayed increases in iNOS levels compared to controls. Taken together, our data suggest that Ron expression in myeloid cells is important for regulating the fine balance of iNOS expression, which may skew the microenvironment towards a tumor-promoting state. Conversely, expression of arginase-1 has been reported to protect tissue from damage. Consistently, our data show that Ron-deficient TAMs exhibited significant decreases in arginase-1 levels. The downregulation of arginase-1 in Ron-deficient TAMs may be significant, as arginase-1 and arginine have been shown to be important in regulating a variety of T-cell activities including proliferation, differentiation and activation (138, 139).

In summary, our data suggest a new role for Ron in regulating tumor intrinsic and extrinsic circuits within the tumor microenvironment. In light of immune impairment in individuals with cancer, short-term blockade of Ron in a controlled manner, such as the infusion of Ron-inhibited macrophages, may reverse the immunosuppressive microenvironment and activate antitumor immune responses. Further research in dissecting bidirectional signaling and the context-dependent role of Ron in cancer may provide a novel therapeutic strategy to improve the efficacy of cancer therapy.

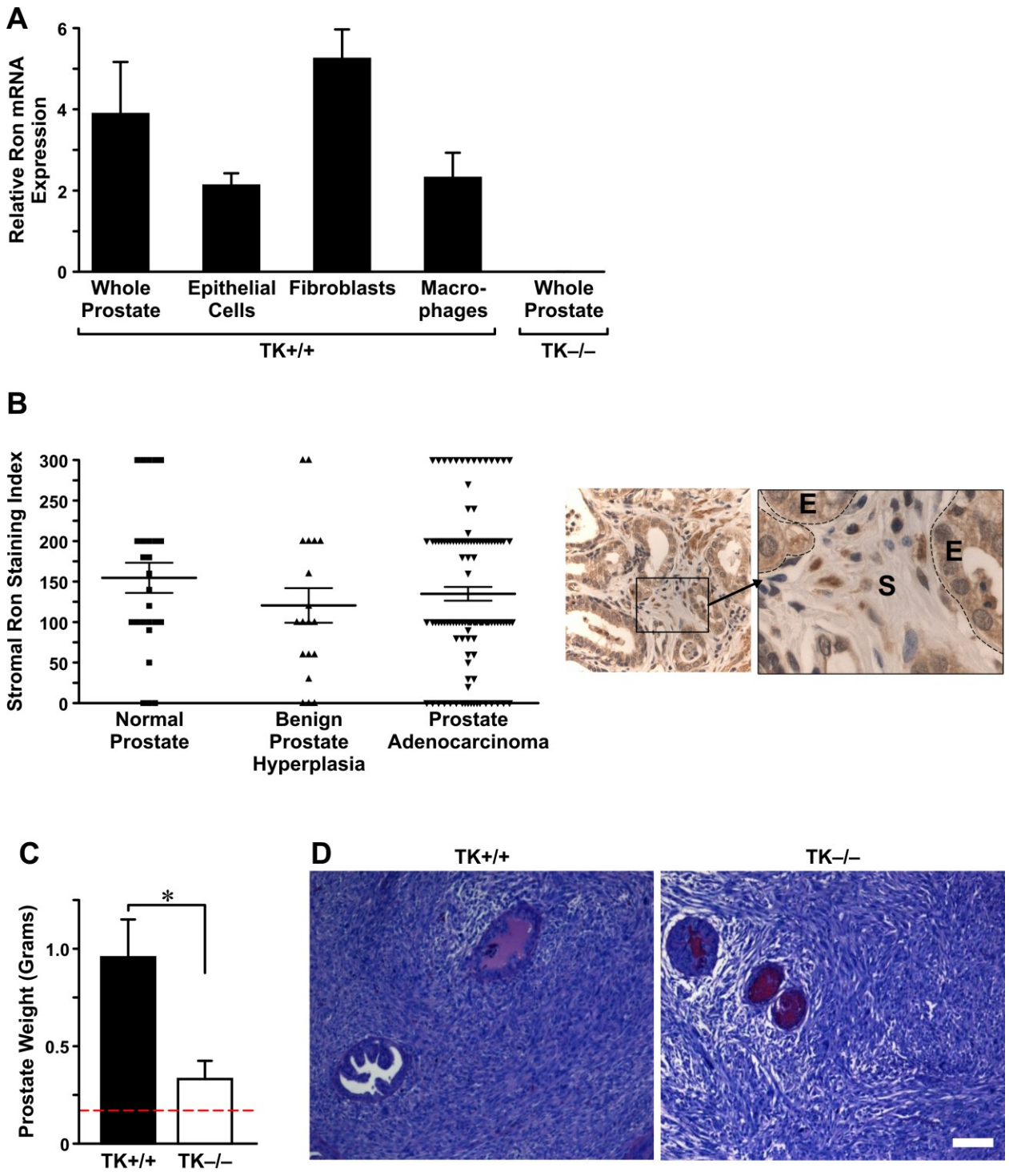


Figure 1. Ron is expressed in stromal cells of normal and tumor-bearing prostates and is important for promoting prostate tumor growth.

(**A**) qRT-PCR analysis of Ron mRNA expression in cells isolated from TK+/+ mice. The expression of Ron in whole TK+/+ and TK-/- prostates is shown for comparison. Expression levels are representative of two independent isolations. (**B**) Ron expression in human prostate tissue specimens. A representative human prostate adenocarcinoma section depicting positive Ron staining in both epithelial (E) and stromal (S) compartments. The horizontal line in the scatter plot represents the mean ± SE. (**C**) TRAMP-C2Re3 cells were injected into the prostates of TK+/+ and TK-/- mice; tumor weights were determined 30 days post-implantation. (TK+/+, n=36; TK-/-, n=33, *P<0.0001). Red line = average weight (0.18±0.012g) of normal prostates from TK+/+ mice. (**D**) Hematoxylin and eosin-stained TK+/+ and TK-/- prostate tumor sections showing similar tissue architecture. Scale bar = 100μm.

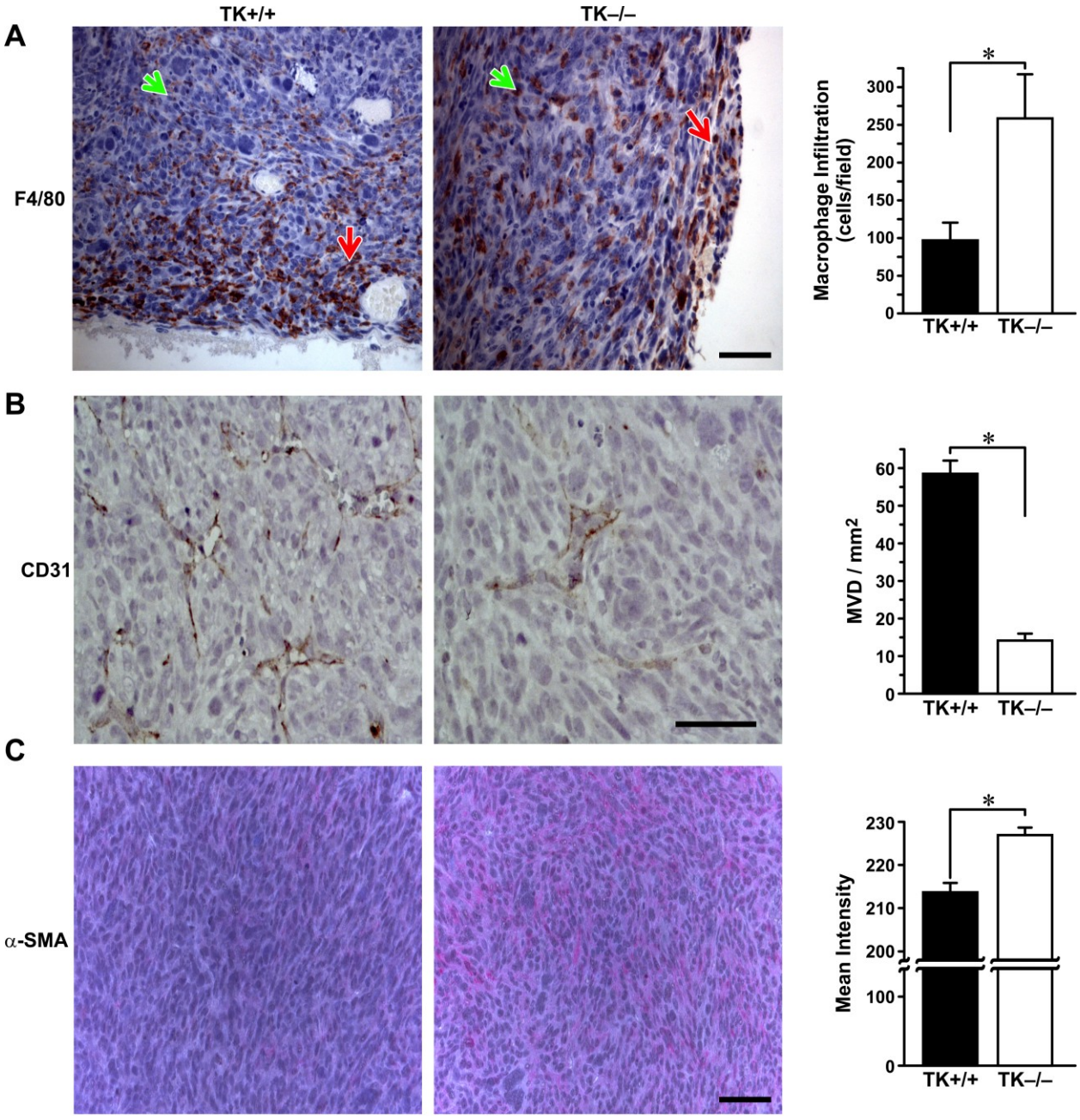


Figure 2. Characterization of stromal cells in TRAMP-C2Re3 tumors from TK+/+ and TK-/- prostates.

(**A**) Representative F4/80 staining of TRAMP-C2Re3 tumors from TK+/+ and TK-/- mice. Differences in the spatial distribution of macrophages are noted with red arrows depicting tumor peripheral infiltration while green arrows depict intratumoral infiltration. Macrophages were

quantitated and the data represents the mean number of $F4/80^+$ macrophages per 40X field (five fields per tumor, TK+/+, n=5; TK-/-, n=5, *P<0.05). Scale bar = 50μm. **(B)** Representative images showing CD31 staining in microvessels and quantification of the microvessel density per tumor area (*P<0.01). Scale bar = 100μm. (**C**) α-SMA–positive fibroblasts in the tumors and the average intensity of α-SMA expression per 40X field (*P<0.01). Scale bar = 50μm.

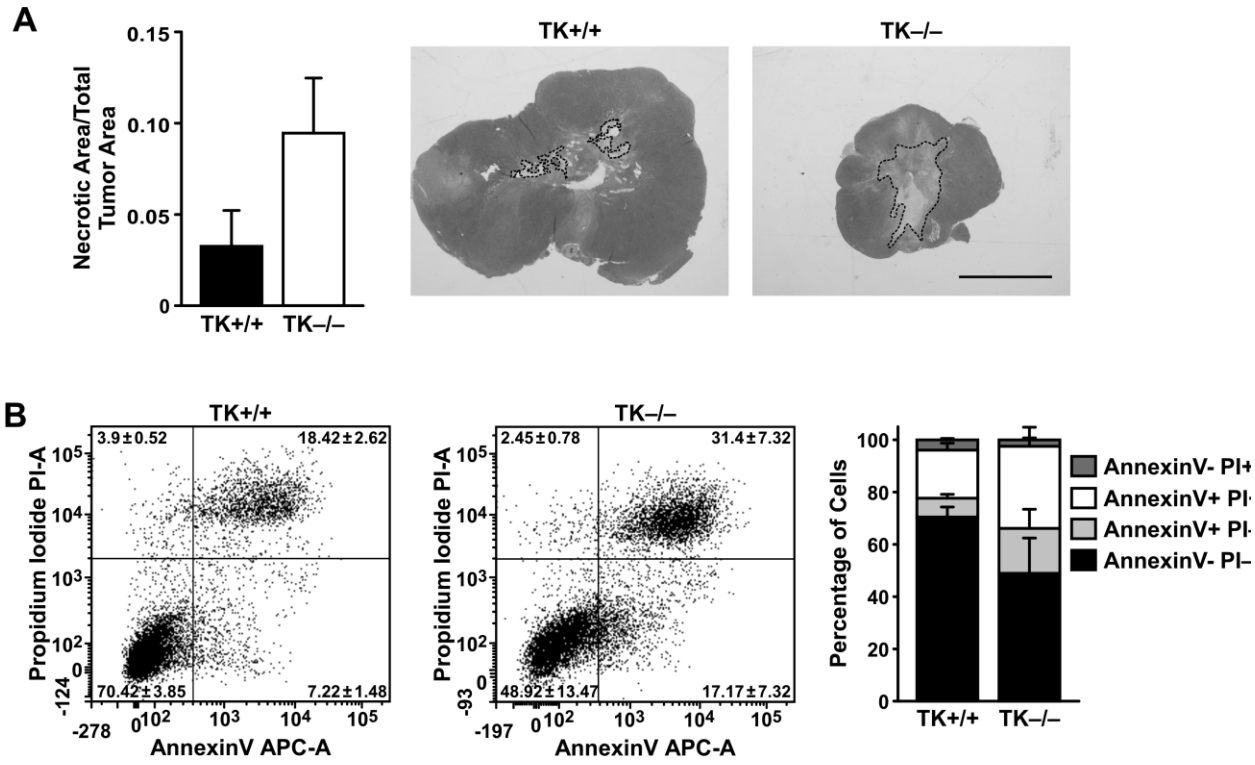


Figure 3. Lack of Ron signaling in the host leads to increases in tumor necrosis and apoptosis.

(**A**) Quantification of necrotic areas in TRAMP-C2Re3 tumor sections from TK+/+ and TK-/- hosts. Representative tissue sections are shown. Scale bar = 1cm. (**B**) Flow analysis for AnnexinV/PI on TRAMP-C2Re3 tumor cells isolated from TK+/+ and TK-/- hosts. A representative flow plot is shown with a histogram depicting data from all experiments (TK+/+, n=4; TK-/-, n=3). Data are the mean percentage of cells in early apoptosis ($AnnexinV^+PI^-$), late apoptosis ($AnnexinV^+PI^+$), dead cells ($AnnexinV^-PI^+$), and live/viable cells ($AnnexinV^-PI^-$) ± SE.

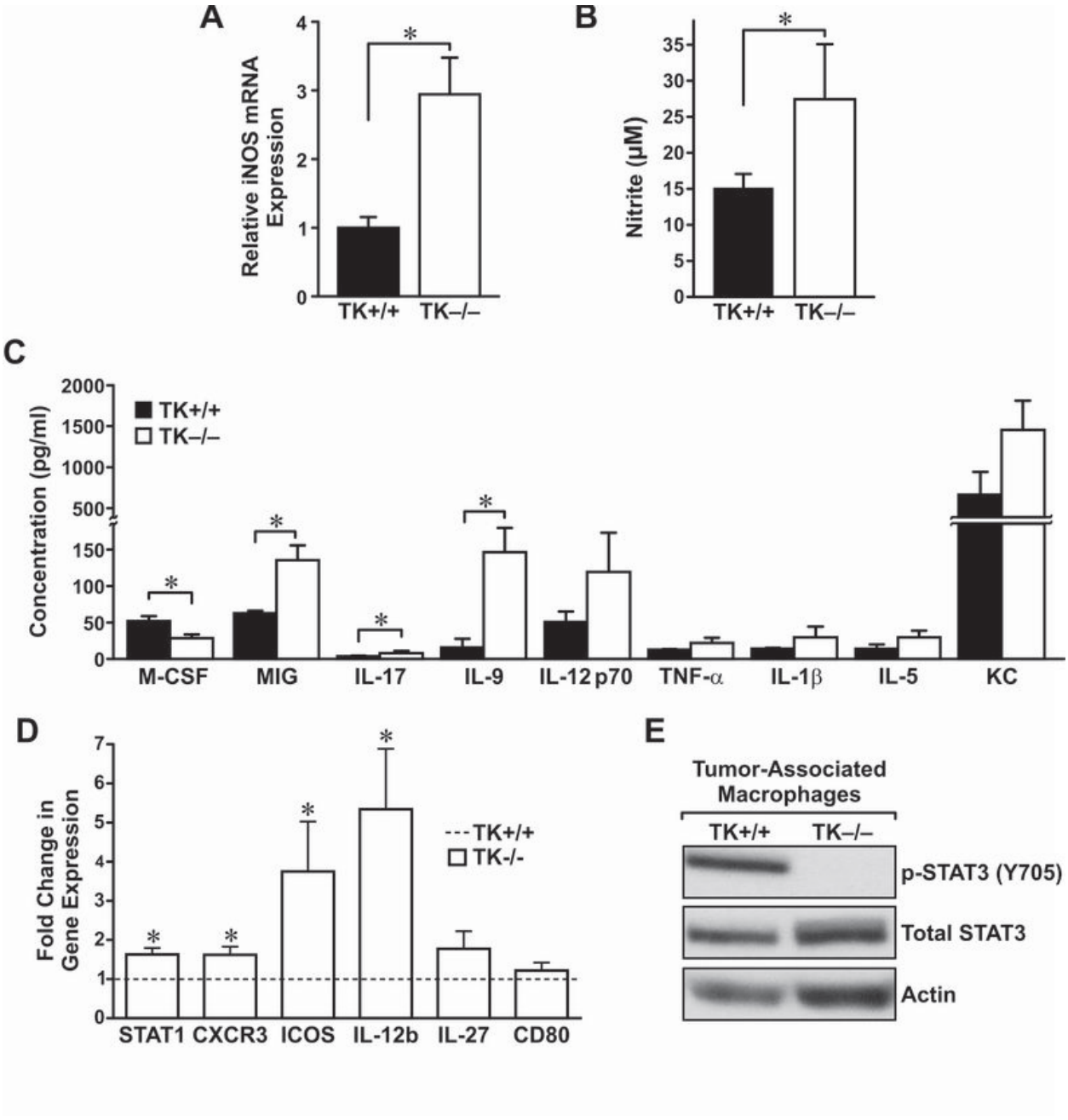


Figure 4. Loss of Ron leads to changes in the inflammatory tumor microenvironment and loss of STAT3 phosphorylation in TAMs.

(**A**) qRT-PCR analyses for iNOS mRNA in TRAMP-C2Re3 tumors from TK+/+ and TK-/- hosts. Data represented as relative change compared to β-glucuronidase (TK+/+, n=5; TK-/-, n=5, *P<0.01). **(B)** Concentration of serum nitrite from TRAMP-C2Re3 tumor-bearing TK+/+ and TK-

/- mice. (TK+/+, n=5; TK-/-, n=5, *P<0.05). **(C)** Levels of inflammatory cytokines in the serum of tumor-bearing mice. (TK+/+, n=5; TK-/-, n=5, *P<0.05) **(D)** Gene expression for T-cell regulatory genes depicted as fold change in TK-/- tumors over controls. **(E)** Western analysis for pSTAT3-Y705 and Total STAT3 in TAMs isolated from orthotopic TRAMP-C2Re3 tumors from TK+/+ and TK-/- mice and is representative of four independent isolations.

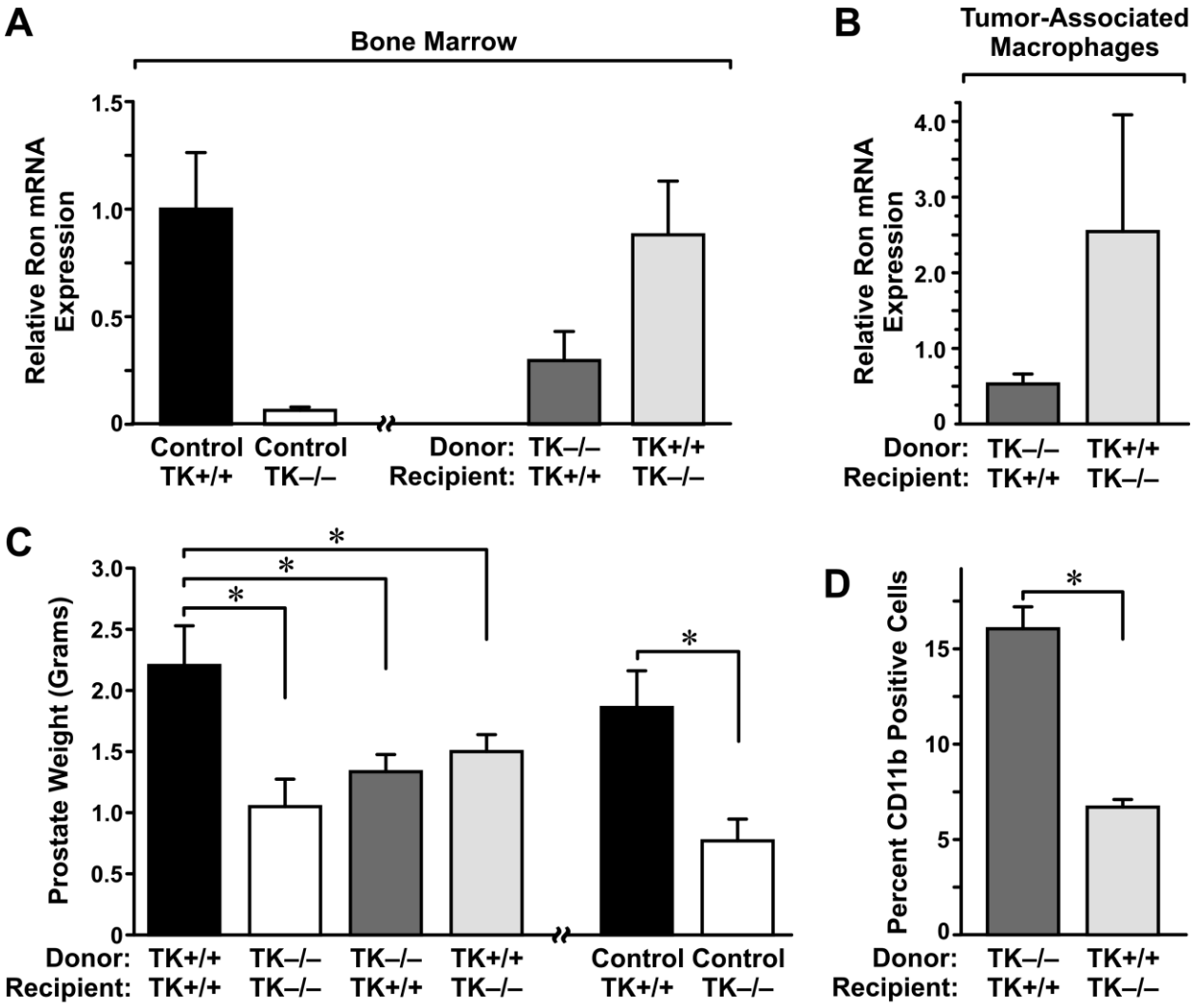


Figure 5. TK-/- bone marrow-derived cells decrease the growth of TRAMP-C2Re3 tumors in TK+/+ chimeras.

(**A**) Ron mRNA in bone marrow cells isolated from tumor-bearing TK+/+, TK-/- or chimeric mice. (**B**) Ron mRNA expression in TAMs isolated from TRAMP-C2Re3 tumors from TK+/+→TK-/- and TK-/-→TK+/+ chimeric mice. (**C**) Tumor weights determined at day 30 after intraprostatic injection of TRAMP-C2Re3 cells into TK+/+, TK-/- and bone marrow transplantation mice. Tumor progression in both chimeras was delayed compared to TK+/+→TK+/+ mice (*P<0.05). Data are pooled from two independent experiments. (TK+/+,

n=7; TK-/-, n=6; TK+/+→TK+/+, n=5; TK-/-→TK-/-, n=5; TK+/+→TK-/-, n=24; TK-/-→TK+/+, n=24; *P<0.05). **(D)** FACS analysis for tumor immune infiltrates in TRAMP-C2Re3 tumors from TK+/+→TK-/- and TK-/-→TK+/+ chimeric mice. Tumors from TK+/+→TK-/- chimeric mice had significantly more macrophages ($CD11b^+$ $Gr1^-$cells) compared to tumors from TK-/-→TK+/+ chimeric mice. Data are mean percentage of cells ± SE (TK+/+→TK-/-, n=2; TK-/-→TK+/+, n=2; *P<0.01).

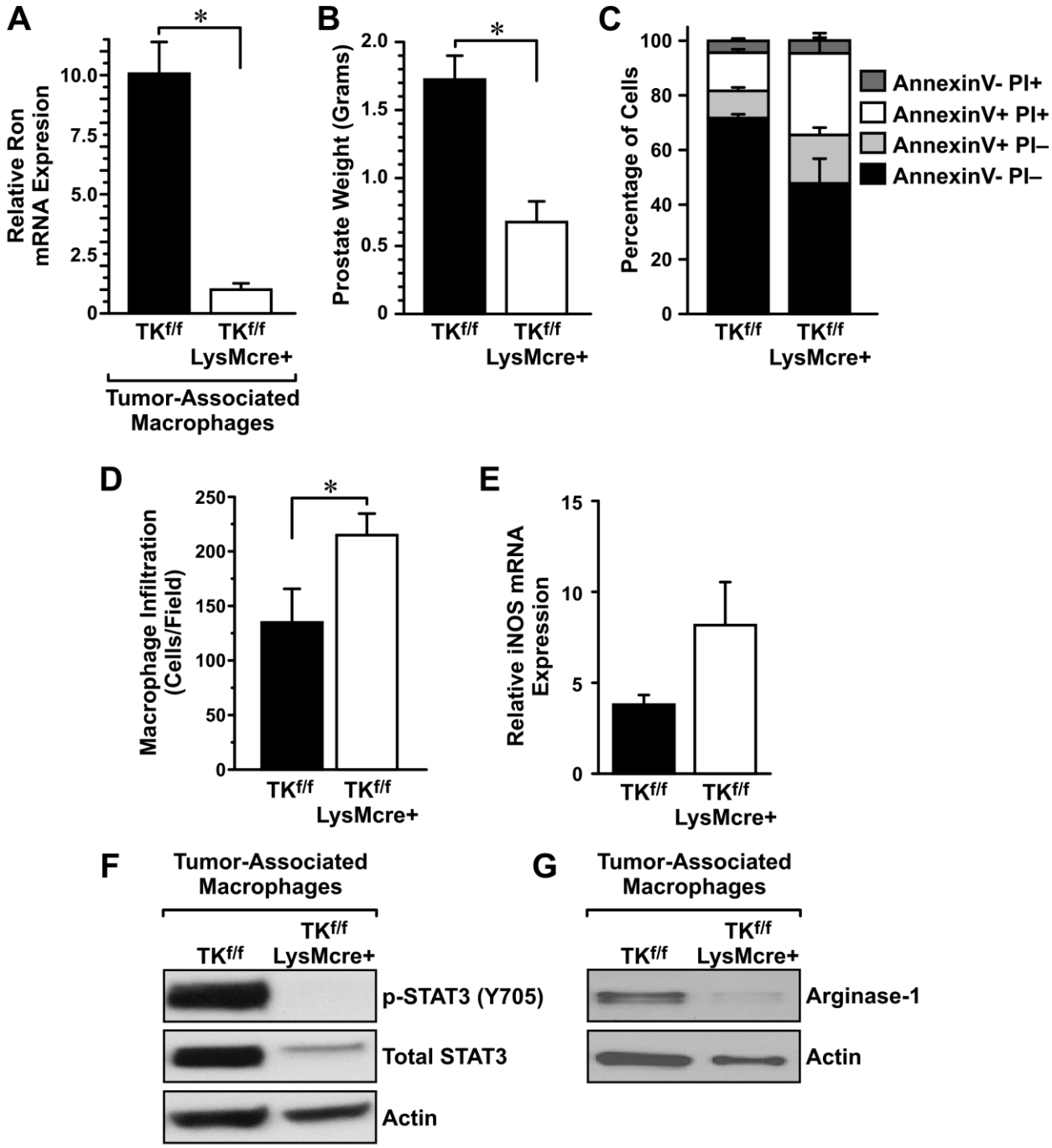


Figure 6. Loss of Ron signaling in myeloid cells is sufficient to abrogate TRAMP-C2Re3 tumor growth.

(**A**) Ron mRNA in TAMs isolated from TRAMP-C2Re3 tumors from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice. Data represented as relative change from two independent TAM isolations. (**B**) TRAMP-

C2Re3 cells were injected into the prostates of $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice and tumor weight determined after 30 days ($TK^{f/f}$, n=15; $TK^{f/f}$LysMcre+, n=13; *P<0.001). Data represent two independent experiments. (**C**) FACS analysis for AnnexinV/PI on epithelial cells isolated from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice 23 days post-tumor cell implantation ($TK^{f/f}$, n=4; $TK^{f/f}$LysMcre+, n=4; *P<0.01). (**D**) Quantification for F4/80 on TRAMP-C2Re3 tumor sections from $TK^{f/f}$ and $TK^{f/f}$LysMcre+. Data represented as mean $F4/80^{+}$ cells per 40X field. ($TK^{f/f}$, n=5; $TK^{f/f}$LysMcre+, n=5; *P<0.05). (**E**) qRT-PCR for iNOS expression in TRAMP-C2Re3 tumors from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice. (**F**) Western analysis of pSTAT3-Y705 and Total STAT3 in TAMs isolated from TRAMP-C2Re3 tumors from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice. (**G**) Western analysis of arginase-1 expression in TAMs isolated from TRAMP-C2Re3 tumors from $TK^{f/f}$ and $TK^{f/f}$LysMcre+ mice. **F** and **G** are representative of four independent TAM isolations.

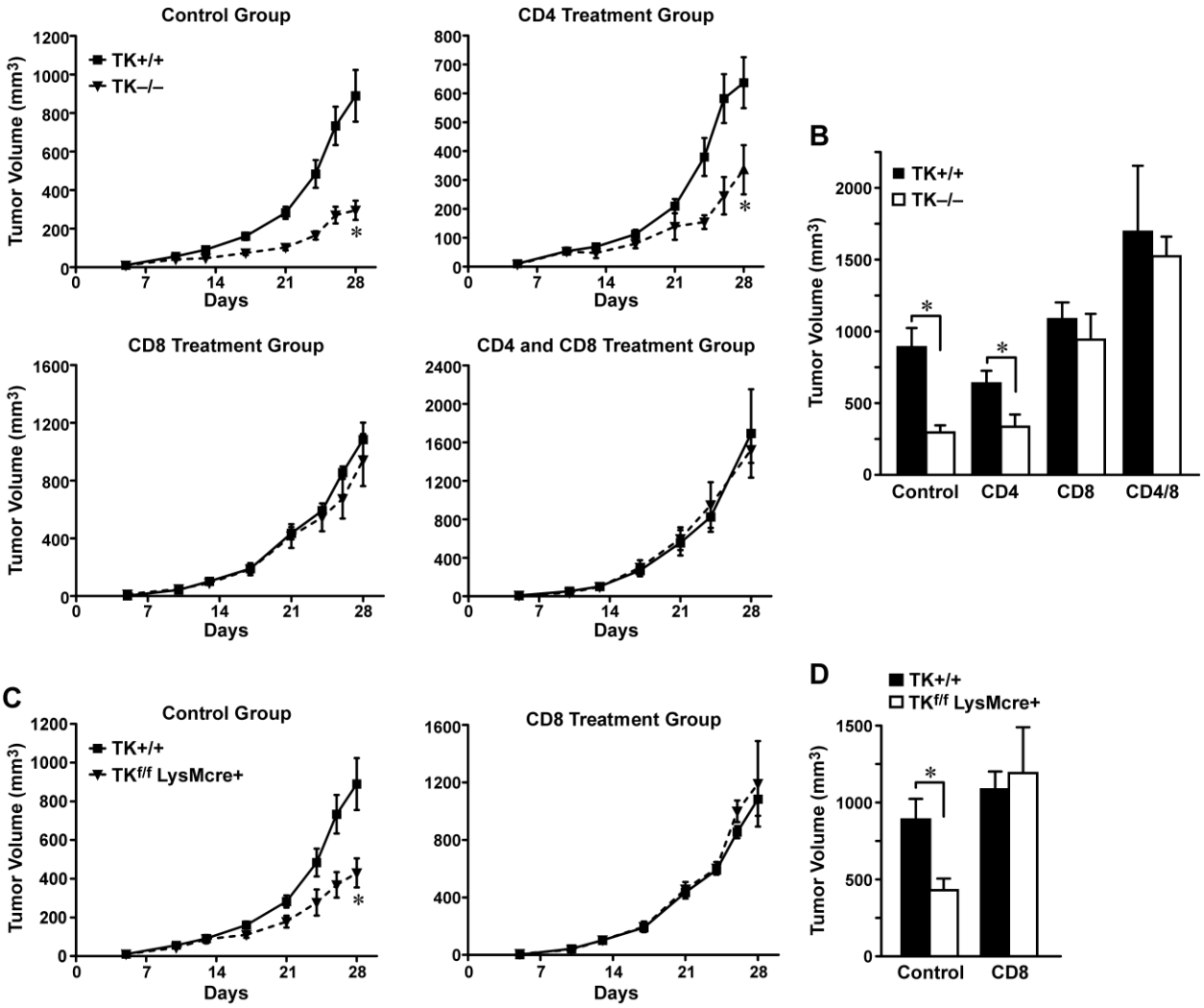
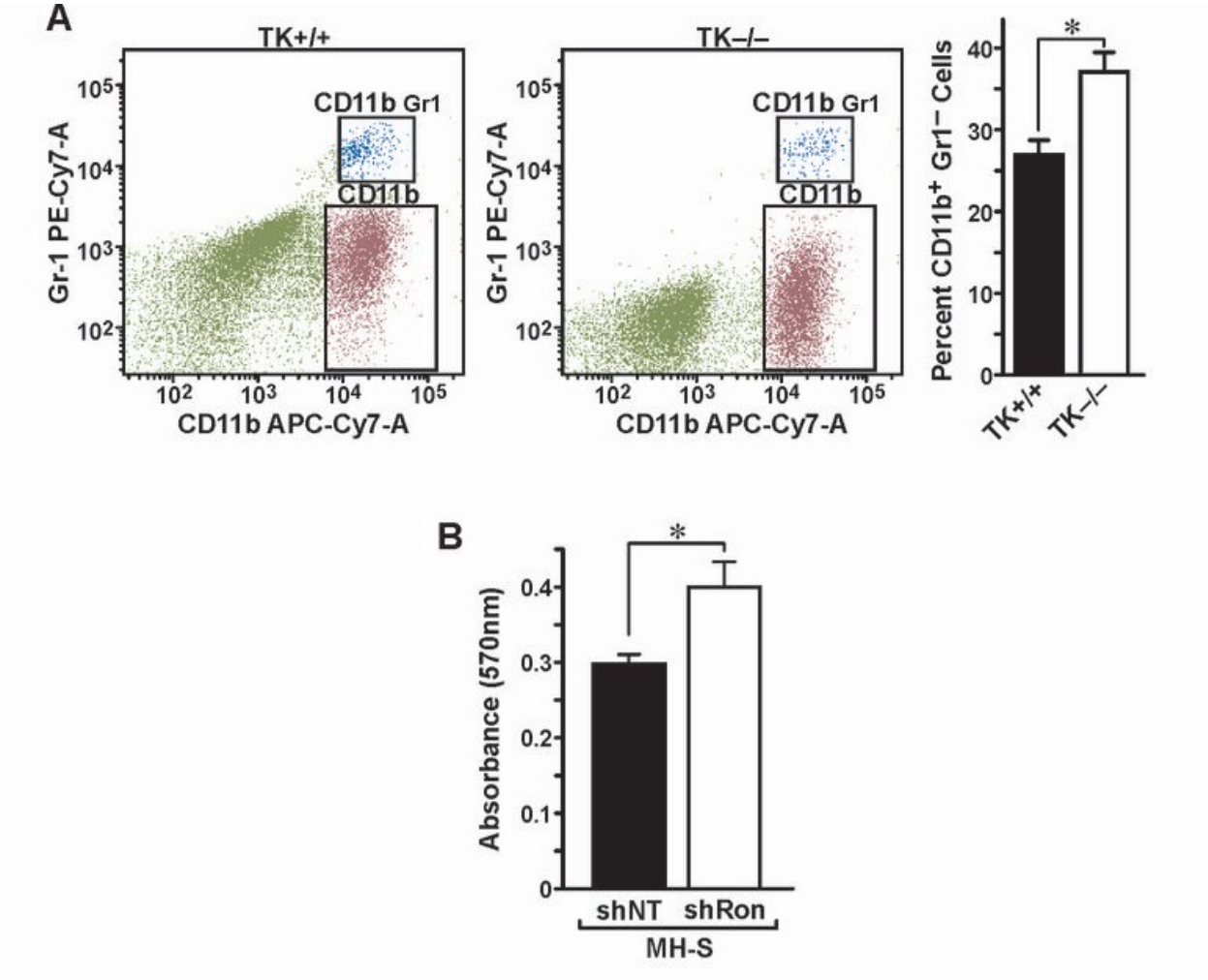


Figure 7. Depletion of CD8+ T-cells restores TRAMP-C2Re3 tumor growth in Ron-deficient mice.

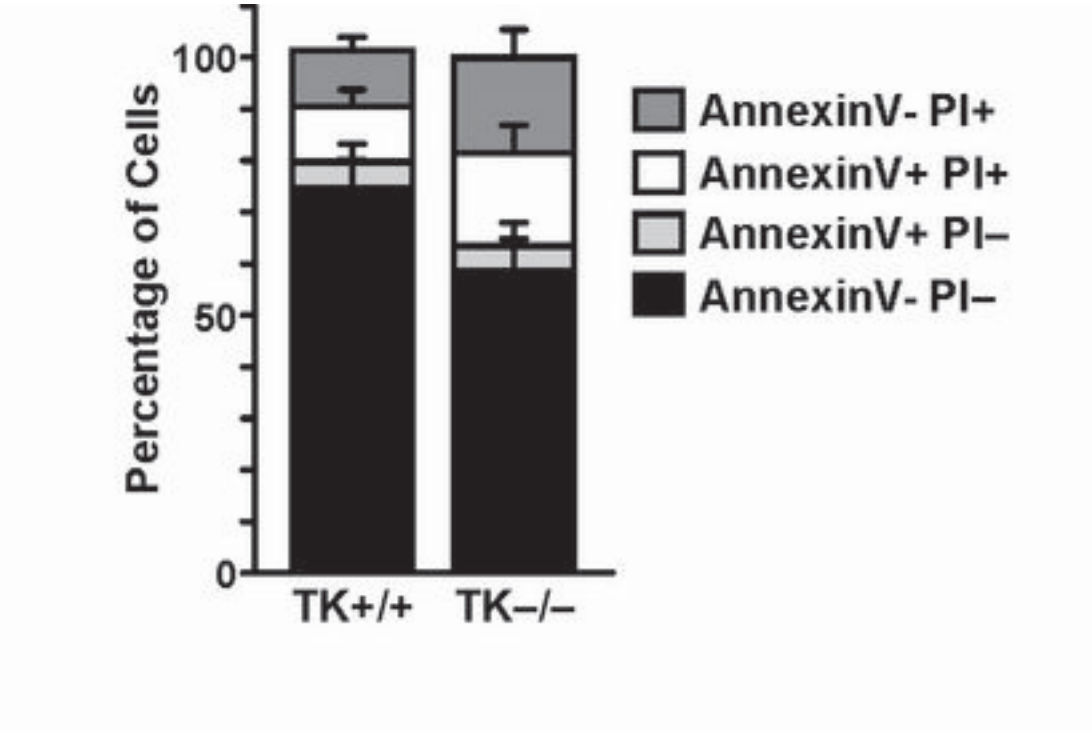
(**A**) Growth curve of subcutaneously injected TRAMP-C2Re3 cells in TK+/+ and TK-/- mice administered with cytotoxic monoclonal antibodies against CD4+ T-cells, CD8+ T-cells, both T-cell subsets, or an isotype control (n=3 per genotype/group). (**B**) Final tumor volume of TRAMP-C2Re3 cells from TK+/+ and TK-/- mice at 28 days post injection. (**C**) Growth of TRAMP-C2Re3 cells in control and $TK^{f/f}$LysMcre+ mice treated with cytotoxic monoclonal antibodies against CD8+ T-cells or an isotype control (n=3 per genotype/group). (**D**) Final tumor volumes from control and $TK^{f/f}$LysMcre+ mice at 28 days.

Supplemental Figures



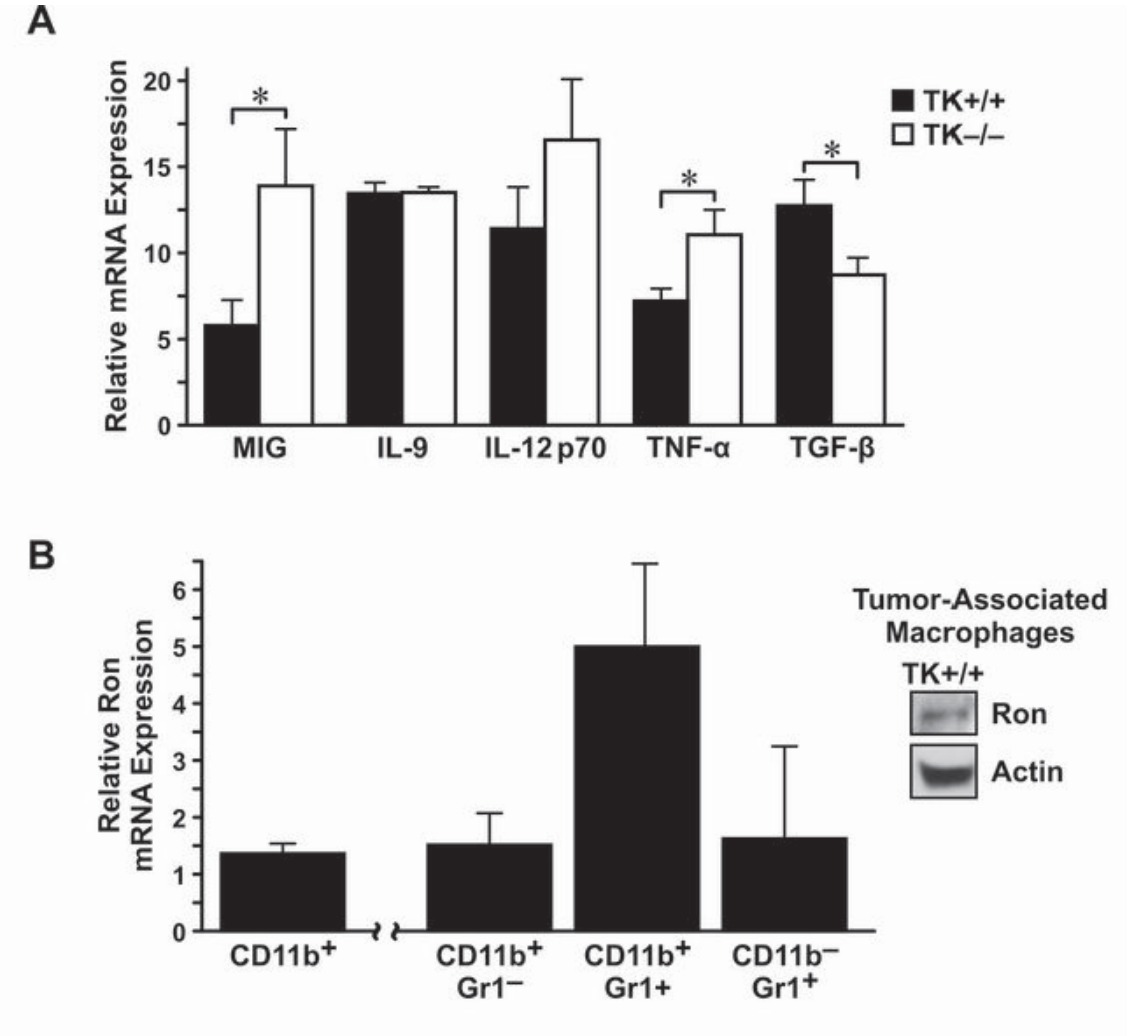
Supplementary Figure S1. Loss of Ron increases the migratory potential of macrophages *in vivo* and *in vitro*.

(**A)** FACS analysis of immune cell infiltrates into TRAMP-C2Re3 tumors harvested 30 days post intraprostatic injection. TRAMP-C2Re3 tumors isolated from TK+/+ mice had significantly less infiltrating macrophages ($CD11b^{+}Gr1^{-}$) compared with tumors isolated from TK-/- mice (TK+/+, n=7; TK-/-, n=5, *P<0.01). Data are represented as the mean percentage of $Gr1^{-}CD11b^{+}$ macrophages from two individual experiments. (**B**) Migration of MH-S control (NTsh) and Ron knockdown (Ronsh) cells towards 10%FBS for 48hours. Migration of MH-S Ronsh cells was significantly increased compared to the control cells.



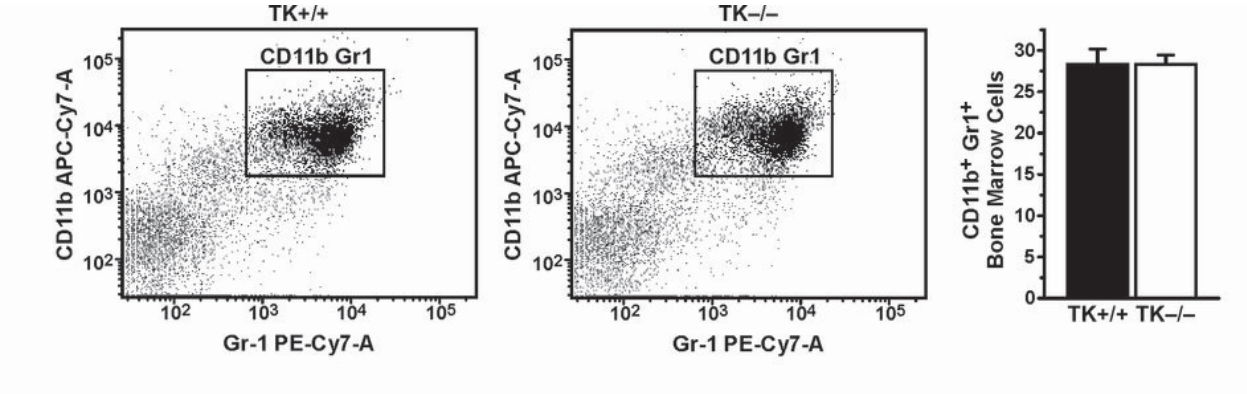
Supplementary Figure S2. Ron loss in the tumor microenvironment increases macrophage cell death.

Apoptotic tumor cell death was assessed on macrophages (F4/80+ cells) isolated from TRAMP-C2Re3 tumors from TK+/+ and TK-/- mice. Data are represented as the mean percentage of cells in early apoptosis (AnnexinV$^+$ PI$^-$), late apoptosis (AnnexinV$^+$ PI$^+$), dead cells (AnnexinV$^-$ PI$^+$), and live/viable cells (AnnexinV$^-$ PI$^-$) ± SE.



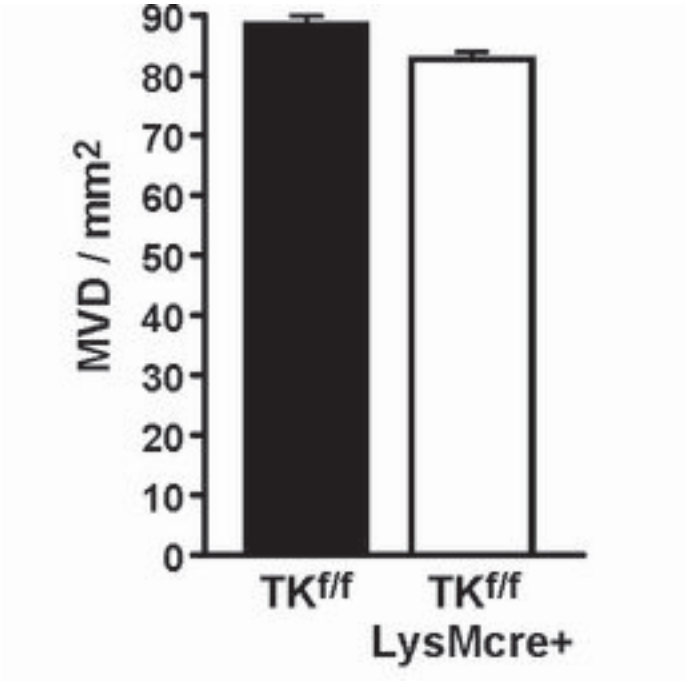
Supplementary Figure S3. qRT-PCR analysis of cytokines in the tumor proper and Ron expression in purified cell populations.

(A) qRT-PCR analyses for MIG, IL-9, IL-12p70, TNF-α and TGF-β mRNA expression in TRAMP-C2Re3 tumors from TK+/+ and TK-/- hosts. Data are represented as the relative change in mRNA levels. (TK+/+, n=7; TK-/-, n=7, *P<0.01). (**B**) qRT-PCR analysis for Ron mRNA expression on CD11b+ cells isolated by magnetic bead pull down assays and by cell sorting of CD11b+Gr1-, CD11b+Gr1+ and CD11b-Gr1+ cells from TRAMP-C2Re3 tumor from TK+/+ mice. Inset shows Western analysis for Ron in CD11b+ cells from TRAMP-C2Re3 tumors from TK+/+ mice.



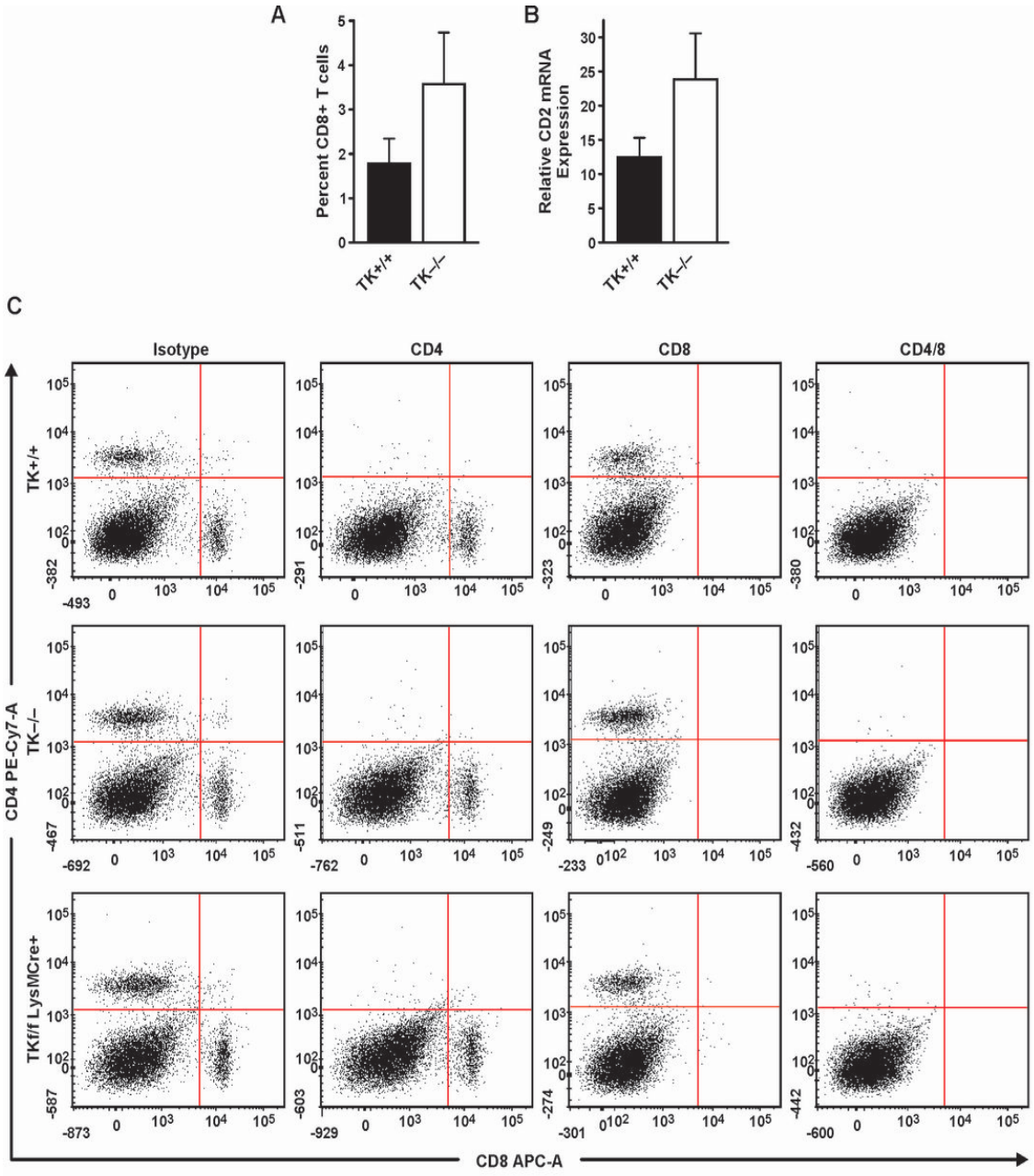
Supplementary Figure S4. FACs analysis of bone marrow derived cells from TK+/+ and TK-/- mice.

FACS analysis of bone marrow cells harvested from the femurs of TK+/+ and TK-/- mice showed no appreciable difference in the numbers of $CD11b^{+}Gr1^{+}$ cells.



Supplementary Figure S5. Microvessel staining in tumors isolated from $TK^{f/f}$ and $TK^{f/f}$ LysMcre+ mice.

Histogram depicting quantification of CD31 microvessel density in TRAMP-C2Re3 tumors from $TK^{f/f}$ and $TK^{f/f}$ LysMcre+ mice.



Supplementary Figure S6. Quantification of T-cells in tumors and spleens.

(**A**) Flow analysis of CD8+ T-cells in TRAMP-C2Re3 tumors from TK+/+ and TK-/- mice. (**B**) qRT-PCR analysis for CD2 mRNA expression in TRAMP-C2Re3 tumors from TK+/+ and TK-/- mice. **(C)** Flow cytometry profiles of spleen cells from TK+/+, TK-/- and $TK^{f/f}$ LysMcre+ mice, administered cytotoxic monoclonal antibodies against CD4+ T-cells, CD8+ T-cells, both CD4+ and CD8+ T-cells or an isotype control antibody (n=3/genotype/treatment group).

Supplemental Methods:

Bone Marrow Transplantation. For bone marrow transplantation experiments, whole bone marrow cells were isolated from the femurs and tibias of TK+/+ and TK-/- male mice by low-density centrifugation using a Histopaque-1077 density gradient (Sigma Aldrich, St. Louis, MO). Mice were irradiated with 7 Gy of ionizing radiation followed by 4.75 Gy of ionizing radiation three hours later. 5×10^6 mononuclear whole bone marrow cells (donor cells) were injected into the tail vein of irradiated mice. The chimeric mice were fed for 4 weeks following bone marrow reconstitution with normal chow containing doxycycline (200 mg/kg) to minimize the risk of infection and the mice were used for intraprostatic injections 4 to 6 weeks post reconstitution.

Primary Cell Isolations. A single-cell suspension of freshly isolated explants of prostate tumor tissue or normal prostates from mice tissue was obtained by mechanical dissociation and enzymatic digestion. Briefly, the tissue was minced into small pieces and enzymatically digested with collagenase IV and protease XIV. The digestion was carried out on a magnetic stirrer at 37^0C for 1 to 2 hours. The cell suspension was then enriched for the epithelial and fibroblastic cells as well as the immune cells by differential centrifugation at 120g for 5min at 4^0C. The supernatants were enriched in immune cells while the pellets were enriched with epithelial and fibroblastic cells. The supernatant obtained from the differential spin was centrifuged at 800g for 11min at 4^0C to pellet the tumor-infiltrating immune cells. The cells were resuspended in plain RPMI medium and strained through a 40µm filter to remove cell clumps. The resulting immune cell-enriched cell fraction was subsequently utilized for magnetic bead separation or flow cytometric analysis. For the isolation of the epithelial and fibroblastic fractions, the pellet obtained from the 120g spin was resuspended in 1X PBS and pulse spun at 1200rpm for 15s, the supernatant removed, and the spins repeated an additional 6 times. The supernatant removed from each of these differential spins was enriched for fibroblasts. The resulting pellet at

the end of the pulse spins contained the epithelial cells. Fibroblasts were then pelleted from the supernatants of the differential spins. The cells were plated at 37^{o}C in DMEM containing 10% FBS. The morphology of these cells was assessed by light microscopy and by staining for cell surface markers (Pan cytokeratin for epithelial cells and F4/80 for macrophages) prior to being utilized for further experiments.

Immunohistochemistry and Immunocytochemistry.

Prostate tumors harvested at the various experimental time points were fixed in 10% formalin and then paraffin embedded. The tissue sections were deparaffinized and rehydrated. Antigen retrieval was carried out using citrate buffer or with proteinase K, depending on the nature of the antigen being detected. The sections were then incubated in 0.5% hydrogen peroxide in methanol for 15min to block endogenous peroxidase activity. The sections were incubated with the primary antibody at room temperature overnight or for 2hrs. The slides were washed in 1X PBS and labeled for 1hr at RT with 1:200 dilution of biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The slides were washed in 1X PBS and incubated in ABC (Vector Laboratories, Burlingame, CA) for 30min. The sections were then developed for color using 3-3'-diaminobenzidine or by using Vector Nova red stain (Vector Laboratories).

In vitro migration assay (modified Boyden chamber migration assay).

Migration of mouse alveolar macrophages, MH-S cells, was measured in a modified Boyden chamber migration assay using transwell inserts with a 5 μm porous membrane (Corning costar corporation, MA). Stable Ron proficient (MH-S shNT) and Ron deficient (MH-S shRon) cell lines were generated as described previously and used for our experiments (51). The migration of cells towards 10% FBS was determined after 48 hours and the number of live cells migrating towards the bottom of the transwell was measured using the 3-(4,5 – dimethythiazol)-2,5 diphenyltetrazolium bromide assay according to the manufacturer's instructions (Sigma, MO) with absorbance read at 570nm.

Real time PCR primer sets PCR primer sets utilized are as follows:

mGus: 5'TTGAGAACTGGTATAAGACGCATCAG3' and

5'TCTGGTACTCCTCACTGAACATGC3';

Ron: 5'TCCCATTGCAGGTCTGTGTAGA3' and 5'CGGAAGCTGTATCGTTGATGTC3';

iNOS: 5'TGCCCCTTCAATGGTTGGTA3' and 5'ACTGGAGGGACCAGCCAAAT3';

iNOS: 5'GTTCTCAGCCCAACAATACAAGA3' and 5'GTGGACGGGTCGATGTCAC3';

IL-12p40: 5'ACCTGTGACACGCCTGAAGAAGAT3' and

5'TCTTGTGGAGCAGCAGATGTGAGT3';

CD80: 5'CTCTTTGTGCTGCTGATTCG3' and 5'GGCAAGGCAGCAATACCTTA3'

CD80: 5'ACCCCCAACATAACTGAGTCT3' and 5'TTCCAACCAAGAGAAGCGAGG3';

STAT1: 5'TCCCGTACAGATGTCCATGAT3' and 5'CTGAATATTTCCCTCCTGGG3';

ICOS: 5'TGACCCACCTCCTTTTCAAG3' and 5'TTAGGGTCATGCACACTGGA3';

IL-12b: 5'TGGTTTGCCATCGTTTTGCTG3' and 5'ACAGGTGAGGTTCACTGTTTCT3';

1L-27: 5'CTGTTGCTGCTACCCTTGCTT3' and 5'CACTCCTGGCAATCGAGATTC3';

CXCR3: 5'TACCTTGAGGTTAGTGAACGTCA3' and 5'CGCTCTCGTTTTCCCCATAATC3';

TNF-α: 5'CATCTTCTCAAAATTCGAGTGACAA3' and 5'CTCCAGCTGCTCCTCCACTT3';

M-CSF: 5'TTGGCTTGGGATGATTCTCAG3' and 5'GCCCTGGGTCTGTCAGTCTC3';

TGF-β: 5'GGTGTGAACTGTCACCGATCA3' and 5'GTTTAGGATGTGAACCTCCCTTG3';

MIG: 5'GGAGTTCGAGGAACCCTAGTG3' and 5'GGGATTTGTAGTGGATCGTGC3';

IL-9: 5'CTCCTGGGCATGACGTTGATT3' and 5'GCATGGCTCTCTTGATCTCGT3';

IL-17A: 5'TTTAACTCCCTTGGCGCAAAA3' and 5'CTTTCCCTCCGCATTGACAC3'.

Chapter 3 – Clinical Implication

RON RECEPTOR TYROSINE KINASE – SWITCH FOR THE REGULATION OF TUMOR IMMUNITY IN MACROPHAGES

Devikala Gurusamy[1] **and Susan E. Waltz**[1,2]

[1]Departments of Cancer and Cell Biology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0521; and [2] Cincinnati Veterans Affairs Medical Center, Cincinnati, OH.

Abstract

The tumor and surrounding microenvironment severely limit the efficacy of various immunotherapies. Recent discoveries by a number of investigators in both mouse models of cancer and humans with cancer have offered evidence that specific immune cell types, effector molecules, and pathways collectively function as extrinsic factors that support tumor progression. Macrophages are components of the innate immune system and play a major role in several aspects of tumor immunity including the processing and presentation of tumor antigens to T-cells, which are then activated to eliminate the tumor cells. However, as the tumors develop they create a microenvironment that not only supports tumor growth and metastasis but also reduces potential adaptive immunity to tumor antigens. We have demonstrated that the Ron receptor tyrosine kinase is a key oncogenic factor that is activated in cancer cells and is important for cancer cell survival. Ron is also expressed on tumor stromal immune cells and myeloid expression of Ron has been shown to be immunosuppressive. Our published studies show that Ron ablation in myeloid cells promotes cytotoxicity by CD8+ T lymphocytes and mediates effective anti-tumor immune responses. Herein we sought to determine if tumor immunity may be reprogramed through the presentation of Ron depleted macrophages. To accomplish this, we show a diminution of tumor growth in mice containing tumor cells transplanted subcutaneously following injection of Ron deficient macrophages. We will discuss our recent findings related to these tumor-associated macrophages and how targeting Ron/MST1R in macrophages may serve to generate a more durable anti-tumor immune response.

Introduction

Macrophages are among the most versatile cells of the immune system and can express different functional programs in response to microenvironmental signals. They exhibit pleiotropic biological roles including target cell cytotoxicity, removal of cellular debris and tissue remodeling

as well as the regulation and induction of anti-tumor immunity (117, 140-143). They are antigen presenting cells [APC] that express HLA class I and class II receptors along with co stimulatory / inhibitory molecules capable of initiating T cell responses, although at a lesser efficiency than dendritic cells (144). Tumor Associated Macrophages [TAMs] represent a major constituent of the tumor inflammatory infiltrates and are one among the early cells of the immune system to infiltrate precancerous lesions and continue to endure throughout the course of development into invasive carcinoma (145). TAMs can either function to suppress or promote tumor growth depending on their functional activation state (117). They represent a heterogeneous population and support to the multifaceted needs of the growing tumor (146). The tumor type and stage of development, as well as the local signals within the tumor microenvironment influence their activation state and hence a thorough understanding of the complex factors that regulate their pro- or anti-tumor affects are necessary.

TAMs exhibit a continuum of polarization states and their functional aspects range from what is known as classically activated macrophages [M1 polarized], associated with acute inflammation and T cell mediated anti-tumor immunity, to alternative activated [M2 polarized] state that is associated with wound healing and tumor promoting responses. The M1 polarized TAMs are characterized by elevated expression of cytotoxic cytokines like *IL-12*, tumor necrosis factor α [*TNFα*] and nitric oxide [*NO*]. In contrast, the M2 polarized TAMs are characterized by the production of growth factors, cytokines, and proteinases (119, 142, 147-149). M2 polarized macrophages have been recently shown to be important during infections for the dampening of immune responses through the polarization of T cells to a Th2 phenotype, promoting parasitic clearance and wound healing (150). Moreover, TAMs that are alternatively activated have been documented to inhibit the proliferation of T cells, as well as exhibit defectiveness in antigen presentation to T cells (124, 139). Several immunotherapeutic strategies have been developed that neutralize factors responsible for polarization and/or recruitment of M2 polarized

macrophages to the tumors; approaches include the attraction and polarization of M1 macrophages to tumors and/or the reprogramming of M2 polarized TAMs to a more anti-tumor M1 polarized state. Such therapies have been relatively ineffective in repolarizing macrophages to a M2 state or mount an effective anti-tumor T cell response. Additionally delivery of the drugs to the TAMs that were localized at the tumors away from the blood vasculature was challenging (151, 152). Therefore, it is necessary to gain a better understanding of the mechanisms that regulates TAMs and TAM mediated T cell responses for targeted therapy.

The Ron receptor tyrosine kinase is an oncogene that is expressed on epithelial cells and terminally differentiated macrophages, but not on mononuclear phagocytes or circulating monocytes; Ron expression is upregulated during macrophage differentiation (22). Ron is expressed on several tissue resident macrophage populations including alveolar, dermal, peritoneal macrophages and microglial cells of the brain (22, 23, 25, 28, 51, 52). The ligand for Ron, hepatocyte growth factor like [HGFL], was initially identified as a serum factor capable of causing macrophage chemotaxis and activation (8). Since its discovery, several studies have documented the importance of the Ron/HGFL pathway in the regulation of inflammatory responses during an inflammatory insult, including the initiation of macrophage activation as well as a its key role in the attenuation of the inflammatory responses to promote tissue repair and healing (68, 91, 153). *In vitro*, HGFL stimulation of Ron in macrophages is sufficient to polarize macrophages from M1 to a M2 polarized state (68, 71, 153). Studies from our laboratory and others have also shown that Ron activation by HGFL in macrophages diminishes LPS induced NF-κB activation and subsequent expression of proinflammatory chemokines/cytokines (42, 47, 48). Ron has also been shown to be a positive regulator of the phosphorylation of the signal transducer and activator of transcription 3 [STAT3] (54, 154), whose signaling in macrophages has been shown to be immunosuppressive during both infection and tumor progression (125). *In vivo,* mice lacking the tyrosine kinase signaling domain

of Ron (TK-/-) are more susceptible to endotoxic shock and produce elevated levels of proinflammatory cytokines including TNFα, IL-6, IFNγ and IL-12 suggesting its key role in the attenuation of infection (50, 51, 58). Taken together, HGFL/Ron signaling in macrophages is important for regulation of inflammation and suggests its potential role in TAMs in regulation of the production pro-tumorigenic factors by the regulation of macrophage immunity.

Recent studies from our laboratory and others have uncovered the importance for Ron signaling in the stromal cells of the tumor microenvironment in supporting tumor growth, in addition to its known oncogenic properties in tumor epithelial cells (72, 75). Our study has confirmed that Ron receptor expression in the tumor stromal cells also regulates the growth of tumors. Our work has confirmed that Ron expression in the hematopoietic compartment, specifically the myeloid cells, is important for the suppression of antitumor immunity in part by attenuating cytotoxic T lymphocytic activity (Gurusamy et al, in press Cancer Research). Our study has provided insights in the potential mechanisms by which CD8+ T lymphocyte activity is suppressed by Ron expression in macrophages, which may include inefficiencies in antigen presentation, induction of T cell anergy, through mechanisms that regulate the number and function of CD8+ T effector lymphocytes.

Based on these results, it is imperative to understand the ability of the Ron modulated macrophages to sustain and activate the immune response in the presence of the Ron derived TAMs present in the tumor bearing patient. In this chapter, we test our hypothesis that Ron deficient macrophages function in a dominant negative fashion to attenuate tumor growth. Based on our previous observations that Ron TK-/- myeloid cells exert increased cytotoxic T cell stimulatory activity and a more pronounced inflammatory anti-tumor environment, we will test this hypothesis by evaluating the ability of Ron TK-/- mouse macrophages to function as an anti-tumor vaccine in tumor bearing TK+/+ animals.

Materials and methods:

Mouse Lines: Wild-type (TK+/+) and Ron tyrosine kinase-deficient mice (TK-/-) were generated and maintained in a C57BL/6 background as described (26, 42). All experiments utilized male mice between 8 to 16 weeks of age. All mice were maintained under specific pathogen-free conditions and were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Cell Lines: Murine prostate cancer cell line TRAMP-C2Re3 were obtained from Dr. Zhongyun Dong (University of Cincinnati, Cincinnati, OH) and grown as previously described (126).

Isolation of mouse peritoneal macrophages: Mouse peritoneal macrophages were harvested from TK+/+ and TK-/- mice. Briefly, the mice were sacrificed and 10mls of ice cold Phosphate bufferd saline (PBS) was injected into the peritoneal cavity of the mouse. The peritoneal lavage fluid was carefully collected and the cells pelleted after centrifugation at 800g for 8min. The supernatant was discarded and the cell pellet was resuspended in 10% FBS DMEM media and allowed to adhere to tissue culture grade plates for an hour. The cells were washed with PBS to remove the non-adherent cell populations. The adherent peritoneal macrophages were used as described below.

Macrophage Vaccination Experiment: A total of 5 x 10^5 TRAMP-C2Re3 cells was injected subcutaneously on to the back of 6-8 week old C57BL/6 mice on day 0. The tumors were allowed to establish for one week and tumor-bearing animals were randomly distributed into three treatment groups. Freshly prepared donor derived peritoneal macrophages from TK+/+ or TK-/- mice were counted and equal numbers were injected into tumor bearing mice subcutaneously close to tumor site on days 7, 10, 14, 17, 24 and 32. The size of the tumors was measured biweekly and the tumor volume was calculated. The animals were harvested on day 34.

Results

Cancer immunotherapy using Ron receptor tyrosine kinase modulated macrophages

Based on our studies, we postulated that Ron deficient macrophages could be used as a potential immunotherapeutic tool to stimulate anti-tumor immune responses in cancer. We hypothesized that vaccination with Ron tyrosine kinase deficient macrophages could induce potent anti-tumor effects in tumor bearing mice and suppress tumor growth. To test this, we performed biweekly injections of Ron proficient or Ron deficient macrophages into prostate tumor bearing wild type (TK+/+) mice. The treatment groups were distributed as follows:

Group 1 – received TK+/+ peritoneal macrophages isolated from Ron proficient WT donor mice;

Group 2 – received TK-/- peritoneal macrophages isolated from Ron tyrosine kinase deficient donor mice; and

Group 3 - Control saline injections.

The tumor weights harvested at the terminal end point indicated that Ron deficient macrophages were able to decrease tumor growth in wild type mice, with a remarkable decrease in tumor weight observed in animals that received Ron TK-/- macrophages (Figure 1). These data suggest that the donor derived Ron deficient macrophages are able to have a dominant negative effect compared to recipients Ron proficient macrophages. Moreover, our experiment indicates that the Ron deficient macrophages can be tested as a nonspecific adoptive cell transfer tool for the treatment of prostate and other cancers. Further experiments are to be performed to identify the use of Ron deficient macrophages from other sources like blood and bone, as this would be a more comparable source of macrophages from patients.

Discussion

Ron is constitutively activated in diverse cancers, promoting tumor cell survival, proliferation, cancer angiogenesis and metastasis. As shown in chapter 3, Ron proves to be a valuable target for tumor therapy as Ron inhibition has the potential to not only directly inhibit tumor growth but

also alter the tumor immunological environment in favor of immunotherapy. The experiments shown in this section provide proof of principle that Ron modulated macrophages can be used as immunotherapeutic tools in the treatment of prostate cancer. Our experiments also define the dominant negative characteristics of the macrophages that lack Ron. Our study listed here also provides insights for the treatment of non-Ron driven tumors by exploiting the ability of Ron deficient macrophages to elicit an anti-immune response. Thus with the emergence of Ron inhibitors, both direct and indirect, we are entering a new era of cancer immunotherapy.

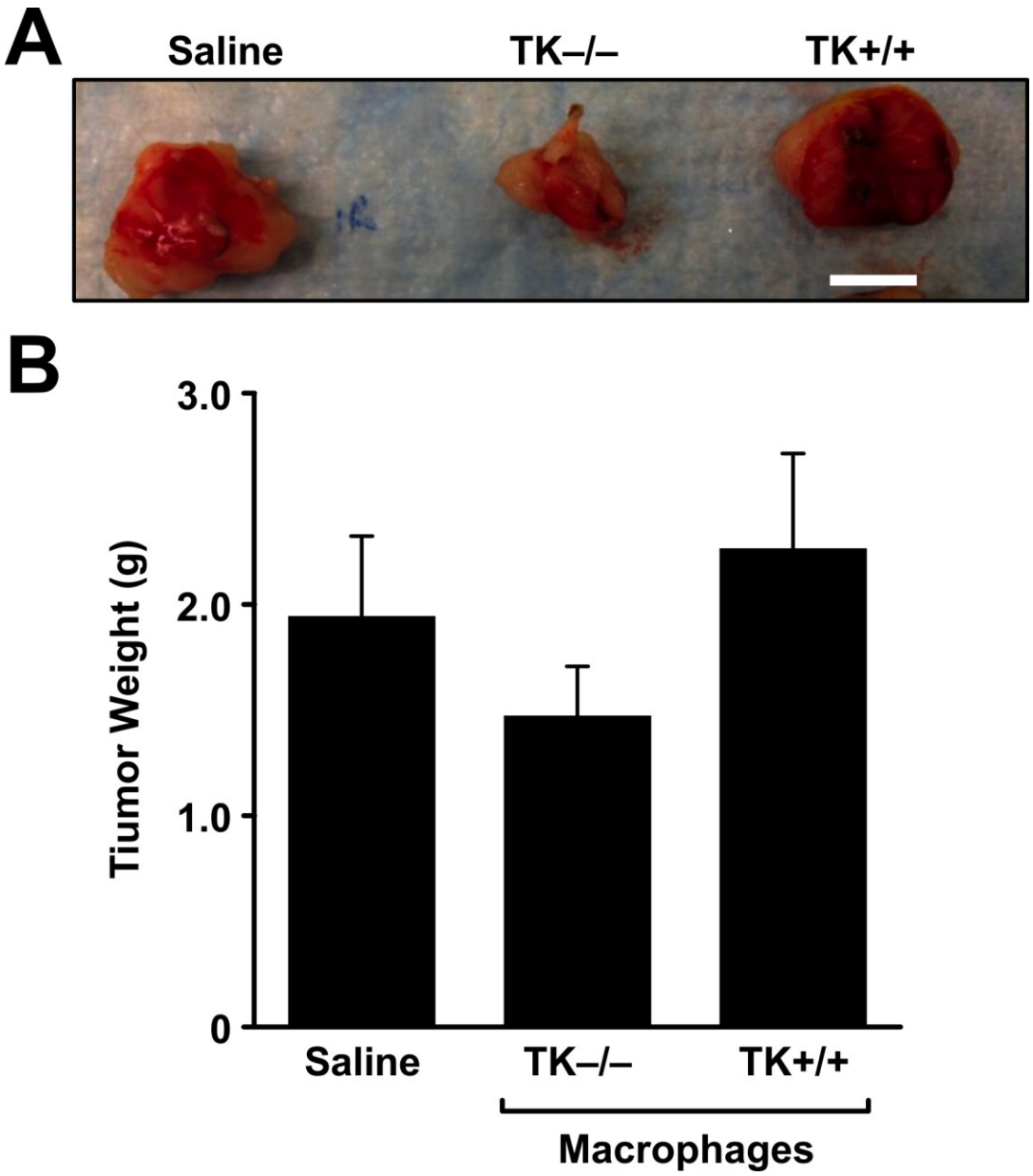


Figure 1: Ron modulated macrophages as immunotherapeutic targets

A. Representative images showing TRAMP-C2Re3 tumors from TK+/+ mice injected biweekly with saline, TK-/- peritoneal macrophages and TK+/+ peritoneal macrophages. **B.** TRAMP-C2Re3 tumor weights from TK+/+ mice injected biweekly with saline, TK-/- peritoneal macrophages and TK+/+ peritoneal macrophages; [n=4 mice in each group].

Chapter 4

FIBROBLAST SPECIFIC RON EXPRESSION SUPPORTS PROSTATE TUMOR GROWTH

Abstract

Tumors are complex tissues comprised of neoplastic cells and a variety of stromal cells, notably mesenchymal cells and inflammatory infiltrates. Tumor associated fibroblasts are mesenchymal cells present during the various stages of tumor development and their functional characteristics evolve with the needs of the tumor to aid in malignant tumor growth. These functions include secretion of extracellular matrix proteins, inflammatory cytokines, growth factors and angiogenic factors. The proteins and the molecular mechanism regulating the reactive functions of the tumor-associated fibroblasts have received limited attention and a better understanding of their role in the tumor microenvironment is needed. The Ron receptor tyrosine kinase is an oncogene that is expressed on neoplastic cells and is upregulated during tumor progression. In this chapter, we report the expression of Ron in fibroblasts isolated from normal and tumor bearing prostate and breast tissues. Furthermore, we show that the expression of this receptor in the fibroblasts is important for accelerating the growth of prostate tumors using an *in vivo* co-culture model system. Our study provides the first evidence for Ron expression in tumor-associated fibroblasts and their functional role in promoting tumor growth.

Introduction

Fibroblasts are abundant stromal cells that help in the formation of the structural framework of tissues through the production of extracellular matrix [ECM] components. Fibroblasts are important for maintaining normal tissue development and homeostasis and resting fibroblasts undergo activation during injury and promote wound healing. On the contrary, tumor-associated fibroblasts [TAF] are perpetually activated throughout the course of cancer progression where they initiate and promote tumorigenic alterations in epithelial cells [reviewed in (155) and (156)]. Understanding the factors that contribute to their activation and hence their effects on neoplastic transformation will aid in the development of treatment targets that can block both initial

neoplastic transformation as well as the establishment and growth of primary or metastatic tumors.

Stromal-epithelial interactions are important to prostate gland development and maintenance (115, 157). During tumor progression, the fibroblastic cells within the human prostate tissues are altered compared to the normal adjacent stroma and exhibit features of an activated stroma (158). The presence of the reactive stroma is observed as early as prostate intraepithelial neoplasia [PIN] lesions and they evolve during cancer progression to effectively replace the normal fibromuscular stroma present in the prostate tissue (115, 158, 159). An elegant study by Wang et al., demonstrated that benign prostatic hyperplastic [BPH] cell lines admixed with TAF were able to form carcinomas in mice compared to BPH co-culture with the normal fibroblasts, suggestive of the tumor inducing properties of the TAFs (160). Moreover, recent mouse models with genetically altered fibroblasts have demonstrated that specific genetic changes in resident fibroblasts are capable of inducing neoplastic transformation in a tissue-specific manner. One such mouse model that is of considerable importance includes mice that have a fibroblast-specific deletion of transforming growth factor beta receptor II [TGFβRII]. These mice developed intraepithelial neoplasia in the prostate tissues and invasive carcinoma in the forestomach (161). Further studies on the TGFβRII knockout fibroblasts revealed that these fibroblasts produced the Ron and cMet ligands HGFL and HGF respectively, which were then capable of inducing tumorigenic signals in Ron and cMET expressing epithelial cells (39, 162). Additionally, reciprocal bone marrow transplantation experiments in mice have shown that Ron expression in both hematopoietic and non-hematopoietic stromal cells is important for supporting prostate tumor growth [Chapter 3, Figure 5; Gurusamy et al, Cancer Research 2013; 73: 1752-1763]. Based on the characterization of Ron expression in stromal cellular compartments of normal prostates, fibroblasts were one such non-hematopoietic stromal cell type where Ron expression was detected. [Chapter 3, Figure 1A; Gurusamy et al, Cancer

Research 2013; 73: 1752-1763]. Investigating the importance of the HGFL/Ron signaling axis in fibroblasts for supporting tumor growth is important so we have a complete understanding of the role of Ron in fibroblasts. In this chapter, we discuss our preliminary results outlining the importance for Ron expression in fibroblasts in supporting prostate tumor growth.

Materials and methods

Mice. Wild-type [TK+/+], Ron tyrosine kinase-deficient mice [TK-/-] were generated and maintained in a C57BL/6 background as described (26, 42). All experiments utilized male mice between 8 to 12 weeks of age. Tumors for our studies were obtained from prostate tumor bearing TRAMP+ TK+/+ mice in the C57BL/6 background (89) and probasin-Ron mice in the FVB background (163) The mammary tumors were obtained from FVB MMTV-Ron mouse tumors (85). All mice were maintained under specific pathogen-free conditions and were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Isolation of normal and tumor associated fibroblasts. A single-cell suspension of freshly isolated explants of normal or tumor bearing prostate or breast tissue was obtained by mechanical dissociation and enzymatic digestion. Briefly, the tissue was minced into small pieces and enzymatically digested with collagenase IV and protease XIV. The digestion was carried out on a magnetic stirrer at 37^0C for 1 to 2 hours. The single cell suspension was then enriched for the epithelial and fibroblastic cells by differential centrifugation at 120g for 5min at 4^0C. The pellet enriched for the epithelial and fibroblastic cells was resuspended in 1X PBS and used for subsequent isolation of fibroblastic cells. The cell suspension was pulse spun at 1200rpm for 15s, the supernatant removed, and the spins repeated an additional 6 times. The supernatant removed from each of these differential spins was enriched for fibroblasts and these cells were cultured or used for further experiments.

Isolation primary Mouse Embryonic Fibroblasts [MEFs] Embryonic day 13.5 embryos were isolated from the uterine horn of pregnant TK+/+ and TK-/- mice and placed in 1X PBS containing antibiotics. The embryos were then isolated from the sac, the heads decapitated and the visceral organs were removed. The embryo was gently minced and trypsinzed for 45min in 0.25% Trypsin EDTA solution at 37^{o}C in a 5% CO_2 incubator. The homogenized embryos were then plated in MEF culture media comprising of DMEM containing 10% Fetal Bovine Serum plus penicillin and cultured in a T75 flask at 37^{o}C in a 5% CO_2 incubator. The cells were used for further experiments within 3 passages.

Western Analysis Cultured primary cells was harvested in protein lysis buffer and subjected to western analysis as per standard procedures using Ron antibody [Santa Cruz Biotechnologies, Santa Cruz, Santa Cruz, CA] and Actin antibody [CCHMC, Cincinnati, OH].

***In vivo* co-culture of MEFs with Tumor cells** TRAMP-C2Re3 cells [2.5 x 10^5] were injected subcutaneously into C57Bl/6 mice alone or admixed with [0.75 x 10^6] early passage primary TK+/+ or TK-/- MEFs. The cells were resuspended in plain DMEM media and injected into syngeneic TK+/+ mice. Animals were harvested on day 34 and the tumor weight was measured.

Statistical Analysis: Unpaired Student's t test [two tailed] analysis was used to determine statistical significance using the Graph Pad Prism software [Graph Pad Software, Inc. La Jolla, CA]. Differences were considered significant at $P < 0.05$.

Results

Ron is expressed in fibroblasts isolated from normal and tumor bearing tissues.

Ron expression was characterized by western analysis on fibroblasts isolated from normal and tumor-bearing wild type prostate and mammary tissues. Ron expression was detected in both normal and tumor-associated fibroblasts isolated from both breast and prostate tumors from different mouse backgrounds [**Figure 1A]**.

Ron expression in fibroblasts increases the in vivo growth of prostate cancer cells

To test the hypothesis that Ron expression in fibroblasts is important for supporting tumor growth, we performed *in vivo* co-culture experiments using TRAMP-C2Re3 and MEFs isolated from Ron proficient [TK+/+] and Ron deficient [TK-/-] mice. As shown in **Figure 4B**, the growth of the prostate cancer cells was enhanced in the presence of the MEFs compared to the tumor cells alone. Our results demonstrated that Ron expression in the MEFs enhanced the growth of the prostate cancer cells compared to growth of TRAMP-C2Re3 cells in the presence of TK-/- MEFs.

Discussion

Our study suggests an important functional role for Ron in fibroblasts in regulating tumor growth *in vivo*. *In vitro* co-culture experiments involving direct contact of TK+/+ and TK-/- MEFs with tumor cells or the use of fibroblast conditioned media on tumor cells are currently ongoing in our laboratory and our preliminary results indicate that loss of Ron in fibroblasts leads to inhibition of the growth of both mammary and prostate cancer cells [data not shown]. A recent study published in cancer cell by Erez et al. showed that cancer associated fibroblasts have an inflammatory gene signature regulated by NF-κB expression and this affected multiple biological processes, including macrophage recruitment, neovascularization and tumor growth (164).

Consistent with the observation in TAFs, recent work by Tong et al. showed that Ron expression in the synovial fibroblasts of rheumatoid arthritis patients is important for the induction of inflammatory genes (165). Given that Ron regulation has been shown to promote NF-κB activation in different cell types (26, 52, 89, 90, 166), evaluating the importance for Ron expression in TAFs and its potential role in TAF-induced inflammation and tumor progression is essential for our understanding of TAF biology. In summary, our data suggest that Ron may be a major player in the regulation of TAF functions and requires further investigation.

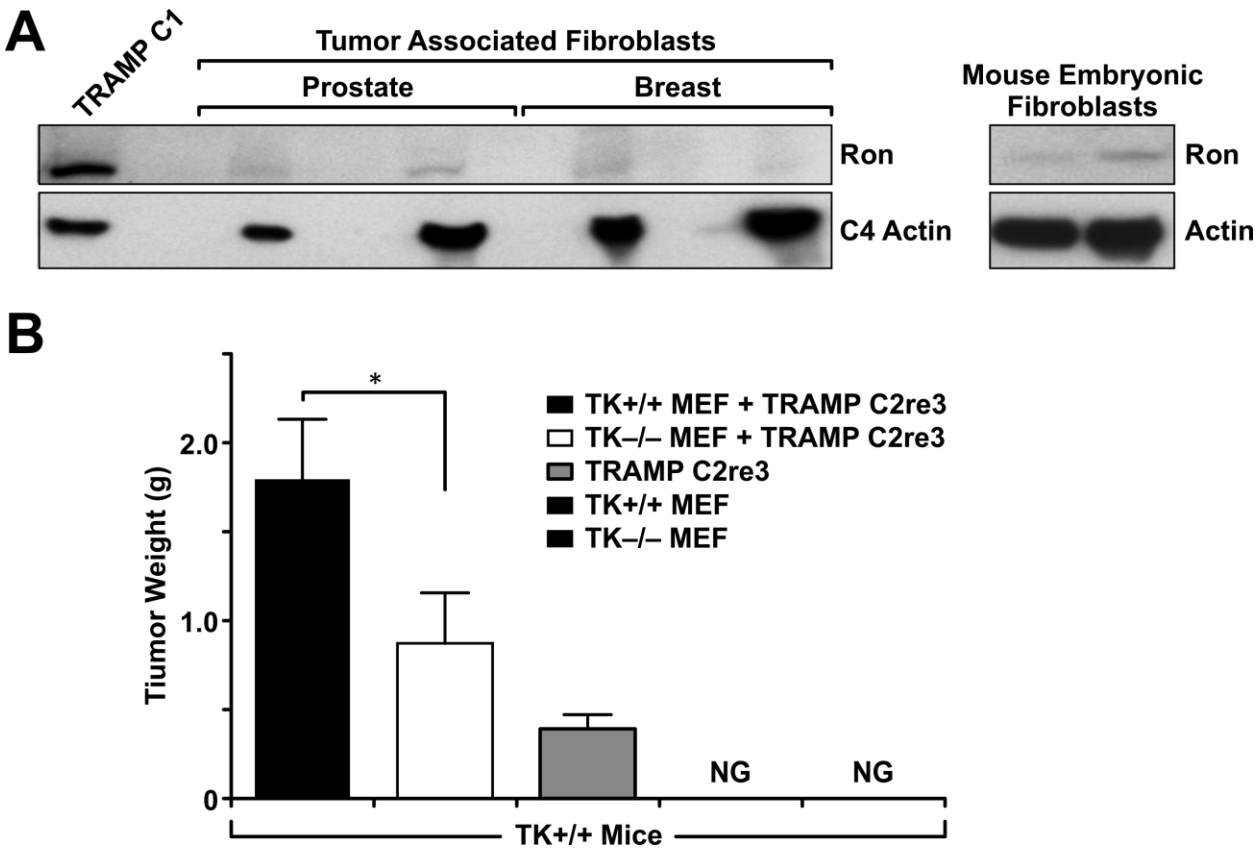


Figure 1. Ron is expressed in tumor-associated fibroblasts and fibroblast Ron expression is important for promoting tumor growth *in vivo.*

A. Ron expression in murine tumor associated fibroblasts isolated from prostate and breast tumors. Ron is also expressed on mouse embryonic fibroblasts isolated from TK+/+ mice. **B.** *In vivo* tumor weights of TRAMP-C2Re3 cells co-cultured with TK+/+ and TK-/- mouse embryonic fibroblasts. *In vivo* tumor weights of TRAMP-C2Re3, TK+/+ and TK-/- MEFs are shown for reference.

Chapter 5

CONCLUSIONS AND FUTURE DIRECTIONS

Prostate cancer is the second leading cause of cancer related death in men in the United States with an estimated 241,740 cases diagnosed in 2012 alone. Although, recent advances in research has led to better treatment and clinical prognosis when diagnosed during the earlier stages of the disease, no effective cure is available for the advanced and metastatic disease stages; the 5 year survival rate is less than 29% in patients with metastatic disease (167). Much of the advanced stages of the disease are characterized by metastasis to the bones and the adjoining lymph nodes and current therapies have very short benefits if any. While much research progress has been made in identifying oncogenic and oncogenic signaling pathways in neoplastic cells for therapeutic targeting, very little is known about the molecular signaling network that governs the cells of the tumor microenvironment in regulating tumor growth. The microenvironment of the neoplastic cells is infiltrated with several stromal cells including mesenchymal cells that include the fibroblasts and the myofibroblasts, pericytes and endothelial cells as well as various cells of the immune system; they are recognized to be a major factor influencing both the growth of the cancer cells and the clinical outcome during therapeutic targeting. While the tumor stromal niche is not malignant per se, their contributions to tumor neoplastic cell growth are vital for the survival of tumor cells and thus are attractive targets for tumor therapy. The work presented in this thesis addresses the contributions of the Ron oncogene in regulating prostate tumor growth through its functional expression in different cellular compartments including the neoplastic cells, the tumor associated macrophages and tumor associated fibroblasts.

Overexpression of the Ron receptor has been documented in several human tumors including > 90% of human prostate tumors. Several studies have outlined the functional role of Ron in epithelial cells and tumors, others and we have also documented important roles for Ron in macrophage activation [Reviewed in (72, 76, 77)]. Two specific studies that were fundamental to the experiments performed in this thesis were the studies in which, mice containing the polyoma

virus middle T antigen [pMT], breast cancer mouse, and Transgenic Adenocarcinoma of the Mouse Prostate [TRAMP], prostate cancer model, were crossed individually to mice that contained the germline deletion of the tyrosine kinase signaling domain of Ron [TK-/-]. In both these studies, a significant decrease in tumor mass and microvessel density with an increase in tumor cell apoptosis was observed (88, 89). Both these studies demonstrated the functional importance of Ron signaling in promoting tumor growth and angiogenesis, however, they did not address the contributions of Ron signaling in the epithelial cells versus the stromal component. In this dissertation, we tested and validated the hypothesis that "***Ron receptor tyrosine kinase signaling in the prostate epithelium and in stromal cells, specifically tumor associated macrophages, is important for promoting prostate tumor growth***". We chose to test the cell type specific contributions of Ron in a prostate tumor model because of the well-established functional importance for epithelium stromal interactions during the development and maintenance of the adult prostate epithelium, and during tumorigenesis (115, 157, 160). Our experiments have provided an important conclusion that Ron expression in both tumor intrinsic and extrinsic compartments are important for regulating prostate tumor growth and survival; with cell type specific functions regulated by Ron expression. Our results also validate Ron as a novel therapeutic target in the treatment of the disease through the targeting of multiple cell types.

The key inferences of our work presented in this thesis are summarized below:

- Ron is expressed in both human and murine prostate epithelial and stromal cells.
- Ron expression in epithelial cells is important for supporting prostate tumor growth and is associated with an increase in pelvic bone metastasis.
- Host derived Ron is critical for prostate tumor growth as tumor cells transplanted into Ron deficient [TK-/-] mice exhibited significantly less growth compared to cells implanted into a Ron expressing microenvironment.

- Loss of Ron in the host increased tumor epithelial cell apoptosis with an increase in the infiltration of macrophages to the tumors.
- An increase in inflammatory cytokine expression was observed in the tumors isolated from Ron deficient mice compared to the controls.
- Absence of Ron in the myeloid cells, specifically in the tumor-associated macrophages leads to the inhibition of prostate tumor growth as demonstrated by: [1] transplantation of bone marrow cells from TK-/- mice into TK+/+ mice and [2] by the conditional deletion of Ron in the myeloid cells by using LysMCre mice.
- Bone marrow transplantation experiments suggested that Ron expression in both hematopoietic and non-hematopoietic stromal cells in the tumor microenvironment is important for promoting tumor growth.
- Ron loss in the myeloid cells was sufficient to induce epithelial cell apoptosis and macrophage infiltration to the tumors.
- Ron deficient macrophages inhibit prostate tumor growth through the activation of cytotoxic CD8+ T lymphocytes to mount an effective anti-tumor immune response.
- Surprisingly, Th1 or Th2 cells were not involved, because CD4+ T-cell depletion did not diminish the anti-tumor effect.
- Ron deficient macrophages act in a dominant negative fashion to elicit an anti tumor response in wild-type animals; a valuable feature that can be exploited in immunotherapy.
- Ron expression in the tumor-associated fibroblasts is also important for supporting prostate tumor progression.

Taken together, our study herein demonstrates for the first time the novel role of the Ron receptor in the myeloid cells in supporting malignant disease, using an orthotopic syngeneic murine model of prostate cancer. These findings have opened new functional roles for Ron in prostate and other cancers that merit further investigation.

Ron signaling in tumor epithelial cells: Several studies, have demonstrated the importance of Ron expression and activity in tumor epithelial cells for promoting cell proliferation, survival, migration, invasion, and Epithelial Mesenchymal Transition [reviewed in (72, 76, 77)]. Gain of function studies in mouse models have shown that activation of Ron in the epithelial cells can be achieved through either the overexpression of Ron (85, 86) or HGFL (84). The activation of Ron through either of these means was sufficient to increase tumor growth as well as the frequency and tissue tropism of metastasis. Essentially, Ron overexpression in the mammary epithelial was sufficient to induce mammary transformation in all animals with 100% penetrance and was associated with a high degree of metastasis to the liver and lungs greater than 86% in the transgenic animals (85). HGFL overexpression in breast cancer cells in a syngeneic orthotopic breast cancer model resulted in an increase in tumor mass as well as osteolytic bone metastasis. Furthermore, the same group had shown that in human patients, the coordinated overexpression of HGFL, Matriptase-1 and Ron resulted in the significant increase in bone metastasis compared to patients without high HGFL/Matriptase-1/Ron expression (84). These studies, in conjunction to ours listed in chapter 2 of this thesis, confirm that Ron expression in the prostate epithelial cells is important for controlling metastasis, specifically to the bones, making it a more therapeutically valuable target in the treatment of patients of with bone metastasis. In addition, to the role of epithelial Ron in supporting metastasis our studies have also identified that the tumors from these animals have a significant decrease in angiogenic blood vessel formation, consistent with the *in vitro* observation that Ron expression in the epithelial cells affect the angiogenic chemokine production (90). Several unanswered questions still remain with respect to Ron's function in tumor epithelial cells and understanding these mechanisms will further advance the field for the development of Ron targeting drugs. Some of these questions are as follows: [1] Is HGFL mediated Ron activation required for the induction of metastasis or ligand independent activation of Ron is sufficient? [2] What are the signaling mediators induced by Ron expression in epithelial cells that affects the metastasis to the bone?

A potential mechanism through which Ron expression can influence bone metastasis could be through the activation of TGF-ß, as referenced in pancreatic cells, where Smad-4 repression of Ron expression was sufficient to block the invasion of pancreatic cancer cells mediated through crosstalk between TGF-ß and Ron activity (168). TGF-ß activity in several cancers has been shown to be important for promoting bone metastasis [reviewed in (169)]; Another potential mechanism would be through the Ron dependent production of bone homing factors that can then facilitate bone marrow homing; and [3] Is Ron/HGFL expression in the stromal cells also important for tumor cell homing to the ossification centers? HGFL mediated Ron activation in human osteoclasts has been previously shown to improve bone resorption (24), a key step involved during bone colonization. Thus, understanding the signaling functions altered by Ron expression in epithelial cells would have important clinical implications in the treatment of metastatic tumors. Furthermore, we have identified a novel bioluminescence assays to track spontaneous metastatic cells to secondary sites after orthotopic tumor establishment, a highly sensitive tool for identification of micro-metastasis to the bones, without the need for extensive bone histology. Future work is aimed at developing luciferase tagged murine prostate cancer cells such that the importance of Ron for tumor progression can be studied in animals with an intact immune environment. Taken together, our results have thus far confirmed that targeting Ron in prostate tumors affects the tumor growth by preventing blood vessel formation and metastasis, validating it to be a candidate for targeting in human tumors.

Ron signaling in the tumor microenvironment: Our studies outlined in chapter 3 and 4 of this thesis have identified new functional roles for Ron signaling during tumorigenesis by virtue of its expression in the stromal cells of the tumor microenvironment. Collectively, our results have shown that prostate tumor growth can be altered significantly by virtue of Ron expression in both tumor-associated macrophages and tumor-associated fibroblasts. The inferences and contributions from our study to the understanding of the biology of Ron to tumor progression, as

well as the use of Ron modulated macrophages as an immunotherapeutic tool for prostate cancer treatment is discussed below. Further future studies that would further our understanding on the functions regulated by Ron expression in TAMs and TAFs are discussed.

The importance of Ron/HGFL signaling during macrophage activation and inflammation has been extensively studied in experimental models of inflammation and injury, and during wound healing [chapter 1 and reviewed in (72, 76)]. Given the importance for Ron signaling in the regulation of macrophage activation and suppression of inflammation, we tested if Ron expression in the tumor microenvironment is important for supporting tumor growth. Our experiments substantiated our hypothesis and showed that Ron expression in both the hematopoietic and non-hematopoietic stromal compartments is important for supporting tumor growth [Chapter 3; Figure 5C]. Reciprocal bone marrow transplantation studies and studies with mice containing a conditional deletion of Ron in myeloid cells have demonstrated the importance of the Ron in myeloid cells in promoting prostate tumor growth [Chapter 3; Figure 5C-6B]. However, our bone marrow transplantation studies in mice also demonstrated the involvement of other Ron expressing non-hematopoietic stromal cells that was necessary for supporting tumor growth, as tumor growth was not restored in TK-/- animals transplanted with TK+/+ bone marrow cells. Our data hereby suggests that the loss of Ron in one or more of stromal cellular compartments can tilt the signaling cues of the microenvironment in favor of or against tumorigenesis. A stromal cell type where significant Ron expression was detected in the normal and tumor-bearing prostates is the fibroblasts [Chapter 1, Figure 1A and Chapter 4, Figure 1A]. Interestingly, *in vivo* co-culture of TRAMP-C2Re3 cells with MEFs demonstrated that Ron loss in the fibroblasts suppresses the growth of the prostate tumors [Chapter 4, figure 1B] identifying that TAFs are another Ron expressing stromal cell type that supports tumorigenesis.
An interesting observation is that loss of Ron signaling in the host cells resulted in a significant decrease in the microvessel density compared to the control tumors. However, our data also

indicated that loss of Ron signaling in tumor-associated macrophages alone was not sufficient to alter the tumor microvessel formation, as no appreciable differences in microvessel density was observed in the tumors from $TK^{f/f}$ $LysMCre^{+}$ mice compared to the control $TK^{f/f}$ animals. Given that Ron signaling in myeloid cells is not important for altering the microvessel density it would be important to evaluate the influence of other Ron expressing stromal sub-cell type, including fibroblasts, for the regulation of angiogenic blood vessel formation in tumors. Cancer associated fibroblasts have been long shown to support the growth and the metastatic progression of tumors. Future studies aimed at the evaluation of the role of Ron signaling in fibroblasts in promoting tumor progression will aid in further dissection of the complexity of the Ron regulated signaling network in the multiple cells types present in the tumor milieu.

In our study, the initial recruitment of macrophages to the tumors from both TK+/+ and TK-/- mice was dramatic and no visual differences in the extent of macrophage infiltration was observed, indicating that Ron expression in the macrophages does not affect their initial recruitment to the tumors [studies using TRAMP-C2Re3 tumors harvested 5 and 10days post injection; data not shown]. However, end stage tumors from Ron deficient mice exhibited a marked increase in macrophage infiltration indicating that loss of Ron in macrophages impacts their temporal accumulation after tumor establishment [Chapter 3, Figure 2A]. An important consideration of this observation is whether the expression of Ron in macrophages is important for mediating antitumor immunity or their ability to infiltrate more to the tumor site that causes the effective removal of tumor cells. Based on our experimental observations in chapter 3-clinical implications, our data indicates that loss of Ron expression in the macrophages renders them to decrease tumor growth in Ron proficient animals by working in a dominant negative fashion against the Ron proficient macrophages. This is an important aspect that needs to be emphasized as Ron modulated macrophages can be used in the clinic in patients without the need to ablate any host derived macrophages that may express Ron. Alternatively, another

explanation for the increased numbers in macrophages would be a consequence of the augmented number of dead cells in the tumor proper in the Ron knockouts, where they function to promote the phagocytosis and scavenging of the dead/dying tumor cells. Further investigation is thus warranted to understand the functional importance for the increase in macrophage numbers to the tumors.

Additional questions that addresses the functional contribution of Ron in TAMs to tumorigenesis are as follows: [1] What are the primary source for the Ron expressing TAMs; are they derived from circulating monocytes or are products of proliferation of resident prostate macrophages or both; [2] Is Ron expression altered in TAMs during the course of tumor progression; [3] Can other truncated variants of the Ron receptor be expressed and regulate the functions of the TAMs and the associated T cell activity? ; [4] Are Ron expressing TAMs important for the regulating of cancer stem cells; and finally [5] is the expression and function of Ron similar in human TAMs as several studies have shown discrepancies within the mouse and the human macrophage sub-cell types and their functions in the various tumors.

In addition to the increased influx of Ron-deficient macrophages to tumors, our data show that a conditional loss of Ron in myeloid cells is sufficient to inhibit tumor growth *in vivo* and our data also suggests that the depletion of cytotoxic T lymphocytes can restore tumor growth in mice lacking myeloid specific Ron expression [chapter 3, Figure 6B-7C-7D]. Based on our experimental data several possible mechanisms can be proposed through which macrophages that are devoid of Ron are able to provoke a strong anti tumor immune response through the cytotoxic CD8+ T lymphocytes. One potential mechanism is through their enhanced ability to present antigen to CD8+ T-cells. An important observation that can support this hypothesis is that the lack of Ron signaling in macrophages results in the decrease in the expression of STAT3, and loss of STAT3 in antigen presenting cells of the tumors have been previously

shown to enhance the cytotoxic T lymphocyte activity and hence overcome the CD8 T cell tolerance (170). Another potential mechanisms through which Ron proficient macrophages can down regulate a cytotoxic T lymphocytic response is through the induction of T cell anergy, Briefly, we observed remarkable decrease in the expression of T cell co-stimulatory genes like ICOS with stromal loss of Ron suggestive that Ron signaling in T cells down regulates T cell activity through the induction of anergy (171, 172). Ron can also potentially influence the number of effector and memory CD8+ T cells through the regulation in the expression of the chemokine receptor CXCR3 and its ligand MIG/CXCL9 [chapter 3 Figure 4C-4D and supplementary figure S3A]. CXCR3/MIG interactions have been reported to regulate the activation and differentiation of T cells into effector CD8+ T cells versus the memory CD8+ T cells (173, 174). In addition to this, the downregulation of Arginase-1 levels in TK-/- macrophages could also be also important, as alternatively activated macrophages that express high levels of arginase-1 have been shown to inhibit the proliferation of T cells (138, 175). Further research is required to understand the mechanism of CD8+ T cell regulation by Ron expressing macrophages and this will provide a better understanding of the mechanisms by which the anti-tumor immunity is abolished.

Ron expression in macrophages also regulates the expression of cytokines and chemokines that promote or suppress tumor growth by indirectly through the regulation of the functional role of the cells in the tumor microenvironment [CXCL9, M-CSF, IL-12, TGF-β] and is discussed below. A majorly discussed pathway regulated by Ron in macrophages is the regulation in the iNOS and Arginase-1 expression. Consistent with these published observations, lack of Ron expression in the tumor microenvironment in our model system increased iNOS expression in the tumor tissues from animals lacking stromal Ron expression in comparison to the controls [Chapter 3, Figure 4A - 4B]. The increase in iNOS expression in these tumors can be partly attributed to the expression of Ron in macrophages, as tumors from $TK^{f/f}$ $LysMCre^{+}$ mice also

had an increase in iNOS expression compared to the controls [Chapter3, Figure 6E]. These results indicate that Ron expression in the host cells including the macrophages are important in the regulation of the fine balance of iNOS expression and hence the skewing of the tumorigenic microenvironment towards either tumor promoting or inhibitory as iNOS expression in the tumors has been previously shown to be either pro or anti tumorigenic depending on its expression level and the duration and delivery of NO (137). Ron loss in the tumor microenvironment also increased the expression of the cytokine IL-12, and increased IL-12 levels in the tumors and the tumor microenvironment has been shown to be important for optimal cytotoxic CD8 T-cell activity (176).

What remains unclear in our studies is the contribution of the Ron ligand, HGFL, to the activation of Ron expressed on tumor-associated macrophages and consequently the regulation of cytotoxic T lymphocytic activity. HGFL is predominantly produced by the hepatocytes in the liver, and is secreted into the blood stream where it circulates in the inactive pro-HGFL form (177). Recent published studies have demonstrated the increased expression of HGFL in both human tumor stromal and epithelial cells (37-39, 63). In addition to become cleaved and activated by membrane bound proteases like Matriptase-1 expressed on macrophages (63), pro-HGFL can also be activated by other proteases of the coagulation cascade (32, 62). Additionally, data mining of studies published by Yu et al., show that the expression of HGFL increases with prostate tumor progression (178). As outlined in chapter 1, HGFL mediated Ron activation is required for the elicitation of the certain functions of Ron, however, it has to be noted that HGFL knockout mice exhibited normal macrophage activation, albeit delays in activation, suggestive of other ligands that might also function to influence Ron activation(43). Furthermore, several studies have also outlined the importance for HGFL in the wound healing process (81, 91) and considering that the tumors have microenvironmental cues as that of a wound, except that it never heals, it is important to validate the importance of HGFL in the

activation of TAMs during tumorigenesis. Thus, future studies aimed at understanding the functional differences in ligand dependent and independent Ron signaling processes in macrophages, and the importance for ligand overexpression and hence the consequent exaggeration or maintenance of a sustained signaling in the tumor milieu, will provide novel insights of the complex signaling network regulated by Ron receptor in the tumor microenvironment.

Given our studies, it would be imperative to study both the temporal and spatial contribution of Ron signaling in the epithelial cells, TAMs and the TAFs during tumor initiation and during tumor progression. Furthermore, the importance of these Ron expressing stromal cells for promoting metastasis to secondary tumor sites will help us understand the complexities of the signaling crosstalk initiated by the Ron expressing cells of the tumor microenvironment.

In conclusion, this thesis has identified cell-type specific functions of the Ron receptor tyrosine kinase in regulating prostate tumor functions. Essentially, Ron expression in both the epithelial and stromal cells is important for tumor progression through the regulation of the multiple arms of tumor progression including proliferation, survival, angiogenesis, metastasis and the evasion of anti-tumor immunity as shown in the model [**Chapter 5; Figure 1**]. These studies have further validated the use of Ron targeting drugs for the treatment of both Ron driven and non-Ron driven tumors. Specifically our work show for the first time that tumor immunity can be regulated by expression of the Ron/MST1R in macrophages whereby the silencing of Ron expression in macrophages enhances anti-tumor immune response and renders as a potential target and tool for immunotherapy.

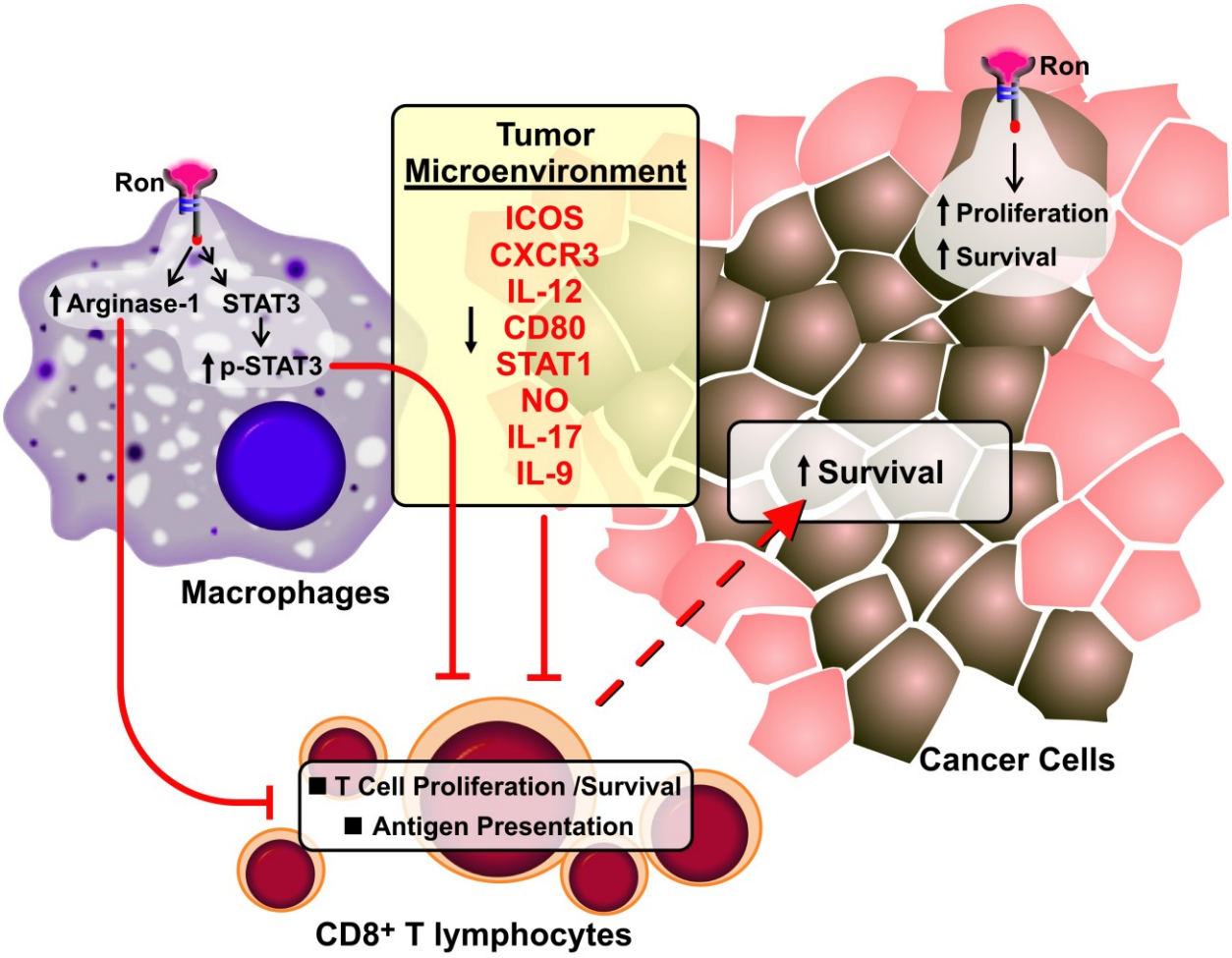


Figure 1. A working model displaying the immunosuppressive role of the Ron receptor in the tumor microenvironment.

Signaling interactions among the different cell types of the prostate tumor microenvironment are shown. Of note, Ron expression in the tumor-associated macrophages promotes the expression of Arginase-1 and the phosphorylation of STAT3. Ron expression is also associated with the modulation of various signaling molecules in the tumor microenvironment. These molecules may inhibit the cytotoxic activity of CD8+ T cells and allow for growth and survival of the tumor cells.

BIBLIOGRAPHY

1. Lapraz F, Rottinger E, Duboc V, Range R, Duloquin L, Walton K, et al. RTK and TGF-beta signaling pathways genes in the sea urchin genome. Developmental biology. 2006;300:132-52.

2. Ronsin C, Muscatelli F, Mattei MG, Breathnach R. A novel putative receptor protein tyrosine kinase of the met family. Oncogene. 1993;8:1195-202.

3. Iwama A, Okano K, Sudo T, Matsuda Y, Suda T. Molecular cloning of a novel receptor tyrosine kinase gene, STK, derived from enriched hematopoietic stem cells. Blood. 1994;83:3160-9.

4. Nakamura T, Aoki S, Takahashi T, Matsumoto K, Kiyohara T. Cloning and expression of Xenopus HGF-like protein (HLP) and Ron/HLP receptor implicate their involvement in early neural development. Biochem Biophys Res Commun. 1996;224:564-73.

5. Huff JL, Jelinek MA, Borgman CA, Lansing TJ, Parsons JT. The protooncogene c-sea encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. Proc Natl Acad Sci U S A. 1993;90:6140-4.

6. Bassett DI. Identification and developmental expression of a macrophage stimulating 1/ hepatocyte growth factor-like 1 orthologue in the zebrafish. Dev Genes Evol. 2003;213:360-2.

7. De Maria R, Maggiora P, Biolatti B, Prat M, Comoglio PM, Castagnaro M, et al. Feline STK gene expression in mammary carcinomas. Oncogene. 2002;21:1785-90.

8. Leonard EJ, Skeel A. A serum protein that stimulates macrophage movement, chemotaxis and spreading. Experimental Cell Research. 1976;102:434-8.

9. Leonard EJ, Skeel AH. Isolation of macrophage stimulating protein (MSP) from human serum. Experimental Cell Research. 1978;114:117-26.

10. Han S, Stuart LA, Degen SJ. Characterization of the DNF15S2 locus on human chromosome 3: identification of a gene coding for four kringle domains with homology to hepatocyte growth factor. Biochemistry. 1991;30:9768-80.

11. Degen SJ, Stuart LA, Han S, Jamison CS. Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: expression during development. Biochemistry. 1991;30:9781-91.

12. Gaudino G, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, et al. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. Embo J. 1994;13:3524-32.

13. Yoshimura T, Yuhki N, Wang MH, Skeel A, Leonard EJ. Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on chromosome 3. J Biol Chem. 1993;268:15461-8.

14. Angeloni D, Danilkovitch-Miagkova A, Ivanov SV, Breathnach R, Johnson BE, Leonard EJ, et al. Gene structure of the human receptor tyrosine kinase RON and mutation analysis in lung cancer samples. Genes Chromosomes Cancer. 2000;29:147-56.

15. Waltz SE, Toms CL, McDowell SA, Clay LA, Muraoka RS, Air EL, et al. Characterization of the mouse Ron/Stk receptor tyrosine kinase gene. Oncogene. 1998;16:27-42.

16. Wei X, Hao L, Ni S, Liu Q, Xu J, Correll PH. Altered exon usage in the juxtamembrane domain of mouse and human RON regulates receptor activity and signaling specificity. J Biol Chem. 2005;280:40241-51.

17. Gaudino G, Avantaggiato V, Follenzi A, Acampora D, Simeone A, Comoglio PM. The proto-oncogene RON is involved in development of epithelial, bone and neuro-endocrine tissues. Oncogene. 1995;11:2627-37.

18. Quantin B, Schuhbaur B, Gesnel MC, Doll'e P, Breathnach R. Restricted expression of the ron gene encoding the macrophage stimulating protein receptor during mouse development. Dev Dyn. 1995;204:383-90.

19. Okino T, Egami H, Ohmachi H, Takai E, Tamori Y, Nakagawa A, et al. Immunohistochemical analysis of distribution of RON receptor tyrosine kinase in human digestive organs. Dig Dis Sci. 2001;46:424-9.

20. Persons DA, Paulson RF, Loyd MR, Herley MT, Bodner SM, Bernstein A, et al. Fv2 encodes a truncated form of the Stk receptor tyrosine kinase. Nat Genet. 1999;23:159-65.

21. Meyer SE, Zinser GM, Stuart WD, Pathrose P, Waltz SE. The Ron receptor tyrosine kinase negatively regulates mammary gland branching morphogenesis. Developmental biology. 2009;333:173-85.

22. Iwama A, Wang MH, Yamaguchi N, Ohno N, Okano K, Sudo T, et al. Terminal differentiation of murine resident peritoneal macrophages is characterized by expression of the STK protein tyrosine kinase, a receptor for macrophage-stimulating protein. Blood. 1995;86:3394-403.

23. Brunelleschi S, Penengo L, Lavagno L, Santoro C, Colangelo D, Viano I, et al. Macrophage stimulating protein (MSP) evokes superoxide anion production by human macrophages of different origin. Br J Pharmacol. 2001;134:1285-95.

24. Kurihara N, Iwama A, Tatsumi J, Ikeda K, Suda T. Macrophage-stimulating protein activates STK receptor tyrosine kinase on osteoclasts and facilitates bone resorption by osteoclast-like cells. Blood. 1996;87:3704-10.

25. Nanney LB, Skeel A, Luan J, Polis S, Richmond A, Wang MH, et al. Proteolytic cleavage and activation of pro-macrophage-stimulating protein and upregulation of its receptor in tissue injury. J Invest Dermatol. 1998;111:573-81.

26. Stuart WD, Kulkarni RM, Gray JK, Vasiliauskas J, Leonis MA, Waltz SE. Ron receptor regulates Kupffer cell-dependent cytokine production and hepatocyte survival following endotoxin exposure in mice. Hepatology. 2011;53:1618-28.

27. Suzuki Y, Funakoshi H, Machide M, Matsumoto K, Nakamura T. Regulation of cell migration and cytokine production by HGF-like protein (HLP) / macrophage stimulating protein (MSP) in primary microglia. Biomed Res. 2008;29:77-84.

28. Tsutsui S, Noorbakhsh F, Sullivan A, Henderson AJ, Warren K, Toney-Earley K, et al. RON-regulated innate immunity is protective in an animal model of multiple sclerosis. Ann Neurol. 2005;57:883-95.

29. O'Toole JM, Rabenau KE, Burns K, Lu D, Mangalampalli V, Balderes P, et al. Therapeutic implications of a human neutralizing antibody to the macrophage-stimulating protein receptor tyrosine kinase (RON), a c-MET family member. Cancer research. 2006;66:9162-70.

30. Wang MH, Lee W, Luo YL, Weis MT, Yao HP. Altered expression of the RON receptor tyrosine kinase in various epithelial cancers and its contribution to tumourigenic phenotypes in thyroid cancer cells. J Pathol. 2007;213:402-11.

31. Leonard EJ, Danilkovitch A. Macrophage stimulating protein. Adv Cancer Res. 2000;77:139-67.

32. Wang MH, Gonias SL, Skeel A, Wolf BB, Yoshimura T, Leonard EJ. Proteolytic activation of single-chain precursor macrophage-stimulating protein by nerve growth factor-gamma and epidermal growth factor-binding protein, members of the kallikrein family. J Biol Chem. 1994;269:13806-10.

33. Kawaguchi M Fau - Orikawa H, Orikawa H Fau - Baba T, Baba T Fau - Fukushima T, Fukushima T Fau - Kataoka H, Kataoka H. Hepatocyte growth factor activator is a serum activator of single-chain precursor macrophage-stimulating protein.

34. Bhatt AS, Welm A, Farady CJ, Vasquez M, Wilson K, Craik CS. Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway. Proc Natl Acad Sci U S A. 2007;104:5771-6.

35. Danilkovitch A, Miller M, Leonard EJ. Interaction of macrophage-stimulating protein with its receptor. Residues critical for beta chain binding and evidence for independent alpha chain binding. J Biol Chem. 1999;274:29937-43.

36. Waltz SE, McDowell SA, Muraoka RS, Air EL, Flick LM, Chen YQ, et al. Functional characterization of domains contained in hepatocyte growth factor-like protein. J Biol Chem. 1997;272:30526-37.

37. Eckerich C, Schulte A, Martens T, Zapf S, Westphal M, K. L. RON receptor tyrosine kinase in human gliomas: expression, function, and identification of a novel soluble splice variant. J Neurochem. 2009;109(4):969-80.

38. Sato S, Hanibuchi M, Kuramoto T, Yamamori N, Goto H, Ogawa H, et al. Macrophage stimulating protein promotes liver metastases of small cell lung cancer cells by affecting the organ microenvironment. Clin Exp Metastasis. 2012.

39. Cheng N, Bhowmick NA, Chytil A, Gorksa AE, Brown KA, Muraoka R, et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. Oncogene. 2005;24:5053-68.

40. Muraoka RS, Sun WY, Colbert MC, Waltz SE, Witte DP, Degen JL, et al. The Ron/STK receptor tyrosine kinase is essential for peri-implantation development in the mouse. J Clin Invest. 1999;103:1277-85.

41. Correll PH, Iwama A, Tondat S, Mayrhofer G, Suda T, Bernstein A. Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. Genes Funct. 1997;1:69-83.

42. Waltz SE, Eaton L, Toney-Earley K, Hess KA, Peace BE, Ihlendorf JR, et al. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. J Clin Invest. 2001;108:567-76.

43. Bezerra JA, Carrick TL, Degen JL, Witte D, Degen SJ. Biological effects of targeted inactivation of hepatocyte growth factor-like protein in mice. J Clin Invest. 1998;101:1175-83.

44. Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T, et al. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature. 1995;373:702-5.

45. Correll PH, Iwama A, Tondat S, Mayrhofer G, Suda T, Bernstein A. Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. Genes Funct. 1997;1:69-83.

46. Waltz SE, Eaton L, Toney-Earley K, Hess KA, Peace BE, Ihlendorf JR, et al. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. J Clin Invest. 2001;108:567-76.

47. Liu QP, Fruit K, Ward J, Correll PH. Negative regulation of macrophage activation in response to IFN-gamma and lipopolysaccharide by the STK/RON receptor tyrosine kinase. J Immunol. 1999;163:6606-13.

48. Ray M, Yu S, Sharda DR, Wilson CB, Liu Q, Kaushal N, et al. Inhibition of TLR4-induced IkappaB kinase activity by the RON receptor tyrosine kinase and its ligand, macrophage-stimulating protein. J Immunol. 2010;185:7309-16.

49. Muraoka RS, Sun WY, Colbert MC, Waltz SE, Witte DP, Degen JL, et al. The Ron/STK receptor tyrosine kinase is essential for peri-implantation development in the mouse. J Clin Invest. 1999;103:1277-85.

50. Lentsch AB, Pathrose P, Kader S, Kuboki S, Collins MH, Waltz SE. The Ron receptor tyrosine kinase regulates acute lung injury and suppresses nuclear factor kappaB activation. Shock. 2007;27:274-80.

51. Nikolaidis NM, Gray JK, Gurusamy D, Fox W, Stuart WD, Huber N, et al. Ron receptor tyrosine kinase negatively regulates TNFalpha production in alveolar macrophages by inhibiting NF-kappaB activity and Adam17 production. Shock. 2010;33:197-204.

52. Nikolaidis NM, Kulkarni RM, Gray JK, Collins MH, Waltz SE. Ron receptor deficient alveolar myeloid cells exacerbate LPS-induced acute lung injury in the murine lung. Innate Immun. 2011;17:499-507.

53. Wilson CB, Ray M, Lutz M, Sharda D, Xu J, Hankey PA. The RON receptor tyrosine kinase regulates IFN-gamma production and responses in innate immunity. J Immunol. 2008;181:2303-10.

54. Ray M, Yu S, Sharda DR, Wilson CB, Liu Q, Kaushal N, et al. Inhibition of TLR4-induced IkappaB kinase activity by the RON receptor tyrosine kinase and its ligand, macrophage-stimulating protein. J Immunol. 2010;185:7309-16.

55. Rampino T, Soccio G, Gregorini M, Guidetti C, Marasa M, Maggio M, et al. Neutralization of macrophage-stimulating protein ameliorates renal injury in anti-thy 1 glomerulonephritis. J Am Soc Nephrol. 2007;18:1486-96.

56. Lu Y, Yao HP, Wang MH. Multiple variants of the RON receptor tyrosine kinase: biochemical properties, tumorigenic activities, and potential drug targets. Cancer Lett. 2007;257:157-64.

57. Wetzel CC, Leonis MA, Dent A, Olson MA, Longmeier AM, Ney PA, et al. Short-form Ron receptor is required for normal IFN-gamma production in concanavalin A-induced acute liver injury. Am J Physiol Gastrointest Liver Physiol. 2007;292:G253-61.

58. Mallakin A, Kutcher LW, McDowell SA, Kong S, Schuster R, Lentsch AB, et al. Gene expression profiles of Mst1r-deficient mice during nickel-induced acute lung injury. Am J Respir Cell Mol Biol. 2006;34:15-27.

59. McDowell SA, Mallakin A, Bachurski CJ, Toney-Earley K, Prows DR, Bruno T, et al. The role of the receptor tyrosine kinase Ron in nickel-induced acute lung injury. Am J Respir Cell Mol Biol. 2002;26:99-104.

60. Lutz MA, Gervais F, Bernstein A, Hattel AL, Correll PH. STK receptor tyrosine kinase regulates susceptibility to infection with Listeria monocytogenes. Infect Immun. 2002;70:416-8.

61. Lutz MA, Correll PH. Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C zeta. J Leukoc Biol. 2003;73:802-14.

62. Skeel A, Leonard EJ. Action and target cell specificity of human macrophage-stimulating protein (MSP). J Immunol. 1994;152:4618-23.

63. Bhatt AS, Welm A, Farady CJ, Vasquez M, Wilson K, Craik CS. Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway. Proc Natl Acad Sci U S A. 2007;104:5771-6.

64. Leonis MA, Toney-Earley K, Degen SJ, Waltz SE. Deletion of the Ron receptor tyrosine kinase domain in mice provides protection from endotoxin-induced acute liver failure. Hepatology. 2002;36:1053-60.

65. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res. 2010;89:219-29.

66. Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci. 2004;9:283-9.

67. Liu QP, Fruit K, Ward J, Correll PH. Negative regulation of macrophage activation in response to IFN-gamma and lipopolysaccharide by the STK/RON receptor tyrosine kinase. J Immunol. 1999;163:6606-13.

68. Morrison AC, Correll PH. Activation of the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase by macrophage-stimulating protein results in the induction of arginase activity in murine peritoneal macrophages. J Immunol. 2002;168:853-60.

69. Morrison AC, Wilson CB, Ray M, Correll PH. Macrophage-stimulating protein, the ligand for the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase, inhibits IL-12 production by primary peritoneal macrophages stimulated with IFN-gamma and lipopolysaccharide. JImmunol. 2004;172:1825-32.

70. Liu H, Chen X, Focia PJ, He X. Structural basis for stem cell factor-KIT signaling and activation of class III receptor tyrosine kinases. Embo J. 2007;26:891-901.

71. Sharda DR, Yu S, Ray M, Squadrito ML, De Palma M, Wynn TA, et al. Regulation of macrophage arginase expression and tumor growth by the ron receptor tyrosine kinase. J Immunol. 2011;187:2181-92.

72. Wagh PK, Peace BE, Waltz SE. Met-related receptor tyrosine kinase Ron in tumor growth and metastasis. Advances in cancer research. 2008;100:1-33.

73. Iwama A Fau - Yamaguchi N, Yamaguchi N Fau - Suda T, Suda T. STK/RON receptor tyrosine kinase mediates both apoptotic and growth signals via the multifunctional docking site conserved among the HGF receptor family.

74. Leonard EJ, Danilkovitch A. Macrophage stimulating protein. Adv Cancer Res. 2000;77:139-67.

75. Leonis MA, Thobe MN, Waltz SE. Ron-receptor tyrosine kinase in tumorigenesis and metastasis. Future Oncol. 2007;3:441-8.

76. Kretschmann KL, Eyob H, Buys SS, Welm AL. The macrophage stimulating protein/Ron pathway as a potential therapeutic target to impede multiple mechanisms involved in breast cancer progression. Current drug targets. 2010;11:1157-68.

77. Benight NM, Waltz SE. Ron receptor tyrosine kinase signaling as a therapeutic target. Expert Opin Ther Targets. 2012;16:921-31.

78. Lee WY, Chen HH, Chow NH, Su WC, Lin PW, Guo HR. Prognostic significance of co-expression of RON and MET receptors in node-negative breast cancer patients. Clin Cancer Res. 2005;11:2222-8.

79. Peace BE, Hill KJ, Degen SJ, Waltz SE. Cross-talk between the receptor tyrosine kinases Ron and epidermal growth factor receptor. Exp Cell Res. 2003;289:317-25.

80. Jaquish DV, Yu PT, Shields DJ, French RP, Maruyama KP, Niessen S, et al. IGF1-R signals through the RON receptor to mediate pancreatic cancer cell migration. Carcinogenesis. 2011;32:1151-6.

81. Santoro MM, Gaudino G, Marchisio PC. The MSP receptor regulates alpha6beta4 and alpha3beta1 integrins via 14-3-3 proteins in keratinocyte migration. DevCell. 2003;5:257-71.

82. Conrotto P, Corso S, Gamberini S, Comoglio PM, Giordano S. Interplay between scatter factor receptors and B plexins controls invasive growth. Oncogene. 2004;23:5131-7.

83. Follenzi A, Bakovic S, Gual P, Stella MC, Longati P, Comoglio PM. Cross-talk between the proto-oncogenes Met and Ron. Oncogene. 2000;19:3041-9.

84. Welm AL, Sneddon JB, Taylor C, Nuyten DS, van de Vijver MJ, Hasegawa BH, et al. The macrophage-stimulating protein pathway promotes metastasis in a mouse model for breast cancer and predicts poor prognosis in humans. Proc Natl Acad Sci U S A. 2007;104:7570-5.

85. Zinser GM, Leonis MA, Toney K, Pathrose P, Thobe M, Kader SA, et al. Mammary-specific Ron receptor overexpression induces highly metastatic mammary tumors associated with beta-catenin activation. Cancer research. 2006;66:11967-74.

86. Chen YQ, Zhou YQ, Fu LH, Wang D, Wang MH. Multiple pulmonary adenomas in the lung of transgenic mice overexpressing the RON receptor tyrosine kinase. Recepteur d'origine nantais. Carcinogenesis. 2002;23:1811-9.

87. Gray JK, Paluch AM, Stuart WD, Waltz SE. Ron receptor overexpression in the murine prostate induces prostate intraepithelial neoplasia. Cancer Lett. 2012;314:92-101.

88. Peace BE, Toney-Earley K, Collins MH, Waltz SE. Ron receptor signaling augments mammary tumor formation and metastasis in a murine model of breast cancer. Cancer research. 2005;65:1285-93.

89. Thobe MN, Gray JK, Gurusamy D, Paluch AM, Wagh PK, Pathrose P, et al. The Ron receptor promotes prostate tumor growth in the TRAMP mouse model. Oncogene. 2011.

90. Thobe MN, Gurusamy D, Pathrose P, Waltz SE. The Ron receptor tyrosine kinase positively regulates angiogenic chemokine production in prostate cancer cells. Oncogene. 2010;29:214-26.

91. Cowin AJ, Kallincos N, Hatzirodos N, Robertson JG, Pickering KJ, Couper J, et al. Hepatocyte growth factor and macrophage-stimulating protein are upregulated during excisional wound repair in rats. Cell Tissue Res. 2001;306:239-50.

92. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315:1650-9.

93. Weinstein IB, Joe AK. Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy. Nat Clin Pract Oncol. 2006;3:448-57.

94. Broekman F, Giovannetti E, Peters GJ. Tyrosine kinase inhibitors: Multi-targeted or single-targeted? World J Clin Oncol. 2011;2:80-93.

95. Huang S, Armstrong EA, Benavente S, Chinnaiyan P, Harari PM. Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. Cancer research. 2004;64:5355-62.

96. Xu XM, Wang D, Shen Q, Chen YQ, Wang MH. RNA-mediated gene silencing of the RON receptor tyrosine kinase alters oncogenic phenotypes of human colorectal carcinoma cells. Oncogene. 2004;23:8464-74.

97. Gunes Z, Zucconi A, Cioce M, Meola A, Pezzanera M, Acali S, et al. Isolation of Fully Human Antagonistic RON Antibodies Showing Efficient Block of Downstream Signaling and Cell Migration. Transl Oncol. 2011;4:38-46.

98. Montero-Julian FA, Dauny I, Flavetta S, Ronsin C, Andre F, Xerri L, et al. Characterization of two monoclonal antibodies against the RON tyrosine kinase receptor. Hybridoma. 1998;17:541-51.

99. Padhye SS, Guin S, Yao HP, Zhou YQ, Zhang R, Wang MH. Sustained expression of the RON receptor tyrosine kinase by pancreatic cancer stem cells as a potential targeting moiety for antibody-directed chemotherapeutics. MolPharm. 2011;8:2310-9.

100. Yao HP, Zhou YQ, Ma Q, Guin S, Padhye SS, Zhang RW, et al. The monoclonal antibody Zt/f2 targeting RON receptor tyrosine kinase as potential therapeutics against tumor growth-mediated by colon cancer cells. MolCancer. 2011;10:82.

101. Li Z, Yao H, Guin S, Padhye SS, Zhou YQ, Wang MH. Monoclonal antibody (mAb)-induced down-regulation of RON receptor tyrosine kinase diminishes tumorigenic activities of colon cancer cells. IntJOncol. 2010;37:473-82.

102. Zhao H, Ou-Yang F, Chen IF, Hou MF, Yuan SS, Chang HL, et al. Enhanced resistance to tamoxifen by the c-ABL proto-oncogene in breast cancer. Neoplasia. 2010;12:214-23.

103. Guin S, Yao HP, Wang MH. RON receptor tyrosine kinase as a target for delivery of chemodrugs by antibody directed pathway for cancer cell cytotoxicity. MolPharm. 2010;7:386-97.

104. Thomas RM, Toney K, Fenoglio-Preiser C, Revelo-Penafiel MP, Hingorani SR, Tuveson DA, et al. The RON receptor tyrosine kinase mediates oncogenic phenotypes in pancreatic cancer cells and is increasingly expressed during pancreatic cancer progression. Cancer research. 2007;67:6075-82.

105. Logan-Collins J, Thomas RM, Yu P, Jaquish D, Mose E, French R, et al. Silencing of RON receptor signaling promotes apoptosis and gemcitabine sensitivity in pancreatic cancers. Cancer Res. 2010;70:1130-40.

106. McClaine RJ, Marshall AM, Wagh PK, Waltz SE. Ron receptor tyrosine kinase activation confers resistance to tamoxifen in breast cancer cell lines. Neoplasia. 2010;12:650-8.

107. Eder JP, Shapiro GI, Appleman LJ, Zhu AX, Miles D, Keer H, et al. A phase I study of foretinib, a multi-targeted inhibitor of c-Met and vascular endothelial growth factor receptor 2. ClinCancer Res. 2010;16:3507-16.

108. Qian F, Engst S, Yamaguchi K, Yu P, Won KA, Mock L, et al. Inhibition of tumor cell growth, invasion, and metastasis by EXEL-2880 (XL880, GSK1363089), a novel inhibitor of HGF and VEGF receptor tyrosine kinases. Cancer research. 2009;69:8009-16.

109. Danilkovitch-Miagkova A. Oncogenic signaling pathways activated by RON receptor tyrosine kinase. Curr Cancer Drug Targets. 2003;3:31-40.

110. Angeloni D, Danilkovitch-Miagkova A, Miagkov A, Leonard EJ, Lerman MI. The soluble sema domain of the RON receptor inhibits macrophage-stimulating protein-induced receptor activation. J Biol Chem. 2004;279:3726-32.

111. Germano S, Barberis D, Santoro MM, Penengo L, Citri A, Yarden Y, et al. Geldanamycins trigger a novel Ron degradative pathway, hampering oncogenic signaling. J Biol Chem. 2006;281:21710-9.

112. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer research. 1997;57:3325-30.

113. Ye L, Kynaston HG, Jiang WG. Bone metastasis in prostate cancer: molecular and cellular mechanisms (Review). Int J Mol Med. 2007;20:103-11.

114. Condon MS. The role of the stromal microenvironment in prostate cancer. Semin Cancer Biol. 2005;15:132-7.

115. G D Grossfeld SWH, T D Tlsty and G R Cunha. The role of stroma in prostatic carcinogenesis. Endocrine-Related Cancer. 1998;5:253-70.

116. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27:5904-12.

117. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39-51.

118. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124:263-6.

119. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, et al. Macrophage polarization in tumour progression. Semin Cancer Biol. 2008;18:349-55.

120. Chen YQ, Fisher JH, Wang MH. Activation of the RON receptor tyrosine kinase inhibits inducible nitric oxide synthase (iNOS) expression by murine peritoneal exudate macrophages: phosphatidylinositol-3 kinase is required for RON-mediated inhibition of iNOS expression. J Immunol. 1998;161:4950-9.

121. Chang CI, Liao JC, Kuo L. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide-mediated tumor cytotoxicity. Cancer research. 2001;61:1100-6.

122. Caldwell CC, Martignoni A, Leonis MA, Ondiveeran HK, Fox-Robichaud AE, Waltz SE. Ron receptor tyrosine kinase-dependent hepatic neutrophil recruitment and survival benefit in a murine model of bacterial peritonitis. Crit Care Med. 2008;36:1585-93.

123. Fu XY. STAT3 in immune responses and inflammatory bowel diseases. Cell Res. 2006;16:214-9.

124. Brayer J, Cheng F, Wang H, Horna P, Vicente-Suarez I, Pinilla-Ibarz J, et al. Enhanced CD8 T cell cross-presentation by macrophages with targeted disruption of STAT3. Immunol Lett. 2010;131:126-30.

125. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009;9:798-809.

126. Olson MV, Lee J, Zhang F, Wang A, Dong Z. Inducible nitric oxide synthase activity is essential for inhibition of prostatic tumor growth by interferon-beta gene therapy.
. Cancer Gene Therapy. 2006;13:676-85.

127. Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z. Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. Cell Stem Cell. 2008;2:90-102.

128. Eckerich C, Schulte A, Martens T, Zapf S, Westphal M, Lamszus K. RON receptor tyrosine kinase in human gliomas: expression, function, and identification of a novel soluble splice variant. Journal of neurochemistry. 2009;109:969-80.

129. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Cancer Gene Therapy. 2001;411:375-9.

130. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nature reviews Cancer. 2008;8:618-31.

131. Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett. 2004;206:193-9.

132. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell. 2009;15:103-13.

133. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med. 2005;11:1314-21.

134. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, DeNardo DG, et al. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. Cancer research. 2010;70:7465-75.

135. Roda JM, Sumner LA, Evans R, Phillips GS, Marsh CB, Eubank TD. Hypoxia-inducible factor-2alpha regulates GM-CSF-derived soluble vascular endothelial growth factor receptor 1 production from macrophages and inhibits tumor growth and angiogenesis. J Immunol. 2011;187:1970-6.

136. Zhang L, Alizadeh D, Van Handel M, Kortylewski M, Yu H, Badie B. Stat3 inhibition activates tumor macrophages and abrogates glioma growth in mice. Glia. 2009;57:1458-67.

137. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6:521-34.

138. Stempin CC, Dulgerian LR, Garrido VV, Cerban FM. Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals. J Biomed Biotechnol.2010:683485.

139. Huber S, Hoffmann R, Muskens F, Voehringer D. Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood. 2010 ;116:3311-20.

140. Hao NB, Lu MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. Clin Dev Immunol. 2012;2012:948098.

141. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol. 2010;22:231-7.

142. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23:549-55.

143. Mantovani A, Sozzani S, Locati M, Schioppa T, Saccani A, Allavena P, et al. Infiltration of tumours by macrophages and dendritic cells: tumour-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Novartis Found Symp. 2004;256:137-45; discussion 46-8, 259-69.

144. Unanue ER. Antigen-presenting function of the macrophage. Annu Rev Immunol. 1984;2:395-428.

145. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, Vonderheide RH. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. Cancer research. 2007;67:9518-27.

146. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer research. 2006;66:605-12.

147. Sica A, Rubino L, Mancino A, Larghi P, Porta C, Rimoldi M, et al. Targeting tumour-associated macrophages. Expert Opin Ther Targets. 2007;11:1219-29.

148. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006;42:717-27.

149. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell.141:39-51.

150. Kreider T, Anthony RM, Urban JF, Jr., Gause WC. Alternatively activated macrophages in helminth infections. Curr Opin Immunol. 2007;19:448-53.

151. CowardJ, H K, P C, D L, V V, DA L, et al. Interleukin-6 as a therapeutic target in human ovarian cancer. 2011.

152. Murdoch C, Giannoudis A, Lewis CE. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood. 2004;104:2224-34.

153. Correll PH, Morrison AC, Lutz MA. Receptor tyrosine kinases and the regulation of macrophage activation. J Leukoc Biol. 2004;75:731-7.

154. Ni S, Zhao C, Feng GS, Paulson RF, Correll PH. A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/Ron receptor tyrosine kinase in response to Friend virus infection. Mol Cell Biol. 2007;27:3708-15.

155. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature. 2004;432:332-7.

156. Tlsty TD, Coussens LM. Tumor stroma and regulation of cancer development. Annu Rev Pathol. 2006;1:119-50.

157. Cunha GR, Ricke WA. A historical perspective on the role of stroma in the pathogenesis of benign prostatic hyperplasia. Differentiation. 2011.

158. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8:2912-23.

159. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer research. 1999;59:5002-11.

160. Wang Y, Sudilovsky D, Zhang B, Haughney PC, Rosen MA, Wu DS, et al. A human prostatic epithelial model of hormonal carcinogenesis. Cancer research. 2001;61:6064-72.

161. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science. 2004;303:848-51.

162. Cheng N, Chytil A, Shyr Y, Joly A, Moses HL. Enhanced hepatocyte growth factor signaling by type II transforming growth factor-beta receptor knockout fibroblasts promotes mammary tumorigenesis. Cancer research. 2007;67:4869-77.

163. Gray JK, Paluch AM, Stuart WD, Waltz SE. Ron receptor overexpression in the murine prostate induces prostate intraepithelial neoplasia. Cancer Lett. 2011.

164. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. Cancer cell. 2010;17:135-47.

165. Tong XM, Wang JC, Shen Y, Xie JJ, Zhang JY, Jin J. Inhibition of inflammatory mediators and related signaling pathways by macrophage-stimulating protein in rheumatoid arthritis synovial fibroblasts. InflammRes. 2011;60:823-9.

166. Nikolaidis NM, Kulkarni RM, Gray JK, Collins MH, Waltz SE. Ron receptor deficient alveolar myeloid cells exacerbate LPS-induced acute lung injury in the murine lung. Innate Immun. 2010.

167. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012;62:10-29.

168. Zhao S, Ammanamanchi S, Brattain M, Cao L, Thangasamy A, Wang J, et al. Smad4-dependent TGF-beta signaling suppresses RON receptor tyrosine kinase-dependent motility and invasion of pancreatic cancer cells. J Biol Chem. 2008;283:11293-301.

169. Juarez P, Guise TA. TGF-beta in cancer and bone: implications for treatment of bone metastases. Bone. 2011;48:23-9.

170. Brayer J, Cheng F, Wang H, Horna P, Vicente-Suarez I, Pinilla-Ibarz J, et al. Enhanced CD8 T cell cross-presentation by macrophages with targeted disruption of STAT3. Immunol Lett.131:126-30.

171. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature. 2001;409:97-101.

172. Tuettenberg A, Huter E, Hubo M, Horn J, Knop J, Grimbacher B, et al. The role of ICOS in directing T cell responses: ICOS-dependent induction of T cell anergy by tolerogenic dendritic cells. J Immunol. 2009;182:3349-56.

173. Groom JR, Luster AD. CXCR3 in T cell function. Experimental Cell Research. 2011;317:620-31.

174. Kurachi M, Kurachi J, Suenaga F, Tsukui T, Abe J, Ueha S, et al. Chemokine receptor CXCR3 facilitates CD8(+) T cell differentiation into short-lived effector cells leading to memory degeneration. J Exp Med. 2011;208:1605-20.

175. Huber S, Hoffmann R, Muskens F, Voehringer D. Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood.116:3311-20.

176. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002;13:155-68.

177. Waltz SE, Gould FK, Air EL, McDowell SA, Degen SJ. Hepatocyte nuclear factor-4 is responsible for the liver-specific expression of the gene coding for hepatocyte growth factor-like protein. J Biol Chem. 1996;271:9024-32.

178. Yu YP, Landsittel D, Jing L, Nelson J, Ren B, Liu L, et al. Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol. 2004;22:2790-9.