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Novel Roles for Ron Receptor Signaling as a Driver of Therapeutic Resistance in Prostate Cancer

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Novel Roles for Ron Receptor Signaling as a Driver of Therapeutic Resistance in Prostate Cancer

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by

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Abstract

Approximately 14% of all men will be diagnosed with prostate cancer. Not only is prostate cancer very prevalent, but as the second leading cause of cancer related deaths in men, it is also very deadly. Many of these deaths result from resistance to androgen deprivation therapy (ADT), defined as Castration Resistant Prostate Cancer (CRPC). The majority of men with CRPC die within 5 years of diagnosis, emphasizing the need for further research for novel CRPC therapeutics. Many prostate cancers become resistant to ADT through reactivation of the Androgen Receptor (AR). Within this dissertation, we demonstrate that the cell surface receptor Ron tyrosine kinase is important in activating AR in prostate cancer under conditions of androgen deprivation, leading to resistance to ADT. Previous studies established the Ron receptor and its ligand (HGFL) as critical players in prostate cancer, with Ron expression levels in prostate cancer correlating with disease severity and loss of Ron or HGFL in the Transgenic Adenocarcinoma of Mouse Prostate model of prostate cancer severely reducing primary tumor growth. These previous studies were critical in demonstrating the importance of Ron in prostate cancer, but had yet to address the potential role of Ron in regulating CRPC. We observed that Ron overexpression was sufficient to drive resistance to castration therapy by overexpressing Ron in androgen sensitive murine and human cell lines for allograft and xenograft models of CRPC. Prostate tumors derived from Ron overexpressing prostate cancer cells display elevated AR activation and require AR to provide growth in androgen depleted conditions. Further, Ron was shown to activate AR in an epithelial cell specific manner through activation of NF-κB and β-Catenin. As we further analyzed the consequences of Ron overexpression in prostate cancer, we discovered increased macrophage recruitment into Ron overexpressing tumors. We show that macrophage infiltration into Ron overexpressing tumors enhances AR, Ron, and Axl activation in prostate cancer cells through the secretion of Gas6, demonstrating a non-cell autonomous role for macrophages in promoting Ron mediated castration resistant growth. Excitingly, as we pursued the role of macrophage secreted Gas6 to drive growth of Ron overexpressing tumors we made the novel discovery that Gas6 binds to and

induces activation of Ron. Discovery of this interaction has the potential to provide clarity to a number of questions that have remained unanswered regarding the role of Ron in the absence of HGFL. As we further investigated roles for Ron in prostate cancer we made the observation that Ron signaling is important for enhancing the effects of diet-induced obesity on prostate cancer. Obese patients have a higher incidence of developing aggressive metastatic prostate cancer compared to lean patients. Herein we provide evidence that adipocyte specific Ron signaling is essential for obesity to enhance prostate cancer growth and metastatic potential. Taken together, this work demonstrates several novel functions for the Ron receptor in driving prostate cancer and provides rationale for targeting this signaling pathway for the treatment of men with aggressive disease.

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Chapter 1

Therapeutic Considerations for Ron Receptor Expression in Prostate Cancer

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Abstract

Introduction: The Ron receptor tyrosine kinase was initially discovered as a protein which played a critical role in regulating inflammatory responses. This effect was primarily determined through studies in various macrophage populations. Since its initial discovery, a role has emerged for Ron as a driver of cancer within epithelial cells. After numerous publications have detailed a role for Ron in promoting tumor initiation, growth, and metastasis, Ron has been designated as an emerging therapeutic option in a variety of cancers.

Areas Covered: This review discusses the current literature regarding the role of Ron in prostate cancer and places special emphasis on the role of Ron in both epithelial cells and macrophages. Whole body loss of Ron signaling initially exposed a variety of prostate cancer growth mechanisms regulated by Ron. With the knowledge that Ron plays an integral part in regulating the function of epithelial cells and macrophages, studies commenced to discern the cell type specific functions for Ron in prostate cancer. A novel role for Ron in promoting Castration Resistant Prostate Cancer has recently been uncovered, and the results of these studies are summarized herein. Furthermore, this review gives a summary of several currently available compounds which show promise at targeting Ron in both epithelial and macrophage populations.

Outlook: Sufficient evidence has been provided for the initiation of clinical trials focused on targeting Ron in both macrophage and epithelial compartments for the treatment of prostate cancer. A number of therapeutic avenues for targeting Ron in prostate cancer are currently available; however, special consideration will need to take place knowing that Ron signaling impacts multiple cell types. Further understanding of the cell type specific functions of Ron in prostate cancer will help inform and shape future clinical research and therapeutic strategies.

1. Introduction

Receptor tyrosine kinases are becoming increasingly prevalent medicinal targets as new information is uncovered revealing their importance in multiple diseases. Specifically, in cancer, receptor tyrosine kinases have been implicated as drivers of disease and there has been success targeting these proteins, such as the use of the monoclonal antibody trastuzumab to target HER2 in breast cancer (1). One such receptor that is receiving increased attention recently due to seminal findings regarding its crucial role in multiple cancers, such as pancreatic, breast, and prostate, is the Ron receptor/MST1R. The Ron receptor is a member of the Ron and c-Met family of cell surface receptor tyrosine kinases and is primarily expressed on epithelial cells and macrophages, although low levels of expression have been detected in other cell types (2-4). Ron and c-Met are the only two members of this family and the two receptors share some similarities in structure and function. Despite these similarities, a number of specific roles have emerged between the two receptors.

The only known ligand for Ron is Hepatocyte Growth Factor Like protein (HGFL), named due to the structural similarities to the ligand for c-Met, HGF, and the two are believed to have evolved from a common ancestor (5). HGFL is produced primarily by hepatocytes and secreted in the blood in a pro-form. Following cleavage, HGFL forms a heterodimer capable of binding to Ron (6-9). Both the Ron receptor and HGFL have been highly associated with multiple cancers (10-13). Specifically, in prostate cancer Ron was highly expressed in over 85% of primary tumors and in 100% of prostate cancer metastasis (14). The exceptionally high correlation with disease progression is one reason why Ron is the focus of numerous studies for the treatment of prostate cancer. With approximately 30,000 deaths annually in the United States from prostate cancer, the identification of novel targets to treat this disease is a crucial task that needs to be completed, and the Ron receptor is an up and coming therapeutic option (15).

1.1 Ron Structure and Function

The Ron receptor is located on chromosome 3p21.31 in humans and has homologs in several other organisms, such as rat (16), chicken (17), feline (18), mouse(16, 19), xenopus (20) and zebrafish (21). Structurally, the Ron receptor originates as an 185kDa precursor protein, which is cleaved into a 35 kDa extracellular alpha chain that is disulfide linked to a 150 kDa transmembrane beta chain. The extracellular portion of Ron contains a Sema-PSI domain required for ligand binding, while the intracellular portion possesses the kinase domain responsible for signal transduction (22). Ron activation results in receptor dimerization leading to autophosphorylation of kinase domain residues Y1238 and Y1239 and subsequent phosphorylation of Y1353 and Y1360, which induces the activation of multiple downstream signaling cascades (23). Recently, it has been discovered that the intracellular portion of Ron contains an acidic region of the juxtamembrane domain responsible for auto-inhibition; however, phosphorylation of Y1198 in the kinase domain relieves this inhibition and facilitates activation (24). A number of splicing and truncation variants have been identified for Ron, which produce differing effects on function/activation of the receptor (25). One isoform of Ron, known as short form Ron (sf-Ron/ RONΔ55), is heavily prevalent in pancreatic cancer and is constitutively phosphorylated, has transforming activity, and is resistant to many therapies targeting the extracellular portion of Ron (26). Structural variants, such as sf-Ron, will need to be taken into consideration during the development of therapeutics targeting Ron signaling.

Initially the ligand for Ron, HGFL, was identified as a protein which induced changes in macrophage shape and spreading (27). Further work indicated that not only did HGFL treatment impact mechanical characteristics of the cell, but it also limited inflammatory responses. HGFL treatment of macrophages reduced nitric oxide production following treatment with a variety of stimuli (8). Shortly thereafter, crosslinking studies were performed to determine that HGFL was binding to Ron at the cell surface (28). Ron was further implicated in mediating inflammatory response as it was determined that Ron signaling

deficient mice (TK-/-, lacking Ron tyrosine kinase domain) have a defect in the ability to regulate nitric oxide levels and incur greater tissue damage following inflammatory responses (29). The regulation of inflammatory responses through Ron signaling is a critical aspect of effective wound healing.

Ron has been shown to have both HGFL-dependent and HGFL-independent functions. Overexpression of Ron specifically in the mammary epithelium was sufficient to drive breast cancer, although, overexpression of Ron in the mammary epithelium of HGFL knockout mice produced tumors with a significant delay in mammary tumor formation (30). In this context, HGFL loss in the tumors altered cell signaling patterns, with decreased NF-κB activation and reduced β-catenin expression. It is interesting to note that genetic knockout of HGFL did not completely prevent tumor formation, indicating that there are HGFL independent functions of Ron that remain oncogenic when Ron is overexpressed. In another breast cancer study, HGFL independent functions of Ron were reported that enhanced cell spreading and survival (31). These reports suggest that Ron activation may function in the absence of HGFL possibly through a yet to be discovered ligand or through cross talk with other receptors. The ideal candidate as an alternative ligand for Ron would be HGF, however, despite having similar structural domains as HGFL, HGF does not appear to activate Ron (32). Ron activation through potential alternative ligand(s) is a current area of investigation. Interaction between Ron and C-Met has been detected and shown to result in direct transphosphorylation of the two receptors (23). Additionally, Ron has been reported to interact with and participate in receptor cross talk with PDGFR-β (33), IGF1-R (34), Plexins (35) and EGFR (36), although whether the activation is direct or through an intermediary is unknown. These prior studies make receptor cross-talk another viable option to explain HGFL independent Ron oncogenic function.

2. Ron in Prostate Cancer

2.1 Ron-Dependent Signaling Mechanisms in Prostate Cancer

Numerous studies within the past decade have expanded upon the role of the Ron receptor in prostate cancer. Our group was the first to demonstrate that Ron is critical for prostate tumor growth (14, 37). We showed that whole-body genetic ablation of Ron signaling in the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mouse model leads to decreased prostate tumor growth (37). Accompanying this research was the discovery of several tumor cell-intrinsic processes regulated by Ron to promote prostate cancer. Prostate tumors isolated from Ron-deficient TRAMP mice exhibited markedly increased tumor apoptosis and decreased microvessel density compared with controls (37). Similarly, loss of HGFL in TRAMP mice led to increased prostate tumor cell death, and this was at least in part due to down regulation of a Ron-STAT3-Bcl2-dependent pro-survival mechanism (38). Thus, Ron signaling provides a survival advantage to prostate tumor cells. Interestingly, neither loss of Ron nor HGFL in TRAMP mice led to changes in tumor cell proliferation, whereas loss of Ron in the polyoma middle T antigen model of breast cancer led to a significant decrease in cellular proliferation (37-39). These studies suggest that Ron provides different oncogenic functions across cancers from different tissues.

Ron has also been identified as an important regulator of tumor-associated macrophages (30, 38, 40, 41). Whole-body loss of Ron signaling in mouse models of breast and prostate cancer leads to increased localization of F4/80-positive macrophages within tumors coupled with altered expression of macrophage activation markers (30, 38). This suggests Ron regulates macrophage recruitment and activation within the tumor microenvironment to promote cancer. As these *in vivo* studies cannot differentiate the contributions of Ron signaling in individual cell compartments to modulation of tumor-associated macrophages, further investigation into cell type-specific mechanisms is necessary to gain a full understanding of the complex roles of Ron in maintaining a pro-tumorigenic microenvironment.

2.2 Epithelial-Specific Roles for Ron in Prostate Cancer

Many studies have demonstrated the importance of epithelial-specific Ron signaling in supporting tumorigenesis in a variety of epithelial cancers. Loss of Ron signaling diminishes tumorigenic activities of several established and primary thyroid (42), colorectal (43-45), and pancreatic (46) cancer cell lines. Furthermore, epithelial-specific overexpression of Ron in the lung, breast, and pancreas induces adenocarcinomas with metastasis in mammary and pancreatic models (47-49). Recent work has similarly uncovered the functions of prostate cancer epithelial specific Ron signaling. Knockdown of Ron in human prostate cancer cell xenografts in immunodeficient mice revealed that loss of Ron in prostate tumor epithelial cells significantly reduces tumor growth (14). Conversely, overexpression of Ron in prostate epithelial cells is sufficient to induce prostate cancer in mice (50). Taken together, this work demonstrates that epithelial Ron expression promotes murine and human prostate tumor growth *in vivo*. Interestingly, Ron overexpression selectively within prostate epithelial cells was associated with increases in cell proliferation and decreases in cell death (50). This contrast in phenotype between whole-body and cell type-specific modulation of Ron signaling may suggest Ron signaling across multiple cell types communicates within a tumor, however further examination is needed to delineate these mechanisms.

While the role of Ron in cancer cell metastasis has been characterized in several cancers, such as breast (13, 39, 49, 51-53) pancreatic (10, 34, 54), and lung (55), few studies have addressed the importance of Ron in prostate cancer cell migration, invasion, and metastasis. Initial *in vitro* studies have suggested an important role for epithelial Ron in regulating some of these phenotypes. Jiang *et al*. showed that inhibition of Ron activation with a neutralizing antibody reduced PC-3 cell migration while stimulation with recombinant HGFL increased cell migration (56). A second study revealed consistent results with these findings by demonstrating that knockdown of Ron in PC-3 and DU-145 cells attenuates cell migration and invasion. Moreover, treatment with HGFL was sufficient to induce migration and invasion.

In these models, ERK1/2 was shown to mediate HGFL-induced cell migration and invasion, suggesting this pathway plays a key role in Ron-mediated prostate cancer cell metastasis (57). These data suggest that epithelial Ron signaling is an important promoter of prostate cancer cell migration and invasion; however additional studies are needed to test the role of Ron in prostate cancer metastasis *in vivo* and expand upon the mediating mechanisms.

Prostate cancer epithelial-specific Ron expression has also been established as a key regulator of the tumor microenvironment. Ron in human prostate cancer cells positively regulates production of angiogenic chemokines through activation of NF-κB (14). Furthermore, Ron expression in these cells was deemed necessary for endothelial cell recruitment and prostate tumor vascularization. Studies performed using TRAMP mice support the role of Ron signaling in angiogenesis, as loss of Ron or HGFL leads to decreased prostate tumor microvessel density as measured by CD31 staining (37, 38).

2.3 Macrophage-Specific Roles for Ron in Prostate Cancer

Continuing the investigation into cell type specific functions for Ron has led to multiple discoveries regarding the role of Ron in macrophages to promote prostate cancer. Research has uncovered that Ron is expressed primarily on tissue resident macrophages and terminally differentiated macrophages, but Ron expression is markedly lower in circulatory monocytes (4, 58-61). This observation was also supported in prostate tumor tissue with Ron expression detected in tumor resident macrophages in an orthotopic model of prostate cancer (41). Using a model of breast cancer, Ron expression was connected specifically to a subset of tumor associated macrophages that express Tie2 (62). The significance of macrophage specific Ron expression for prostate tumor growth was first directly examined when mice harboring a myeloid specific deletion of the Ron tyrosine kinase domain (LysMCre TKf/f) were orthotopically injected with syngeneic murine C2RE3 prostate cancer cells. In this model, mice with a myeloid specific Ron loss developed significantly smaller prostate tumors and exhibited

increased tumor cell apoptosis compared to transplantation into Ron proficient counterparts (41). Interestingly, prostate tumors in Lys-M-Cre TKf/f mice had an increase in the number of tumor-infiltrated macrophages. This observation was consistent with whole body loss of Ron signaling in both prostate and breast cancer murine models (30, 38), suggesting that macrophage-specific Ron expression is at least partly responsible for regulating macrophage tumor infiltration.

Research regarding Ron expression in macrophages has established that Ron is capable of promoting a M2 macrophage phenotype, as Ron expression promotes arginase expression and inhibits inducible nitric oxide synthase (iNOS) expression (30, 41, 62-64). Macrophage activation can be characterized as a continuum between M1 and M2, with M1 traditionally being inhibitory toward tumor growth and M2 being tumor promoting (65). M2 macrophages are anti-inflammatory in nature and are known to promote angiogenesis and matrix remodeling in cancer (65, 66). Thus, loss of Ron in macrophages suppresses the M2 phenotype and produces a macrophage that is capable of infiltrating tumors and suppressing tumor growth.

Macrophages are known to impact the function of other cells within the tumor microenvironment. Given that Ron in macrophages has been shown to suppress inflammatory responses in several injury and infection models, macrophage Ron signaling may play a crucial role in regulating the tumor microenvironment (7, 60, 67-70). Moreover, Ron activation in macrophages suppresses TLR4 signaling, which could limit the activation of neighboring immune cells within the tumor thereby suppressing tumor immune surveillance (71). Indeed, myeloid specific loss of Ron resulted in reduced cytotoxic T-cell function in prostate tumors (41). This result is consistent with what was observed in a murine model of breast cancer, where T-cells isolated from Ron signaling deficient mice had increased proliferation rates, increased expression of T-cell activation markers, and increased *in vitro* cytotoxicity when co-cultured with breast cancer cells. Furthermore, the increased cytotoxic T cell response seen in tumor-bearing Ron

deficient mice was correlated with reduced tumor growth and metastasis (30). Taken together, these studies implicate Ron signaling in macrophages as a key regulator of the antitumor immune response.

2.4 Ron in Castration Resistant Prostate Cancer

Ron signaling had been established as a critical player in prostate cancer growth and development, but until recently the role of this signaling pathway had not been evaluated in the most deadly form of prostate cancer, Castration Resistant Prostate Cancer (CRPC). Ron mRNA and protein expression in patients was determined to be elevated in hormone refractory prostate cancer samples relative to hormone naïve samples (72, 73). Further, recent data from our laboratory has shown that Ron is functionally important for the development of castration resistance in several murine allograft and human xenograft mouse models (72). The ability of Ron to promote castration resistance is, at least in part, dependent on activation of β-catenin, NF-κB, and the androgen receptor. Activation of β-catenin through Ron in prostate cancer had yet to be detected, however, in breast cancer Ron has been shown to activate β-catenin for promotion of growth and in the regulation of cancer stem cells (49, 74, 75). Under androgen deprivation, Ron activation of the androgen receptor appears to be dependent on β-catenin and NF-κB. Interestingly, Ron has been reported to have differential effects on the androgen receptor depending on the presence or absence of androgens. When androgens are present, the relationship between Ron and the androgen receptor may be inhibitory (73). However, under androgen deprived conditions the relationship appears to be mutually active as Ron overexpression was shown to induce activation of the androgen receptor (72, 73) and re-expression of the androgen receptor in PC-3 cells was shown to induce transcription of Ron (73).

Understanding the differential effects of Ron on the androgen receptor is critical for the treatment of patients with CRPC, because the majority CRPCs have low levels of androgens due to treatment with androgen deprivation therapy. Additionally, several androgen receptor variants have been uncovered

which play pivotal roles in prostate cancer, most notably AR-variant 7 (76). Further studies should focus on determining if Ron expression alters androgen receptor variant expression, and if so under what conditions. Moreover, reports have shown that macrophage androgen receptor expression plays an important role in the development/initiation of prostate cancer (77). With this information, understanding the impact that Ron inhibition may have on macrophage function and on androgen receptor signaling during the treatment of CRPC patients may prove to be crucial. CRPC is a devastating disease with limited treatment options and these initial studies established the scientific underpinnings for targeting Ron in CRPC.

3 Available Therapeutic Options for RON

3.1 Small Molecules

Possessing an intracellular kinase domain has made targeting Ron with a small molecule inhibitor (SMI) a realistic possibility. As such, several inhibitors have been developed that show efficacy against Ron. Specifically, Foretinib (EXEL-2880) is a SMI with high specificity against Ron, c-Met, and VEGF and was shown to reduce proliferation in cancer cells (78). A clinical trial with Foretinib has yet to be completed for prostate cancer; however, a phase II clinical trial was completed in Triple Negative Breast Cancer showing a clinical benefit rate of 46% (79). Clinical benefit rate was defined as the percentage of patients who exhibited a partial response or stable disease following treatment. Recent pre-clinical work in prostate cancer cell lines indicates that Foretinib treatment may be beneficial in prostate cancer as treatment suppressed metastasis and reversed epithelial to mesenchymal transition (57).

Another intriguing SMI is the compound known as ASLAN002/BMS-777607. ASLAN002 is a dual Ron/c-Met tyrosine kinase inhibitor, but is one of the few compounds available that has preferential action against Ron over c-Met (80). A phase 1 clinical trial of ASLAN002 recently completed in patients with metastatic solid cancers and showed that the inhibitor is well tolerated and suggested that a phase 2

clinical trial begin with the treatment of 300mg twice daily (81). Recently, preclinical work from our laboratory with ASLAN002 illustrated that treatment in combination with castration therapy for castration resistant prostate tumors in a murine model of CRPC inhibits tumor growth (72). Additionally, bone metastases are frequent occurrences in metastatic prostate cancer patients and work by Andrade *et al* showed that treatment with ASLAN002 limits cancer-mediated bone destruction in murine models (82). The numerous reports demonstrating that ASLAN002 is safe and possibly effective at treating prostate cancer warrants further clinical study regarding use of this compound in prostate cancer patients.

A more recently developed inhibitor for Ron/c-Met is Merestinib/LY2801653 (83). Preclinical work with this compound showed its ability to inhibit cancer cell proliferation and cell scattering, and showed potent in vivo antitumor effects in xenograft mouse models (83). Merestinib recently completed a phase 1 clinical trial to determine tolerability in humans and the results have yet to be released (trial I3O-MC-JSBA, NCT01285037). Each of these SMI compounds targeting Ron has the potential to benefit prostate cancer patients. However, knowing that Ron promotes prostate cancer through its expression in both epithelial cells and macrophages, further research into how treatments should be targeted in patients is warranted.

3.2 Antibodies

A number of monoclonal antibodies have been generated toward the Ron receptor, with some making progress in clinical trials. Monoclonal antibodies against Ron can be used to directly target Ron signaling in cancer or they can be fused to a cytotoxic agent and used to guide that agent toward the tumor with Ron overexpression. Narnatumab/IMC-RON8 is a fully humanized monoclonal antibody that binds with high affinity to Ron, subsequently preventing the association of Ron with HGFL. A phase 1 clinical trial of Narnatumab has completed, determining that Narnatumab is well tolerated and provides limited

antitumor activity (84). This study produced less than ideal results, however, with only 1 patient reaching the trough concentration at which Narnatumab produced antitumor activity in animal models.

A different approach to target Ron has been used by M.H. Wang's group, where these investigators developed three antibodies that target the Maturation Required Sequence of Ron located on the extracellular domain of this receptor. These antibodies are known as Zt/g4, Zt/f2, and Zt/c9 and rather than preventing ligand binding, this interaction induces receptor internalization and degradation (45, 85, 86). The induction of receptor internalization has been exploited by this group as a means to transport cytotoxic compounds inside the cell. These antibodies have been successfully coupled to doxorubicin, 5-fluorouracil, Gemcitabine, as well as other compounds, and have shown preclinical efficacy in colon and breast cancer models (45, 85, 87). These proofs of concept studies establish the utility of Ron antibodies in cancer therapy, although none of these current antibodies have been tested in preclinical models for the treatment of prostate cancer. Furthermore, no studies have examined the impact of antibodies targeting Ron in the epithelial versus macrophage compartments.

4. The Outlook of Ron in Prostate Cancer

Significant progress has been made detailing the important role of Ron in prostate cancer since Ron was initially discovered. This progress has laid a solid groundwork for future studies to catapult the idea of the treatment of Ron signaling for prostate cancer into mainstream therapeutics. New areas of research will need to focus on the different possibilities for Ron to be used as a biomarker and the mechanism for directly targeting Ron in patients with prostate cancer. As a biomarker, plasma levels of HGFL have already been shown to correlate with prostate cancer progression and Ron expression shown to correlate with Gleason score and response to hormone therapy (73, 88). As a direct target, several compounds outlined previously are already available to begin testing in patients. Additionally, a major problem with treating prostate tumors is that they are known to have low immunogenicity, making

many immunotherapies ineffective. Knowing that macrophage loss of Ron impacts macrophage infiltration and alters T-cell function provides a basis for changing that constraint. Although not in prostate cancer, it has recently been shown that Ron inhibition in breast cancer enhances response to anti-CTLA-4 immunotherapy in murine models (89). This suggests that Ron inhibition may be able to increase antitumor immunity in prostate cancer to increase immunogenicity and sensitize tumors to immunotherapies.

With the number of studies demonstrating that Ron can impact the prostate tumor microenvironment, it is imperative to determine what role the tumor microenvironment plays in regulating castration resistant prostate cancer and whether or not targeting Ron alone or in combination with therapeutics that focus on the tumor microenvironment will be efficacious. Work within this thesis demonstrates novel roles for Ron in driving castration resistance directly within prostate cancer cells and by increasing macrophage recruitment into prostate tumors. Additionally, this thesis details a novel role for Ron in adipocytes for promoting prostate cancer growth and metastatic potential. With this knowledge, Ron has now been implicated in impacting endothelial cells, macrophages, T-cells, and adipocytes of the prostate tumor microenvironment through various mechanisms. With Ron signaling playing a significant role in regulating the structure and function of the tumor microenvironment to promote prostate cancer, coupling Ron to other therapies which target the microenvironment may prove effective in Ron overexpressing CRPC tumors. Further clinically focused research is essential to discerning what patients will benefit from Ron directed therapy and what method will be best suited for targeting Ron in prostate cancer.

Chapter 2

Tumor Cell Autonomous Ron Receptor Expression Promotes Prostate Cancer Growth Under Conditions of Androgen Deprivation

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Abstract

Current treatment strategies provide minimal results for patients with castration resistant prostate cancer. Attempts to target the androgen receptor have shown promise, but resistance ultimately develops, often due to androgen receptor reactivation. Understanding mechanisms of resistance, including androgen receptor reactivation, are crucial for development of more efficacious CRPC therapies. Here, we report that the RON receptor tyrosine kinase is highly expressed in the majority of human hormone refractory prostate cancers. Further, we show that exogenous expression of RON in human and murine prostate cancer cells circumvents sensitivity to androgen deprivation and promotes prostate cancer cell growth in both in vivo and in vitro settings. Conversely, RON loss induces sensitivity of castration resistant prostate cancer cells to androgen deprivation. Mechanistically, we demonstrate that RON overexpression leads to activation of multiple oncogenic transcription factors (namely β-catenin and NF-κB), which are sufficient to drive androgen receptor nuclear localization and activation of AR responsive genes under conditions of androgen deprivation and support castration resistant growth. In total, this study demonstrates the functional significance of RON during prostate cancer progression and provides a strong rationale for targeting RON signaling in prostate cancer as a means to limit resistance to androgen deprivation therapy.

Introduction

In spite of recent advances in the knowledge and understanding of prostate cancer, effective therapeutic strategies for men with aggressive castration resistant prostate cancer (CRPC) remain elusive, resulting in an estimated 26,730 deaths in the United States in 2017(90). Men diagnosed early with localized prostate cancer have positive clinical responses when treated with surgery and radiation, however, a large population of men either experience recurrence following surgery or are diagnosed with metastatic disease. A primary driver of prostate cancer is the androgen receptor (AR), and for men with recurrent and/or metastatic disease, the treatment is often Androgen Deprivation Therapy (ADT). ADT serves as a form of chemical castration to limit the supply of androgens available for activation of AR signaling. The timeframe in which ADT is effective varies between patients, however, once resistance develops (termed CRPC) the outlook is poor and median survival is only ~19 months (91). The advent of new compounds targeting AR signaling in CRPC, such as Abiraterone or Enzalutamide, have provided a slight increase in overall survival; however, resistance inevitably develops and leads to death of the patient (92, 93). New approaches are needed for treating men with prostate cancer at this stage of disease, where the majority of deaths from prostate cancer occur and currently all available therapeutic options have been ineffective at providing long term benefits to patients.

Numerous reports have cited a crucial role for receptor tyrosine kinases (RTKs) in promoting cancer, and subsequently the use of small molecule RTK inhibitors have been proposed as a novel treatment for men with CRPC (94). An example of this is Cabozantinib, which was shown to reduce the progression of some pathological variables in select CRPC patients, but was unable to significantly improve overall survival (94). An explanation for this lack of efficacy is that many RTK inhibitors (like Cabozantinib) have several targets, and this minimal specificity prevents effective doses to be reached due to unacceptable adverse events in the patient (94). By understanding the importance of the various targets of such inhibitors, we

have the capability to formulate compounds with enhanced specificity for the critical players that promote CRPC, which can in return provide more promising clinical outcomes.

An emerging target of several RTK inhibitors is the RON receptor tyrosine kinase/Macrophage Stimulating 1 Receptor (MST1R). RON is a cell surface RTK that is primarily expressed on epithelial cells and select macrophage populations, where activation of RON will function to reduce inflammation and promote wound healing (4, 41, 60, 67, 70, 95). Overexpression of RON has been observed in a number of solid cancers, and within cancer RON promotes phenotypes such as survival, proliferation, migration/invasion, angiogenesis, and stemness (14, 38, 39, 74). Specifically, in prostate cancer previous studies have established the RON receptor as critical for cancer development and progression (14, 37, 41, 50). Selective overexpression of RON in the prostate epithelium of mice induces prostate intraepithelial neoplasia with invasion or adenocarcinoma in the majority of animals, indicating RON as sufficient to drive prostate cancer (50). The necessity of RON signaling for prostate cancer growth was determined through genetic loss of RON signaling in the probasin driven T-antigen (PB-TAg/TRAMP) murine model of prostate cancer, where mice deficient in RON signaling developed significantly smaller prostate tumors compared to control mice (37, 38). Furthermore, plasma levels of the ligand for RON (Hepatocyte Growth Factor-Like Protein/Macrophage Stimulating Protein) positively correlate with prostate cancer progression in patients and was observed to be significantly elevated in CRPC patients (88). Mechanistically, our laboratory has connected RON to activation of several oncogenic signaling pathways in prostate cancer, such as NF-κB/RELA, STAT3, and BCL-2 signaling (14, 38, 74, 75). Despite the strides made in understanding the function of RON in prostate cancer, research regarding its role in promoting CRPC has been minimal, but is of the utmost importance due to the high number of deaths each year resulting from this form of disease

In this report, we demonstrate that the RON receptor is highly expressed in CRPC. We have investigated the consequences of RON expression for prostate cancer growth *in vivo* in response to castration using murine transplantation models and *in vitro* in response to androgen deprivation using sphere forming assays. Strikingly, we show that RON expression is sufficient for prostate tumor growth following androgen withdrawal. Notably, we provide direct evidence of RON mediated AR activation through the autonomous stimulation of two transcriptional effectors, β-catenin and NF-κB, highlighting the impact of RON signaling as a mechanism of resistance to ADT. The conclusions of this study establish the importance of RON signaling in CRPC and provide strong rationale for the development of specific inhibitors targeting the RON receptor signaling pathway as treatment for this devastating disease.

Results

RON expression is elevated in CRPC patient samples and is critical for tumors to develop castration resistance. RON receptor signaling has been established as a central factor in promoting prostate cancer in several murine models (14, 37, 38, 41, 50). To assess the significance of RON expression in human prostate cancer, multiple human data sets were analyzed using the Cancer Outlier Profile Analysis (COPA) method (96-104). COPA identifies novel oncogenic drivers by taking into account the heterogeneity of tumors through assessing overexpression in subsets of cancers, as opposed to performing a simple t-test over an entire class of cancer (103). Analysis indicates that RON has comparable COPA scores to the prominent oncogene ERG in prostate cancer, highlighting the potential importance of RON in this disease (**Table 1**).

When assessing the expression of RON in human CRPC, data sets comparing hormone naïve vs hormone refractory prostate cancers were analyzed. RON expression was found to be significantly higher in hormone refractory samples relative to hormone naïve samples, and this was true whether the hormone refractory tissue was from the prostate or isolated from a metastatic site (**Fig. 1A**) (105, 106). To examine whether RON mRNA expression correlated with increased protein expression, RON immunohistochemistry was performed on human prostate tissue microarrays and expression levels were compared across normal, localized, and hormone refractory samples. Previous reports have shown RON protein expression is elevated during prostate tumorigenesis, although it has not been reported whether or not RON protein expression changes in response to hormone therapy (14). In support of previous studies, RON expression was elevated in localized prostate cancer tissue relative to normal tissue (**Fig. 1B**). Strikingly, however, RON expression was drastically higher in hormone refractory patient samples than both normal and localized samples, suggesting that RON may play a causal role in hormone refractory disease (**Fig. 1B**).

Several reports have established a role for RON in the development and growth of prostate cancer (14, 37, 38, 41, 50). However, mechanisms linking RON to castration resistant prostate cancer, which leads to the death of thousands of men annually, have not been investigated. To experimentally evaluate the requirement of RON in CRPC, multiple *in vitro* and *in vivo* CRPC models were assessed. Western blot analysis demonstrated that RON expression was elevated in the castration resistant C4-2B cell line relative to the parental castration sensitive human LNCaP cell line (**Fig. 1C**) (107, 108). To directly determine the requirement of RON in CRPC, intact FVB male mice were injected with Myc-CaP cells to establish androgen sensitive Myc-CaP prostate tumors (109). Mice bearing tumors of 1000mm^3 were castrated and outgrowth of castration resistant Myc-CaP tumors was monitored temporally. **Figure 1D** demonstrates that Myc-CaP tumors upregulate RON expression following the development of castration resistance in this model. Furthermore, western blot analysis of a cell line derived from a castration resistant tumor, termed Myc-CaP-C, showed increased RON expression relative to parental Myc-CaP cells, similar to what was observed with human cell lines (**Fig. 1C**). Knockdown of RON in Myc-CaP-C cells (**Fig. 1D**, Myc-CaP-C sh*Ron*) led to a delay in the growth of castration resistant tumors following subcutaneous implantation into pre-castrated FVB mice (**Fig. 1E**). Further, while the growth of Myc-CaP-C tumors were not sensitive to castration following implantation into intact FVB male mice, RON knockdown led to Myc-CaP-C tumor regression following castration (**Fig. 1F**). Overall, these data demonstrate a causal function for RON in driving prostate tumor growth under conditions of androgen deprivation and suggests that RON may be a biomarker for aggressive prostate cancers.

RON overexpression mediates castration resistant growth *in vivo*. To investigate whether RON overexpression is sufficient to confer castration resistant growth to Myc-CaP tumors *in vivo*, RON expression was modulated in Myc-CaP and LNCaP cells. RON was either exogenously expressed (RON OE, **Fig. 2A**) or CRISPR/Cas9 technology was used to delete RON in Myc-CaP cells (RON KO1 and RON KO2, **Fig. 2A**). RON modulated and control cells were implanted subcutaneously into intact male FVB

(for Myc-CaP cells) or immunodeficent (for LNCaP cells) mice and once tumors established, mice were surgically castrated and tumor growth was monitored. Strikingly, we observed that exogenous RON expression, in either murine or human prostate cancer cell lines, conferred castration resistant growth to established tumors compared to control tumors which were sensitive to castration (**Figs. 2B** and **2C**). Moreover, we found that genetic RON loss resulted in sustained sensitivity of established Myc-CaP prostate tumors to androgen deprivation for at least three weeks following castration, compared to control cells which allowed androgen-independent growth of tumors during this same time frame (**Fig. 2B**). Considering the importance of RON in CRPC, we next evaluated the effects of targeting RON under castration conditions utilizing Myc-CaP RON OE tumors implanted into precastrated FVB mice. Once tumors reached 100mm^3, mice were treated daily by oral gavage with either vehicle or BMS-777607, a small molecule inhibitor of RON/c-MET family receptor tyrosine kinases (80). As shown in **Figure 2D**, pharmacologically inhibiting RON in Myc-CaP RON OE tumors with BMS-777607 blocked castration resistant tumor growth. These data provide the first direct evidence that therapeutic targeting of RON restores sensitivity to castration *in vivo*.

RON expression mediates castration resistant growth *in vivo* through oncogenic signaling pathways that enhance tumor cell proliferation and reduce apoptosis. As RON expression levels correlate with the ability of tumors to grow in the context of castration, we next evaluated whether RON modulation would potentiate the effects of castration. As shown in **Fig. 3**, Myc-CaP prostate tumors with RON expression displayed a higher proliferative index as seen by increased numbers of Ki67-positive cells. Further, RON expression was inversely correlated with tumor cell apoptosis as judged by TUNEL staining. The most dramatic effects of RON modulation were observed under castration conditions, with a 3.2-fold decrease in apoptosis observed in Myc-CaP RON OE tumors and a 1.9-fold increase following RON knockdown in Myc-CaP-C tumors. Correspondingly, under castration conditions there was a 1.7-fold

increase in proliferation in Myc-CaP RON OE tumors and a 3.3-fold decrease in proliferation following RON knockdown in Myc-CaP-C tumors.

Having established a crucial role for RON expression in prostate cancer growth under castration conditions, we sought to identify the oncogenic signaling pathways mediated by RON (**Fig. 4**). As persistence of AR signaling has been shown to be important in castration resistance, we determined whether RON expression influences AR signaling through the activation of reported AR target genes (**Fig 4A**) and AR nuclear localization (**Fig. 4B**). The AR target genes *Tmprss2* and *Klkb1* were both expressed higher in Myc-CaP RON OE castrated tumors compared to Ctrl castrated tumors (**Fig. 4A**). Correspondingly, exogenous RON expression also induced the nuclear localization of AR from Myc-CaP castrated tumors (**Fig. 4B**). We further explored the possibility that RON dependent signaling pathways may mediate AR activation under conditions of androgen deprivation. As shown in **Fig. 4B**, RON expression facilitated the nuclear localization of two transcription factors, β-CATENIN and NF-κB, that have been associated with aggressive prostate cancers and activation of the AR (110-113). To substantiate data from our murine CRPC model, we queried patient samples from the cancer genome atlas (TCGA) database and found a positive correlation between gene expression levels of RON (*MST1R*) with both β-CATENIN (*CTNNB1*) and NF-κB (*RELA*/*RELB*) (**Fig. 4C**). Collectively, the data suggests that RON-mediated activation of these transcription factors may be responsible for castration resistant tumor growth.

Activation of oncogenic β-CATENIN, NF-κB, and AR is required for RON to promote growth under androgen deprivation. Given the elevated activation of β-CATENIN, NF-κB, and AR in response to RON overexpression, we tested whether RON-mediated activation of these transcription factors is necessary for castration resistant growth using 3D cell culture assays. Cells were grown in 3D culture conditions under androgen deprived (Charcoal Stripped Serum, CSS) or in androgen containing (Complete)

conditions for 10 days prior to assessing growth. Strikingly, Myc-CaP RON OE cells formed 2.8-fold more spheres compared to Myc-CaP Ctrl cells under conditions of androgen deprivation (**Fig. 5A**). No difference in sphere formation was observed when these same cells were in androgen containing media (**Fig. 5A**). In addition to Myc-CaP cells, identical results were obtained following ectopic RON expression in the human LNCaP cells wherein RON expression lead to significantly greater sphere formation compared to LNCaP Ctrl cells under conditions of androgen deprivation (**Fig. 5B**). Correspondingly, *RON* knockdown in human C4-2B cells reduced sphere formation relative to control shNT C4-2B cells under androgen deprivation (**Fig. 5B and 5E**). These findings suggest that RON expression is essential for sphere formation selectively under conditions of androgen deprivation and provide further rationale for therapeutic targeting of RON in CRPC. To more broadly understand the scope of therapeutic targeting of RON, several androgen responsive and non-androgen responsive cell lines were treated with the RON/c-MET family receptor tyrosine kinase inhibitor BMS-777607 under androgen containing and androgen depleted conditions. Strikingly, we observed that AR expressing cell lines (Myc-CaP, Myc-CaP RON OE, LNCaP, LNCaP RON OE, and C4-2B) were only sensitive to the RON inhibitor under androgen depleted conditions (**Figs. 5A**, **5B**). Alternatively, cell lines without AR expression (PC-3, DU145) were sensitive to the inhibitor under both androgen containing and androgen depleted conditions (**Fig. 5C**). These findings suggest that RON inhibition is a viable therapeutic option for patients with CRPC that is both AR+ and AR-, however, in AR+ patients RON inhibition should be coupled with androgen deprivation therapy.

As our data demonstrate that RON expression promotes AR nuclear localization and the expression of AR target genes under conditions of androgen deprivation (**Fig. 4**), we next sought to examine the requirement of β-CATENIN and NF-κB activation downstream of RON for androgen independent prostate cancer growth. Similar to tumors, Myc-CaP RON OE cells also exhibited increased nuclear localization of β-CATENIN and NF-κB (**Fig. 5D**). To examine the requirement of β-CATENIN and NF-κB, we ectopically expressed either IKKβ, for activation of NF-κB, or β-CATENIN (**Fig. 5E**) in Myc-CaP cells. Clonal

pools of expressing prostate cancer cell lines were then examined for 3D growth under conditions of androgen deprivation. Interestingly, both β-CATENIN and IKKβ expression promoted sphere formation to levels comparable to RON expression in media devoid of androgens (**Fig. 5A**). No changes in sphere formation were observed when the same cells were cultured in complete media (**Fig. 5A**), demonstrating the selectivity of tumor growth for these transcription factors under conditions of androgen deprivation. Having established that RON expression induces nuclear localization of β-CATENIN and NF-κB, we next sought to test whether IKKβ and/or β-CATENIN expression was sufficient to induce prostate cancer cell sphere formation under androgen deprivation. For these studies, Myc-CaP RON KO2 cells were generated which ectopically expressed IKKβ or β-CATENIN (**Fig. 5E**). Overexpression of either protein augmented sphere formation in the RON deficient cell line compared to control cells under androgen deprived conditions (**Fig. 5A**). Interestingly, knockout of RON did not reduce sphere formation under androgen deprived conditions relative to Myc-CaP control cells (**Fig. 5A**).

To further validate β-CATENIN and NF-κB as crucial RON-dependent downstream mediators of AR activation and prostate cancer cell sphere formation under conditions of androgen deprivation, Myc-CaP RON OE cells either had β-CATENIN knocked down or were treated with the NF-κB signaling inhibitor Bay-11-7085 (114). Remarkably, either β-CATENIN knockdown or NF-κB inhibition significantly reduced sphere formation in Myc-CaP RON OE cells under androgen deprivation conditions (**Fig. 5F**). While no changes were observed in sphere formation in Myc-CaP RON OE cells treated with Bay-11-7085 in complete media (**Fig. 5F**), knockdown of β-CATENIN in Myc-CaP RON OE cells led to a reduction in sphere formation when the cells were cultured in complete media. However, the reduction in sphere formation in complete media was modest (1.3 fold) compared to the large reduction (3.5-fold) observed with androgen depletion (**Figs. 5F**), suggesting the importance of β-CATENIN signaling for prostate cancer growth in both the presence and absence of androgens.

Furthermore, we confirmed that Myc-CaP RON OE cells require AR activation to promote sphere formation under androgen deprived conditions by treating Myc-CaP RON OE cells with Casodex (CDX) [a well-established AR inhibitor (115)]. Treatment of parental Myc-CaP cells with casodex reduced sphere formation under both androgen deprived and androgen containing conditions; however, sphere formation for Myc-CaP RON OE cells was only reduced under androgen deprived conditions (**Figs. 5A and 5F**). This information suggests that RON expression is able to bypass the need for ligand dependent AR activation but that the AR is required downstream of RON for prostate cancer cell growth during androgen deprivation. Moreover, this advocates that early treatment of RON expressing tumors with combined androgen blockade and RON inhibition may be a successful therapeutic approach.

β-CATENIN and NF-κB independently induce AR activation to promote growth under androgen deprivation *in vitro.* We found activation of β-CATENIN, and NF-κB to be crucial for RON to promote sphere formation under androgen deprivation (**Fig. 5**). We next investigated whether these transcription factors are capable of activating AR during androgen withdrawal. To assess AR activation, Myc-CaP β-CATENIN OE and Myc-CaP IKKβ OE cells were grown in charcoal stripped serum and subsequently fractionated into nuclear and cytoplasmic fractions. As depicted in **Figure 6A**, ectopic β-CATENIN expression led to increased accumulation of nuclear β-CATENIN as well as induced the nuclear localization of AR. Correspondingly, ectopic IKKβ expression induced both NF-κB and AR nuclear localization (**Fig. 6B**). These findings indicate that both β-CATENIN and NF-κB induce AR nuclear localization, but may do so independently of one another. Consistent results were obtained when lithium chloride was used to activate β-CATENIN in Myc-CaP Ctrl cells and when Bay-11-7085 was used to inhibit NF-κB pharmacologically in Myc-CaP RON OE cells. Lithium chloride strongly induced both β-CATENIN and AR nuclear localization in Myc-CaP Ctrl cells (**Fig. 6C**) while Bay-11-7085 treatment reduced AR and NF-κB nuclear localization in Myc-CaP RON OE cells (**Fig. 6D**). In addition to nuclear localization, AR reporter activity was elevated upon β-CATENIN OE, IKKβ OE, and RON OE compared to Myc-CaP Ctrl

cells as demonstrated by increased expression of the ARR2PB luciferase reporter construct which contains two androgen response elements (**Fig. 6E**). Lastly, to determine if AR activation was required for β-CATENIN or NF-κB to promote growth under androgen deprivation, we treated Myc-CaP β-CATENIN OE and Myc-CaP IKKβ OE cells with casodex and performed an *in vitro* 3D growth assay under androgen deprived and androgen containing conditions. **Figure 6F** demonstrates that β-CATENIN OE and IKKβ OE prostate cancer cells exhibited a significant reduction in 3D sphere formation under conditions of androgen deprivation when treated with Casodex compared to vehicle treated controls (**Fig. 6F**). The cell lines exhibited similar sphere formation in complete media regardless of treatment with Casodex. These data illustrate the necessity of β-CATENIN and NF-κB activation to induce AR re-activation during androgen independent growth. Taken together, these data demonstrate that both β-CATENIN and NF-κB function downstream of RON as a means to promote AR reactivation in times of androgen deprivation for sustained prostate cancer growth.

Discussion

Castration Resistant Prostate Cancer (CRPC) is a devastating disease with limited treatment options. Our data reveals the RON receptor signaling pathway as a novel target for the treatment of CRPC. We and others have recently reported that RON protein levels are elevated in castration resistant prostate cancer cell lines and that RON mRNA expression is elevated in castration resistant human prostate tumors (14, 73). Consistent with these reports, we observed that RON protein levels are greatly elevated in hormone refractory prostate cancer patient samples compared to hormone naïve samples and that this expression is critical for castration resistant tumors to maintain resistance (**Fig. 1**). Not only is RON expression vital for tumors that are castration resistant, but we show that RON overexpression can drive resistance to castration therapy (**Fig. 2**). These studies are the first to show elevated RON protein levels in hormone refractory patient samples and to demonstrate the *in vivo* significance of RON in supporting CRPC. These findings build on prior studies showing that RON is capable of promoting mechanical changes in prostate cancer cells which may be advantageous for survival under androgen deprived conditions (73). Based on multiple reports demonstrating the significance of RON in prostate cancer, we postulate that elevated RON levels may serve as a novel biomarker for aggressive prostate cancers, which may require more aggressive treatment strategies.

We further showed that RON expressing prostate tumors exhibit enhanced tumor cell proliferation and decreased cell death both prior to and following castration conditions *in vivo* (**Fig. 3**), with the most profound changes occurring post castration. Mechanistically, we found that under castration conditions, RON overexpressing Myc-CaP cells formed tumors with increased activation of AR, β-CATENIN and NF-KB based on elevated nuclear localization compared to control Myc-CaP cells (**Fig. 4**). Consistent with AR activation, an increase in AR target gene expression was observed (**Fig. 4**). Interestingly, a previous report showed that the relationship between RON and AR may vary between activating or inhibitory, although the exact details for each scenario remained unclear (73). Our data show that under castrate

conditions, RON has an activating function on AR. Identifying this connection is intriguing, because although several therapeutics have been used to limit AR activation in prostate cancer, the ability of providing sustained AR inhibition has remained elusive (116). Some therapies, such as abiraterone acetate, orteronel and galeterone, have been used to reduce systemic androgen production to limit AR activation, but have yet to provide sustained results (116). In combination with traditional ADT, these therapies help limit ligand mediated AR activity, but fail at preventing ligand-independent AR activation. Our data suggests that overexpression of the RON receptor may function as a major alternative ligand-independent pathway used by aggressive prostate cancers to activate the AR. This makes targeting the RON receptor signaling pathway a strong candidate for reducing the activation of the AR and eliminating the growth of CRPC.

Previous studies have detailed an association for both β-CATENIN and NF-κB activation with poor clinical prognosis in prostate cancer patients (110, 111); however, the studies herein discover dual activation of these two transcription factors downstream of RON in the context of CRPC (**Fig. 5**). This information makes targeting RON an appealing approach because targeting this single pathway may have broad negative consequences for tumor progression. Prior work from our group in breast cancer illustrated a similar relationship, with RON activation leading to β-CATENIN and NF-κB activation. However, in breast cancer, a linear relationship was observed with RON activation of NF-κB being dependent on β-CATENIN (74). It is interesting to note, that in CRPC we observed independent activation of β-CATENIN and NF-κB downstream of RON under conditions of androgen deprivation, suggesting that this relationship may be a product of cancer cells being under intense stress brought on by androgen withdrawal. The independent activation of these transcription factors through RON may function as a survival mechanism for cancer, and it would be interesting to test whether or not this mechanism exists in other cancers following different stress conditions, such as after chemotherapy treatment or in conditions of hypoxia. This concept is substantiated by other studies in breast cancer wherein RON expression was

shown to be a predictor of recurrent disease (40, 117, 118). The broad potential of RON as a driver of resistance and recurrence makes targeting RON a viable option for treating aggressive cancers, potentially of many different types, that have risen up following treatment.

Consistent with published studies, we also found that activation of either β-CATENIN or NF-κB leads to enhanced AR activation observed by an increase in AR nuclear localization and ARR2PB promoter luciferase activity (**Fig. 6**) (112, 113). Further, we observed that RON-dependent activation of either β-CATENIN or NF-κB requires the AR to promote sphere formation under androgen deprived conditions (**Fig. 6**). This data is consistent with our experiments showing that RON overexpression is unable to overcome inhibition of the AR under androgen deprivation *in vitro,* suggesting that RON functions upstream of β-CATENIN, NF-κB and the AR (**Fig. 5**). Interestingly, others have shown β-CATENIN and AR nuclear localization to be significantly correlated in human CRPC, but not in Hormone Naïve Prostate Cancer (HNPC) (119). In another study examining NF-κB and the AR, a strong positive correlation between NF-κB and AR protein expression was observed in human CRPC samples (112). These prior studies are consistent with our data wherein RON expression is elevated in CRPC samples relative to HNPC samples, indicating that an increase in RON expression may be driving the correlations between β-CATENIN, NF-κB, and AR.

Identifying an activating role for RON upstream of AR was informative as a previous report showed that RON may inhibit AR signaling in the AR expressing C4-2B cell line when androgens are present (73). Our data build upon this report and show that in AR expressing cells, under conditions of androgen deprivation, RON functions to drive castration resistance and is associated with AR activation. This information is significant as a large number of patients undergo ADT for the treatment of CRPC. Additionally, we have determined that prostate cancer cells which have adapted to grow in the absence of AR expression still have a requirement for RON overexpression to promote growth. This is illustrated

in **Fig. 5** wherein the growth of AR expressing cells was reduced by RON inhibition only under androgen depleted conditions, while in AR-negative cell lines, prostate tumor cell growth was inhibited under both androgen containing and depleted conditions. We hypothesize this is due to a reprogramming of cells to more heavily rely on other growth pathways activated by RON, like NF-κB, β-Catenin, STAT3, or AKT, as a response to the loss of AR. Determining which signal transduction pathways are necessary for RON to promote growth when AR expression is lost is an area that requires further study. Together, these reports show that RON plays a critical role in promoting growth of AR expressing and AR-negative cells and further establishes RON as a viable target for men with aggressive prostate cancer.

Further work is needed to establish the mechanism(s) by which RON induces β-CATENIN and NF-κB activation and to uncover how these two proteins facilitate AR activation. Prior work from our group has shown that, in breast cancer, RON induces tyrosine phosphorylation of β-CATENIN at residues 654 and 670, facilitating its activation (75). Additional studies will be needed to confirm if this relationship exists in the context of CRPC. In regards to NF-κB, RON activation may facilitate activation of upstream members of the NF-κB pathway, such as IKKα/β and/or NIK kinase, as these proteins have been shown by others to be activated by receptor tyrosine kinases (120). Lastly, others have shown that there are multiple mechanisms possible for β-CATENIN and NF-κB to induce AR activation. For example, one group demonstrated that β-CATENIN can interact with the ligand binding domain of the AR to promote AR transcriptional activity (113, 121). Regarding NF-κB, others have shown that expression of NF-κB can function to maintain AR expression levels and promote AR transactivation activity (112). Whether or not these mechanisms are operant downstream of RON activation remain unclear.

In summary, our laboratory and others have demonstrated that RON is a key player in the growth and progression of prostate cancer. Here we show that RON overexpression is sufficient to drive resistance to castration therapy in multiple mouse models and uncovered a crucial role for β-CATENIN, NF-κB, and

the AR in this process. This broad activation of several important oncogenic growth pathways makes RON an attractive target for CRPC therapy as opposed to targeting a single pathway downstream; targeting RON will prevent tumors from sustaining growth due to activation of remaining oncogenic pathways. This novel therapeutic approach should be further studied as it may help provide hope to CRPC patients currently without effective therapeutic options.

Materials and Methods

Immunohistochemistry. RON immunohistochemistry was performed on human prostate cancer tissue microarrays from the Prostate Cancer Biorepository Network. Tissue staining and scoring was performed as described (14, 39, 75). Tumor tissue was fixed, paraffin embedded, and 5μm sections were stained for Ki67 (ThermoFisher Scientific) and TUNEL (Millipore). Counts were performed using ImageJ software (National Institutes of Health).

Western Blot Analyses. Cells were homogenized in RIPA buffer as described (74). Nuclear and cytoplasmic extracts were isolated by centrifugation and hypotonic lysis as described (75). Antibodies for analyses included: RON (SC-322), ANDROGEN RECEPTOR, (SC-815) and TUBULIN (SC-5286) from Santa Cruz Biotechnology; β-CATENIN (#9582S), phospho-NF-κB s536 p65 (#3033S), NF-κB (#8242S), IKKα (#2682S), IKKβ (#2678S), and LAMIN A/C (#4777S) from Cell Signaling Technologies; and ACTIN (Cincinnati Children's Hospital Medical Center, Clone C4). Peroxidase-conjugated secondary antibodies (Jackson Laboratories) were applied and membranes were developed using Pierce ECL2 Western Blotting substrate (ThermoFisher Scientific). Membranes were stripped using Restore Western Blot Stripping Buffer (ThermoFisher Scientific) before re-probing.

Mouse Models. Mice were maintained under specific pathogen-free conditions and experimental protocols approved by the University of Cincinnati IACUC. For murine cell injections, $1.0x10^6$ cells were injected subcutaneously into the flanks of 6-8 week old male FVB mice as described (109, 122). Tumor growth was measured via calipers and volume was determined by the formula $0.5xLengthxWidth^2$ [123]. Surgical castration was performed as described when tumors reached $1000mm^3$ (109, 122). Pre-castrated animals were surgically castrated at 6 weeks of age and allowed to recover for 10 days before injection of cells, as described previously (124). For *in vivo* kinase inhibitor studies, pre-castrated mice were treated with 50mg/kg/day BMS-777607 (Selleck Chemicals) or methocellulose (vehicle) via oral

gavage once tumors reached 100mm^3. For human LNCaP and LNCaP RON OE cells, 5X10^6 cells were injected subcutaneously into athymic nude mice (NCr-Foxn1^{nu}) from Charles Rivers; mice were castrated when tumors reached 500mm^3.

Cell Models. Myc-CaP cells (109) were developed in the laboratory of Charles Sawyers and were obtained from Memorial Sloan Kettering Cancer Center. For tumor formation, 1x10^6 Myc-CaP cells were injected subcutaneously into wild type FVB male mice; once tumors reached 1000 mm^3, the mouse was castrated. Myc-CaP-C cells were generated from a castration resistant tumor formed following injection of Myc-CaP cells. All Myc-CaP cells were maintained in DMEM with 10% Cosmic Calf Serum and 1% gentamycin (109). The human prostate cancer cell lines LNCaP and C4-2B were obtained from ATCC and were maintained in RPMI-1640 with 10% Fetal Bovine Serum and 1% gentamycin. The human prostate cancer cell line PC-3 was obtained from ATCC and maintained in F-12 media with 10% Fetal Bovine Serum and 1% gentamycin. The human prostate cancer cell line DU145 was obtained from ATCC and maintained in MEM with 10% Fetal Bovine Serum and 1% gentamycin. All cells were maintained at 37^{o}C and 5.0% CO_2.

Cell Transfections. Stable polyclonal cell lines were generated by performing transfection with Lipofectamine 2000 (ThermoFisher Scientific) and selection in puromycin (Invitrogen, 5μg/ml) or G418 (Invitrogen, 500 μg/ml). LNCaP Ctrl and LNCaP RON OE cells overexpressing human RON were generated as described (14). The *Ron* gene was deleted in Myc-CaP cells using CRISPR/Cas9 technology. RON knockout guide RNA was cloned into the PX458 plasmid (Addgene #48138) using the following primers: 5'-caccgCAGAGACTTGATGGCACAGT-3', 5'-aaacACTGTGCCATCAAGTCTCTGC-3'. Overexpression of β-CATENIN was performed using wild-type murine β-CATENIN cloned into the p3XFLAG-CMV vector as previously described (75). Overexpression of IKKβ was performed using the pCDNA-IKK-β construct (Addgene, #23298). The ARR2PB luciferase construct was developed in the laboratory of Robert J.

Matusik and was obtained from Vanderbilt University (125). Luciferase assay was performed as previously described using the pRL-TK renilla plasmid as an internal control (14).

Viral transduction. Lentivirus short hairpin RNA (Open Biosystems) was used to target murine sh*Ron* (RMM3981-9590952), human sh*RON* (RHS3979-9571732), murine sh *β-Catenin* (RMN1766-96879831), and nonsense shNT (RHS1764). The pCDH backbone and pCDH-CMV-EF1-PURO-RON full-length mouse RON cDNA expression vectors were utilized for control and RON overexpression. Transduction was performed as described (14, 75).

***In Vitro* Cell Growth Assays and Treatments.** 3D growth assays were performed as described with the substitution of agarose to prevent cell adhesion (38). Briefly, $2x10^4$ cells were plated on top of 1.0% agarose in 6 well plates in media supplemented with cosmic calf serum (Complete, Thermofisher Scientific) or charcoal stripped serum (CSS, Midsci). Cells were left untreated, treated with DMSO (vehicle), Bay-11-7085 (Enzo Life Sciences, 1μM), Casodex (Selleck Chemicals, 10 μM) or BMS-777607 (Selleck Chemicals, 5 μM) daily. After 10 days, images of spheres were taken using a Zeiss Axiovert S100TV inverted microscope (Carl Zeiss Microscopy) and spheres >25μm in diameter were counted using ImageJ software. The 25 μm threshold was established based on the average sphere size obtained for the control cells. For 2D growth, $2.5x10^4$ cells were plated on 12 well plates and counted every 24 hours. Myc-CaP Ctrl or Myc-CaP Ron OE cells were grown in 2D and treated with DMSO (vehicle), LiCl (Sigma, 10mM, 4 hours), or Bay-11-7085 (Bay 11, 5μM, 4 hours) when cells were ~70% confluent prior to fractionation.

Quantitative Real-Time (qRT)-PCR. RNA was extracted with TRIzol (Invitrogen) and cDNA was prepared using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Quantitative PCR was performed with 2X SYBR Green Master Mix (Roche Diagnostics) on a Mastercycler ep realplex4

(Eppendorf). Data were normalized to an 18S reference gene and analyzed by ΔΔCT. Primer sequences included: Murine *Tmprss*2 (Forward: 5'-AAGTCCTCAGGAGCACTGTGCA-3'; Reverse: 5'-CAGAACCTCCAAAGCAAGACAGC-3'). Murine *Klkb*1 (Forward: 5'-AAAGTCAGCGGACAACCTGGTG-3'; Reverse 5'-AGATGGTGCGACACACAAAGGC-3'). 18S (Forward: 5'-AGTCCCTGCCCTTTGTACACA-3'; Reverse: 5'-GATCCGAGGGCCTCACTAAAC-3').

Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by performing Student's t-test for pairwise comparisons or ANOVA for comparison of multiple groups using GraphPad Prism software. All *in vitro* experiments represent the average of at least triplicate experiments. Spearman's rank sum test was utilized to determine the P-values of correlation for different quantiles of RON expression for data taken from the TGCA data base. All *in vitro* experiments represent the average of at least triplicate experiments. Significance was set at *P<0.05.

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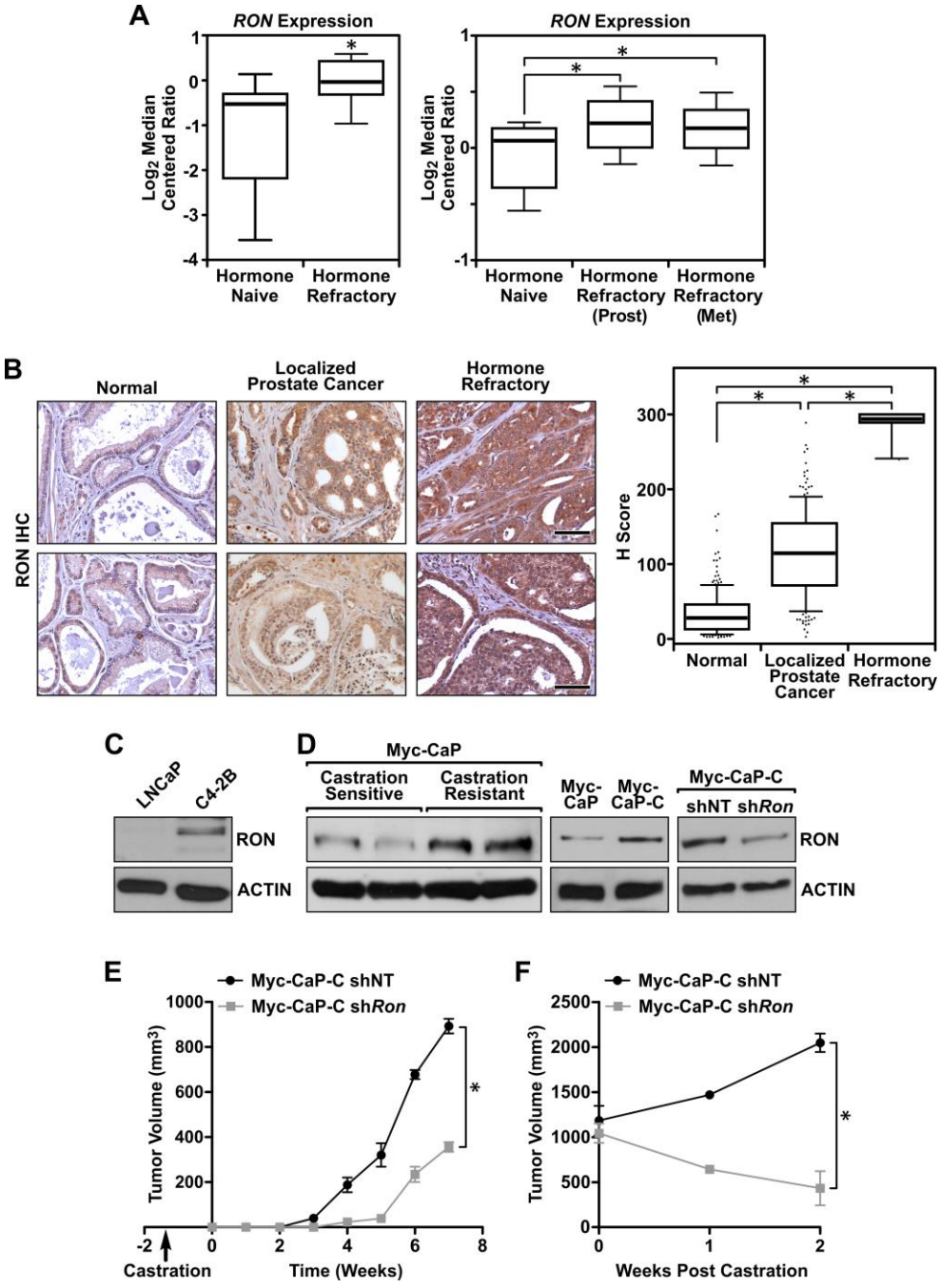


Figure 1. RON expression is elevated in CRPC patient samples and critical for tumors to develop castration resistance. **A**, Boxplot depicting relative *RON* expression from the Best (left) and Tamura (right) prostate cancer datasets. Boxplot lists 25th and 75th percentiles along with the group median. Whiskers display 10th and 90th percentiles. **B**, Representative images of normal matched (n=206), Hormone Naïve (n=186), and Hormone Resistant (n=20) patient prostate tissues stained for RON, along with boxplot showing H scores of RON staining intensity. Boxplot lists 25th and 75th percentiles along

with the group median. Whiskers display 10th and 90th percentiles. Scale bar=50μm. **C**, Western blot of human LNCaP and C4-2B cell lysates depicting RON expression levels. **D**, Western blot of lysates from Myc-CaP tumors following implantation into male FVB mice before (castration sensitive) and after the development of castration resistance, and Myc-CaP and Myc-CaP-C cells depicting RON expression levels following RON knockdown (sh*Ron*). **E**, Average tumor volume formed from control Myc-CaP-C shNT (black line, n=4) and Myc-CaP-C sh*Ron* (grey line, n=4) cells grown in pre-castrated FVB mice. **F**, Average tumor volume formed from Myc-CaP-C shNT (black line, n=4) or Myc-CaP-C sh*Ron* (grey line, n=4) cells following implantation into intact FVB mice and castration at 1000mm^3. Data represent mean values ± SEM. *P < 0.05.

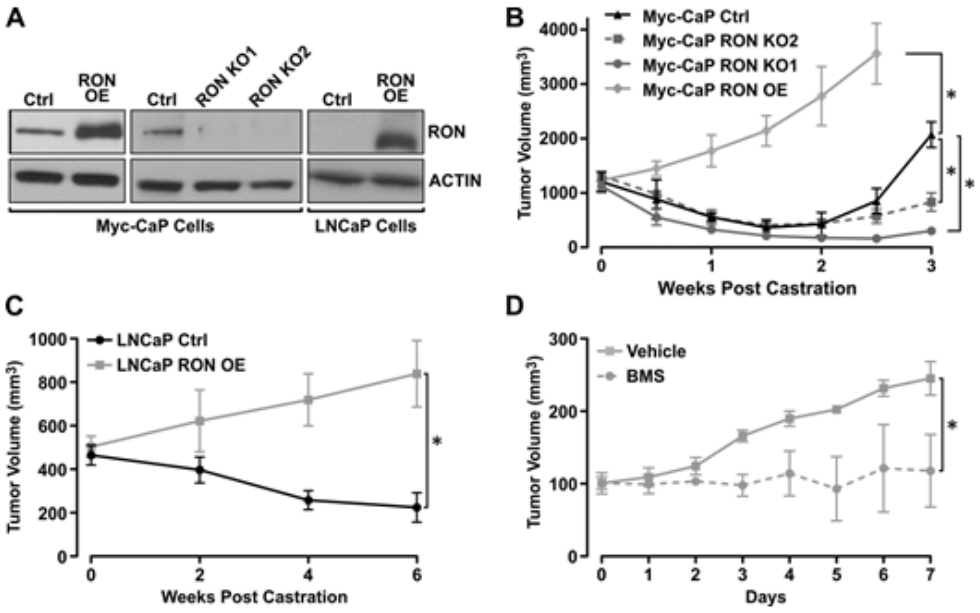


Figure 2. RON overexpression mediates castration resistant growth *in vivo*. A, Western blot of murine Myc-CaP cells depicting expression levels of RON in control (Ctrl), RON overexpressing (RON OE) or CRISPR/Cas9 deleted RON (RON KO1 and RON KO2) and human LNCaP cell lysates depicting expression levels of RON in control (Ctrl) or RON overexpressing (RON OE) cells. **B**, Average subcutaneous tumor volume of Myc-CaP Ctrl (black line, n=4), Myc-CaP RON OE (grey line, n=8), Myc-CaP RON KO1 (dark grey line, n=3), and Myc-CaP RON KO2 (dark grey dashes, n=3) cells following castration at 1000mm^3 in FVB mice. **C**, Average tumor volume of LNCaP Ctrl (black line, n=3) and LNCaP RON OE (grey line, n=4) cells following castration at 500mm^3 in athymic nude mice. **D**, Average tumor volume of Myc-CaP RON OE cells grown in precastrated FVB mice and treated with DMSO (Vehicle, grey line, n=4) or 50mg/kg/day BMS-777607 (BMS, grey dashes, n=4) once castration resistant tumors reached 100mm^3. Data represent mean values ± SEM. *P<0.05.

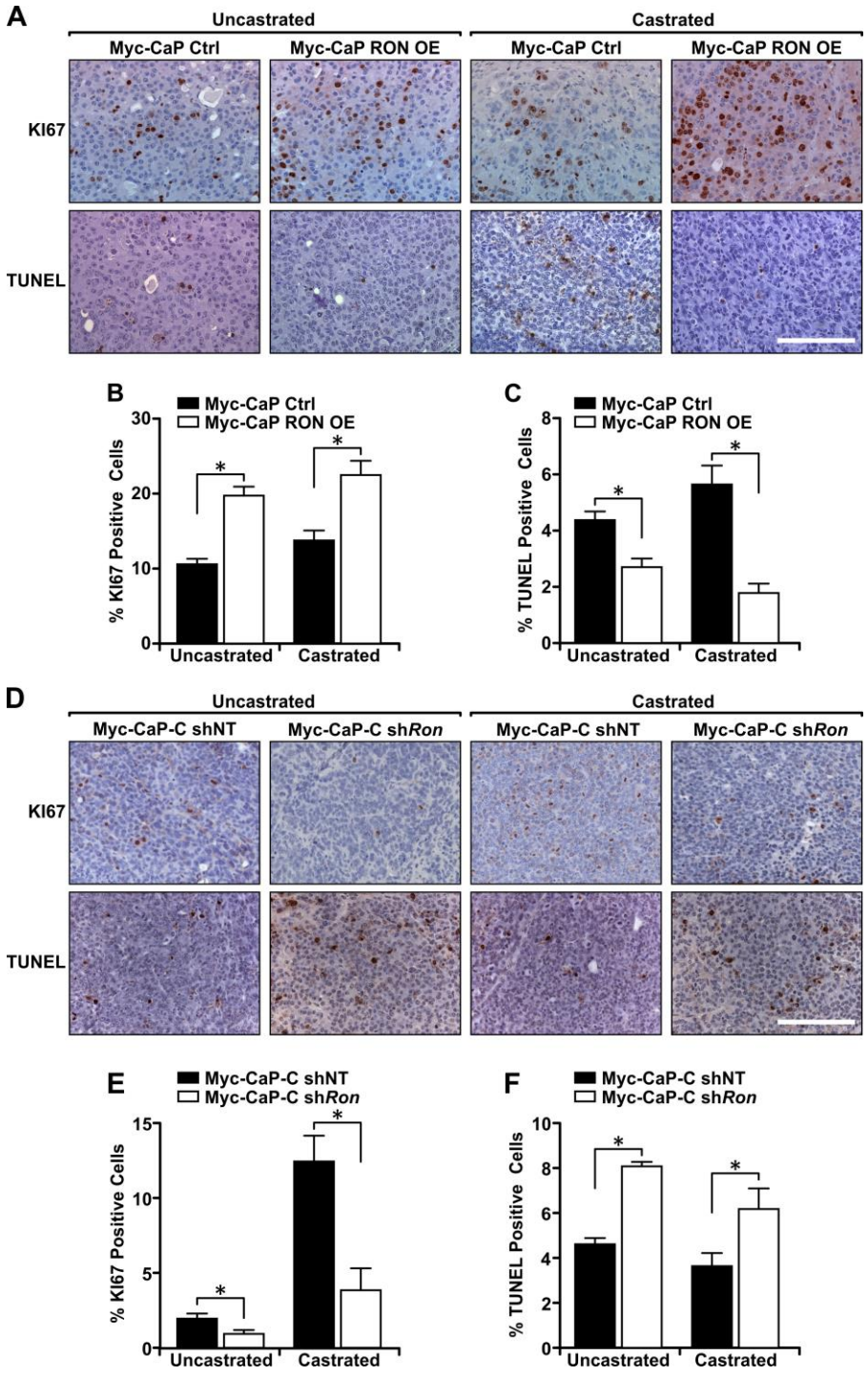


Figure 3. RON expression mediates castration resistant growth *in vivo* by enhancing tumor cell proliferation and reducing apoptosis. A, Representative images of Myc-CaP Ctrl and Myc-CaP RON OE tumors from FVB mice, before and after castration, stained for KI67 and TUNEL. Scale bar=50µm. **B&C**,

Percentage of cells staining positively for KI67 (**B**) or TUNEL (**C**) in tumors from Myc-CaP Ctrl and Myc-CaP RON OE cells before and after castration (n=3-5 independent tumor samples with quantification of at least 3 fields/sample). **D**, Representative images of tumors from Myc-CaP-C shNT and Myc-CaP-C sh*Ron* cells before and after castration stained for KI67 and TUNEL. Scale bar=50µm. **E&F**, Percentage of cells staining positively for KI67 (**E**) or TUNEL (**F**) in tumors from Myc-CaP-C shNT and Myc-CaP-C sh*Ron* cells before and after castration (n=3-5 independent tumor samples with quantification of at least 3 fields/sample). Data represent mean values ± SEM. *P<0.05.

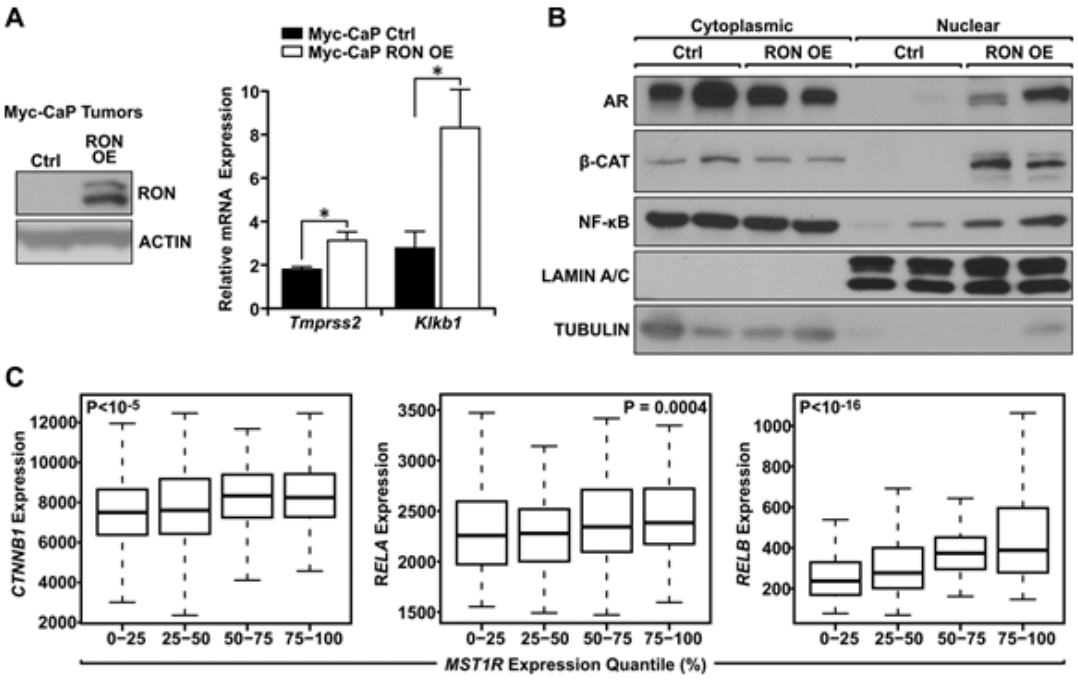


Figure 4. RON expression mediates castration resistant growth *in vivo* through oncogenic AR, β-CATENIN, and NF-κB. A, Western blot analysis and qRT-PCR of tumor samples from Myc-CaP Ctrl and Myc-CaP RON OE cells depicting RON protein levels and AR target gene expression of *Tmprss*2 and *Klkb*1. Data represent mean values from three independent experiments ± SEM, *P<0.05. **B**, Western blot analysis of tumors from Myc-CaP Ctrl and Myc-CaP RON OE cells following separation into cytoplasmic and nuclear fractions depicting nuclear localization of the AR, β-CATENIN, and NF-κB. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls. Each lane represents an independent tumor sample. C, mRNA expression levels of RELA, RELB and CTNNB1 (β-CATENIN) in different quantiles of MST1R (RON) expression in primary prostate adenocarcinoma samples (from TCGA). The P-values of correlation (Spearman's rank sum) are indicated in the legend of each plot.

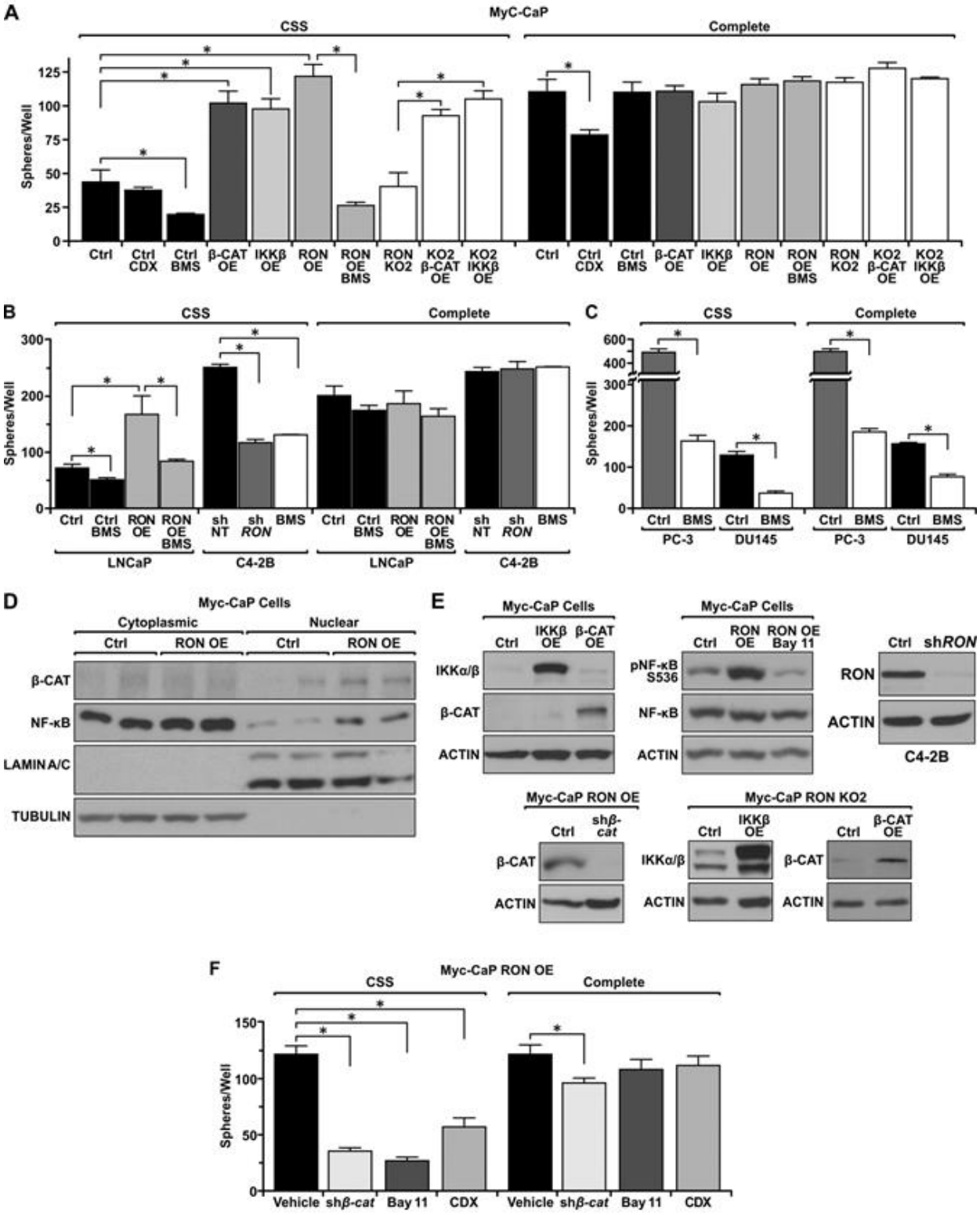


Figure 5. Activation of oncogenic β-CATENIN, NF-κB, and AR is required for RON to promote growth under androgen deprivation. A, Number of prostate cancer cell spheres formed per well (n=3 per

group) from Myc-CaP Ctrl, RON OE, β-CATENIN OE, IKKβ OE, RON KO2, RON KO2 β-CATENIN OE, and RON KO2 IKKβ OE cells grown in charcoal stripped serum (CSS) or complete media for 10 days. CDX indicates cells treated Casodex; BMS indicates cells treated with BMS-777607. **B**, Number of spheres formed per well (n=3 per group) of LNCaP Ctrl, LNCaP RON OE, C4-2B shNT, and C4-2B sh*RON* cells following 10 days growth in CSS or complete media. BMS indicates cells treated with BMS-777607. **C**, Number of spheres formed per well (n=3 per group) of PC-3 (treated with vehicle or BMS) and DU145 (treated with vehicle or BMS) cells following 10 days growth in CSS or complete media. **D,** Western blot of Myc-CaP Ctrl and Myc-CaP RON OE cells separated into cytoplasmic and nuclear fractions depicting nuclear localization of the β-CATENIN and NF-κB. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls. **E**, Western blot of Myc-CaP cells with ectopic IKKβ or β-CATENIN expression. Western blots of Myc-CaP RON KO2 cells depicting ectopic β-CATENIN and IKKβ expression. Western blot for s536 phosphorylated NF-κB and total NF-κB in Myc-CaP Ctrl and Myc-CaP RON OE cells treated with vehicle (DMSO) or Bay-11-7085 (Bay11) for 4 hours. Western blot depicting β-CATENIN knockdown in Myc-CaP RON OE shβ-*Catenin* cells relative to Myc-CaP RON OE cells. Western blot depicting RON expression levels following knockdown of RON in C4-2B cells. **F**, Number of spheres formed per well (n=3 per group) in Myc-CaP RON OE cells treated with Vehicle (DMSO), Bay11-7085 (Bay11), Casodex (CDX), or following knockdown of β-CATENIN (sh*β-cat*) and Myc-CaP Ctrl cells treated with Vehicle (DMSO) or CDX following 10 days growth in CSS or complete media. Data represent mean values from three independent experiments ± SEM, *P<0.05

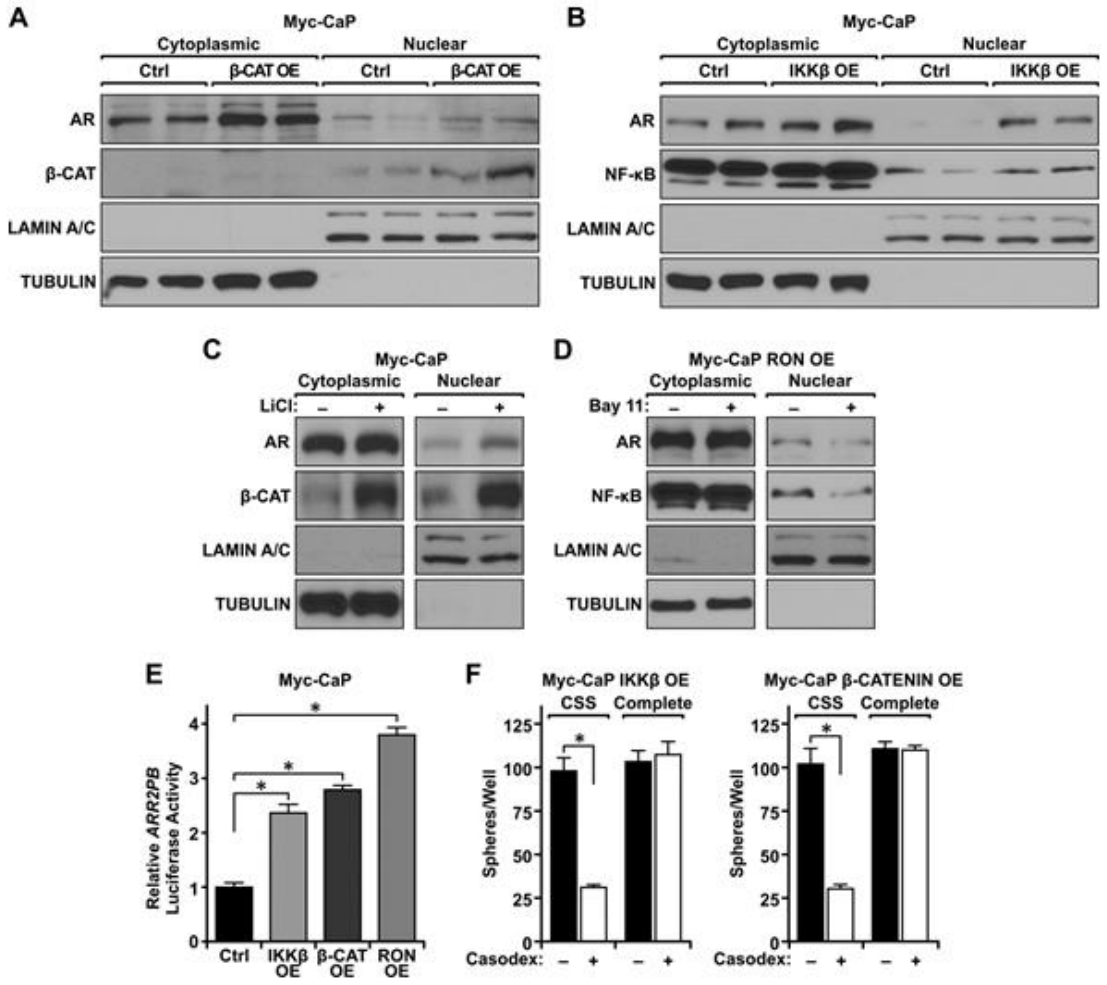


Figure 6. β-CATENIN and NF-κB independently induce AR activation to promote growth under androgen deprivation *in vitro.* **A**, Western blot of Myc-CaP Ctrl and Myc-CaP β-CATENIN OE cells from cytoplasmic and nuclear fractions depicting nuclear localization of the AR and β-CATENIN. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls for each fraction. **B**, Western blot of Myc-CaP Ctrl and Myc-CaP IKKβ OE cells from cytoplasmic and nuclear fractions depicting nuclear localization of the AR and NF-κB. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls for each fraction. **C**, Western blot of Myc-CaP Ctrl cells treated with DMSO (-) or LiCl (+, 4 hours) and fractionated into cytoplasmic and nuclear fractions depicting nuclear localization of the AR and β-CATENIN. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls for each fraction. **D**, Western blot of Myc-CaP RON OE cells treated with DMSO (-) or Bay-11-7085 (Bay 11, +, 4

hours) and separated into cytoplasmic and nuclear fractions depicting nuclear localization of the AR and NF-κB. TUBULIN (Cytoplasmic) and LAMIN A/C (Nuclear) are shown as loading controls for each fraction. **E,** Relative luciferase activity normalized to renilla luciferase for Myc-CaP Ctrl cells, Myc-CaP β-CATENIN OE cells, Myc-CaP IKKβ OE cells, and Myc-CaP RON OE cells following 48 hours growth in CSS (n=3 per group). **F**, Number of spheres formed per well (3 per group) of Myc-CaP β-CATENIN OE and Myc-CaP IKKβ OE cells treated with vehicle (-) or Casodex (+) grown in CSS or complete media. Data represent mean values from three independent experiments ± SEM, performed with three technical replicates, *P<0.05.

Table 1. COPA Analysis for RON and ERG in Prostate Cancer Datasets.

RON			*ERG*			
COPA Score	**Rank**	**%**	**COPA Score**	**Rank**	**%**	**Data Set**
3.801	**5%**	**95**	**3.781**	**5%**	**95**	**Barwick**
13.789	**6%**	**75**	**4.3**	**8%**	**75**	**Welsh**
8.972	**4%**	**95**	**6.894**	**9%**	**95**	**Varambally**
1.94	**6%**	**75**	**1.727**	**9%**	**75**	**Chandran**
1.282	**10%**	**75**	**3.343**	**1%**	**75**	**LaPointe**
7.73	**10%**	**95**	**NA**	**NA**	**NA**	**Demichelis**
NA	**NA**	**NA**	**76.976**	**3%**	**95**	**Magee**
3.322	**10%**	**90**	**5.472**	**1%**	**90**	**Grasso**

COPA identified RON and ERG as significantly overexpressed in a subset of tumors from the Oncomine 4.4 database (threshold by: top 10%, fold change >2 and P<1E-4). RON was shown to have a higher COPA score than ERG in 4 of the 6 databases in which RON and ERG were both identified. NA, not applicable. Rank is relative to other outlier genes identified in the data set. % is the percentile for gene expression intensity within the dataset.

Chapter 3

Prostate Epithelial Ron Mediates Macrophage Recruitment to Drive ADT Resistance in Prostate Cancer

Abstract

Despite the recent surge in the use of immunotherapies for the treatment of cancer, treatment with immunotherapies for castration resistant prostate cancer remains unsuccessful. The prostate tumor microenvironment has been referred to as immunologically cold, limiting the potential effectiveness of several currently available T-cell activating agents. Interestingly, research has shown that macrophages play a significant role in promoting prostate cancer growth and progression toward an aggressive castration resistant disease. We have discovered a novel role for the Ron receptor in promoting resistance to androgen deprivation therapy through the recruitment of macrophages into prostate tumors. Ron has previously been described to promote castration resistant prostate cancer, although a role for Ron in restructuring the tumor microenvironment to do so was not known. Macrophages provide Gas6 to further enhance Ron activation and induce activation of Axl to drive castration resistant growth. Understanding this mechanism in prostate cancer helps provide clarity for why the effectiveness of current immunotherapies is limited and provides a means to increase efficacy for future treatments.

Introduction

Prostate cancer continues to be a major health issue despite the advent of novel cancer therapies. Androgen deprivation therapy (ADT) has remained the primary treatment for men with metastatic disease since it's use was discovered in 1941 (126). The recent surge in the use of immunotherapies has become effective across a number of cancers; however, prostate cancer stands out because the majority of patients are not responsive to immunomodulating agents. A phase III study of the anti-CTLA4 monoclonal antibody Ipilimumab in patients with metastatic castration resistant prostate cancer (mCRPC) following chemotherapy resulted in only a subgroup of patients showing improved overall survival (127). This subgroup required patients to have normal alkaline phospatase, normal hemoglobin, and no visceral metastases, effectively eliminating a large number of patients with CRPC. An additional study was performed for Ipilimumab in patients with asymptomatic metastatic chemotherapy naive CRPC and again showed no difference in overall survival, although a slight increase in progression free survival was observed (128). Inhibition of PD-1/PD-L1 is another immunotherapeutic approach that has gained a lot of traction in a number of cancers; however, a number of studies have been conducted regarding the potential of PD-1/PD-L1 inhibitors or monoclonal antibodies in prostate cancer yet none have achieved success (129). Although targeting PD-1 and PD-L1 has become effective for a broad range of cancers, prostate cancers may not be as responsive because studies have shown that prostate cancers tend to express very little PD-L1 (130). Alternative means for altering the immune system in prostate cancer are necessary to either work as stand-alone therapeutics or enable some of the currently available immunotherapies to be used in men with CRPC.

The majority of immunotherapies attempt to alter T-Cell activity and neglect to address the function macrophages have on tumor progression. Increased presence of tumor associated macrophages (TAMs) was shown to correlate with prostate cancer progression in patients (131). Additionally, other studies have shown that patients with higher infiltration of TAMs have significantly shorter disease free survival

following radical prostatectomy compared to patients with low TAM infiltration (132, 133). A number of papers have detailed several roles for macrophages in prostate cancer. Recently, myeloid cells were shown to inhibit T-Cell responses in a murine model of CRPC through protein nitration (134). Macrophage effects on neighboring T-cells of the tumor microenvironment may be preventing the efficacy of current immunotherapies targeting T-cells. Macrophages have also been shown to directly promote tumor progression by supporting prostate tumor growth and metastatic potential (135, 136). Innovative immunotherapeutic approaches that focus on targeting macrophage function may address current issues with immunotherapies in prostate cancer patients.

A novel driver of prostate cancer, which may suppress the effectiveness of immunotherapies, is the Ron receptor. Ron has been established as a critical driver of prostate cancer, and recent work has detailed a function for Ron in driving resistance to ADT (14, 38, 41, 50, 72). Ron has been reported to be overexpressed in the majority of CRPC patients, and herein we have identified a novel mechanism for Ron to promote CRPC through recruitment of macrophages in prostate tumors. Ron overexpressing prostate cancer cells both recruit macrophages and are more effective at utilizing macrophages to promote growth under androgen deprived conditions compared to low Ron expressing prostate cancer cells. We also provide preclinical evidence that combining Ron inhibition with immunomodulating agents sensitizes castration resistant tumors to castration therapy, providing a novel treatment option for men with CRPC.

Results

Ron overexpression enhances F4/80+ myeloid cell recruitment into tumors in murine models of prostate cancer. In order to examine the effects of epithelial Ron overexpression in a genetic model of prostate cancer, we crossed mice which overexpress Ron in the prostate epithelium (ARR_2Pb-Ron) with the well-established Hi-Myc model of prostate cancer, termed Hi-Myc Pb-Ron. Overexpression of Ron significantly increased prostate tumor weight at 30 weeks of age (**Fig. 1A**) and resulted in increased incidence of prostate adenocarcinoma (**Fig. 1B**). Upon further characterization, Hi-Myc Pb-Ron mice had prostates which exhibited increased proliferation as marked by BrdU staining and decreased cell death as shown by TUNEL staining (**Fig. 1C**). Interestingly, we also observed changes to the tumor microenvironment in this model, as Hi-Myc Pb-Ron mice displayed an increased presence of F4/80+ myeloid cells within their prostates (**Fig. 1C**). To determine whether this change in the tumor microenvironment was broadly applicable to other Ron overexpressing prostate tumors, we assessed two previously characterized murine subcutaneous prostate cancer models (72). Ron overexpression in Myc-CaP cells (Myc-CaP Ron OE) also resulted in increased presence of F4/80+ myeloid cells in subcutaneous tumors (**Fig. 1D**). Conversely, knockdown of Ron in castration resistant Myc-CaP-C cells resulted in decreased presence of F4/80+ myeloid cells in the tumor (**Fig. 1E**). Additional immunohistochemical and qPCR analysis revealed that Hi-Myc Pb-Ron prostates have elevated expression of the M2 macrophage marker Arginase relative to Hi-Myc control prostates (**Suppl. Fig. 1A and 1B**).

Ron overexpression enhances CCL2 production and requires CCL2 to promote macrophage migration. Ron overexpression has previously been established to regulate the synthesis and secretion of a number of cytokines (14). One of which, CCL2/MCP-1, has been established as a key regulator of macrophage chemotaxis. CCL2 mRNA expression was observed to be elevated in Hi-Myc Pb-Ron prostates relative to Hi-Myc prostates (**Fig. 2A**) and in Myc-CaP Ron OE tumors relative to Myc-CaP Ctrl tumors (**Fig. 2B**). To

assess if production of CCL2 has a function for influencing macrophage infiltration into Ron overexpressing tumors ,CCL2 was knocked down in Myc-CaP Ron OE cells and cells were used for an *in vitro* migration assay measuring migration of bone marrow derived macrophages (BMDMs) toward prostate cancer cells (**Fig. 2C**). Remarkably, knockdown of CCL2 reduced macrophage migration toward Myc-CaP Ron OE cells to levels comparable to Myc-CaP ctrl cells (**Fig. 2D**), suggesting CCL2 as a key player in promoting macrophage migration into Ron overexpressing prostate tumors.

Ron overexpressing prostate cancer cells require Axl to promote growth following castration. Macrophages have been established to play a critical role in the growth of prostate cancer and in the development of castration resistance (134-136). Further, Ron overexpressing tumors, which exhibit increased macrophage infiltration, have previously been shown to be resistant to castration therapy (72). To assess whether Ron overexpressing tumors utilize macrophages for growth in the absence of androgens, a previously established 3D-sphere culture assay was used to assess growth in the absence of androgens for Myc-CaP cells with Ron modulation with and without bone marrow derived macrophages (BMDMs) (72). The addition of BMDMs increased sphere formation for both Myc-CaP Ctrl and Myc-CaP Ron OE cells; however, there was not a significant increase in sphere formation for Myc-CaP cells where Ron was genetically deleted using CRISPR/Cas9 technology (**Fig. 3A**). Additionally, the fold change for growth with and without the addition of macrophages was highest for Myc-CaP Ron OE cells (2.42 for Ron OE, 1.7 for Ctrl), suggesting that Ron expression impacts the ability of prostate cancer cells to utilize macrophages for growth promotion.

An unbiased phosphokinase array was used to determine what major signaling changes occur following Ron overexpression in tumors which may be a result of changes in the tumor microenvironment that allow growth under androgen deprivation (**Table 1**). The strongest phosphorylation induction in response to Ron overexpression was for the Axl receptor (**Fig. 3B**). Axl has been established as a driver

of prostate cancer and in oral cancer was shown to be activated through myeloid cells of the tumor microenvironment (137). Myc-CaP Ron overexpressing tumors were confirmed to have increased Axl signaling relative to Myc-CaP Ctrl tumors as shown by increased Src and AKT phosphorylation (**Fig. 3C**). To determine if Axl plays a functional role in Ron overexpressing tumors for promoting castration resistance, Axl was knocked down in Myc-CaP Ron OE cells and growth *in vivo* following castration was observed (**Fig. 3D and 3E**). Tumors derived from Myc-CaP Ron OE cells with Axl knockdown (sh*Axl*1/2) were observed to be sensitive to castration therapy, unlike their Myc-CaP Ron OE ctrl counterparts continuing to grow upon castration (**Fig. 3E**). These data indicate that Axl is a key player for Ron overexpressing tumors to grow in the context of castration; however, the mechanism for how Axl is activated remained to be determined.

Gas6 binds to and induces activation of Ron signaling. Receptor tyrosine kinases can be activated through ligand binding or through receptor crosstalk with other receptors. In fact, Ron has specifically been shown to crosstalk with c-Met, PDGFR-β, IGF1-R, and others (23, 33, 34). In order to determine if Ron overexpression induces Axl activation through receptor crosstalk, Myc-CaP and LNCaP cells were treated with Gas6 (ligand for Axl) and HGFL (ligand for Ron) and phosphorylation of Axl and/or Ron was measured. Interestingly, HGFL addition induced Ron phosphorylation but did not induce Axl phosphorylation, indicating crosstalk is not a likely option (**Figs. 4A**). However, addition of Gas6 induced phosphorylation of both Ron and Axl (**Figs. 4A**). To determine if the phosphorylation of Ron upon Gas6 stimulation requires Axl to be present, 293T cells, which have undetectable levels of Axl and Ron, were treated with HGFL and Gas6. Following stimulation with either HGFL or Gas6 in ctrl 293t cells, Ron phosphorylation was not observed; however, stimulation with either HGFL or Gas6 in 293T cells where Ron was exogenously overexpressed resulted in comparable levels of Ron phosphorylation between the two ligands (**Fig. 4B**).

It has previously been shown that Ron activation drives nuclear localization and activation of the androgen receptor under conditions of androgen deprivation, and stimulation of Myc-CaP cells with Gas6 under conditions of androgen deprivation resulted in increased AR nuclear localization (**Fig. 4C**). Increased expression of AR target genes in both murine Myc-CaP and human LNCaP cells was also observed, with the greatest induction occurring in Ron overexpressing cells (**Figs. 4D and 4E**). These results indicate that addition of Gas6 increases Ron phosphorylation and activation; however, this does not appear to be through receptor crosstalk with Axl. An alternative option is that Gas6 binds directly to Ron to facilitate activation. In order to test this mechanism, microscale thermophoresis was used to measure binding potential of both HGFL and Gas6 to a GFP-labeled Ron receptor. As shown in **Figure 4F**, HGFL was shown to bind to Ron with an EC50 of 0.0619 ± 0.0065 nM, which is similar to what has been published using sandwich ELISA by others (138). Shockingly, Gas6 was also shown to bind to Ron, with an EC50 of 0.5672 ± 0.0689 nM (**Fig. 4G**). These results are the first to show Gas6 binding to Ron, and further tests are needed to further identify the other kinetic variables associated with this interaction.

Macrophage produced Gas6 increases growth of Ron overexpressing prostate cancer cells under androgen deprived conditions. After observing that receptor crosstalk was not likely the mechanism for Axl activation in the context of Ron overexpression, we hypothesized that increased presence of Gas6 in tumors may be responsible for inducing Axl activation. Our prior data indicates that tumors derived from Ron overexpressing cells have more F4/80+ myeloid cells (Figure 1). Moreover, Ron overexpressing cells are more responsive to macrophages for promotion of growth under castrate conditions (Figures 3). Further, others have shown that macrophages produce Gas6 (139). Gas6 was observed to be up regulated at the mRNA level in Myc-CaP Ron OE tumors relative to Myc-CaP ctrl tumors (**Fig. 5A**). Interestingly, there was no difference in the expression of Gas6 in Myc-CaP ctrl and Myc-CaP Ron OE cells, suggesting that Gas6 must be coming from another cell of the tumor microenvironment. To test if macrophage derived Gas6 is capable of promoting growth of Ron OE cells under androgen deprived

conditions, we added macrophage conditioned media to various cells with and without Gas6 depletion under androgen deprived conditions (**Fig. 5B and 5C**). We observed that, similar to the co-culture experiments in Figure 3A, the addition of BMDM conditioned media promoted sphere formation of Myc-CaP Ctrl and Ron OE cells, but no significant changes were observed in Myc-CaP Ron KO1 cells (**Fig. 5C**). Strikingly, Gas6 depletion reduced sphere formation in Myc-CaP Ron OE cells to levels comparable to cell with no addition of BMDM conditioned media (**Fig. 5C**). Additionally, exogenous addition of Gas6 to Myc-CaP Ron OE cells exhibited a similar phenotype to the addition of BMDM conditioned media, with no significant changes occurring in Myc-CaP Ctrl or Myc-CaP Ron KO1 cells (**Fig. 5C**). These data indicate Gas6 as a critical component secreted from macrophages for Ron overexpressing cells to grow in the absence of androgens.

To determine the extent that macrophage secreted Gas6 supports prostate sphere formation of Myc-CaP Ron OE cells through Axl activation, Myc-CaP Ron OE sh*Axl* cells were also treated with Gas6 and BMDM conditioned media with/without Gas6 depletion. Interestingly, Gas6 addition and BMDM conditioned media still promoted growth in Myc-CaP Ron OE sh*Axl* cells (1.7 fold), although not to the same extent as in Myc-CaP Ron OE cells maintaining endogenous levels of Axl (2.1 fold) (**Fig. 5C**). Additionally, Gas6 depletion from BMDM conditioned media still resulted in a decrease in sphere formation to comparable levels to non-BMDM conditioned media treated cells (**Fig. 5C**). These data demonstrate that Gas6 secreted from macrophages is capable of activating both Ron and Axl on prostate cancer cells for the promotion of growth under androgen deprived conditions.

Macrophage depletion and Ron inhibition sensitizes castration resistant tumors to castration therapy. After establishing *in vitro* that Ron overexpressing prostate cancer cells are capable of utilizing macrophages to promote growth under androgen deprived conditions, we next sought to test the therapeutic implications of this relationship *in vivo*. This concept was tested through depletion of

macrophages with clodrosome and/or inhibition of Ron/Axl with BMS777607/ASLAN002 (BMS) in combination with castration therapy in mice with Ron OE tumors. BMS is a kinase inhibitor with specificity for both Ron and Axl (80). Shockingly, Myc-CaP Ron OE tumor growth is dramatically reduced when macrophages are depleted in combination with castration therapy (**Fig. 6A**). Treatment with clodrosome significantly reduced the number of F4/80+ myeloid cells in tumors as judged by F4/80 staining and resulted in increased tumor cell death (**Figs. 5B and 5C**). Interestingly, the reduction in tumor growth in the clodrosome treated group was similar to the reduction in tumor growth in the BMS treated group (**Fig. 6A**). However, when clodrosome treatment was combined with BMS treatment the response of tumors derived from Myc-CaP Ron OE cells was the greatest. The reasoning for this result is unclear, however, this may be due macrophage depletion and/or Ron/Axl inhibition not being 100% efficient in the individual treatment groups (as shown in Fig. 6B). The combination of the two treatments may be able to effectively reduce activation of both Ron and Axl to sufficient levels for maximum tumor regression. These data indicate that immunomodulatory agents targeting macrophages may have success in Ron overexpressing castration resistant tumors and efficacy may be enhanced when used in combination with Ron/Axl inhibitors.

Discussion

Prostate cancer is estimated to claim the lives of over 350,000 men worldwide in 2018 (140). Currently there is no curative therapy for men with CRPC and the promising novel immunotherapies that focus on altering T-cell activity have yet to produce significant results in CRPC patients, illustrating the need for novel approaches to treat men with CRPC. Recent studies have shown a pivotal role for tumor infiltrating myeloid cells in the promotion of CRPC. Additionally, work from our laboratory and others have identified the Ron receptor as a highly relevant driver of resistance to castration therapy. Herein we demonstrate that Ron receptor overexpression in prostate epithelial cells results in a restructuring of the tumor microenvironment with increased presence of myeloid cells (**Figure 1**). Further, we show that the increased presence of myeloid cells may be in part due to elevated synthesis and secretion of the macrophage chemoattractant protein CCL2 (**Figure 2**). Prior work from our group illustrated a novel role for Ron in promoting endothelial cell recruitment to the prostate tumor microenvironment through the secretion of angiogenic chemokines, however, CCL2 was not implicated in this process (14). Other reports have shown CCL2 production in prostate cancer cells induces infiltration of myeloid cells and promotes CRPC; however, CCL2 induction was driven by stimulation of WNT5a through MAPK signaling (141). It would be intriguing to investigate whether Ron signaling crosses with WNT5a signaling or utilizes MAPK signaling for increasing CCL2 expression. Together, our recent data and prior work demonstrate that Ron overexpressing prostate tumors structure the microenvironment to have higher presence of endothelial cell and myeloid cells compared to low Ron tumors, and this structure increases prostate tumor growth potential and primes the tumor for resistance to ADT.

Our data also reveal a novel role for macrophages in prostate cancer, in facilitating the activation of both Ron and Axl signaling through the secretion of Gas6 (**Figures 3 and 4**). Axl has been shown to play a significant role in prostate cancer, as silencing Axl in prostate cancer cell lines resulted in reduction of proliferation, migration, and invasion properties (142). Additionally, Gas6 stimulation has been shown to

induce AR activation through activation of the non-receptor tyrosine kinase Ack1 (143). Interestingly, the cell surface receptor responsible for mediating the effects of Gas6 on AR through Ack1 was not detailed. Consistent with this report, we also observed that Gas6 stimulation induces activation of AR signaling, although we observed that this effect was enhanced with Ron overexpression (**Figure 4**). Our prior work demonstrates that Ron activation facilitates AR activation through both NF-κB and β-Catenin (72). Interestingly, Ack1 kinase is capable of activating NF-κB signaling (144). Although Ron has not been shown to activate Ack1 kinase, Ack1 has been previously shown to be activated by other RTKs. It is therefore plausible that Gas6 stimulation results in a Ron-Ack1-NF-κB-AR signaling cascade that is capable of driving growth in the absence of androgens.

Through our analysis of the interplay between Ron and Axl signaling, we made the novel discovery that Gas6 binds to and induces activation of Ron (**Figure 4**). Multiple receptors have been identified which bind to Gas6 (Axl, Tyro3, Mer), however, the only known ligand for Ron had been HGFL (145). This novel relationship may help explain a number of previously unknown functions of Ron, which up until this point have simply been referred to as HGFL-independent functions. Previous studies in both breast and prostate cancer have utilized HGFL knockout mice to show that HGFL has specific roles in Ron driven tumorigenesis (30, 38). Interestingly, in breast cancer, cell spreading and survival have been determined to be HGFL independent functions (31). Further work will be needed to determine if any of these functions are Gas6 specific functions of Ron. It is interesting to note that when comparing the MST data between the HGFL/Ron and Gas6/Ron interactions it was determined that Gas6 has an almost 10 fold higher EC50 than HGFL. There are several potential reasons for this, each of which needs to be further evaluated. The HGFL used for MST contains a cysteine to alanine substitution at position 672, which increases the bioactivity of the recombinant protein. Although, our measurements for the Gas6-Ron ec50 are still 3 fold higher than published studies using a non-C672A substituted form of HGFL (0.5672 nm vs 0.18 nm) (138). For example, HGFL is secreted as an inactive precursor protein that needs to be

cleaved in order to bind to Ron and Gas6 does not require a cleavage event (7). When cell surface proteases are limited, binding of Gas6 to Ron may be preferential to HGFL. Additionally, Tyro3 and Mer also bind to Gas6; however, their binding affinity is greatly improved in the presence of phosphatidyl serine (146). It is possible that the presence of another molecule may aid in Gas6 binding to Ron. These potential avenues will need to be explored as the relationship between Gas6 and Ron is further characterized.

With the current rise in immunotherapies, CRPC has stood out as a form of cancer not responsive to immunomodulatory agents. Our pre-clinical data indicates that Ron overexpressing prostate tumors have a unique reliance on myeloid immune cells for castration resistant growth (**Figure 6**). Myeloid cells interact with and regulate a number of immune cells within the microenvironment. Tumor associated macrophages, for example, have been shown to limit CD8+ T-cell responses toward antitumor immunity (41). With this in mind, targeting Ron has potential to sensitize CRPC tumors to T-cell focused immunomodulatory agents through its regulation of macrophages. Prior work has shown that a large proportion of CRPC patients have high Ron expression, making this approach broadly applicable (72). Additionally, directly targeting macrophages in CRPC patients with Ron overexpressing tumors may also provide therapeutic success.

The need for novel CRPC therapeutics is tremendous, and our data has demonstrated the Ron receptor as a novel target for CRPC. The multi-kinase inhibitor BMS777607 used herein has recently completed a phase 1 clinical trial for advanced solid tumors, and our pre-clinical work illustrates a need to pursue the use of drugs such as this in prostate cancer patient studies (81). We have shown that Ron overexpression in prostate epithelial cells creates a unique tumor microenvironment that is capable of promoting growth following castration therapy. We hypothesize that disruption of this

microenvironment through the inhibition of Ron has the potential to improve patient outcomes for a number of those suffering with CRPC.

Materials and Methods

Immunohistochemistry and scoring: Murine subcutaneous tumor tissues were fixed in 10% formalin, paraffin embedded, and cut into 5µm sections prior to staining for BrdU (52925S, cell signaling) Ki67 (MA5-14520, ThermoFisher Scientific), TUNEL (Millipore *In Situ* Apoptosis Detection Kit), F4/80 (14 4801 85, eBiosciences), Arginase (610708, Becton Dickinson), iNOS (610328, Becton Dickinson) was performed as previously described (1). Hematoxylin and Eosin (H&E) staining was performed as previously described (30). For BrdU analysis, mice were injected intraperitoneally with 200 µl 3mg/ml BrdU in 0.9% normal saline 2 hours prior to euthanasia. Samples with immunoglobulin G control antibody were used as negative controls. Images were taken of at least 3 fields per slide and all cells in the field were counted. Cells staining positively were divided by the total number of cells and then multiplied by 100 to obtain % positive cells/field. The average of at least 3 fields per slide was used as the % positive cells/field for that sample, at least 3 samples were used per genotype.

Western blot analyses: Cells were homogenized in RIPA buffer as described (74). Nuclear and cytoplasmic extracts were isolated by centrifugation and hypotonic lysis as described (75). Antibodies for analyses included: Ron (SC-322), Androgen Receptor, (SC-815), Axl (SC-1096), Gas6 (SC-1935), and Tubulin (SC-5286) from Santa Cruz Biotechnology; Src (2110S), phosphor-Src y416 (2101S), Akt (4691S), phosphor-Akt s473 (4060S), phospho-Axl y702 (5724S) and LAMIN A/C (4777S) from Cell Signaling Technologies; phospho-Ron y1238/y1239 (AF1947, R&D); ACTIN (Cincinnati Children's Hospital Medical Center, Clone C4). Peroxidase-conjugated secondary antibodies (Jackson Laboratories) were applied and membranes were developed using Pierce ECL2 Western Blotting substrate (ThermoFisher Scientific). Membranes were stripped using Restore Western Blot Stripping Buffer (ThermoFisher Scientific) before re-probing. The Proteome Profiler Mouse Phospho-RTK Array Kit (ARY014, R&D) was used on whole tumor lysates and performed according to manufacturer's instructions.

Mouse Models. Mice were maintained under specific pathogen-free conditions and experimental protocols approved by the University of Cincinnati IACUC. For prostate cell injections, $1.0x10^6$ cells were injected subcutaneously into the flanks of 6-8 week old male FVB mice as described (109, 122). Tumor growth was measured via calipers and volume was determined by the formula $0.5xLengthxWidth^2$ (123). Surgical castration was performed as described when tumors reached $1000mm^3$ (109, 122). For *in vivo* kinase inhibitor studies, mice were treated with 50mg/kg/day BMS-777607 (Selleck Chemicals) or methylcellulose (vehicle) via oral gavage. Mice treated with clodrosome (Encapsula Nanosciences CLD8909) for macrophage depletion were injected with 200 µl intraperitoneally every 3 days. Hi-Myc mice (FVB-Tg(ARR2/Pbsn-MYC)7Key/Nci, Strain # 01XK8) were obtained through the mouse repository at the National Cancer Institute and crossed with ARR_2Pb-Ron strain B mice which were described previously by our laboratory (50, 147). Mice were euthanized and tissues were collected for analysis at 30 weeks of age.

Cell Models. Myc-CaP cells (109) were developed in the laboratory of Charles Sawyers and were obtained from Memorial Sloan Kettering Cancer Center. For tumor formation, $1x10^6$ Myc-CaP cells were injected subcutaneously into wild type FVB male mice; once tumors reached 1000 mm^3, the mouse was castrated. Myc-CaP-C cells were generated from a castration resistant tumor formed following injection of Myc-CaP cell (72). All Myc-CaP cells were maintained in DMEM with 10% Cosmic Calf Serum and 1% gentamycin (109). The human prostate cancer cell lines LNCaP and C4-2B were obtained from ATCC and were maintained in RPMI-1640 with 10% Fetal Bovine Serum and 1% gentamycin. All cells were maintained at 37^oC and 5.0% CO_2. Bone marrow derived macrophages (BMDMs) were isolated as previously described and cultured in DMEM supplemented with 10% FBS, 1% glutamine, 1% gentamycin, and 20ng/ml M-CSF (95).

Cell Transfections. Stable polyclonal cell lines were generated by performing transfection with Lipofectamine 2000 (ThermoFisher Scientific) and selection in puromycin (Invitrogen, 5μg/ml) or G418 (Invitrogen, 500 μg/ml). LNCaP Ctrl and LNCaP RON OE cells overexpressing human RON were generated as described (14). The *Ron* gene was deleted in Myc-CaP cells using CRISPR/Cas9 technology as described previously to generate Myc-CaP Ron KO1 cells (72). Short Hairpin RNA (shRNA) constructs were purchased from Cincinnati Children's Hospital Medical Center (CCHMC) for knockdown of Axl (TRCN00000023311 for sh*Axl* 1, TRCN00000023313for sh*Axl* 2) or CCL2 (TRCN00000034470 for shCCL2-1, TRCN00000034473 for shCCL2-2) and knockdown was performed as previously described (72).

Viral transduction. Lentivirus short hairpin RNA (Open Biosystems) was used to target murine sh*Ron* (RMM3981-9590952), and nonsense shNT (RHS1764). The pCDH backbone and pCDH-CMV-EF1-PURO-RON full-length mouse RON cDNA expression vectors were utilized for control and RON overexpression. Transduction was performed as described (14, 75).

***In Vitro* Cell Growth Assays and Treatments.** 3D growth assays were performed as described previously (38, 72). Briefly, $2x10^4$ cells were plated on top of 1.0% agarose in 6 well plates in media supplemented with cosmic calf serum (Complete, Thermofisher Scientific) or charcoal stripped serum (CSS, Midsci). Cells were left untreated, treated with PBS (vehicle), Gas6 (R&D, 1 nM daily), or macrophage conditioned media (R&D, 1:10 every other day) daily. After 10 days, images of spheres were taken using a Zeiss Axiovert S100TV inverted microscope (Carl Zeiss Microscopy) and spheres >25μm in diameter were counted using ImageJ software. The 25 μm threshold was established based on the average sphere size obtained for the control cells.

Conditioned media and Co-cultures. Macrophage conditioned media was collected from $2X10^4$ BMDMs seeded in a 6 well plate. 24 hours prior to collection, BMDM media was removed and serum free media

with 1% gentamycin was added. Media was aspirated, centrifuged at 200g for 10 minutes, and then passed through a 40 μM filter prior to being placed on cells at a ratio of 1:10. Gas6 depletion from BMDM conditioned media was performed by rotating BMDM conditioned media with 1μg/ml Gas6 antibody (SC-1935) for 2 hours at 4^{o}C then by adding 30μl/ml protein A/G agarose beads (SC-2003) and rotating for 2 hours at 4^{o}C. Following incubations with antibody and beads, the mix was centrifuged at 1000g for 5 minutes and the supernatant removed and used for treatment. Incubation with beads only served as the control. For sphere forming co-culture assays, 2*10^{4} epithelial cells and 2*10^{4} BMDM cells were placed in a single well of a 6 well plate.

Quantitative Real-Time (qRT)-PCR. RNA was extracted with TRIzol (Invitrogen) and cDNA was prepared using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Quantitative PCR was performed with 2X SYBR Green Master Mix (Roche Diagnostics) on a Mastercycler ep realplex4 (Eppendorf). Data were normalized to an 18S reference gene and analyzed by ΔΔCT. Primer sequences included: Murine *Tmprss*2 (Forward: 5'-AAGTCCTCAGGAGCACTGTGCA-3'; Reverse: 5'-CAGAACCTCCAAAGCAAGACAGC-3'). Murine *Klkb*1 (Forward: 5'-AAAGTCAGCGGACAACCTGGTG-3'; Reverse 5'-AGATGGTGCGACACACAAAGGC-3'). 18S (Forward: 5'-AGTCCCTGCCCTTTGTACACA-3'; Reverse: 5'-GATCCGAGGGCCTCACTAAAC-3'). Murine *Gas6* (Forward: 5'-AGAACTTGCCAGGCTCCTACTCTTG-3'; Reverse: 5'-TCGCCCATCACAGTGGCAGGTATAG-3'). Murine *Vegf* (Forward: 5'-GCAGAAGTCCCATGAAGTGA-3'; Reverse: 5'-TCCAGGGCTTCATCGTTA-3'). Murine *Ccl2* (Forward: 5'-TTAAAAACCTGGATCGGAACCAA-3'; Reverse: 5'-GCATTAGCTTCAGATTTACGGGT-3'). Murine *Arginase* (Forward: 5'-AGCATGAGCTCCAAGCC-3'; Reverse: 5'-CAGACCAGCTTTCCTCAGTG-3'). Murine *iNos* (Forward: 5'-GTTCTCAGCCCAACAATACAAGA-3'; Reverse: 5'-GTGGACGGGTCGATGTCAC-3'). Human *PSA* (Forward: 5'-TGCCCACTGCATCAGGAA-3'; Reverse: 5'-GCTGACCTGAAATACCTGGCC-3'). Human *VEGF* (Forward: 5'-GACAAGAAAATCCCTGTGGGC-3'; Reverse: 5'-AACGCGAGTCTGTGTTTTTGC-3'). Human *TMPRSS2* (Forward: 5'-CCTCTAACTGGTGTGATGGCGT-3'; Reverse: 5'-TGCCAGGACTTCCTCTGAGATG-3').

Microscale Thermophoresis. Ron protein was tagged with GFP as described. After labeling, the protein was collected in lysate from 293T cells overexpressing the RON-GFP fusion protein using RIPA buffer. Non-fluorescent Gas6 (R&D, 885-GSB-050) or HGFL (R&D, 4306-MS) was titrated in a 1:1 dilution series (concentrations between 0.05 – 27.00 nM for Gas6, 0.004nM - 4.00nM for HGFL). Samples were loaded into Monolith™ NT.115 MST Standard Capillaries (NanoTemper Technologies) and measured using a Monolith NT.115 and MO.Control software at room temperature (LED/excitation power setting 50%, MST power setting 60%). Data was analyzed using MO.Affinity Analysis software (NanoTemper Technologies). Data were normalized using fraction bound binding (148).

Migration Assays. $2X10^4$ BMDMs were plated on top of a 24 well plate Transwell Permeable Support with an 8 µM pore size (Costar, 3422). $1x10^5$ prostate cancer cells were placed in the bottom of the 24 well plate. BMDMs were allowed to migrate for 20 hours, fixed in methanol for 20 min, then stained with 0.1% Crystal violet. Images were taken of the transwell insert and the total number of cells migrated were counted using Image J software.

Statistical Analysis: Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by performing Student's t-test for pairwise comparisons or ANOVA for comparison of multiple groups using GraphPad Prism software (GraphPad Software). All *in vitro* experiments represent the average of at least triplicate experiments. Significance was set at *P<0.05.

Acknowledgements

We would like to thank Glenn Doermann for his assistance in figure preparation and Madison Nashu and Andrew Paluch for technical assistance.

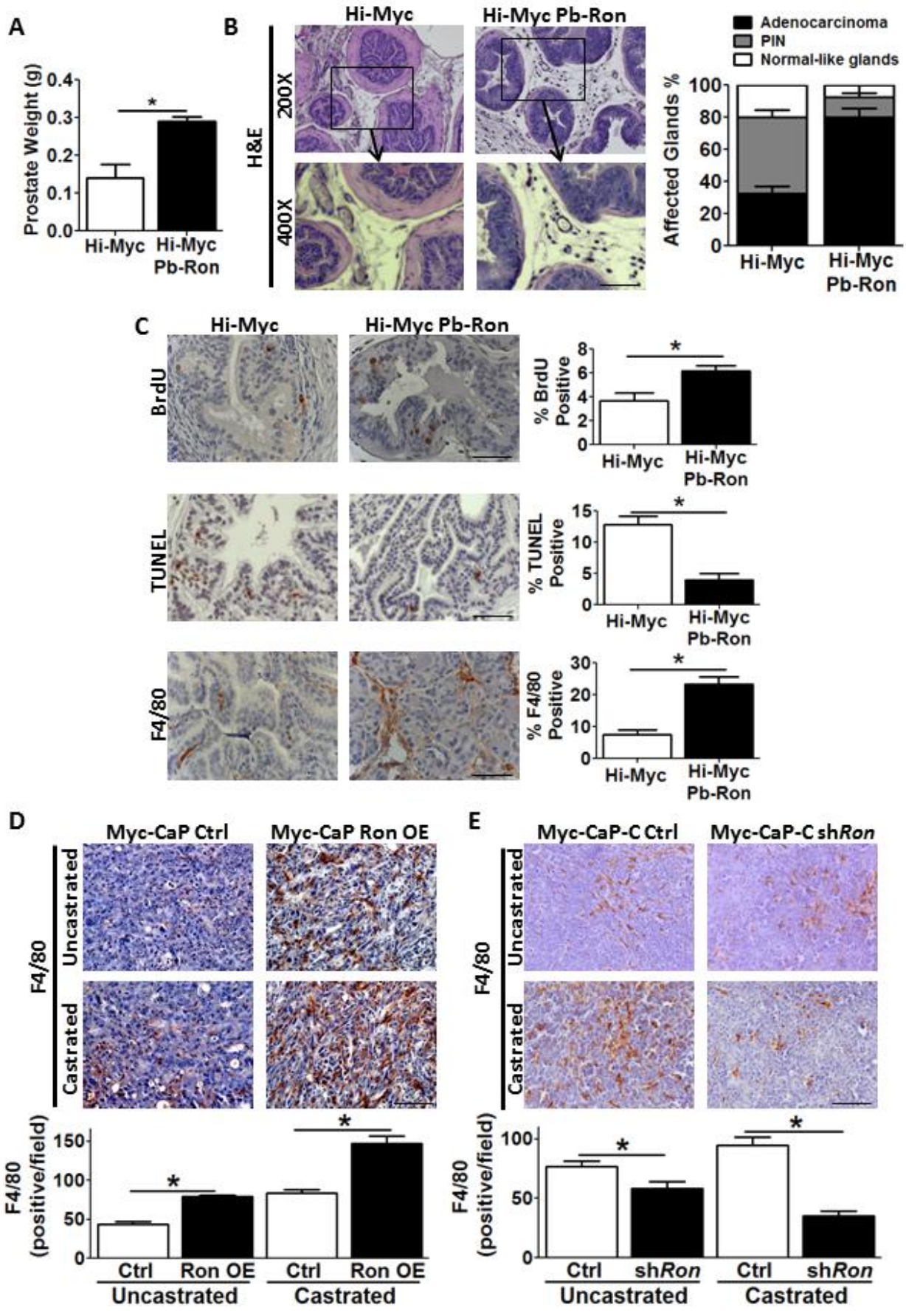

A
Prostate Weight (g)
0.4
0.3
0.2
0.1
0.0
*
Hi-Myc
Hi-Myc Pb-Ron
B
Hi-Myc
Hi-Myc Pb-Ron
H&E
200X
400X
Adenocarcinoma
PIN
Normal-like glands
Affected Glands %
100
80
60
40
20
0
Hi-Myc
Hi-Myc Pb-Ron
C
Hi-Myc
Hi-Myc Pb-Ron
BrdU
% BrdU Positive
8
6
4
2
0
*
Hi-Myc
Hi-Myc Pb-Ron
TUNEL
% TUNEL Positive
15
10
5
0
*
Hi-Myc
Hi-Myc Pb-Ron
F4/80
% F4/80 Positive
30
20
10
0
*
Hi-Myc
Hi-Myc Pb-Ron
D
Myc-CaP Ctrl
Myc-CaP Ron OE
F4/80
Uncastrated
Castrated
F4/80 (positive/field)
150
100
50
0
*
*
Ctrl
Ron OE
Ctrl
Ron OE
Uncastrated
Castrated
E
Myc-CaP-C Ctrl
Myc-CaP-C shRon
F4/80
Uncastrated
Castrated
F4/80 (positive/field)
100
50
0
*
*
Ctrl
shRon
Ctrl
shRon
Uncastrated
Castrated

Figure 1. Ron overexpression enhances F4/80+ myeloid cell recruitment into tumors in murine models of prostate cancer. A, Prostate weights from 30 week old Hi-Myc (n=4) and Hi-Myc Pb-Ron mice (n=4). **B**, Representative images of H&E staining of prostates from 30 week old Hi-Myc and Hi-Myc Pb-Ron mice (left, scale bar = 50μm) with quantitation of prostate tumor staging (right, n=4 mice per group). **C**, Representative images and quantitation of immunohistochemical staining of prostates from Hi-Myc and Pb-Ron Hi-Myc mice for BrdU (n=3 per group, scale bar = 50μm) as a marker of proliferation, TUNEL (n=3 per group, scale bar = 50μm) as a marker of apoptosis, and F4/80 (n=3 per group, scale bar = 50μm) as a marker of myeloid cell infiltration. **D**, Representative images and quantitation of immunohistochemical staining for F4/80 as a marker of myeloid cell infiltration in subcutaneous tumors formed from Myc-CaP Ctrl cells before (n=12) and after (n=8) castration and Myc-CaP Ron OE cells before (n=8) and after (n=8) castration (scale bar = 50μm). **E**, Representative images and quantitation of immunohistochemical staining for F4/80 in subcutaneous tumors formed from Myc-CaP-C shNT cells before (n=12) and after (n=8) castration and Myc-CaP-C shRon cells before (n=8) and after (n=7) castration (scale bar = 100μm). Data represent mean values ± SEM. *$P < 0.05$.

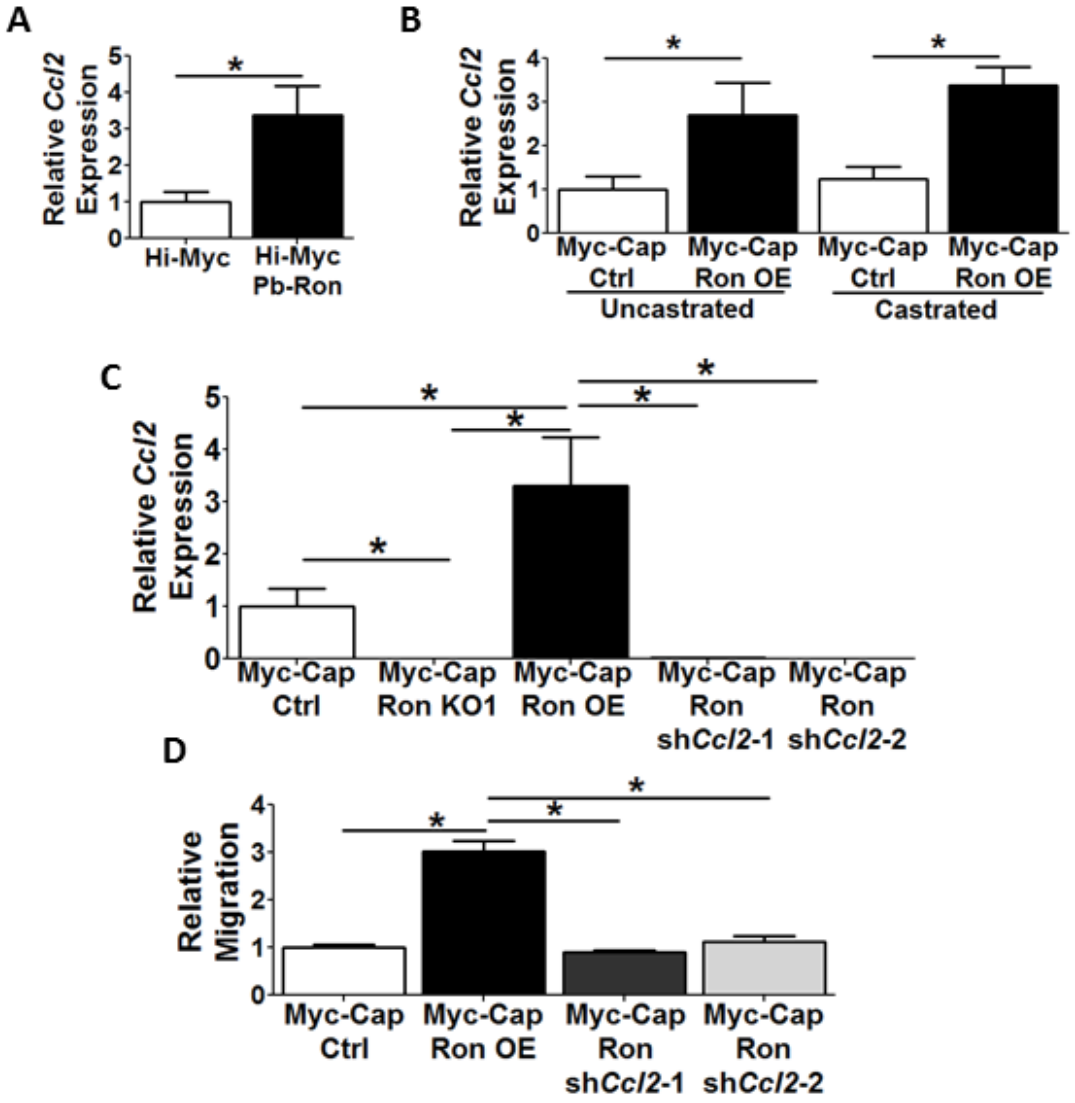


Figure 2. Ron overexpression enhances CCL2 production and requires CCL2 to promote macrophage migration. A, qRT-PCR of prostate samples from 30week old Hi-Myc (n=) and Hi-Myc Pb-Ron mice depicting gene expression of *Ccl2.* **B**, qRT-PCR from subcutaneous tumors derived from Myc-CaP Ctrl and Myc-CaP RON OE cells before and after castration depicting gene expression of *Ccl2* (n=4 per group). **C**, qRT-PCR from Myc-CaP Ctrl (n=5), Myc-CaP Ron KO1 (n=3), Myc-CaP Ron OE (n=5), Myc-CaP Ron sh*Ccl*2-1 (n=3), and Myc-CaP Ron sh*Ccl2*-2 (n=3) cells depicting gene expression of *Ccl2*. **D**, Relative migration of BMDMs toward Myc-CaP Ctrl, Myc-CaP RON OE, Myc-CaP Ron sh*Ccl2*-1, and Myc-CaP Ron sh*Ccl2*-2 cells (n=3 per group). Data represent mean values ± SEM. *P < 0.05.

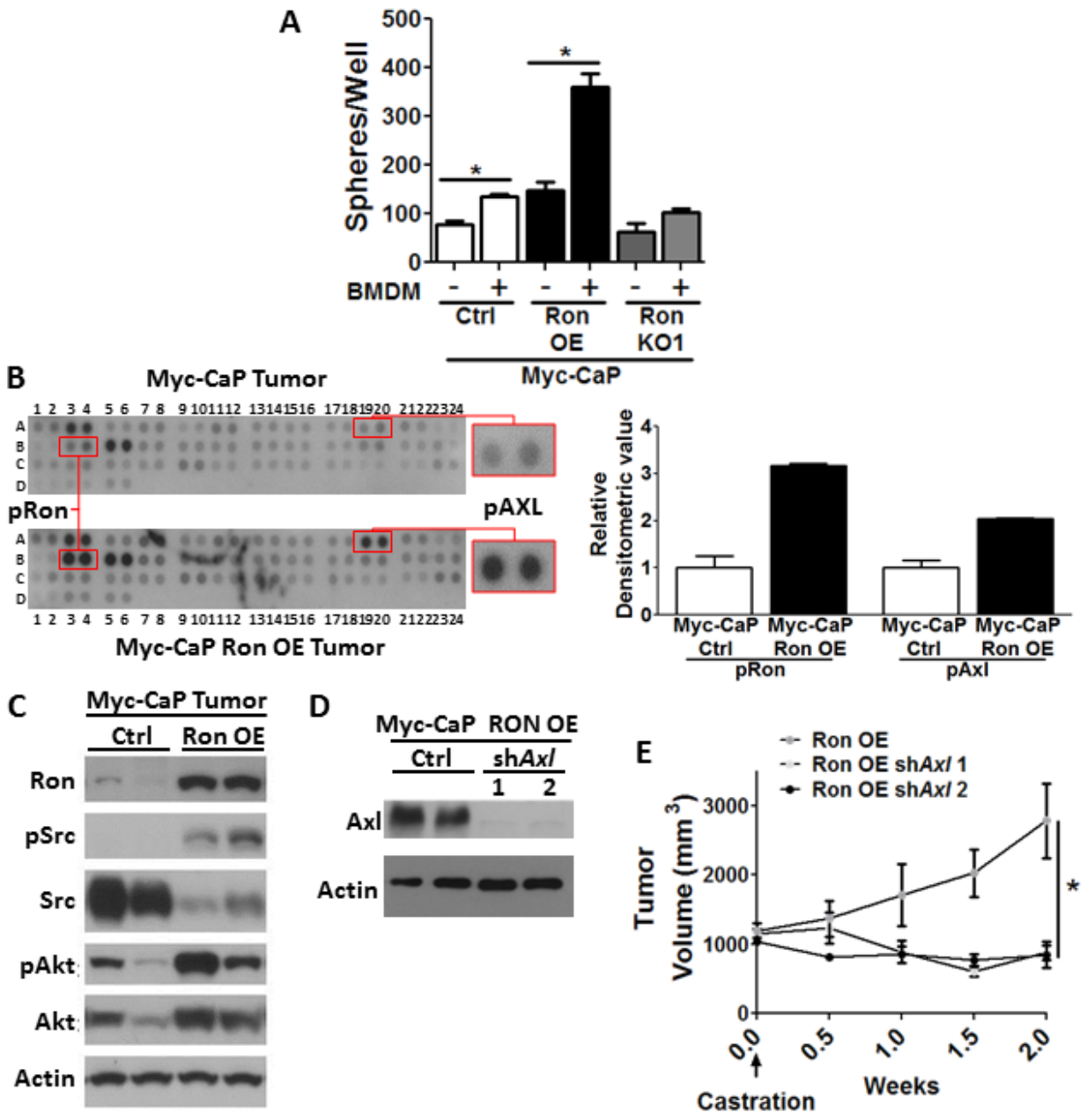


Figure 3. Ron overexpressing prostate cancer cells require Axl to promote growth following castration.

A, Total spheres produced from 2*10^4 Myc-CaP Ctrl, Myc-CaP Ron OE, and Myc-CaP Ron KO1 cells following 10 days growth in CSS with (n=3 per group) or without (n=4 per group) co-culture with 2*10^4 BMDMs. **B**, Phosphokinase array performed on lysates from Myc-CaP Ctrl and Myc-CaP Ron OE tumors depicting phosphorylation status of 39 receptor tyrosine kinases with densitometric values of pRon (B3, B4) and pAxl (A19, A20) portrayed on right (n=2). **C,** Western blot for Ron, y416 phosphorylated Src, Src, s473 phosphorylated Akt, and Akt in tumors derived from Myc-CaP Ctrl and Myc-CaP Ron OE cells. Actin

is shown as a loading control. Each lane represents an independent tumor sample. **D**, Western blot showing Axl expression following knockdown in Myc-CaP Ron OE sh*Axl*-1 and Myc-CaP Ron sh*Axl*-2 cells compared to Myc-CaP Ron OE cells. Actin is shown as a loading control. **E**, Average subcutaneous tumor volume of tumors derived from Myc-CaP Ron OE (grey circles, n=4), Myc-CaP RON shAxl-1 (light grey circles, n=4), and Myc-CaP Ron sh*Axl*-2 (black circles, n=4) cells following castration at 1000mm^3 in FVB mice. Data represent mean values ± SEM. *P < 0.05.

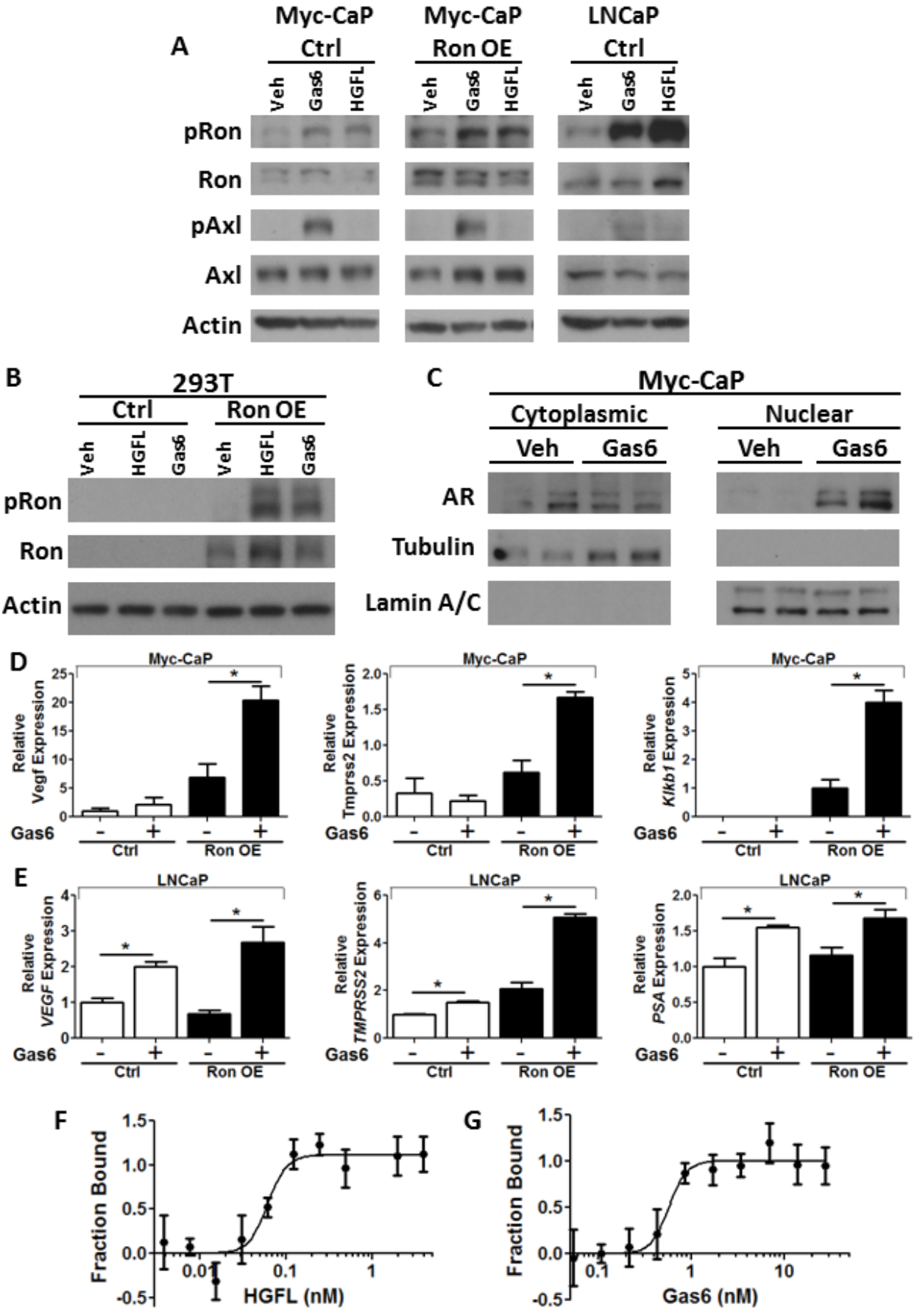

A
Myc-CaP Ctrl
Myc-CaP Ron OE
LNCaP Ctrl
Veh
Gas6
HGFL
pRon
Ron
pAxl
Axl
Actin
B
293T
Ctrl
Ron OE
Veh
HGFL
Gas6
pRon
Ron
Actin
C
Myc-CaP
Cytoplasmic
Nuclear
Veh
Gas6
AR
Tubulin
Lamin A/C
D
Myc-CaP
Relative Vegf Expression
Relative Tmprss2 Expression
Relative Klkb1 Expression
Gas6
Ctrl
Ron OE
E
LNCaP
Relative VEGF Expression
Relative TMPRSS2 Expression
Relative PSA Expression
F
Fraction Bound
HGFL (nM)
G
Fraction Bound
Gas6 (nM)

Figure 4. Gas6 binds to and induces activation of Ron signaling. A, Western blot analysis for y1238 phosphorylated Ron, Ron, y702 phosphorylated Axl, and Axl in 12 hour serum starved Myc-CaP Ctrl, Myc-caP Ron OE, and LNCaP cells 15 minutes following treatment with vehicle, Gas6 (100ng/ml), or HGFL (100ng/ml). Actin is shown as a loading control. **B**, Western blot analysis for y1238/y1239 phosphorylated Ron and Ron in 12 hour serum starved 293t Ctrl and 293t Ron OE cells 15 minutes following treatment with vehicle, Gas6 (100ng/ml), or HGFL (100ng/ml). Actin is shown as a loading control. **C**, Western blot analysis of Myc-CaP Ctrl cells following 4 hours treatment with vehicle or Gas6 (100ng/ml) then separated into cytoplasmic and nuclear fractions depicting nuclear localization of the AR. Tubulin (Cytoplasmic) and Lamin A/C (Nuclear) are shown as loading controls. Each lane represents an independent sample. **D**, qRT-PCR of Myc-CaP Ctrl and Myc-CaP Ron OE cells treated for 6 hours with vehicle or Gas6 (100ng/ml) depicting expression of AR target genes *Vegf, Tmprss*2 and *Klkb*1 (n=6 per gene per group). **E**, qRT-PCR of LNCaP Ctrl and LNCaP Ron OE cells treated with vehicle or Gas6 depicting expression of AR target genes *VEGF, TMPRSS*2 and *PSA* (n=6 per gene per group). **F**, Purified HGFL was incubated at 1:1 dilution ranging from 0.004nM - 4.00nM with a constant amount of whole cell lysate from 293T GFP-tagged Ron OE cells and binding of HGFL to Ron was measured using microscale thermophoresis (n=5, 0.0619 ± 0.0065 nM). **G**, Purified Gas6 was incubated at 1:1 dilution ranging from 0.05 – 27.0 nM with a constant amount of whole cell lysate from 293T GFP-tagged Ron OE cells and binding of Gas6 to Ron was measured using microscale thermophoresis (n=5, 0.5672 ± 0.0689 nM). Data represent mean values ± SEM. *P < 0.05.

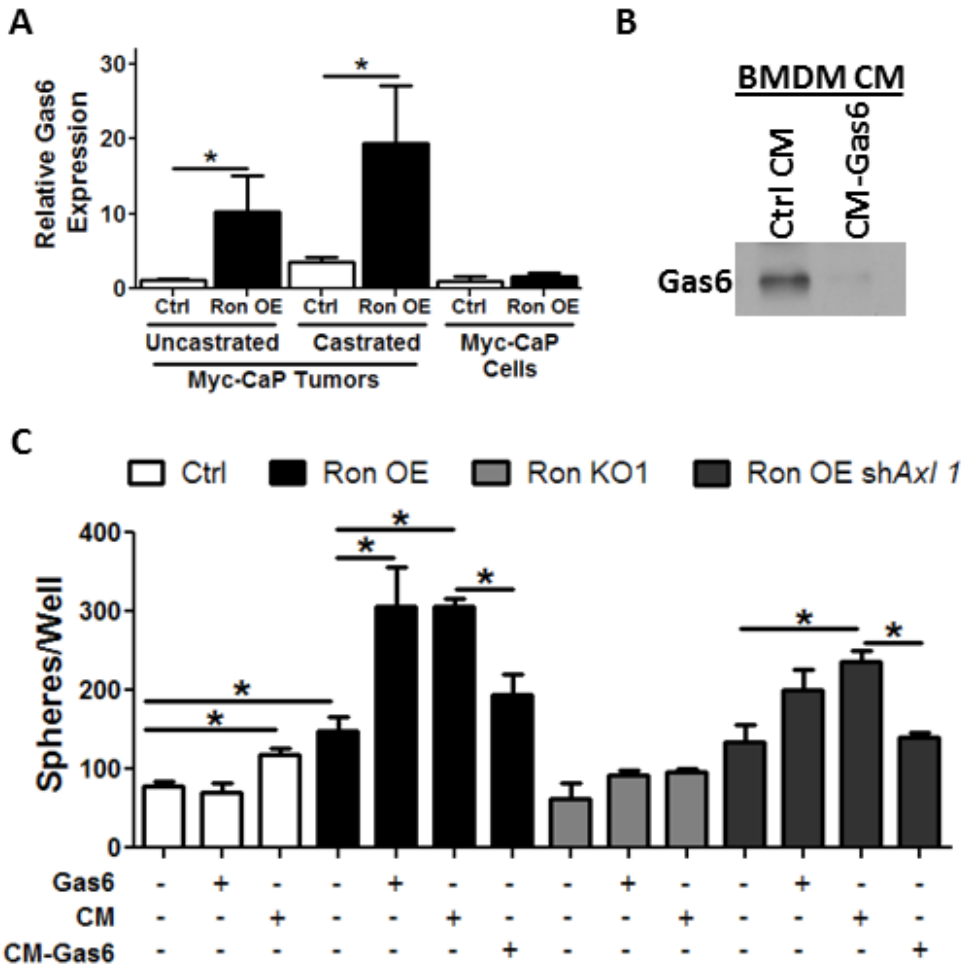


Figure 5. Macrophage produced Gas6 increases growth of Ron overexpressing prostate cancer cells under androgen deprived conditions. A, qRT-PCR from tumors derived from Myc-CaP Ctrl cells before (n=4) and after (n=5) castration and derived from Myc-CaP Ron OE cells before (n=3) and after (n=5) castration and Myc-CaP Ctrl (n=3) and Myc-CaP Ron OE (n=3) cells grown in 2D depicting gene expression of *Gas6.* **B**, Western blot analysis for Gas6 expression in Bone Marrow Derived Macrophage (BMDM) conditioned media (CM) with and without Gas6 depletion. **C**, Number of spheres formed from Myc-CaP Ctrl, Myc-CaP Ron OE, Myc-CaP Ron KO1, and Myc-CaP Ron OE shAxl 1 cells following 10 days growth in CSS and treated with either vehicle, Gas6 (1nM), or BMDM CM with or without Gas6 depletion(n=4 per group).

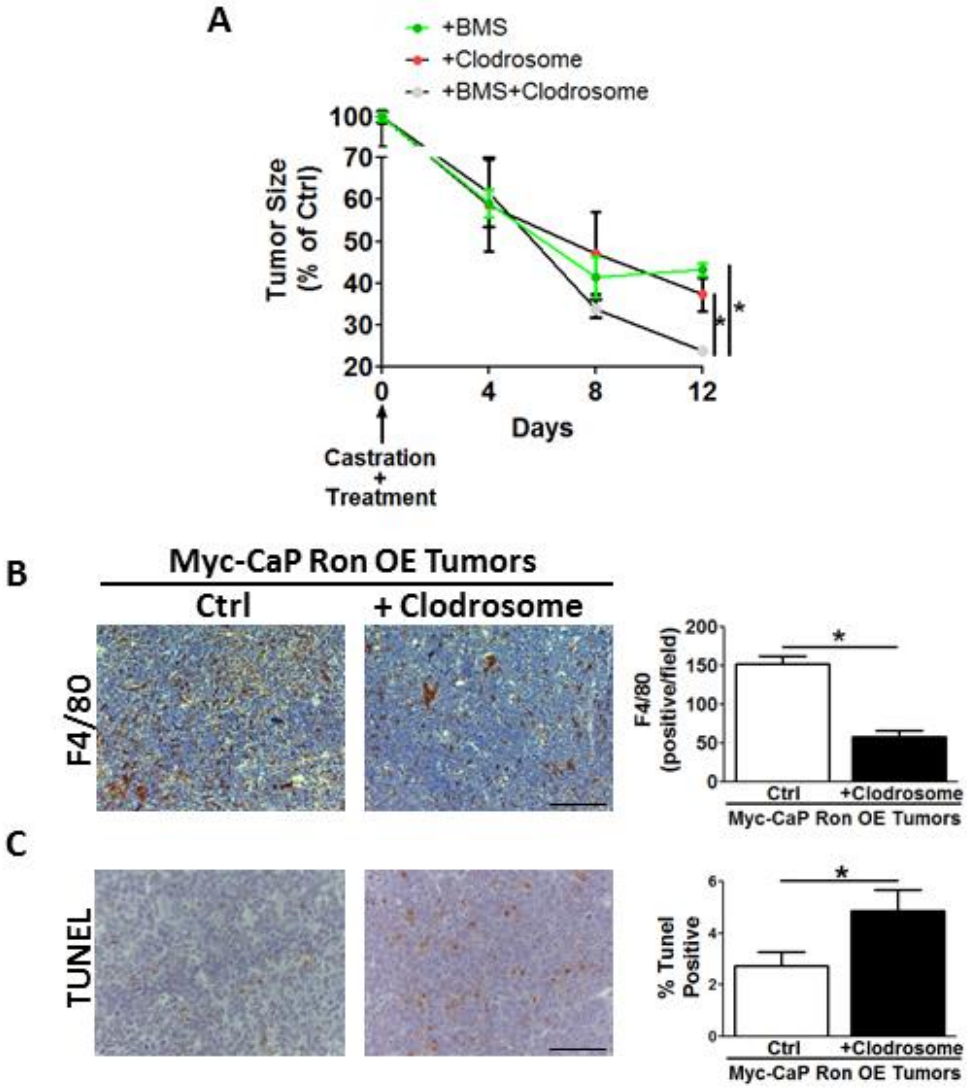
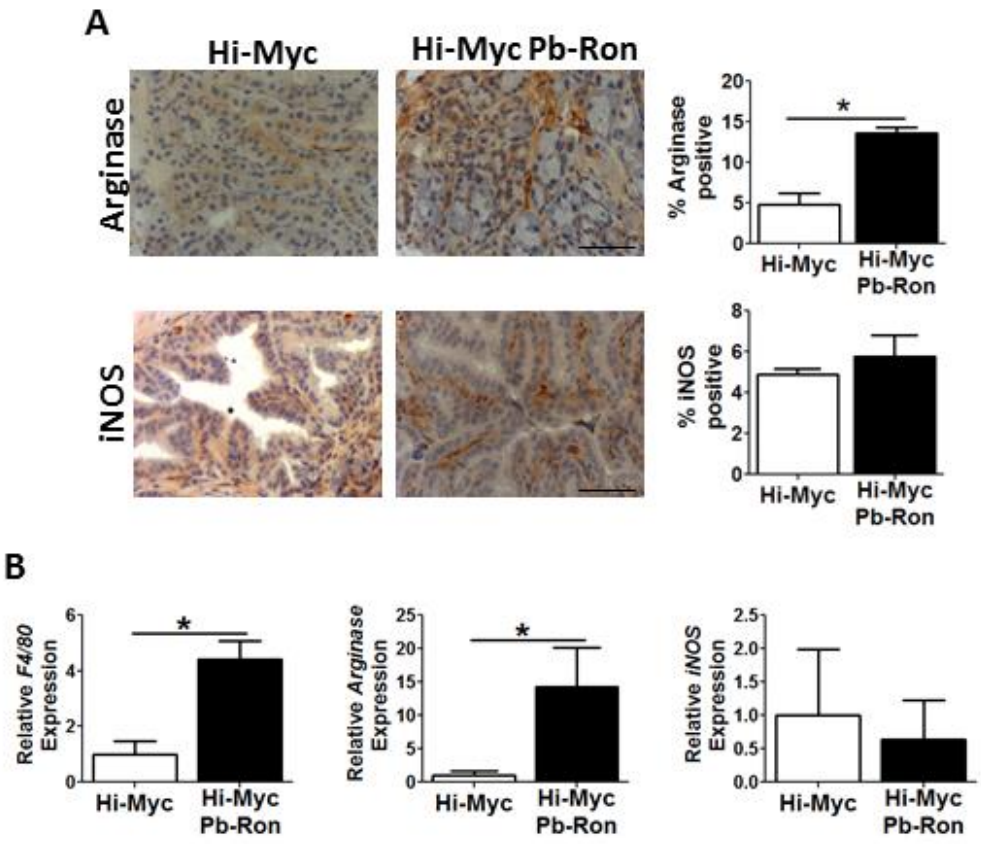


Figure 6. Macrophage depletion and Ron inhibition sensitizes castration resistant tumors to castration therapy. A, Average subcutaneous tumor volume of tumors derived from Myc-CaP Ron OE cells castrated at 1000mm^3 in FVB mice and treated with clodrosome (red squares, n=4), BMS (green squares, n=4) or BMS + Clodrosome (light grey squares, n=4). **B**, Representative images and quantitation of immunohistochemical staining of tumors in A for F4/80 (n=9 Ctrl, n=3 +Clodrosome, scale bar = 50µm) as a marker of myeloid cell infiltration. **C**, Representative images and quantitation of immunohistochemical staining of tumors in A for TUNEL (n=4 per group, scale bar = 50µm) as a marker of apoptosis. Data represent mean values ± SEM. *P < 0.05.



Supplemental Figure 1. Hi-Myc Pb-Ron prostates have elevated F4/80 and Arginase expression. A, Representative images and quantitation of immunohistochemical staining of prostates from Hi-Myc and Pb-Ron Hi-Myc mice for Arginase (n=3 per group, scale bar = 50µm) and iNOS (n=3 per group, scale bar = 50µm). **B**, qRT-PCR from Hi-Myc (n=4) and Hi-Myc Pb-Ron (n=4) prostates for gene expression of F4/80, Arginase, and iNOS.

Table 1. Densitometry results from RTK Phosphoarray.

Coordinates	Receptor	Fold Change	Coordinates	Receptor	Fold Change
A1, A2	EGFR	1.073708023	B17, B18	Tie-1	1.390132
A3, A4	ErbB2	0.839397091	B19, B20	Tie-2	1.068679
A5, A6	ErbB3	1.133019869	B21, B22	TrkA	1.4348
A7, A8	ErbB4	1.759371593	B23, B24	TrkB	1.882351
A9, A10	FGF R2	1.08292947	C1, C2	TrkC	0.802591
A11, A12	FGF R3	0.793624668	C3, C4	VEGF R1	0.721265
A13, A14	FGF R4	1.264547344	C5, C6	VEGF R2	0.818611
A15, A16	Insulin R	0.978055335	C7, C8	VEGF R3	0.851168
A17, A18	IGF-1R	1.013540153	C9, C10	MuSK	0.679367
A19, A20	Axl	2.044932296	C11, C12	EphA1	1.137668
A21, A22	Dtk	0.75016072	C13, C14	EphA2	1.152849
A23, A24	Mer	0.50532455	C15, C16	EphA3	0.533973
B1, B2	c-Met	1.609679	C17, C18	EphA6	0.896544
B3, B4	Ron	3.16731	C19, C20	EphA7	1.125449
B5, B6	PDGF Ra	1.231418	C21, C22	EphA8	0.924373
B7, B8	PDGF Rb	1.57125	C23, C24	EphB1	0.961356
B9, B10	SCF R	1.768606	D1, D2	EphB2	0.922031
B11, B12	Flt-3	1.498765	D3, D4	EphB4	1.611381
B13, B14	M-CSF R	0.969688	D5, D6	EphB6	0.872447
B15, B16	c-Ret	1.185835			

Coordinates indicate the dot location for each receptor on the phosphoarray shown in figure 3. Fold change normalizes the densitometric value from the phosphoarray performed using a Myc-CaP Ron overexpressing tumor relative to the densitometric value from the phosphoarray performed using a Myc-CaP Ctrl tumor. A value greater than 1 indicates an increase of signal with Ron overexpression, a value less than 1 indicates a reduction in signal with Ron overexpression.

Chapter 4

Ron Receptor Signaling Enhances the Effects of Obesity on Prostate Tumor Growth and Metastatic Dissemination

Abstract

As the prevalence of obesity continues to rise, the number of people impacted by its associated co-morbidities will rapidly grow. The link between obesity and a number of its co-morbidities has been well defined; however, the relationship with cancer remains vague. Specifically, in prostate cancer obese men are at higher risk of developing aggressive, metastatic forms of prostate cancer. A major characteristic of obesity is the accumulation of excess adipose tissue. We have shown expression of the Ron receptor tyrosine kinase in human adipose tissue and have identified a novel role for adipocyte specific expression of Ron to promote prostate cancer cell growth and metastatic potential. Further, we show in a genetic model of prostate cancer that Ron signaling deficient mice are resistant to the effects of diet induced obesity on prostate cancer progression. The Ron receptor has long been studied as an oncogene in epithelial cells; however, we show that Ron may be playing a significant role in tumorigenesis from a different cell type.

Introduction

It is estimated that over one third of all adults in the U.S. are obese, with many suffering from co-morbidities such as cardiovascular disease, diabetes, and cancer (149). Incidence of obesity has dramatically risen in recent years, and this is attributed to changes in dietary patterns and shifts toward sedentary lifestyles (150). Incidence is anticipated to rise as high calorie fast foods are becoming more frequently consumed and physical activity is becoming less required for most daily tasks. The link between cancer and obesity is the least characterized of the major co-morbidities associated with obesity. Currently, there is strong evidence showing the association of obesity with endometrial, esophageal adenocarcinoma, colorectal, postmenopausal breast, prostate, and renal cancers. Further investigation estimates that obesity accounts for approximately 20% of cancer deaths in women and 14% of cancer deaths in men over the past 25 years (151-153). Specifically, the link between obesity and prostate cancer has been very perplexing. A study published by the American Association for Cancer Research in 2011 explained that obesity had low risk for prostate cancer incidence, yet severely obese men had almost a fourfold higher risk of developing aggressive, metastatic prostate cancer (154). Patients with metastatic prostate cancer have a median survival of only 19 months (91). Although there are many interesting new therapies for treatment of aggressive, metastatic prostate cancer, most only increase survival a few months (155). With obese men at a higher risk of developing metastatic prostate cancer, it is imperative to determine the mechanisms which connect obesity to promoting this lethal disease.

Obesity is characterized by the accumulation of excess adipose tissue coupled with chronic low level inflammation (156). The primary cell type in adipose tissue are adipocytes, and research has indicated a substantial role for adipocytes and their secreted adipokines in cancer growth (157). Multiple studies have shown adipocytes are capable of promoting cancer cell growth and metastasis in prostate and breast cancer (158-160). Serum levels of the adipocyte produced adipokine adiponectin are reduced in

endometrial and hepatocellular carcinoma patients. Additionally, serum levels of the adipocyte produced adipokines leptin, visfatin, TNFα, IGF's, and IL-6 were detected to be elevated in endometrial, hepatocellular carcinoma, and colorectal cancer patients. (161, 162). These pieces of information make adipocytes and adipokines very relevant potential players when studying the ability of obesity to enhance prostate cancer. To date, there has yet to be a therapy focused on adipocytes or adipokines in the treatment of prostate cancer, making targeting them a novel approach.

A novel role has recently been described for the Ron receptor in regulating adipocyte function and development (163). The Ron receptor is a member of the c-Met family of cell surface receptor tyrosine kinases. Ron is known to be expressed primarily on select epithelial and macrophage populations, however, recent work has shown that Ron is also expressed in adipocytes and expression increases in the context of obesity (163). Additionally, whole body loss of Ron in a murine model of prostate cancer showed that Ron plays a functional role in promoting prostate tumor growth (37). However, the specific cell types responsible for Ron to promote prostate tumor growth have yet to be fully uncovered. With the new information that Ron plays a functional role in regulating adipocyte function and knowledge that adipocytes are the primary cell type impacted by obesity, we tested whether Ron signaling in adipocytes is critical for obesity to enhance prostate cancer growth and metastasis.

Results

Total body loss of Ron signaling reduces the effects of obesity on prostate cancer growth and metastasis *in vivo*. Prostate cancer in the context of obesity was modeled by using the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model on a high fat diet (45% calories from fat) (163, 164). TRAMP mice were crossed with mice harboring a whole body deletion of the Ron tyrosine kinase signaling domain (Ron TK-/-) in order to determine whether Ron plays a role in enhancing prostate cancer growth in the context of obesity (37). Shockingly, the prostates from Ron positive (Ron TK+/+) TRAMP mice were significantly larger than prostates from Ron TK-/- TRAMP mice (**Fig. 1A**). Previously, Ron has been shown to regulate the growth of prostate cancer in non-obese mice fed a regular diet (163). To confirm the decrease in tumor burden which accompanied Ron loss was a phenomenon related to obesity, we compared the weights of the mouse prostates on a high fat diet to the weights of prostates of non-obese TRAMP mice on a regular diet. It was observed that high fat diet fed Ron TK+/+ TRAMP mice exhibited a 7.5 fold increase in tumor growth relative to TK+/+ TRAMP mice on a regular diet (**Fig. 1B**). Conversely, high fat diet fed Ron TK-/- TRAMP mice only exhibited a 1.4 fold increase in tumor growth relative to TK-/- TRAMP mice on a regular diet (**Fig. 1B)**. The slight change in prostate weight for Ron TK-/- TRAMP mice under high fat diet indicates that Ron TK-/- mice are partially resistant to the effects of diet induced obesity on prostate tumor growth. Additionally, high fat diet has been shown to induce metastasis in the TRAMP model, and 100% of Ron TK+/+ mice possessed overt Lung metastasis (**Fig, 1C**). This contrasts with Ron TK-/- TRAMP mice, of which only 20% had overt lung metastasis (**Fig, 1C**). The body weights between the two groups were unchanged at 30 weeks of age, indicating that these drastic changes were not due to Ron TK-/- TRAMP mice resisting becoming obese (**Fig. 1D)**.

Ron signaling in adipocytes promotes growth and metastatic potential of C2RE3 prostate cancer cells *in vitro.* A common characteristic of diet-induced obesity is the expansion in the size and number of

adipocytes (165). Our prior work illustrated an important role for Ron in regulating adipocyte function and development (163). The ability of Ron signaling in adipocytes to promote prostate cancer growth was assessed by culturing the murine C2RE3 prostate cancer cell line with conditioned media from Ron TK+/+ and Ron TK-/- adipocytes. Interestingly, Ron TK-/- adipocyte conditioned media was unable to promote growth of C2RE3 prostate cancer cells to the same extent as Ron TK+/+ adipocyte conditioned media (**Fig. 2A**). In addition to growth, our *in vivo* model of obesity enhanced prostate cancer indicated that Ron signaling loss impaired metastatic potential. The ability of Ron signaling in adipocytes to enhance metastatic potential of C2RE3 prostate cancer cells was assessed by performing a migration assay and wound healing assay with C2RE3 cells cultured in Ron TK+/+ or Ron TK-/- adipocyte conditioned media. C2RE3 cells migrated 2.1 fold more when cultured in Ron TK+/+ adipocyte conditioned media relative to Ron TK-/- adipocyte conditioned media (**Fig. 2B**). Consistently, C2RE3 wound closure was 65% over 16 hours in Ron TK+/+ adipocyte conditioned media compared to only 43% wound closure in Ron TK-/- adipocyte conditioned media (**Fig. 2C**). These data reproduce what was observed *in vivo* and suggests that Ron signaling in adipocytes may regulate the secretion of factor(s) responsible for obesity to enhance prostate cancer growth and metastasis.

Ron signaling in adipocytes regulates secretion of cancer associated adipokines. The nature of the adipocyte secreted factor(s) regulated by Ron signaling for the promotion of prostate cancer cell growth was assessed by heat denaturation or size fractionation of adipocyte conditioned media prior to assessing growth potential. To determine if the factor(s) secreted by adipocytes which promote growth of prostate cancer cells are heat labile, conditioned media from Ron TK+/+ and Ron TK-/- adipocytes was heated to 95^{o}C for 10 minutes prior to being placed on C2RE3 cells. Following heat denaturation, neither Ron TK+/+ or Ron TK-/- adipocyte conditioned media promoted growth of C2RE3 cells (**Fig. 3A**). Heat labile factors which may be secreted by adipocytes can be a variety of sizes. The size range of the heat labile factors secreted by adipocytes for the promotion of prostate cancer cell growth was assessed by

size fractionating Ron TK+/+ conditioned media into fractions that were <3, <30, or >30 kDa. It was observed that adipocytes secret component(s) >30kDa to support growth of C2RE3 cells, as no other fraction was able to promote growth (**Fig. 3B**).

Proteins make up a large portion of adipocyte secreted factors which are heat labile and greater than 30 kDa in size. A variety of proteins secreted by adipocytes were assessed using an adipokine array with Ron TK+/+ adipocyte conditioned media and Ron TK-/- adipocyte conditioned media (**Fig. 3C**). Interestingly, secretions of a number of factors were observed to be altered following loss of Ron signaling. Specifically, DPP-IV (**Fig. 3D**) and ICAM-1 (**Fig. 3E**) secretions were decreased upon loss of Ron signaling and RBP4 (**Fig. 3F**), Resistin (**Fig. 3G**), and Adiponectin (**Fig. 3H**) secretions were increased following loss of Ron signaling in adipocytes. Up and down regulation of these identified factors have been implicated in key functions related to prostate cancer and will need to be further assessed to determine if these are some or all of the factors responsible for Ron to promote obesity enhanced prostate cancer through adipocytes.

Discussion

Obesity is a growing problem as advances in technology continually reduce demands for physical labor and fast foods continue to be a staple for many individuals. The large number of people affected by obesity and the major medical concerns associated with it have led the American Medical Association to officially classify obesity as a disease, highlighting the importance for research to be conducted in this area (166). Studies show that obese men are at higher risk of developing aggressive, fatal prostate cancer, yet no mechanism exists explaining this relationship. Prior work from our laboratory has shown expression of the Ron receptor tyrosine kinase in human adipose tissue and gene array data shows this expression is increased in adipose tissue from obese individuals, suggesting an important role for Ron in this tissue (163). Additional studies implicated a specific role for Ron in regulating the function of adipocytes within adipose tissue. The Ron receptor has been studied for its role as an oncogene in epithelial cells; however, research regarding its ability to promote cancer through adipocytes, the primary cell type in adipose tissue, had not been studied until now.

When Ron TK+/+ and Ron TK-/- TRAMP mice were placed on a high fat diet, both sets experienced an increase in the size of their tumors resulting from the change in diet (**Fig. 1**). However, this change was minimal in Ron TK-/- TRAMP mice. Not only was there lower tumor growth for Ron TK-/- TRAMP mice, but metastatic incidence was dramatically reduced. These data indicate that Ron signaling plays an important role in the ability of obesity to enhance the aggressiveness of prostate tumors. This *in vivo* data laid a solid foundation to begin studying cell type specific functions for Ron in enhancing prostate cancer growth in the context of obesity. Adipocytes, the primary cell type within adipose tissue, were shown to rely on Ron signaling for their ability to promote growth and migration of neighboring prostate cancer cells *in vitro* (**Fig. 2**). These phenotypes mimicked what was observed in our *in vivo* model for a total body knockout of the Ron tyrosine kinase signaling domain. It would be interesting to observe whether a conditional deletion of Ron in adipocytes (adiponectin-cre) would result in the same

phenotypic changes *in vivo*. These studies would require that Ron signaling be maintained in other cell types, such as adipose tissue resident macrophages, since Ron has been shown to promote tumor growth from some cells of the tumor microenvironment (41).

The noteworthy result that Ron signaling in adipocytes is capable of promoting growth and migration of prostate cancer cells *in vitro* led us to investigate what secreted factors might be altered upon loss of Ron signaling. Through this analysis, DPP-IV, ICAM-1, RBP4, Resistin and Adiponectin arose as potential candidates regulated by Ron to promote growth and migration. DPP-IV has been shown to correlate with increased adipocyte size and increased serum levels of DPP-IV were observed in obese patients relative to lean patients (167). DPP-IV secretion was higher in Ron TK+/+ adipocytes conditioned media relative to Ron TK-/- adipocyte conditioned media. This result is consistent with prior work showing that Ron loss reduces adipocyte size (163). Interestingly, DPP-IV was shown to be downregulated as prostate cancer progresses, which is contrary to our observations for the function of Ron in adipocytes (168). ICAM-1 was similarly observed to be higher in Ron TK+/+ adipocyte conditioned media relative to Ron TK-/- adipocyte conditioned media. ICAM-1 is a regulator of cell motility and increased ICAM-1 expression was shown to correlate with prostate cancer metastasis (169). This result is consistent with our observation that prostate cancer cells migrate more in Ron TK+/+ adipocytes conditioned media relative to Ron TK-/- adipocyte conditioned media.

Both RBP4 and Resistin were observed to be lower in Ron TK+/+ adipocytes conditioned media relative to Ron TK-/- adipocyte conditioned media. Interestingly, RBP4 is a retinol transporter protein and its expression has been associated with prostate cancer cell growth, although the exact mechanism for its role in prostate cancer remains unclear (170). Resistin is a low molecular weight protein (known to exist in oligomers) that functions in mediating insulin resistance (171). Similar to RBP4, the expression of Resistin was increased in the prostate epithelium of prostate cancer patients relative to normal tissue

and it has been shown to induce prostate cancer cell proliferation (172). These levels are contrary to the phenotypic observations we observed *in vivo* and *in vitro* following Ron signaling loss. Interestingly, however, Adiponectin, a protein involved in the regulation of glucose levels, was also lower in Ron TK+/+ adipocytes conditioned media relative to Ron TK-/- adipocyte conditioned media. Low levels of Adiponectin correlate with high levels of prostatic oxidative stress, which plays a significant role in the development and progression of prostate cancer (173). This result is consistent with our *in vivo* and *in vitro* data, making adiponectin regulation a potential novel role for Ron in mediating prostate tumorigenesis. With conflicting results observed from our adipokine array with literature reports regarding the specified adipokines and prostate cancer, it would be interesting to perform immunodepletion assays for the specified adipokines to determine which may play a functional role in Ron mediated obesity enhanced prostate cancer growth and metastasis.

The report herein details a novel role for a cell that is often neglected when studying tumorigenesis, adipocytes. Further research should be pursued into discerning the exact mechanisms by which Ron promotes obesity enhanced prostate cancer through adipocytes, as this study is still in its infancy and requires additional mechanistic analysis before major conclusions can be made. Once uncovered, these mechanisms may have major implications for a wide range of cancers that are impacted by obesity.

Materials and Methods

Mouse Models. Mice were maintained under specific pathogen-free conditions and experimental protocols approved by the University of Cincinnati IACUC. C57Bl/6 TRAMP mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice containing a germline deletion of the Ron tyrosine kinase domain (TK-/-) have been characterized previously (29). TRAMP mice were crossed with TK-/- mice to generate Ron TK-/- TRAMP mice as previously described (37). Only hemizygous TRAMP male mice were used for experimental analyses. Wild-type (TK+/+) and TK−/− mice were maintained on regular mouse chow until 14 weeks of age then fed a high-fat, lard-based diet with 45% calories from fat (D12451; Test Diet, Richmond, IN) or regular mouse chow (control) ad libitum. For tissue harvest, mice were killed by CO2 asphyxiation followed by cervical dislocation. For obtaining weight measurements for the G complex and the prostate, mice were euthanized and the GU complex (consisting of prostate, seminal vesicles, urethra and bladder) was first removed and weighed. The prostate was then dissected from the GU complex and weighed. Overt lung metastasis was observed visually upon dissection.

Migration Assays. 2X104 C2RE3 cells were plated on top of a 24 well plate Transwell Permeable Support with an 8µM pore size (Costar, 3422). 0.5 ml of adipocyte conditioned media was placed in the bottom layer. C2RE3 cells were allowed to migrate for 20 hours, fixed in methanol for 20 min, then stained with 0.1% Crystal violet prior to quantification. Images were taken of the transwell insert and the total number of cells migrated/field were counted using Image J software. At least 3 fields per Transwell were counted and averaged for 3 different transwells for each type of conditioned media.

MTT Growth Assays. C2RE3 cells were seeded at a density of 10,000 cells per well and MTT assays (Sigma, Cat#298-93-1) were performed following 72 hours growth in adipocyte conditioned media. Briefly, 200ul of 5mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) in PBS was added for

2 hours at 37°C. Formed formazan crystals were dissolved in 200ul of DMSO and absorbance was taken at the wavelength of 570nm as previously described (14).

Cell Models and conditioned media. Murine TRAMP-C2Re3 cells were obtained from Dr. Zhongyun Dong (University of Cincinnati) and grown as previously described (174). Adipocytes were isolated from epididymal adipose tissue that was minced with a razor blade in a sterile petri dish, passed through a 1-mm tissue sieve, and digested with collagenase by rotating at 120 revolutions/min for 30 min at 37°C. Adipocytes were floated by centrifugation at 50 g and retrieved. Preadipocytes were separated from the stromal vascular pellet by differential centrifugation. Adipocyte conditioned media was isolated by culturing equal number of adipocytes under serum free conditions for 24 hours. Media was then aspirated, centrifuged, and passed through a 40 µM filter. Conditioned media was heat denatured by heating for 10 minutes at 95^{o}C. Conditioned media fractionation was performed using Amicon Ultra Centrifugal filters per the manufacturer's instructions. Roche proteome profiler mouse adipokines array (ARY013) was used for adipokines analysis of conditioned media per manufacturer's instructions. Densitometry was performed using Image J software.

Wound healing Assay. C2RE3 cells were seeded in a 24 well plate. Once cells reached ~80% confluency, a wound was produced using a 200ul pipette tip (corning, 4111). Dead cells were washed away and media was replaced with serum free media supplemented with 10% adipocyte conditioned media. Images were taken immediately after the wound was made and then again after 16 hours. Image J software was used to measure the area of the wound before and after the 16 hour period for calculation of % wound closure.

Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by performing Student's t-test for pairwise comparisons using GraphPad

Prism software. All in vitro experiments represent the average of at least triplicate experiments. Significance was set at *P<0.05.

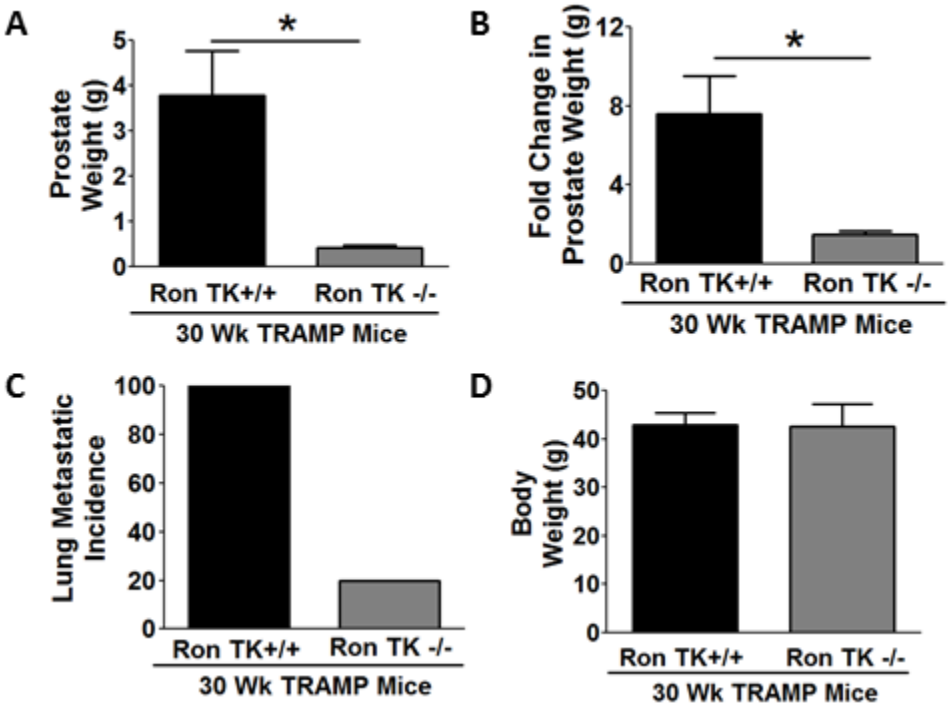


Figure 1. Total body loss of Ron signaling reduces the effects of obesity on prostate cancer growth and metastasis *in vivo*. A, Prostate weight in grams (g) from 30 week old Ron TK+/+ TRAMP (n=9) and Ron TK-/- TRAMP (n=5) mice following 16 weeks on high fat diet. **B,** Fold change in prostate weight for Ron TK+/+ TRAMP (n=9) and Ron TK-/- TRAMP (n=5) mice following 16 weeks on high fat diet compared to respective prostate weight from mice on regular diet recorded in previously published study (37). **C,** Overt lung metastatic incidence between 30 week old Ron TK+/+ TRAMP (n=9) and Ron TK-/- TRAMP (n=5) mice following 16 weeks on high fat diet. **D,** Body weights from 30 week old Ron TK+/+ TRAMP (n=5) and Ron TK-/- TRAMP (n=4) mice following 16 weeks on high fat diet. Data represent mean values ± SEM. *P<0.05.

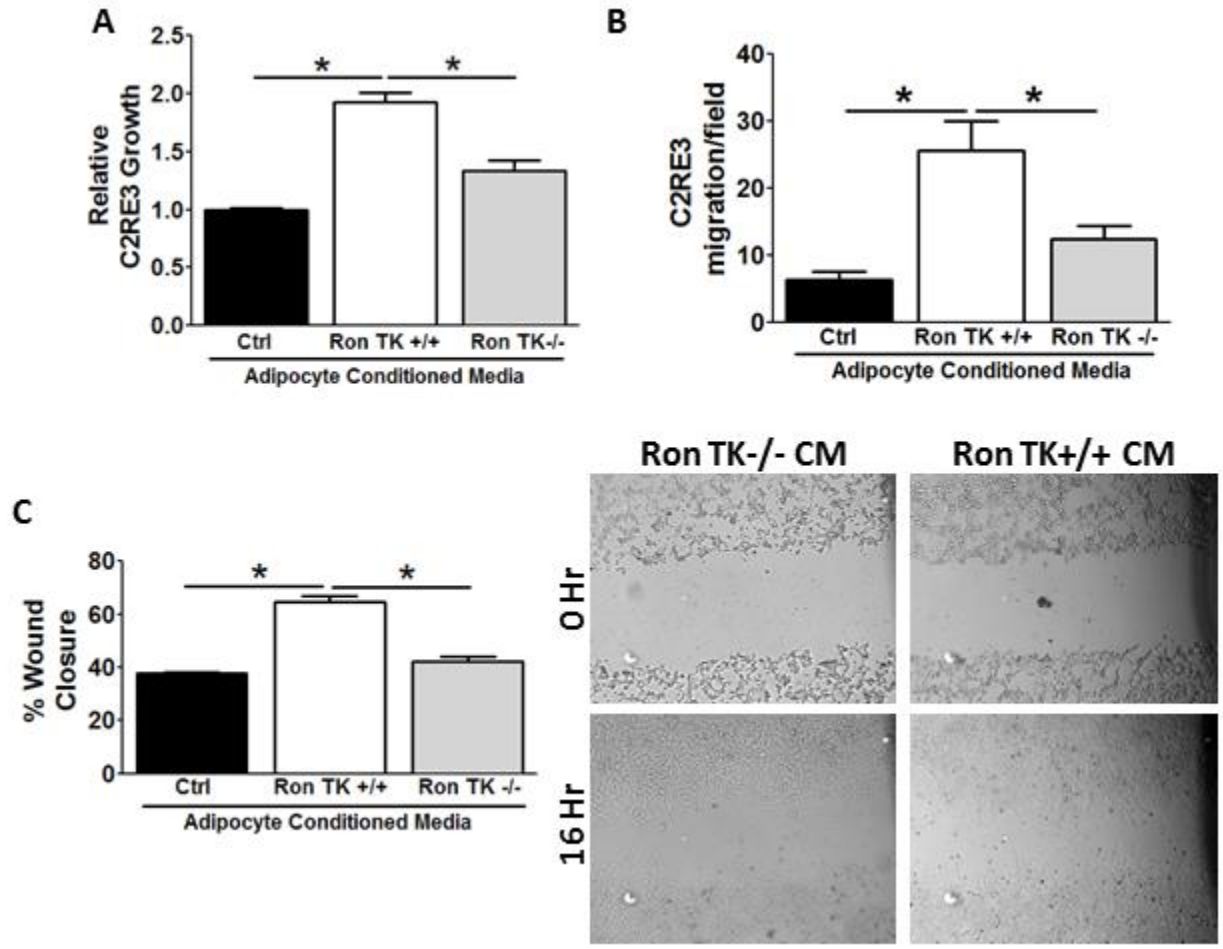
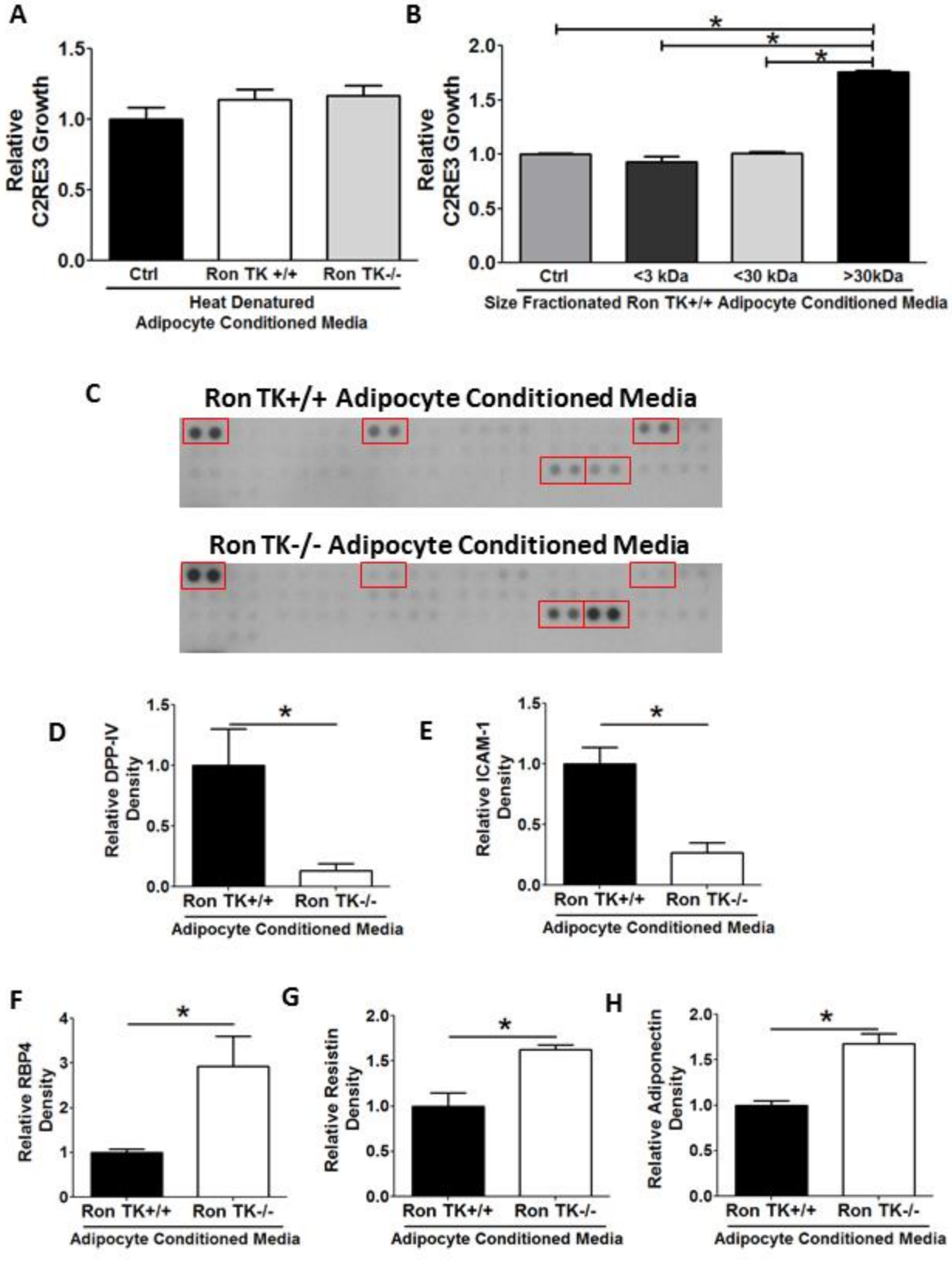


Figure 2. Ron signaling in adipocytes promotes growth and metastatic potential of C2RE3 prostate cancer cells *in vitro.* A, Growth of C2RE3's as measured by MTT assay following 72 hours culture in serum free media supplemented with 10% Ron TK+/+ (n=4) or Ron TK-/- (n=4) adipocyte conditioned media. Growth normalized to growth of serum free only control (ctrl, n=6). **B,** Number of C2RE3 cells per field which migrated over a 20 hour period toward serum free media (n=3) or serum free media supplemented with 10% Ron TK+/+ (n=6) or Ron TK-/- (n=6) adipocyte conditioned media. **C,** (**LEFT**) Percent wound closure of C2RE3 cells 16 hours post wound formation with cells in serum free media (n=4) or in serum free media supplemented with 10% Ron TK+/+ (n=4) or Ron TK-/- (n=4) adipocyte conditioned media. (**RIGHT**) Representative images at time 0 and 16 hours post wound formation with cells supplemented with 10% Ron TK+/+ or Ron TK-/- adipocyte conditioned media. Data represent mean values ± SEM. *P<0.05.


A
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Ron TK +/+
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Relative C2RE3 Growth
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0.0
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<3 kDa
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Size Fractionated Ron TK+/+ Adipocyte Conditioned Media
C
Ron TK+/+ Adipocyte Conditioned Media
Ron TK-/- Adipocyte Conditioned Media
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Adipocyte Conditioned Media
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2.0
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Adipocyte Conditioned Media

Figure 3. Ron signaling in adipocytes regulates secretion of cancer associated adipokines. A, Growth of C2RE3's as measured by MTT assay following 72 hours culture in serum free media supplemented with 10% heat denatured Ron TK+/+ (n=4) or heat denatured Ron TK-/- (n=4) adipocytes conditioned media. Growth normalized to growth of serum free only control (ctrl, n=5). **B,** Growth of C2RE3's as measured by MTT assay following 72 hours culture in serum free media supplemented with 10% Ron TK+/+ adipocyte conditioned media fractionated into <3 (n=4), <30 (n=4), and >30 kDa fractions (n=4). Growth normalized to growth of serum free only control (ctrl, n=6). **C,** Images from proteome profiler adipokine array analyzing Ron TK+/+ or Ron TK-/- adipocyte conditioned media with identified candidates in red boxes. Relative densitometric values for DPP-IV (**D, n-=3**), ICAM-1 (**E n=3**), RBP4 (**F, n=3**), Resistin (**G, n=3**), and Adiponectin (**H, n=3**) from adipokine array pictured in C. Data represent mean values ± SEM. *P<0.05.

Chapter 5

Conclusions, Discussion, and Future Directions

For some patients, surgery and/or radiation are sufficient treatment options for prostate cancer. These treatments can be effective; however, they come with a number of risks and harsh side effects. For others, these treatments are not enough and patients are required to undergo androgen deprivation therapy (ADT) in an attempt to prevent the disease from progressing. For many of these patients ADT often provides only a temporary relief from the disease before it becomes resistant and progresses to castration resistant prostate cancer (CRPC). Despite the immense knowledge gained regarding the mechanisms which drive CRPC, patients remain with minimal treatment options. Many prostate cancers become resistant to ADT through reactivation of the Androgen Receptor (AR). AR is a transcription factor that translocates into the nucleus after being bound by androgens and promotes transcription of many genes, several of which code for metabolic proteins that regulate lipid and protein biosynthesis required for cell division. Following ADT, the androgen supply is decreased and growth of the cancer slows down due to decreased AR activity, but cells almost certainly develop resistance.

There are a number of mechanisms by which prostate cancer becomes resistant to ADT, and many of them involve reactivation of AR. Studies have shown that although ADT decreases systemic androgen levels, prostate cancer cells can upregulate enzymes which allow for the production of androgens locally within the prostate tumor (175, 176). Additionally, mutations have been detected in AR which allow for binding of ligands other than androgens for activation (177). AR can also be modified post-translationally by phosphorylation, methylation, acetylation, sumoylation, and or ubiquitination to induce nuclear localization and transcriptional activity (178). With all of these potential mechanisms for AR reactivation, it is critical to understand the upstream mechanisms regulating AR which exist as viable therapeutic targets.

We have identified the Ron receptor as a major mediator of resistance to ADT and promoter of AR activation. In chapter 2 of this thesis we demonstrate a novel function for the Ron receptor to drive AR activation through activation of β-Catenin and NF-κB. In Chapter 3 of this thesis we discuss novel ways in which Ron promotes resistance to castration therapy through the recruitment of macrophages. In chapter 4 we have illustrated a novel role for Ron signaling in adipocytes to enhance the effects of diet-induced obesity on prostate cancer growth and metastatic potential. Overall, the work presented herein provides novel insights into the role of the Ron receptor in prostate tumor cells and in regulating neighboring cells of the tumor microenvironment. These newly discovered roles for Ron are uniquely tied to therapeutic resistance in prostate cancer and have the potential to be exploited for novel prostate cancer treatments.

Prior work regarding Ron's role in prostate cancer was critical in leading us toward studying the role of Ron in CRPC. When the transgenic adenocarcinoma of mouse prostate (TRAMP) model was crossed with mice with total body deletion of the Ron tyrosine kinase domain (TK-/-) the result was a decrease in prostate tumor size (37). Prostates from TRAMP Tk-/- mice exhibited decreased microvessel density and decreased cellular survival relative to TRAMP TK+/+ controls. In another study, Ron overexpression driven by the probasin promoter in mice allowed for the effects of elevated Ron expression specifically in the prostate epithelium to be observed (50). This model showed that Ron overexpression induced prostate intraepithelial neoplasia in 100% of mice, with some mice progressing to prostate adenocarcinoma. Immunohistochemistry analysis of the prostates revealed that proliferation had increased and cell death decreased upon Ron overexpression, showing Ron's ability to impact multiple aspects of cancer. In order to further establish the epithelial cell type specific functions of Ron in prostate cancer, another study utilized knockdown or exogenous overexpression of Ron in a number of human prostate cancer cell lines (14). Interestingly, knockdown of Ron in human PC-3 and DU145 cells resulted in decreased production of angiogenic chemokines and overexpression in LNCaP cells induced

production of the angiogenic chemokine CXCL8. Additionally, when PC-3 cells with Ron knockdown were orthotopically injected into mouse prostates the resulting tumors had decreased tumor vasculature relative to control tumors. This result corresponded well to the decrease in microvessel density observed in TRAMP TK-/- mice and were some of the first pieces of data indicating that epithelial Ron is capable of influencing cells of the tumor microenvironment. Another interesting finding from this study was that Ron was highly expressed in androgen-independent prostate cancer cell lines relative to less aggressive androgen-dependent cell lines, highlighting a potential role for Ron in resistance to hormonal therapies. These previous studies prompted us to test the hypothesis that Ron signaling promotes CRPC by both cell intrinsic and extrinsic mechanisms.

<u>The key findings of our study related to CRPC are outlined below and shown in Illustration 1:</u>

Chapter 2

- RON expression is elevated in CRPC patient samples and is critical for tumors to develop castration resistance in murine CRPC models.
- RON expression mediates castration resistant growth *in vivo* through oncogenic signaling pathways that enhance tumor cell proliferation and reduce apoptosis.
- Activation of oncogenic β-CATENIN, NF-κB, and AR is required for RON to promote growth under androgen deprivation.
- β-Catenin and NF-κB independently induce AR activation to promote growth under androgen deprivation *in vitro.*

Chapter 3

- Ron overexpression enhances F4/80+ myeloid cell recruitment into tumors in murine models of prostate cancer.
- Ron mediated CCL2 production is required for Ron to promote macrophage migration.
- Axl is required by Ron overexpressing prostate cancer cells to promote growth following castration.

- Gas6 binds to and induces activation of Ron and activation of AR.
- Macrophage produced Gas6 increases growth of Ron overexpressing tumors under androgen deprived conditions.
- Macrophage depletion and Ron inhibition sensitizes castration resistant tumors to castration therapy.

Discussion and future directions regarding chapters 2 and 3

These findings reveal a fascinating relationship between Ron and castration resistance. Ron overexpression induces several critical changes which allow for tumors to continue growing in the absence of androgens. There are a number of different proteins which Ron activates in order to achieve growth in the absence of androgens, and whether or not activation of these signaling proteins converge or impact one another will require further study. A recent study in melanoma revealed that AR overexpression significantly increases the expression of Axl (179). Interestingly, a separate study in esophageal squamous cell carcinoma revealed that Axl mediates tumorigenesis by activating NF-κB and repressing an inhibitor of β-Catenin, GSK3β (180). Both NF-κB and β-Catenin have been identified to be transcriptional regulators of CCL2 (181, 182). These findings complement the studies performed in chapters 2 and 3 and illustrate that the mechanisms described in each may have some crossover. All of these questions will require further study in order to adequately detail the mechanism for Ron to drive castration resistance.

Relating our recent findings for Ron in prostate cancer to previous work on Ron brings up a number of thought-provoking questions. We describe a novel relationship between Ron and AR in prostate cancer; however, previous reports showed that Ron is highly expressed and important for the growth of AR negative PC-3 and DU145 cells (14). The exact role for Ron in the absence of AR has not been completely defined, although, the ability of Ron to activate NF-κB in AR negative cells was observed and highlighted as critical for allowing secretion of angiogenic chemokines. In the absence of AR, prostate cancer cells

may become heavily reliant on Ron to activate other proteins or transcription factors, such as NF-κB or β-Catenin, for sustaining growth. Knowing that Ron is important both in the presence and absence of AR may require targeted therapeutics to be tailored to AR expression patterns. Additionally, all current studies regarding Ron and prostate cancer have specifically focused on Ron's role in relation to full length AR, however, a number of patients also express AR variants which are critical in driving disease (183). The role for Ron in regulating expression patterns or function of AR variants will require further evaluation for adequate discernment of treatment options for patients.

Our in depth analysis regarding the ability of Ron to promote therapeutic resistance has unique correlations to a recent paper in breast cancer describing a novel role for Ron in promoting cancer stem cell phenotypes (74). Activation of NF-κB and β-Catenin in breast cancer enhanced cancer stem cell self-renewal, numbers, and tumorigenic potential. Cancer stem cells are known for promoting therapeutic resistance and recurrence in cancer through their ability to survive treatments and repopulate a tumor once treatment has ceased (184). We have implicated Ron in promoting activation of both NF-κB and β-Catenin for therapeutic resistance in prostate cancer and show that both promote AR activation in prostate cancer. Recent work has shown that AR functions in breast cancer to support cancer stem cell phenotypes (185). AR status in Ron mediated breast cancer has yet to be assessed and neither has Ron's ability to promote cancer stem cell phenotypes been assessed in prostate cancer. Evaluating each of these possibilities may reveal another layer to the mechanism in which Ron drives therapeutic resistance in both breast and prostate cancer.

Another interesting observation that prior reports have made in both breast cancer and prostate cancer is that HGFL plays distinct roles in Ron driven tumorigenesis. Whole body genetic knockout of HGFL in both the TRAMP model of prostate cancer and the MMTV-Ron model of breast cancer model resulted in decreased tumor growth and reduction of cancer cell survival abilities (30, 38). Interestingly, in the

MMTV-Ron model, breast tumorigenesis is driven by breast epithelial specific overexpression of Ron. Loss of HGFL in this model delayed mammary tumor initiation but did not completely prevent it, indicating that Ron activation can occur through an HGFL independent mechanism. We made the significant finding that Gas6 is capable of binding to and activating Ron. The presence of Gas6 in Ron driven breast tumors has yet to be determined, but it would be very interesting to see if the remaining oncogenic functions of Ron following HGFL loss were due to remaining Gas6 signaling. Whether or not there are differential roles for HGFL and Gas6 mediated Ron activation will require further study, however, HGFL independent functions of Ron for enhancing cell spreading and survival of breast cancer cells has already been reported (31).

In chapter 3 we detailed a novel role for Ron to recruit macrophages into the tumor microenvironment to drive resistance to ADT. This phenomenon was exciting to observe, and is even more intriguing knowing the role Ron has been demonstrated to play in macrophages. As described previously, Ron has been shown to be expressed in both epithelial cells and macrophages. In fact, the expression of Ron in macrophages was established to play a functional role in promoting prostate cancer growth (41). When murine C2RE3 prostate cancer cells were orthotopically injected into the prostates of mice with a myeloid specific deletion of the Ron tyrosine kinase signaling domain, the resulting tumors were smaller and had a significantly higher percentage of apoptotic cells relative to tumors formed in wild type control mice. Macrophage Ron expression has been shown to promote a pro-tumor, anti-inflammatory M2 phenotype, demonstrated by the promotion of arginase expression and inhibition of iNOS expression (30, 41, 62-64). Within our studies, all of the macrophages maintained exogenous levels of Ron and were shown to exhibit an M2 phenotype. It would be interesting to determine the significance in macrophage Ron expression on the ability for epithelial Ron overexpression to drive resistance to castration therapy. Losing Ron expression in the macrophages might allow for only M1 anti-tumor macrophages to be recruited into tumors, resulting in reduced tumor growth.

The key findings of our study concerning diet-induced obesity and prostate cancer are outlined below:

Chapter 4

- Total body loss of Ron signaling reduces the effects of obesity on prostate cancer growth *in vivo*.
- Ron signaling in adipocytes promotes growth and metastatic potential of C2RE3 prostate cancer cells *in vitro.*
- Ron signaling in adipocytes regulates secretion of cancer associated adipokines.

Discussion and future directions regarding chapter 4

This brief analysis of the importance of Ron in prostate cancer enhanced by diet-induced obesity highlights an ever growing need in regards to the harmful effects of obesity on health. It is estimated that by 2030 approximately 50% of people in the United States will be obese, emphasizing the need to increase our understanding of the harmful effects of obesity and increase our ability to treat it's associated co-morbidities (186). Prior to this study, Ron's role in regulating the effects on diet-induced obesity was beautifully demonstrated through a study detailing the major phenotypic changes between Ron TK+/+ and Ron TK-/- mice on a high fat diet (163). This report showed that Ron TK-/- mice were leaner, more insulin sensitive and glucose tolerant, and had less hepatic steatosis than Ron TK+/+ control mice when fed a high fat diet. Importantly, this study also demonstrated that Ron plays a functional role in regulating the function of adipocytes. Adipocytes are the primary cell type of adipose tissue, which has been established to have major endocrine and paracrine functions (187). Importantly, when adipocytes are in close proximity with prostate cancer cells they have been shown to stimulate prostate cancer growth (158). The importance of Ron for adipocyte function and the role adipocytes can play for promoting prostate tumor growth led us to examine the hypothesis that Ron signaling is important for adipocytes to promote prostate cancer growth and metastasis in the context of obesity.

While pursuing this hypothesis, we observed that TRAMP TK-/- mice developed significantly smaller tumors and had a decrease in overt lung metastasis compared to TRAMP TK+/+ mice when both were fed a high fat diet. The phenotypic changes in this model were remarkable; however, they occurred when looking at a whole body loss of the tyrosine kinase signaling domain of Ron. Our *in vitro* data demonstrates that Ron signaling in adipocytes can contribute to these phenotypes, although, we will need to perform an adipocyte specific knockout *in vivo* to confirm the *in vivo* significance of Ron's role in this cell type. With obese men being at greater risk of developing aggressive prostate cancer, it is imperative to further define the role of Ron in adipocytes for promoting prostate cancer in the context of obesity to discern if this population of patients is likely to respond to a Ron related therapy. Specifically, with the role Ron has been established to play in regulating CRPC, it would be advantageous to identify whether or not adipocyte specific Ron expression plays a role in regulating response to ADT.

Important considerations for Ron related therapies

Work presented in this thesis and in past publications has demonstrated that Ron plays a role in multiple cell types for the promotion of prostate cancer. This knowledge suggests that it might be advantageous to globally inhibit Ron in order to achieve maximum therapeutic effect. This idea, while intriguing, does come with the potential for adverse side effects. One of the primary functions for Ron signaling is the regulation of inflammatory responses (29, 188). Ron is known to limit inflammatory responses, and mice hemizygous for Ron (Ron +/-) were shown to be highly susceptible to endotoxic shock following lipopolysaccharide (LPS) administration (189). Additionally, mice with a global deletion of the Ron tyrosine kinase signaling domain (Ron TK-/-) exhibited enhanced inflammatory responses relative to control mice to acute irritant induced contact dermatitis and allergic contact dermatitis (29). These prior reports indicate that long term global inhibition of Ron may result in exaggerated inflammatory responses that may be difficult for patients to tolerate.

With global inhibition of Ron posing potential adverse events in patients, cell type specific Ron directed therapies may be desirable. Prior work has demonstrated that loss of Ron signaling in myeloid cells in a murine model of prostate cancer results in an increase in macrophage infiltration into prostate tumors and decreased prostate tumor growth (41). Loss of Ron signaling in macrophages has been shown to induce an M1 pro-inflammatory macrophage phenotype, capable of suppressing tumor growth (41, 62, 63). Work presented in this thesis shows that Ron overexpression in prostate epithelial cells also results in an increase in macrophage infiltration into prostate tumors (Chapter 3). Taken together, inhibition of Ron specifically in macrophages for epithelial Ron overexpressing tumors might result in an influx of anti-tumor macrophages into the prostate tumor. Clinically, this could be performed by isolating a patient's own macrophages, deleting or reducing Ron expression, and infusing the Ron modulated macrophages back into the patient similar to the process used with CAR T-cell therapy.

Targeting Ron signaling therapies directly to prostate tumors may also be advantageous knowing that Ron promotes prostate tumor growth cell intrinsically (Chapter 2). Different prostate cancer specific delivery methods have been developed around The Prostate Specific Membrane Antigen (PSMA). These delivery approaches have potential because PSMA is highly expressed in most prostate cancer cells but expressed at very low levels in other cells (190). A PSMA RNA aptamer has been used as a targeting ligand capable of delivering siRNA or miRNA to prostate cancer cells (191). Utilizing this technology with Ron siRNA may prove capable of silencing Ron in the prostate tumor while limiting adverse events associated with global Ron inhibition. An additional method uses the folate hydrolysis function of PSMA for enhancing drug deliver to the prostate tumor (190). Researchers have shown that conjugating folate to liposomes increases their uptake in prostate cancer cells allowing for delivery of drugs contained in liposomes (192). Conjugating folate to liposomes containing a Ron inhibitor may limit side effects sufficiently to allow for long term inhibition of Ron signaling.

With the abundance of adipocytes in obese patients, inhibiting Ron specifically within this cell type will have some difficulties. If the specific adipokines regulated by Ron signaling which promote prostate cancer are identified, using an antibody mediated approach to reduce the serum concentration of these adipokines may be a successful alternative means to directly targeting Ron. Further research will need to be conducted in order to determine whether global, cell type specific, or a combination of cell type specific Ron focused therapies provides the best efficacy with the least amount of adverse events.

Concluding Remarks

The research portrayed within this thesis demonstrates the major impact the Ron receptor has on driving prostate cancer growth and resistance to ADT (shown in illustration 1). The currently available therapeutic options for men with prostate cancer that has become resistant to ADT provide minimal hope for a lasting recovery. Our research indicates that Ron mediated therapy has the potential to alter cell intrinsic and cell extrinsic mechanisms which drive resistance to ADT, resulting in broad consequences for the tumor. Ron directed therapies in combination with other therapies or as standalone treatments may provide hope to patients who are in desperate need.

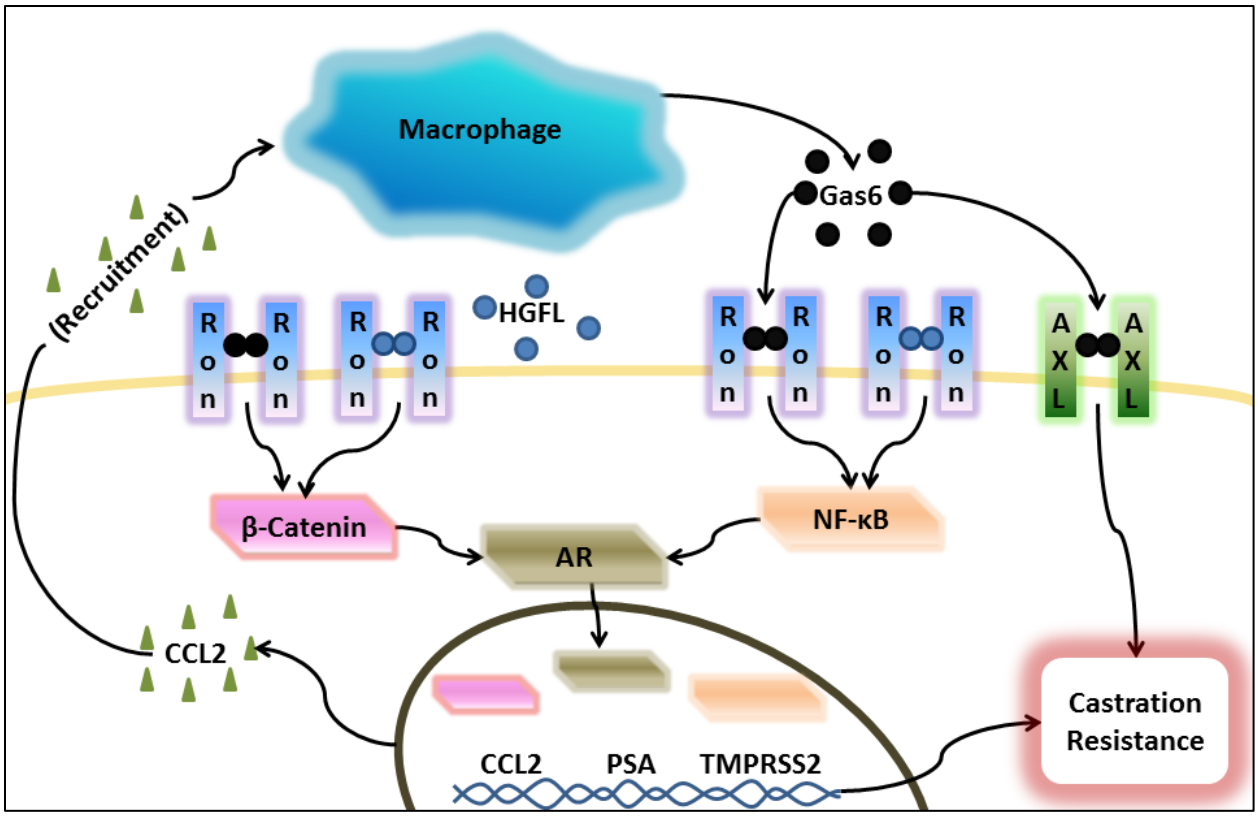

Illustration 1. Working model displaying the multiple mechanisms for Ron to drive castration resistance in prostate cancer.

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