I, Andrew M Paluch, hereby submit this original work as part of the requirements for the degree of Master of Science in Cancer and Cell Biology.

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The Ron Receptor Tyrosine Kinase as a Mediator of Inflammation and Tumorigenesis

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THE RON RECEPTOR TYROSINE KINASE AS A MEDIATOR OF INFLAMMATION AND TUMORIGENESIS

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by

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Abstract

Prostate cancer (PCa) is one of the leading causes of death among men in the United States, particularly given that many patients will present with or develop castration-resistant prostate cancer. Identifying novel biomarkers is therefore essential to develop new therapeutics for the treatment of this disease. While current therapeutics have targeted the androgen receptor, a major driver in the development and progression of PCa, this has been met with limited success. Understanding the role of other potent drivers of PCa is paramount to successful patient outcomes. One potential driver may be the Ron receptor tyrosine kinase. Chronic inflammation has been suggested to be a precursor for the development of PCa and interestingly, Ron has been shown previously to be a regulator of macrophage inflammatory responses. In addition, new studies have suggested its role in oncogenesis. While there are Ron inhibitors in clinical trials for the treatment of several malignancies, there have been no trials looking at Ron inhibition in PCa. Therefore, understanding how Ron functions to mediate inflammatory responses and drive oncogenic events are crucial to potentially utilizing Ron inhibitors for the treatment of prostate cancer.
**Prostate Cancer**

Prostate cancer is a leading cause of cancer-related death among men in the United States with more than 230,000 men expected to be diagnosed in 2014 (1). Initially dependent upon androgens, treatment for prostate cancer typically involves a combination of surgical removal of the tumor as well as an anti-androgen therapy, such as bicalutamide or flutamide, which antagonizes androgen receptor (AR) signaling. Patients presenting with early prostate cancer undergoing this therapy typically respond positively. However, many patients will regress into a hormone-refractory (or castration-resistant) state wherein the tumor is no longer dependent upon androgens for growth and the tumor becomes metastatic, leading to a high incidence of mortality. For these patients, the treatment options are limited.

Chemotherapeutics such as docetaxel are typically the first-line treatment for these patients given its effectiveness in clinical trials, though decreases in quality of life are often outweighed by the moderate increase in overall survival (2). Recently, however, a multitude of new drugs in clinical trials have showed increases in patient overall survival, though it is noted that these increases are moderate (average of 2-4 months increase over placebo). Therefore, there is a critical need to identify new biomarkers and therapeutic targets in the treatment castration-resistant prostate cancer.

**Prostate Tissue Architecture and Prostate Cancer Histopathology**

The normal prostate is comprised of both glands and surrounding stromal cells. In humans, the glandular prostate arises from endodermal origin and the stromal prostate arises from mesenchyme. The human prostate can be further classified into zones: The Peripheral Zone, which surrounds the urethra and from which a majority of prostate cancers arise, the
Central Zone which surrounds the ejaculatory ducts and accounts for a small percentage of prostate cancer and the Transitional Zone which surrounds the prostatic urethra and from which benign prostatic hyperplasia (BPH) typically arises (3). In mice, however, the prostate is comprised of three/four distinct lobes: the dorsal, lateral (often combined as dorsolateral), ventral and anterior.

Histologically, the prostate is comprised of ducts that are lined with secretory epithelial cells as well as basal cells and neuroendocrine cells. It was initially thought that the basal cells gave rise to the secretory epithelium, however, it was demonstrated that both the secretory and basal epithelial cells are independent cell lineages with self-renewal capacity (4). In mice, the lobes differ in their appearance of epithelium that lines the glands with the dorsal prostate containing simple cuboidal epithelium, the ventral prostate containing columnar epithelium exhibiting focal folding and the anterior lobe containing cuboidal/columnar epithelium with folds extending into the lumen. The vast majority of prostate cancers arise from the luminal epithelium; however, studies recently have suggested that prostate cancer can also arise from the basal cell compartment (5, 6). In contrast, neuroendocrine-arising prostate cancers in humans are considered rare but are aggressive in nature.

Clinically, prostate cancer is suspected based upon high serum prostate-specific antigen (PSA) expression (>10ng/ml), an AR-dependent gene product (7). Prostate cancer is classified based upon a histopathology grading system described by Donald Gleason (8). Briefly, prostate tumors are given a Gleason score from 2-7 wherein the less differentiated and the more aggressive the tumor, the higher the score. A score from 2-3 consists of a tumor which progresses from one with well-delineated foci and no obvious infiltration into the surrounding stroma to a tumor that has irregularly sized and amplified numbers of ducts with moderate
infiltration. A score from 4-5 delineates a tumor that progresses from one with complex, confluent, cribriform epithelium without any intervening stroma to one in which there is diffuse, confluent growing sheets of tumor cells or large areas with obvious central necrosis. A Gleason score of 7 (3+4) indicates a tumor in which the predominant pattern is a score of 3 and a secondary, or less prominent, pattern corresponds to a score 4.

**Androgen Receptor Signaling and the Transition to Castration-Resistant Prostate Cancer (CR-CaP)**

Early in their development, prostate cancers are reliant upon androgens for their growth. Therefore, currently, the mainstay therapy for patients presenting with prostate cancer is androgen deprivation, wherein AR is antagonized using a variety of methods from surgical removal of the testes to anti-androgens that compete for binding with AR. Many prostate cancers that initially respond well to this treatment eventually stop responding and become more aggressive. This type of prostate cancer is called castration-resistant prostate cancer and results in aberrant AR activation in the absence of ligand and occurs through a variety of mechanisms.

The gene for AR is located on the X chromosome, contains eight exons and encodes a 110 kDa protein. Similar to other nuclear receptors, AR structurally contains an N-terminal domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD). The DBD of AR is a cysteine-rich region that is evolutionarily conserved among steroid hormone receptors and is comprised of four cysteine residues and a coordinate zinc finger region. In its unliganded state, AR is associated with heat shock proteins in the cytoplasm. Upon ligand activation, AR disassociates with heat shock proteins and, similar to other hormone receptors, is translocated to the nucleus where it dimerizes and binds to DNA promoter regions utilizing the N-terminal zinc
finger that binds to androgen response elements (ARE) in the DNA major groove. The nuclear localization signal (NLS) is located at the junction of the DBD and the hinge region and it has been suggested that ligand binding of AR exposes the NLS and thus allows it to interact with nuclear importin α resulting in nuclear import (9). Following receptor dimerization and DNA binding, AR then associates with coordinate coactivators and general transcription machinery to drive mRNA expression of AR-dependent genes such as PSA and TMPRSS2 to induce growth, survival and metastasis. AR is exported from the nucleus upon ligand withdrawal by utilizing the nuclear export signal located in the LBD in a mechanism that is not clearly understood (10).

The mechanisms of AR deregulation following androgen withdrawal can be classified into several categories: AR gene amplification, mutation, proteolysis, activation by non-steroid growth factors, an increase in AR co-regulators or local production of androgens. Amplification of AR is frequent mechanism for inducing castration-resistant growth, wherein the very low levels of androgens that remain following androgen withdrawal are sufficient to activate AR. Consistent with this, several xenograft models have demonstrate that AR gene expression is increased following the progression from androgen-dependent growth to androgen-independent growth (11).

Several AR mutations have been described in patients that have undergone anti-androgen therapy (12-15). Of these, many are gain-of-function mutations that localize to the LBD sensitizing it to preferentially bind androgens with greater affinity. In addition, some mutations allow for the binding of other steroids and it is thought that anti-androgens provide a selective pressure to allow for this mutation to drive cellular proliferation.

Activation of AR by non-steroid growth factors is another mechanism driving CR-CaP. For instance, it has been reported that insulin-like growth factor and keratinocyte growth factor
induce AR transcriptional activity and in addition, inhibition of insulin-like growth factor activity reduces androgen-independent prostate cancer cell growth (16, 17). Moreover, Wnt/β-Catenin has also been demonstrated to transactivate AR through both physical interactions as well as transcriptional upregulation of AR itself (18-21). Overexpression of receptor tyrosine kinases such as Her2 has also been shown to stabilize AR protein expression as well as activate AR dependent gene transcription (22, 23). The overall result of aberrant AR activation is the induction of ligand-independent growth and metastasis, which accounts for the decrease in survival seen in patients presenting with CR-CaP. Therapeutic targeting of AR signaling has been a major focus in the treatment of CR-CaP, leading to the development of next-generation inhibitors such as MDV3100 (Enzalutamide), which has been approved by the FDA for the treatment of CR-CaP. There is limited data, however, with regard to the clinical efficacy of enzalutamide alone or in combination with chemotherapy or the antiandrogen Abiraterone (Zytiga) to suggest a beneficial patient outcome in targeting AR signaling alone. Therefore, there is a critical need to identify other targets that may provide clinical benefit for patients with CR-CaP and these targets may be receptor tyrosine kinases.

Receptor Tyrosine Kinases and Prostate Cancer

The first receptor tyrosine kinase (RTK) was described in 1979 with the discovery of v-Src, and since then, more than 90 receptor and non-receptor tyrosine kinases have been described in the human genome (24). RTKs are cell surface receptors for growth factors, hormones and cytokines and as such, translate external stimuli into a functional cellular response through the activation of a highly conserved catalytic kinase domain leading to the phosphorylation of downstream substrates or through the docking of adapter proteins using the structurally
conserved Src Homology 2 (SH2) domains. In this regard, RTKs can activate a variety of
downstream signal transduction pathways such as Ras/MAPK, PI3K/Akt, Stat3, etc. In prostate
cancer, tumor microarrays (TMA), phosphotyrosine proteomics, RNAi screening and genomic
and cDNA arrays have yielded extensive knowledge on the expression of RTKs (25-28). The
Epidermal Growth Factor/Erythroblastic leukemia viral oncogene homolog (EGFR/ErbB) family
of RTKs has been shown to play a role in prostate cancer development and progression. For
instance, the expression of ErbB2 and ErbB3 is limited to the basal cell layer in benign glands;
however, strong ErbB2 and ErbB3 cytoplasmic luminal expression is seen in PIN lesions
suggesting a molecular switch activating this pathway following the development of prostate
cancer (29). Moreover, higher expression of Her2/ErbB2 was noted during the transition to
castration-resistance in tumors following androgen withdrawal (30). In the absence of AR ligand,
it was found that Her2 could activate AR through the stimulation of MAPK and the involvement
of AR coactivators thus enhancing AR protein stabilization and DNA binding (22, 31). In
addition, other RTK families such as the TAM family (Tyro3, Axl and Mer), specifically Axl,
have been noted in mediating the tumorigenic properties of prostate cancer cells (32, 33).
Moreover, the c-Met family of RTKs has been implicated in prostate cancer through the
observation that Met is repressed by AR activation and ligand stimulation of Met induces
epithelial-to-mesenchymal transitions in prostate cancer cells (34, 35). As a result of the
extensive knowledge gained in understanding the human kinome and the various roles of RTKs
in mediating prostate tumorigenesis, they are attractive therapeutic targets. Indeed, inhibitors to
RTK families such as EGFR (Gefitinib, Lapatinib, Trastuzumab), platelet-derived growth
factor/vascular endothelial growth factor (PDGFR/VEGFR) (Bevacizumab, Sunitinib),
Met/PDGFR (Cabozantinib) and others are at various stages in clinical trials with some increase in overall survival in metastatic prostate cancer patients as a measure of success.

**Ron Receptor Structure and Function**

The receptor tyrosine kinase Recepteur d'Origine Nantais (Ron), also known as human Macrophage stimulating 1 Receptor (MST1R), is a cell surface receptor that belongs to the Hepatocyte growth factor (HGF)/Met receptor family of receptor tyrosine kinases. As a novel tyrosine kinase, Ron was first identified from a library of human tumors. Ron full-length cDNA was first identified in 1993 from a cDNA library of human foreskin fibroblasts (36). The murine Ron ortholog was first cloned from hematopoietic stem cells in 1994 and was therefore called stem cell derived tyrosine kinase (STK) (37). Since then, several orthologs of Ron in different species have been identified, including rat, chicken, and feline (38-40). In addition, Ron orthologs have been identified in non-mammalian species such as xenopus, puffer fish and sea urchin (41-44), suggesting its importance through evolutionary conservation.

Ron and Met are structurally homologous as both contain an extracellular ligand-binding domain, a hydrophobic single-pass membrane spanning domain and an intracellular tyrosine kinase domain (45). Ron is synthesized as a single-chain 185 kDa glycosylated precursor protein that is further proteolytically cleaved by furin-like proteases before membrane translocation (45). Once at the cell surface, Ron exists as a heterodimeric protein consisting of a 35 kDa alpha chain and a 150 kDa beta chain that are linked by disulfide bonds. Entirely extracellular, the alpha chain contains the ligand-binding domain whereas the beta chain contains the extracellular, transmembrane and intracellular region containing the tyrosine kinase domain of Ron (45). With regard to sequence homology, Ron is 34% identical to Met overall with 80% homology seen in
the kinase domain (46). At the transcript level, the human Ron receptor consists of 20 exons whereas murine Ron has 19 exons. An analysis of the mouse Ron promoter region identified putative binding regions for transcription factors such as Ets-1, NF-κB, Sp-1 and AR, among others [(47) and unpublished observations]. A truncated form of the Ron receptor wherein the C terminus is deleted (leaving only the transmembrane and intracellular regions) has been reported in mice leading to an enhanced susceptibility to Friend virus-induced erythroleukemia (48, 49). Moreover, a short form of Ron has been identified in humans as well, where it induces epithelial-to-mesenchymal transition and enhances cell motility (50). In addition, several mRNA splice variants of Ron have also been described with varying degrees of oncogenic potential (51-53).

Developmentally, expression of the Ron receptor is required for peri-implantation, as mice with total loss of the Ron receptor (Ron−/−) are viable through the blastocyst stage but fail to survive past implantation (54). In situ hybridization analysis of Ron has showed that expression seems to be a late event during embryogenesis with its earliest expression seen in liver tissue at p.c. day 12.5 (55). Following liver, Ron expression is seen in neural tissue from days 14.5-16.5. High Ron expression was also observed at day 16.5 in the glandular epithelium of the gut (colon, small intestine, stomach), indicating that Ron expression is a hallmark of the developing gut. At day 18.5, Ron transcripts are seen in in the skeleton, with highest expression noted in areas of active bone remodeling, suggesting Ron expression is limited to differentiated osteoclasts (55, 56). In adult tissue, Ron expression is further maintained in the glandular epithelium of the gastrointestinal tract, adrenal glands, kidneys, mammary gland, lung, pancreas, liver, testis, ovaries and hematopoietic cell lineages (56, 57).

**Ron Ligand Structure and Function**
The ligand for the Ron receptor was initially identified in 1976 as a human serum factor that was capable of stimulating macrophage chemotaxis and changes in both shape and phagocytic activity (58). It was purified from human serum in 1978 and named Macrophage Stimulating Protein (MSP), given its ability to stimulate macrophage migration (59). The gene for MSP was cloned in 1991 following a human genomic screen for characteristic kringle domains similar to prothrombin and those found in the blood coagulation cascade (60). This newly identified protein was predicted to be 50% similar to the ligand for Met, Hepatocyte Growth Factor (HGF) and hence, MSP was further named Hepatocyte Growth Factor-Like Protein (60, 61). Both HGF and HGFL are serine proteases involved in blood coagulation and fibrinolysis and lack protease activity (60, 62). The murine HGFL gene and cDNA were isolated from mouse liver and predicted to be 80% homologous to human HGFL (62). The primary tissue expression of HGFL was determined to be liver, with minimal expression in placenta, adrenal gland and lung. Using a cDNA library prepared from HepG2 human hepatocellular carcinoma cells, MSP cDNA was cloned and characterized showing that the gene for MSP is located on chromosome 3 (63). At the structural level, MSP was predicted to have an N-terminal hairpin loop, four kringle domains and a serine protease-like domain. Following purification and functional assays, it was determined that MSP is identical to HGFL (61).

Despite the fact that HGF and HGFL are approximately 45% homologous at the sequence level, they do not, however, cross-react with either Ron or Met, respectively, suggesting their receptor specificity (45). HGFL is secreted from the liver into circulation at a concentration of 400 ng/ml and acts in an endocrine manner at distant sites to activate the Ron receptor. In contrast, HGF, which is primarily produced by mesenchymal cells, activates Met in a paracrine fashion.
Similar to HGF, HGFL is synthesized as an 80 kDa single chain precursor that has no biological activity and is proteolytically cleaved by blood coagulation cascade proteases such as kalikrein, factor XIIa and factor XIa into a 50 kDa alpha chain and a 35 kDa beta chain that are linked by disulfide bonds (64). The alpha chain, which contains the kringle domains, is important for regulating the biological functions of the Ron receptor, such as proliferation and macrophage cell scattering (65). The beta chain, containing the serine protease-like domain, is required for receptor binding (66). Cleavage of pro-HGFL into active HGFL can also occur at the cell surface by local membrane-bound proteases produced by macrophages (64).

At the transcript level, it was found that hepatocyte nuclear factor-4 (HFN4) is primarily responsible for the liver-specific expression of HGFL, as mapping the promoter region of HGFL demonstrated the necessity and sufficiency of HNF4 to HGFL transcription and DNA binding (67). In addition, it was further shown that HGFL gene expression requires indirect coactivation of HNF4 with CREB binding protein while in contrast, retinoic acid suppresses HGFL transcription (68). Moreover, cis-acting elements within the first exon and intron regulate liver and, to a lesser extent, kidney expression of HGFL (69).

**Ron Expression in Macrophages: Inflammation and Cancer**

The role of the Ron/HGFL signaling axis was originally characterized using resident peritoneal macrophages where it was shown that stimulation with HGFL induced shape change, chemotaxis and stimulated phagocytosis (58, 70, 71). Ron expression has since been described on several different resident macrophage populations including human alveolar, peritoneal and monocyte-derived macrophages; however its expression was not noted on circulating monocytes, suggesting its upregulation is linked to terminal macrophage differentiation (37, 72). Ron
activation by HGFL on macrophages has been shown to activate a variety of downstream pathways such as Ras, Erk and PI3K/Akt, where it regulates several important macrophage activities such as phagocytosis, anti-apoptosis and pro-inflammatory cytokine production (72-74).

Inflammation plays a vital role in both normal cellular functions as well as pathogenesis. The role of macrophage-specific Ron expression in mediating inflammation was first characterized in the observation that mice deficient in Ron signaling displayed defects in inflammation (75). These mice, which lacked the tyrosine kinase domain of the Ron receptor were fertile, but displayed a hyper inflammatory macrophage response upon challenge (75, 76). In these studies, peritoneal macrophages isolated from Ron-deficient mice were shown to produce excess nitric oxide (NO) upon interferon-gamma (IFNγ) stimulation and in vivo, these mice had higher serum NO levels upon lipopolysaccharide (LPS) injection (76). Consistent with this, in another study, peritoneal macrophages were examined for NO production upon LPS stimulation and it was found that cells from Ron-deficient mice had a significant upregulation of NO, and in addition, these mice were more susceptible to sub-lethal LPS-induced endotoxic shock (75). Furthermore, macrophage Ron activation was shown to limit LPS-induced NF-κB activation diminishing cytokine/chemokine expression (77, 78). In total, these studies suggest that macrophage-specific Ron expression is a negative regulator of inflammation and thus serves to ameliorate the potential tissue damaging effects of macrophage-produced inflammatory responses.

Further studies have since characterized macrophage-specific Ron expression in other models of inflammation such as acute lung injury (ALI). ALI is a heterogeneous pathological condition in which there is a severe triggering of local and/or systemic inflammation and from
which few therapeutic options exist. In a model of ALI induced by exposure to nickel, Ron-deficient mice displayed greater lung damage and decreased survival and moreover, exhibited greater increases in cytokines such as IL-6, MCP-1, MIP-2 and NO than their wild-type counterparts (79). Gene expression profile analysis of Ron-deficient mice during nickel-induced ALI demonstrated an increase in genes responsible for pro-inflammatory signals and lymphocyte activation, potentially accounting for the increased polymorphonuclear leukocyte infiltration observed in the lungs of Ron-deficient animals following injury (80).

As described previously, Ron-deficient mice and macrophages significantly upregulate NO production in response to LPS, and in a study by Lentsch, et al., intrapulmonary administration of LPS to wild-type and Ron tyrosine kinase domain-deficient (TK−/−) mice showed that TK−/− mice had significantly greater lung damage that correlated with increased NF-κB activation and subsequent increased NO and TNF-α production (81). Consistent with this, decreases in NF-κB transcriptional activity, TNF-α production and expression of Adam17, the metalloproteinase responsible for TNF-α release, were described in alveolar macrophages stimulated with HGFL (82). In a follow up study, crossing Ron tyrosine kinase floxed mice to mice expressing LysozymeM Cre recombinase generated mice in which Ron had been deleted from the myeloid lineage, it was shown that myeloid Ron expression is necessary to suppress NF-κB activation and subsequent TNF-α production, and protect against enhanced lung injury upon LPS administration (83).

Ron signaling has also been described in a model acute liver failure wherein LPS is given to mice sensitized with D-galactosamine (GalN). Paradoxically, it was shown that, unlike the ALI model, the Ron-deficient animals are protected from liver failure compared to wild-type mice. The Ron knockout mice displayed marked decreases in hepatocyte apoptosis while
cytokines such as TNF-α were significantly elevated and IL-10 and IFNγ were reduced (84). The authors speculated that in this model, the high levels of TNF-α produced by Ron-deficient mice are, in fact, protective. To understand the cell type-specific role of the Ron receptor in mediating this phenotype, Stuart, et al. observed that hepatocyte-specific deletion of Ron protects against the effects of LPS/GalN through anti-apoptosis, whereas myeloid-specific deletion of Ron is detrimental to hepatocyte survival via a mechanism involving NF-κB-mediated TNF-α production (85).

As a mediator of inflammation in response to LPS challenge, Ron expression has also been linked to decreased susceptibility to infection with Listeria monocytogenes. In these studies, Ron-deficient peritoneal macrophages exhibited a significant increase in NO production in response to heat killed Listeria monocytogenes, while in vivo, overall survival was decreased in Ron-deficient animals compared to wild-type in response to infection (86). Consistent with this, a role for Ron in innate immunity was described by Lutz, et al. In this study, Ron-deficient peritoneal macrophages displayed a defect in complement-mediated phagocytosis of erythrocytes and in addition, it was found that HGFL/Ron-mediated phagocytosis requires PI3K and protein kinase C zeta downstream of Ron’s kinase activity (74). This data therefore suggests a role for the HGFL/Ron signaling axis in resolving bacterial infection through enhanced clearance and limiting inflammatory responses.

Ron expression has also been linked to macrophage polarization. Macrophage polarization is a term used to describe the perturbation of macrophages in response to environmental cues such as cytokines, microbial products, infection or other signal that can be recognized by a macrophage (87). In this regard, macrophages can be polarized either toward an M1 or M2 state, a definition that was proposed by Mills in 2000, wherein the dichotomy between
M1/M2 is dependent upon a macrophage’s role in either promoting inflammation or healing (88). Ron’s role in this dichotomy is such that Ron promotes an M2, or anti-inflammatory state, in macrophages, characterized by increases in the expression of Arginase-1, Macrophage Scavenger Receptor-A and IL-1 receptor antagonist (89, 90). In addition to promoting M2 markers, Ron expression also suppresses M1, or pro-inflammatory, markers such as iNOS, TNF-α, COX-2 and IL-12p40 in primary macrophages stimulated with IFN-γ and LPS (89-91).

The role of inflammation in mediating oncogenesis is becoming widely accepted. For instance, chronic inflammation has been shown to be a precursor to the development of prostate cancer (92). Areas of local inflammatory cell aggregation, known as proliferative inflammatory infiltrate (PIA), were observed in prostate regions that developed into prostate adenocarcinoma (93). Recent studies have shown that macrophage-specific Ron expression is intimately involved in modulating inflammation that promotes tumorigenesis. In one study, tumor growth was abrogated in Ron signaling-deficient mice, presumably due to tumor-associated macrophage (TAM)-specific Arg-1 expression loss (94). Gurusamy, et al further showed that total body loss of the Ron tyrosine kinase domain inhibits prostate tumor growth, marked by increases in TAM recruitment and areas of necrosis and changes in microenvironment inflammation. Furthermore, they showed that myeloid-specific Ron loss is sufficient to inhibit prostate tumor growth, as loss of Ron in this cell type induced significant tumor cell apoptosis as well as increasing iNOS expression and markedly diminishing Arg-1 expression in TAMs (95). In total, this data demonstrates the unique anti-inflammatory and pro-tumorigenic properties of Ron signaling in macrophages.

**Epithelial Ron Expression in Normal Physiology and Cancer**
Activation of the Ron receptor involves binding of its ligand hepatocyte growth factor-like protein/macrophage stimulating protein (HGFL/MSP) to the extracellular ligand-binding domain of Ron, resulting in receptor dimerization and trans-autophosphorylation of tyrosine residues 1238, 1239 within the tyrosine kinase domain that leads to phosphorylation of key tyrosine residues in the C-terminus (Y1353,Y1360) (45). Once activated, Ron serves as a multifunctional docking site for downstream kinases such as Src, MAPK, PI3K, FAK and JNK, through the interaction of SH2 domains (96, 97). Mutational analysis has shown that Y1353,Y1360 are required for Ron signaling, as both Y1353F and Y1360F failed to engage SH2 domain-containing signaling proteins leading to a loss of downstream kinase activity and transforming ability (97). Further studies have suggested an autoinhibitory role for the C-terminus of Ron, as work by Yokoyama, et al. demonstrated that by introducing a wild-type dityrosine C-terminus into cells, Ron kinase activity was blunted and moreover, the Y1353F, Y1360F C-terminus introduced into cells had a greater inhibitory affect (98). Recent studies have also suggested an autoinhibitory role for the transmembrane domain, as it was proposed that Y1198 phosphorylation in the kinase domain is a key event that leads to Ron activation, presumably by relieving the inhibition imposed by the transmembrane domain (99).

The Ron receptor can also be activated through heterodimerization with other receptors. For instance, EGFR has been shown to cross talk with the Ron receptor, wherein stimulation of either receptor with its ligand induces the phosphorylation of both receptors and PI3K downstream (100). In further studies, it was found that Ron can also interact with c-Met, the insulin-like growth factor 1 receptor (IGF1R), integrins and B-type plexins (101-103). Ron’s interactions with these receptors occur through transactivation within the kinase domain or binding of the SH2 domain docking sites, and in both ligand-dependent and ligand-independent
manners. In this regard, activation of the Ron receptor can provide a wider cellular response that is not limited to ligand-dependent signaling alone.

As mentioned previously, in situ tissue surveys during development and in adult tissue found Ron to be expressed on several epithelial cell populations, however its function in normal physiology and disease pathology had only been speculated. Earliest studies of HGFL/MSP signaling focused primarily on resident peritoneal macrophages, but following the identification of Ron in keratinocytes as the cognate receptor for MSP (104), the focus shifted to identifying what role Ron played in epithelial cells. One of the possible normal physiological functions of epithelial Ron may be to coordinate wound healing. Wound healing, specifically re-epithelialization, is a complex process involving the migration of keratinocytes that is coordinated by growth factors, cytokines, etc. (105) Stimulation of murine keratinocytes with MSP induced significant proliferation as well as migration and chemotaxis (106). In a follow-up study, it was determined that PI3K was bound to the Ron receptor following ligand stimulation, and that MSP treatment induced the migration of three separate epithelial cell lines that was abolished upon PI3K inhibition with wortmanin (107). Work by Medico, et al. further implicated the Ron signaling pathway in proliferation and cell migration as they described that stimulation with MSP elicited a unique response known as “cell scattering”, or the motile dispersion of colonies of epithelial cells involving actin cytoskeletal reorganization and membrane ruffling (108, 109). Furthermore, in a dose-responsive manner, activation of the Ron receptor by ligand not only induced proliferation and cell migration, but also invasion through matrix, further implying a role for Ron signaling in wound healing. To further substantiate a role in wound healing, it was found that MSP stimulation of the Ron receptor enhances integrin β1-mediated cell adhesion that is dependent upon PI3K activity and furthermore, that even in the absence of
ligand, Ron and β1 integrin form a protein complex (110). In total, this work demonstrated that, in addition to its macrophage functions, Ron signaling is also a motility factor on epithelial cells.

In addition to acting as an enhancer of cell motility, epithelial MSP/Ron signaling is also a potent inducer of cell growth and survival, particularly under anchorage-independent conditions. When extra cellular matrix (ECM)-Integrin interactions are inhibited, cells are no longer attached and often undergo apoptosis, termed anoikis. The ability of cells to survive this anoikis is an integral process and has been described during embryogenesis, in normal skin, mammary gland, and digestive tract (111-114). However, the ability of neoplastic epithelial cells to survive anchorage independence is a consequence of their resistance to anoikis and was one of the earliest recognized hallmarks of metastatic potential (115, 116). MSP/Ron signaling was shown to contribute to epithelial cell anoikis resistance in a study by Danilkovitch, et al., in which they demonstrated that both MAPK and PI3K/Akt are stimulated by MSP independently, and inhibition of either pathway is compensated for by activation of the other, leading to survival under apoptosis-inducing conditions (117).

The ability of epithelial-specific MSP/Ron signaling to promote cell motility, invasion, proliferation and apoptosis resistance is therefore crucial to understanding this pathway’s role in oncogenesis. Ron has been shown to be overexpressed in a variety of human cancers (118, 119). One of the first links of Ron to cancer was in the description of high MSP and Ron expression during a chromosomal screening of injured lungs, and the speculation that this pathway might influence tumor cell phenotype (120). In a follow-up study, both MSP and Ron mRNA expression were identified in several non-small cell lung cancer (NSCLC) cell lines where it was shown that Ron receptor activation induced cell motility leading to the hypothesis of an autocrine/paracrine loop (121). Furthermore, using transgenic animals, in which Ron is
specifically overexpressed in the lung epithelium, Ron was found to drive tumorigenesis in mice as young as 2 months of age that developed into adenocarcinomas (122).

In breast cancer, mouse mammary epithelium-specific overexpression of the Ron receptor resulted in 100% penetrance of cellular transformation and importantly, Ron overexpression induced a far greater percentage of metastasis and was associated with increased \(\beta\)-Catenin activity (123). In another study, Ron overexpression was also found to correlate with increased metastasis and decreased survival in humans (124). Work by Wagh, et al. further characterized the role of Ron’s tyrosine phosphorylation of \(\beta\)-Catenin as being necessary to mediate the growth and metastasis of breast cancer cells both \textit{in vitro} and \textit{in vivo} (125, 126).

Ron is overexpressed in 90% of all prostate cancers and its expression has been shown further to correlate with disease progression with little to no staining observed in benign hyperplasia and higher staining observed in prostate adenocarcinomas and metastatic tissue (118, 127). The Ron receptor is also highly expressed in androgen-independent epithelial prostate cancer cell lines such as DU-145 and PC-3, whereas its expression is very low in androgen-dependent cell lines such as LNCaP and CWR22Rv1 (127). High Ron expression in these cell lines has been demonstrated to regulate angiogenic chemokine secretion through NF-\(\kappa\)B pathway activation. Furthermore, mouse models have demonstrated a requirement for Ron expression in prostate tumorigenesis (95, 128). In these studies, mice with total body loss of the Ron receptor were crossed to Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP or PbTA\textsubscript{g} (129)) resulting in an abrogation of prostate tumor size, with marked decreases in metastasis and angiogenesis noted. Mechanistically, total body loss of the Ron receptor also reduced NF-\(\kappa\)B activity in the tumor and prostate epithelial cells isolated from Ron-deficient prostate tumors. In
addition, shRNA knockdown of the Ron receptor in PC-3 cells was found to be sufficient to inhibit growth and metastasis in vivo [(127) and unpublished observations].

In addition to the tumor proper, a requirement for the Ron receptor in the prostate tumor microenvironment was also identified (95). In this study, Gurusamy, et al. show that total body loss of the Ron receptor inhibits the growth of prostate tumors in a syngeneic orthotopic model of prostate cancer using mouse TRAMP-C2Re3 cells, which were isolated from TRAMP mice, injected into the prostates of mice. They further showed that tumors in Ron-deficient hosts displayed enhanced cell apoptosis, changes in stromal inflammation and increases in macrophage recruitment. Moreover, this study also showed a requirement for myeloid-specific Ron expression for tumor growth, as tumors in myeloid-deficient hosts had increased apoptosis, decreased macrophage Stat3 activation, Arginase-1 expression and inversely, increased inducible nitric oxide synthase (iNOS) expression. In a novel mechanism, they also showed that CD8+ cytotoxic T-cell depletion restored tumor growth in both total body and myeloid-deficient Ron mice, suggesting that Ron expression inhibits CD8+ cytotoxic T-cell recruitment and activity. These studies were the first to not only identify that Ron is upregulated in prostate cancer and its expression is critical to the development and progression of tumors, but that there are potential cell type-specific functions for Ron in mediating the growth and metastasis of prostate cancer. Further work is therefore required to identify the cell type-specific functions of Ron in a single model. Moreover, the observation of androgen-independent prostate cancer cell-limited Ron expression suggests that Ron may play a pivotal role in the development and/or growth of CR-CaP and therefore warrants further study. In total, the aforementioned data demonstrates many of the functions of the Ron receptor as a mediator of both normal physiological functions such as
wound healing and how aberrant expression and activation can lead to the development of several cancers.

**Conclusions**

In summary, there is abundant and growing evidence to suggest a vital role for the Ron receptor in human cancer. The data summarized in the previous sections outline the cell-type specific functions for Ron receptor signaling in both the myeloid and epithelial cell compartments and identifies its critical role as a mediator of inflammation and oncogenesis. What is lacking in these studies, however, is a comprehensive analysis of both cell intrinsic and cell extrinsic functions for Ron in each cell type, as it pertains to prostate cancer development and progression in a single model. In addition, the finding of androgen-independent PCa cell-specific Ron expression warrants further study into how and why Ron is preferentially expressed in these cells and what the functional consequences may be with regard to CR-CaP development and growth.
REFERENCES


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