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It is entitled:
Ron Receptor Activation in Breast Cancer

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Ron receptor activation in breast cancer

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ABSTRACT

The Ron receptor tyrosine kinase is a member of Met family of receptor tyrosine kinases. The ligand for Ron is hepatocyte growth factor like-protein (HGFL). Previous studies from our laboratory have shown that Ron overexpression in the mammary epithelium of mice (referred to as the MMTV-Ron) leads to mammary tumors with 100% incidence and were associated with a high degree of metastasis to the lung and liver. Mammary tumors from MMTV-Ron mice exhibit elevated levels of β-catenin and its target genes. However, the requirement of β-catenin and HGFL in tumor formation and metastatic dissemination has not been directly examined. We show that Ron and β-catenin are coordinately elevated in human breast cancers. We also show that Ron activation induces the tyrosine phosphorylation of β-catenin. HGFL, induced Ron activation leads to β-catenin nuclear localization and transcriptional activity, with tyrosine residues 654 and 670 of β-catenin being critical for these processes. We also found that HGFL-dependent Ron activation mediates upregulation of the β-catenin target genes cyclin D1 and c-myc. Finally, we show that genetic ablation of β-catenin in Ron-expressing breast cancer cells decreases cellular proliferation in vitro, as well as mammary tumor growth and metastasis following orthotopic transplantation into the mammary fat pad. To determine the significance of HGFL in these processes, we examined mammary tumor growth and metastasis in MMTV-Ron expressing mice with or without a targeted deletion of HGFL. Our studies show that HGFL loss significantly delayed mammary tumor initiation in this model. These changes were associated with a decrease in Ron receptor kinase activity based on kinase assays, and a reduction in mammary tumor proliferation. In addition we show for the first time that HGFL is produced in the tumor microenvironment of MMTV-Ron mice. Also blockade of HGFL, using an HGFL neutralizing antibody decreased mammary tumor growth that was associated with decreased levels in the Ron downstream signaling targets cyclin D1 and MAPK activation. Taken together, our data demonstrates that HGFL is required for Ron mediated tumor initiation, growth and metastasis and suggests that the HGFL/Ron/β-catenin pathway is a potential therapeutic target for breast cancer.
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Chapter I Introduction
Targeted overexpression of the Ron Receptor in mouse mammary gland induces mammary tumor formation with upregulation of beta-catenin in tumor tissue as compared to non-transgenic control. However the precise role of beta-catenin downstream of Ron receptor remains unknown. Here we investigated the mechanism of beta-catenin regulation and activity in human as well as mouse breast cancers that have Ron overexpression. We show that beta-catenin expression is elevated in patients that have high Ron expression. Treatment with Ron ligand, hepatocyte growth factor like protein (HGFL) led to beta-catenin tyrosine phosphorylation at residue 654 and 670. Ligand treatment induced nuclear translocation, increased beta-catenin reporter activity and expression of beta-catenin target genes. Finally deletion of beta-catenin in cells that have Ron overexpression dramatically reduced mammary tumor growth in vivo. Deletion of HGFL in mice that have Ron overexpression also dramatically increased tumor latency. This delay in tumor formation was associated with decreased mammary hyperplasia and decreased Ron kinase activity. HGFL null mammary tumors exhibited decreased proliferation and increased cell death. Most importantly, HGFL was detected in mammary gland that was produced mainly by macrophages. In tumor tissue, HGFL was expressed by both epithelial cells as well as macrophages. We show that both epithelial as well as macrophage derived HGFL is important for growth of Ron driven mammary tumors.
Breast Cancer

Breast cancer is the second leading cause of cancer death among women. Around 230, 480 new cases of invasive breast cancer and 39, 520 deaths are expected to occur in 2011 in women of US (1). In the last decade breast cancer deaths have decreased by 2.2% largely because of early detection and improved treatment options. The current therapy that has benefited a subset of breast cancer patients is use of Estrogen Receptor (ER), Progesterone receptor (PR) and epidermal growth factor receptor-2 (HER-2) antagonist. However there is still a need for cancer biomarkers and improved chemotherapeutics for targeting disease growth and metastasis as triple negative breast cancers account for more than 20% of breast cancers. The grading system that affects breast cancer prognosis and treatment options includes histopathology, grade, stage and receptor status. The current regimen for breast cancer treatment includes surgery, hormonal therapy, chemotherapy, use of monoclonal antibodies (targeted therapy) and radiation therapy. The six hallmarks of cancer cells are evasion of apoptosis, self-sufficiency, insensitivity to anti-growth signals, invasion/metastasis, angiogenesis and limitless replication potential (2).

Breast Tissue Architecture and Tumor Histopathology

Mammary gland development occurs primarily postnatally. Mammary gland remodeling occurs during the lifetime of the animal with continued cycles of growth, differentiation and apoptosis. Both hormonal and growth factor signaling can regulate the structure and functional changes that are observed in the breast tissue during development, pregnancy, lactation and involution. The two major compartments in the breast tissue are the epithelia and the stroma. The breast stroma is comprised of fibroblast, inflammatory cells, muscle cells and adipocytes comprise the stroma. Epithelial-stromal crosstalk tightly regulates normal mammary gland development.

Breast tissue has a bi-layered epithelium comprised of luminal and myoepithelial cells. Premalignant lesion forms when genetic or epigenetic changes induce formation of an abnormal cell mass in the within the duct or the lobule. Atypical ductal hyperplasia (ADH) can progress to form ductal carcinoma in-situ.
(DCIS). With a risk of developing malignant disease, the DCIS can progress to form invasive breast cancer (IBC) (3) (Figure 1).

Histopathologically, breast cancer is graded into two major categories. First is the ductal carcinoma that originates within cells of mammary ducts and accounts for 85% of the breast cancers. The second is the lobular carcinoma that begins in cells that line the lobule and accounts for 15% of breast cancer. Breast cancers can then be graded as Invasive or Non-invasive. Ductal Carcinoma In-Situ and Lobular Carcinoma In-situ are two types of Non-invasive breast cancers. The more common aggressive forms of breast cancers are Invasive Ductal Carcinoma (IDC) and Invasive lobular Carcinoma (ILC). IDC occurs in about 55% of breast cancers after diagnosis and ILC accounts for 10% of breast cancers. The other types of breast cancers that are permissive are tubular, medullary, mucinous, metaplastic, cribiform and papillary carcinoma (American Cancer Society, 2011).

Based on various histopathological parameters and microarray classification, breast cancer stem cells and normal stem cells of breast could be distinguished by properties such as proliferation, survival, differentiation and migration. Such cells more appropriately called cancer stem cells or tumor initiating cells drive breast cancer through epithelial-stromal interactions (3).

**Receptor Tyrosine Kinase and Breast Cancer**

Receptor tyrosine kinases (RTK) play important role in signal transduction, regulation of cell growth, differentiation, survival, metabolism, migration and cell-cycle control. Cell surface RTKs have similar molecular structure with extracellular ligand binding domain, single transmembrane helix, and cytoplasmic region that contains the tyrosine kinase domain and juxtamembrane regulatory region (4). In addition to their role in regulating normal cellular functions, many RTK’s are overexpressed in cancer cells and are involved in tumor progression and metastasis. (5). Some of the RTKs that are deregulated in breast cancer are HER-2, HER-1 (EGFR)R and c-MET. (6-7). Receptor tyrosine kinases are attractive therapeutic candidates because inhibiting these proteins has led to efficient clinical treatment. For
example, treatment of about 25% IDC patients that have overexpression of HER-2 oncogene with trastuzumab has provided benefit in patients with metastatic breast cancer (7-8).

The hallmark of self-sufficiency displayed by cancer cells is largely reflected by growth factor receptor signaling. Growth factor receptors overexpressed in cancer cells respond less likely to GF cues from surrounding normal tissue and rely heavily on GF’s that are self-produced thus creating an autocrine signaling loop, promoting cell survival. Some of the signaling pathways that are commonly activated downstream of RTKs are RAS, MAPK, PI3K/Akt, STAT and beta-catenin (9).

**Ron Receptor and Breast Cancer**

Ron (MST-1R) is a receptor tyrosine kinase overexpressed in 50% of human breast cancers (10). Ron is expressed at very low in normal mammary gland and in benign lesions but is significantly higher in primary breast carcinomas, ductal carcinoma in-situ and infiltrating ductal carcinoma (10-11). Ron receptor is highly expressed in epithelial breast cancer cells including T47D and ZR 75-1 cells (12). HGFL is able to induce Ron activation in T47D cells, and stimulation of the Ron receptor in ZR 75.1 cells causes increased cell proliferation, invasion and about a 12-fold increase in migration in response to Ron ligand (10, 12). Interestingly, a feline form of Ron was found to be overexpressed in about 33% of archival feline mammary carcinoma samples tested (13). Mouse models have also demonstrated an important role for Ron in mammary tumorigenesis (14-15).

**Ron structure and function**

Cell surface growth factor receptors play a vital role in translating signals from the extracellular environment into an intracellular biologic response. One such receptor is the Ron receptor tyrosine kinase. Ron, also referred to as macrophage stimulating 1-receptor (MST1R), is a receptor tyrosine kinase (RTK) of the hepatocyte growth factor (HGF)/Met receptor family. Ron was first identified as a novel protein tyrosine kinase by screening a library prepared from a mixture of human tumors. The full-length Ron cDNA was then identified using a human foreskin keratinocyte library (16). The Ron ortholog in the
mouse was first cloned from hemapoietic stems cells and is also referred to as stem cell derived tyrosine kinase (STK) (17). Met and Ron are the only two members of this RTK family, in contrast to other receptor tyrosine kinase families with multiple members (18). Ron was identified by its homology to Met and also by its homology to the Sea receptor found in chicken. The c-Sea receptor is the cellular homolog of the avian oncoprotein v-sea, and is structurally similar to Ron and Met (19-20). Sea is activated by chicken macrophage stimulating protein (21). Homologs of Ron and its ligand have been identified by sequence analysis in the total genome sequence information that is becoming increasingly available for many mammalian species, including Rattus norvegicus (rat), Canis lupus (dog), Bos taurus (cow), Equus caballus (horse) and Macaca mulatta (rhesus monkey) (22). Homologs of Ron have also been found in non-mammalian species, including Fugu rubripes (puffer fish) and Strongylocentrotus purpuratus (sea urchin) (23-24).

The Ron and Met receptors are structurally very similar. Both Ron and Met receptors contain an extracellular ligand binding domain, a single pass hydrophobic membrane spanning domain, and an intracellular region containing a tyrosine kinase domain. Ron is synthesized as a 185 kDa pre-cursor glycosylated protein and is further processed by furin-like proteases before delivering the mature receptor to the cell surface (12). On the cell surface, Ron exists as a heterodimeric receptor, consisting of a 35 kDa alpha chain and 150 kDa beta chain. The alpha chain is entirely extracellular whereas the beta chain consists of extracellular, transmembrane and intracellular region of the receptor (12). The 50-amino acid tyrosine kinase domain of Ron shares 80% identity to the Met tyrosine kinase domain and overall the receptors exhibit 34% identity (BLAST, 2007) (Figure 2). Human Ron cDNA and murine Ron cDNA share about 74% identity overall, with about 88% identity in the intracellular domains (17). The human Ron transcript consists of 20 exons while murine Ron codes for 19 exons. Altered splicing of the murine Ron gene creates a deletion of a small juxtamembrane region that is present in the human Ron gene (25). An analysis of the mouse Ron gene promoter region showed the presence of a number of putative
transcription factor binding sites important in tumor progression, including NF-kB, Ets-1, and estrogen receptor (26).

**Ron ligand structure and function**

The ligand for Ron is hepatocyte growth factor-like (HGFL) protein and is also known as macrophage stimulating protein (MSP). HGFL was originally cloned from a human genomic library by screening for the characteristic kringle domains present in prothrombin and several other proteins in the blood coagulation system (27). The protein sequence of the gene that was isolated was predicted to contain four kringle domains followed by a serine protease-like domain. On the basis of domain structure, this protein was predicted to be similar to hepatocyte growth factor. By sequence comparison, however, HGF and HGFL are only about 45% identical (BLAST, 2007) (Figure 2). This newly identified protein was localized to human chromosome 3p21, a region that often displays loss of heterozygosity in cancerous tissue. The mouse gene and cDNA for HGFL were then isolated from mouse liver (28). The mouse homolog of HGFL was predicted to display the same domain structure as human HGFL and to be about 80% identical. The expression of HGFL was determined in pregnant rat. By Northern analysis, the primary site of expression for HGFL was the liver, with low levels detected in lung, adrenal gland, and placenta. Another group similarly cloned a cDNA for macrophage stimulating protein (MSP) from a library prepared from HepG2, a human hepatocarcinoma cell line (29). The probe for this clone was derived from the peptide sequence of MSP that had previously been isolated from human serum. The predicted amino acid sequence of MSP also included four kringle domains and was subsequently found to be most similar to hepatocyte growth factor, the ligand for the Met receptor, and HGFL and MSP were determined to be identical (30). Two independent groups later determined that HGFL is the ligand for the Ron receptor and that, in spite of sequence similarities, no cross activation is seen between HGFL and Met, or HGF and Ron (12, 31).
In spite of the structural similarity of HGF and HGFL, their production is different. HGF is generally produced by mesenchymal cells and primarily activates the Met receptor in a paracrine fashion. HGFL is primarily produced by hepatocytes and is secreted from the liver into the blood at a concentration of about 400ng/ml, and works in an endocrine fashion at distant sites to activate Ron (Figure 2). These differences in ligand activation may reflect the localization of Met and Ron in normal tissue. In an analysis of normal bronchiolar ciliated epithelium of the lung, the Met receptor was localized to the basolateral cell membrane, while the Ron receptor was localized on the apical cell membrane (32). Disregulation of the spatial localization of Ron and HGFL, as well as disregulation in the quantity of the receptor and ligand, may be important in tumor tissue growth.

Promoter analyses suggested that the transcription factor hepatocyte nuclear factor-4 is important for the liver-specific expression of HGFL (33). Specific elements in the first intron of HGFL were also found to regulate liver- and kidney-specific expression (34). In addition, mutant p53 has been shown to associate with the HGFL promoter and repress its transcriptional activity, leading to a decrease in HGFL mRNA and secreted protein and increased cell survival after exposure to a chemotherapeutic agent (35). However, these experiments were performed in only one cell type (HT1299), derived from a large-cell lung carcinoma, and it remains to be determined if this effect is cell type-specific.

Like HGF, HGFL is secreted as a single chain inactive precursor molecule of 80 kDa. The pro-HGFL molecule exhibits no biological activity, nor does it bind the receptor. Proteolytic cleavage results in the formation of a disulfide linked heterodimer of HGFL composed of an alpha (50kDa) and a beta (35 kDa) chain. The alpha chain of HGFL contains four kringle domains while the beta chain contains a serine protease like domain. The two protein chains have distinct functions. The alpha chain is important for regulating in the functional activities of Ron whereas the beta chain is important for binding of HGFL to its receptor (36-37). Proteases of the coagulation cascade, such as kallikrein, factor XIIa and factor Xla, are capable of cleaving pro-HGFL into HGFL (38). Membrane bound proteases produced by macrophages were shown to have specific and non-specific pro-HGFL proteolytic activity, such that both
activation and degradation of pro-HGFL occurred at the cell surface (39). The inhibitor of the HGFL degrading enzyme was identified as alpha 1-antichymotrypsin (40). Suggestively, increased expression of alpha 1-antichymotrypsin in human breast tumors, that might allow increased activation of HGFL, was associated with significantly poorer prognosis of patients with grade 2 and 3 tumors (41). Estradiol treatment of breast cancer cells has also been shown to increase the production of alpha 1-antichymotrypsin (42). Recently, the specific membrane-bound protease that is responsible for the activation of pro-HGFL at the cell surface has been identified by transcriptional profiling of normal tissues, cancer cell lines, and multiple types of cancer tissues, and validated by biochemical and functional testing. This enzyme is known as membrane type serine protease 1 (MT-SP1) or matriptase (43). This multifunctional approach was developed to determine novel substrates of this enzyme that had been identified as an important determinant in cancer. An examination of 330 node-negative breast cancer specimens showed an association between expression of matriptase and poor patient outcome (44). Matriptase is highly expressed in many breast, ovarian, prostate, and colon cancer cell lines (45). An analysis of microarray gene expression data from 162 primary tumors was also analyzed for expression of Ron, HGFL, and matriptase. Overexpression of all three was associated with significantly shorter relapse-free survival compared to other patients. The overexpression of all three genes also significantly improved the accuracy of prediction of a 70-gene signature predicting poor outcome (46).

Overexpression of HGFL has recently been shown to promote breast tumor growth and promote metastasis to multiple sites in a model of oncogene-induced mouse mammary tumors (46). In this model system, orthotopically transplanted cells expressed the polyoma middle T antigen under the control of the mouse mammary tumor virus promoter, with or without the addition of HGFL overexpression. The additional HGFL expression significantly increased the initial growth rate of the mammary tumors, but the most striking effect of ligand overexpression was the increased range of metastasis. Cells overexpressing HGFL metastasized not only to the lung, but also to lymph nodes, spleen, liver, and bone.
Ron chromosomal location and cancer

Interestingly, each receptor-ligand pair consisting of Met and its ligand HGF, and Ron and its ligand HGFL, is located close together on the same chromosomes. Met is located on 7q31.2, and HGF is located on 7q21.11. Ron and HGFL are both located on 3:p21.31 (47). The murine Ron gene and the HGFL murine counterpart are also both located on chromosome 9qF2 (48). The human chromosome 3p.21 region has been frequently observed to undergo loss of heterozygosity in cancer specimens and cell lines. This region may harbor tumor suppressor genes. Using the sensitive detection method of quantitative real-time PCR to examine cervical carcinoma, it was recently shown that aberrations in the 3p21 region are complex and may involve gene amplification as well as deletion (49). Aberrations in the 3p21 chromosome region were also examined in lung cancer cell lines, and renal cell and breast carcinoma biopsy material. It was discovered that amplification of 3p was common event in these cancers, occurring in 15-42.5% of the samples examined (50). This amplification of the chromosome region containing Ron and HGFL is consistent with the overexpression of Ron seen in many human tumor types, although direct evidence for the amplification of Ron has not been produced at this time.

Ron in macrophages, inflammation and cancer

The determination of the expression of Ron in normal tissues and cells has helped to define its normal roles and the signaling pathways that are activated during transformation from normal cell to tumor cell. The initial characterization of the effect of HGFL was on mouse resident peritoneal macrophages. Stimulation by this ligand caused shape change, response to chemoattractant, and stimulated phagocytosis (51). Through absorption studies, it was determined that HGFL was binding to a receptor and activating mature resident macrophages (52). Through functional assays, it was found that Ron was expressed on human alveolar, peritoneal macrophages, and monocyte-derived macrophages, but not on circulating human monocytes (53). Ron, through HGFL stimulation, was shown to play an inhibitory role in regulating nitric oxide production by macrophage (54). In macrophages, the various
activities including superoxide anion production, apoptotic resistance, and phagocytosis were induced through interactions with diverse signal molecules, including Src, Erk, p38 and PI3-K (53, 55-56).

The link between Ron, inflammation, and cancer has had little attention. However, it is becoming increasingly evident that chronic inflammatory processes contribute to the development of cancer (57-58). Mice with a defect in Ron signaling had altered inflammatory responses (59). Ron has been shown to be a negative regulator of nitric oxide in epithelial cells as well as macrophages (60). Nitric oxide was shown to suppress tumorigenesis in vivo in one report, but both pro-and anti-neoplastic effects have been noted (61). The effect of overexpression of the Ron receptor on inflammation and modulation of immune responses has not been determined at this time.

Recently it was reported that Ron activation on tumor associated macrophages induces arginase I (Arg1) expression through c-Fos binding on AP-1 site in Arg-1 promoter. High levels of Arg I skews macrophages towards a M2 phenotype (pro-tumorigenic). Further Ron expression is observed in Tie-2 expressing M2 activated macrophages in tumor microenvironment that promotes tumor growth (62).

Developmental roles of Ron and tumor properties

The expression of Ron in normal development also may indicate some future role in tumorigenesis. The expression of Ron mRNA was determined in normal mouse tissues at different stages of development (12, 63-64). Expression of Ron was found in the liver as early as day 12.5, but expression in other tissues appeared at later stages of development, from day 13.5-16.5, and was present in the adult. There have been some contradictory reports concerning the expression of Ron in different tissues and in cell lines. One reason for this discrepancy may be the very low level of Ron that is present in normal tissue. An estimation of the number of Ron receptors per cell was calculated by determining the saturation kinetics of binding of HGFL to BK-1 cells, a normal keratinocyte cell line. Keratinocytes had been shown to express Ron and were responsive to HGFL stimulation in functional assays. The estimated receptor number per cell using this method was about 600-1200 (65). Several other keratinocyte cell lines showed
equivalent binding levels. In contrast, a receptor binding study estimated that the number of epidermal growth factor receptors in NIH3T3 fibroblasts were about 70,000 per cell (66).

Ron expression was seen in the glandular epithelium of the gastrointestinal tract, including stomach and colon; adrenal glands; testis; kidney; the central and peripheral nervous system; and ossification centers of developing bone. Ron is expressed in ovaries and in mammary tissue (60, 67). Ron protein is also expressed in tumor cells from the breast, colon, pancreas, liver, gastric system, kidney and lung, and haematopoietic cells (12). Ron appears to be expressed in nearly every tissue tested, at low levels, and good agreement from several studies finds that Ron is expressed in most epithelial tissues.

Although the role that Ron plays in tumor formation and growth are still under investigation, some of the functions of Ron in normal development suggest mechanisms by which Ron may influence cancer progression. Ron is expressed in reproductive, hormone-dependent mouse tissues including uterus, placenta, testis, and epididymis, and HGFL transcripts are present in the cervix, placenta, epididymis, and testis. Ron has been shown to be expressed during the process of mouse embryo implantation and placentation. In vivo, Ron was expressed in the invading ectoplacental cone and trophoblast giant cell regions surrounding the implanting embryo. Using several murine trophoblast cell lines, HGFL stimulation was shown to increase invasion through a basement membrane component material (Matrigel) and to enhance cell survival (68). In liver progenitor cells, the Ron receptor induces additional cell responses after ligand stimulation including cell scattering (motility), and DNA synthesis, in addition to extracellular matrix invasion (69). These normal cellular responses are also mechanisms by which tumor cells propagate, invade, and metastasize.

Epithelial to mesenchymal transition (EMT)

Another hallmark of the progression from normal epithelium to tumor development is termed the epithelial to mesenchymal transition (EMT). EMT is a process that is characterized by loss of epithelial differentiated morphology and reversion to mesenchymal phenotype. The cell undergoing EMT is
characterized by a transition from a cuboidal to a spindle-shaped morphology, a reorganized actin cytoskeleton, and the expression of mesenchymal cellular marker proteins. Ron activation by HGFL has been shown to induce a motile-invasive phenotype marked by dissociation or cell scattering, and matrix invasion, and these characteristics resemble EMT. The characteristics that mark EMT were also evaluated in MDCK cells expressing Ron. Constitutive expression of Ron was shown to induce EMT, marked by phenotypic changes and cell motility (70). A collaborative effect of HGFL and TGF-β1 in EMT was also demonstrated. These results demonstrate that Ron overexpression alone or in combination with HGFL stimulation can induce traits that promote tumorigenic properties such as EMT, cell migration, and matrix invasion.

**Oncogenic potential of the Ron receptor**

The oncogenic potential of Ron and its role in cellular transformation has been investigated with in vitro and in vivo experimental systems. Stable expression of wild-type and constitutively active murine Ron mutants in NIH3T3 mouse fibroblast cells were investigated for transforming potential. The point mutations in the Ron gene were analogous to those found in the Met receptor tyrosine kinase in hereditary papillary renal carcinoma (HPRC), and had also been found in somatic mutations in renal carcinoma. Two of the point mutations were also analogous to activating mutations in the Ret and Kit oncogenes. Both overexpression of wild-type murine Ron and the activating mutations induced receptor phosphorylation and transformation of the fibroblasts, as determined by phenotypic changes and foci formation. These transformed cells also demonstrated increased proliferation rates and increased motility. The NIH3T3 cells overexpressing wild-type or mutant Ron formed tumors when injected into nude mice. A point mutation in the kinase domain (M1231T) and wild-type Ron showed equivalent tumor latency and 100% tumor formation in the nude mice. To determine if these transformed cells exhibited metastatic potential in vivo, NIH3T3 cells injected into nude mice were tested for both spontaneous and experimental metastasis. Mutation M1231T was the most aggressive form, and showed spontaneous and experimental metastasis to lungs (71).
The oncogenic potential of similar point mutations in the human Ron gene has also been investigated (72). The point mutations D1232V and M1254T in the tyrosine kinase domain of the Kit and Ret receptor respectively are found in human malignancies like mastocytosis and multiple endocrine neoplasia type 2B. Mouse NIH3T3 fibroblasts transfected with these Ron mutants produced transformed cells that formed foci. Constitutive phosphorylation of Ron and kinase activity of the receptor was shown for both the mutants and for the wild-type overexpressed receptor, although the mutant forms were more active. These same mutant forms were also examined for tumor formation when injected into nude mice. Both mutant forms produced tumors in nude mice and were highly metastatic. Overexpression of both of these mutant receptors in fibroblasts induced constitutive Ron receptor phosphorylation. Phosphorylation and constitutive activation of Ron also lead to activation of its downstream target, the mitogen activated protein kinase (MAPK) (73).

A constitutively active form of Ron was also produced as a Tpr-Ron chimera that mimics the oncogenic form Tpr-Met (74). The properties of this constitutively active Ron were also examined after transfection into NIH3T3 fibroblasts. The constitutive activation of Ron produced by this chimera produced a phenotype that is highly relevant to tumor progression and metastasis, marked by cell scattering, cellular motility, and invasion of an extracellular matrix.

**Loss of function mouse models for Ron**

To dissect the function of Ron in vivo, two different groups produced mouse models in which the Ron gene was knocked out. A mouse model with total loss of Ron protein was produced by a global deletion of exon 1-14 of the mouse Ron gene. This knock-out strategy completely eliminated the extracellular domain, the transmembrane domain, and a portion of the intracellular domain of the Ron gene. Strikingly, homozygous deletion of Ron is lethal at an early stage of embryo development (e7.5) and leads to death at the peri-implantation stage (75). Implantation of an embryo is a process that parallels tumor invasion, and demonstrates a normal function of Ron that may be co-opted for tumor progression.
The mice that were hemizygous for the knock-out of Ron were viable and fertile, but displayed an enhanced response to inflammation. The hemizygous mice were more susceptible to endotoxic shock and displayed an impaired ability to regulate nitric oxide, demonstrating the role of Ron in regulating these functions. Nevertheless, the lethality of this homozygous deletion made it impossible to dissect the role of Ron in different tissues in vivo. Therefore, a mouse model in which the signaling function of Ron could be ablated was designed and produced.

A mouse model was produced in which the extracellular and transmembrane domains of Ron are preserved, along with 8 amino acids of the intracellular domain, while the ablation of the remainder of the cytoplasmic domain of Ron results in complete loss of Ron intracellular signaling (59). Homozygous mice with this germline deletion are viable, fertile, and display no gross phenotypic abnormalities. However, the Ron receptor plays an important role in macrophage mediated inflammatory response by limiting nitric oxide production and thereby attenuating its harmful effects. In the absence of Ron signaling, the Ron TK−/− mice show an enhanced response to both acute and cell-mediated inflammatory stimuli. This model has been used to examine the role of Ron signaling in oncogene-mediated tumorigenesis.

A similar enhanced response to inflammation was observed in another knockout model (76). In this case, the gene targeting strategy inserted a beta-galactosidase gene into exon 1 of the mouse Ron gene so that transcription of the reporter would arise from the endogenous reporter, and be translated from its own start site. With this strategy, homozygous −/− mice were produced that were viable and that were phenotypically normal. This seemingly discrepant result may have arisen from the different targeting strategies used. The disruption of the majority of the gene sequence produced a true null animal. The insertion in exon 1 probably produced a functionally hypomorphic allele. Although this insertion ablated the activity of Ron arising from ligand binding, it is probable that some functions of Ron were still preserved by the production of known alternate splicing forms that did not require exon 1. Nevertheless,
the preponderance of evidence showed that the Ron gene plays a significant role in negative regulation of
the inflammatory response.

The expression of the ligand for Ron, HGFL, has also been deleted in a mouse model (HGFL<sup>−/−</sup>) (77). The global deletion of HGFL in mice leads to no gross phenotypic abnormalities, and the mice were fertile. Histological examination of mouse tissues revealed the presence of lipid filled vacuoles in hepatocytes in the HGFL<sup>−/−</sup> mice, but the significance of these vacuoles has not been determined at this time. The impact of ligand-mediated signaling in Ron-overexpressing tumors has not been determined at this time.

**Loss of Ron function and tumorigenesis**

To examine the significance of Ron in mammary tumorigenesis and metastasis, mice with a global deletion of the Ron tyrosine kinase intracellular signaling domain (Ron TK<sup>−/−</sup>) were crossed with mice pre-disposed to mammary cancer through expression of polyoma virus middle T antigen (pMT) under the control of the mouse mammary tumor virus (MMTV) promoter (MMTV-pMT) (14). The MMTV-pMT mouse is a well-characterized model in which 100% of the mice develop mammary tumors by three months of age. The mammary tumors in MMTV-pMT mice metastasize to the lung. In this model, loss of Ron signaling (MMTV-pMT / Ron TK<sup>−/−</sup>) dramatically impacted mammary tumor latency, tumor growth, and metastasis compared to mice with intact Ron signal function (MMTV-pMT / Ron TK<sup>+/+</sup>). Loss of Ron signaling significantly delayed tumor initiation and growth, and reduced metastasis. Loss of Ron signaling reduced tumor angiogenesis, decreased cell proliferation, and increased tumor apoptosis. The experiments also demonstrated that Ron impacted tumorigenesis through the MAPK and Akt signal pathways.

Loss of Ron signal function was also examined in the context of skin carcinogenesis using a model of chemically-induced Ras-mediated skin cancer. Mice expressing a mutated Ras transgene (v-Ha-Ras; Tg.AC) were crossed to mice deficient in the Ron tyrosine kinase domain (TK<sup>−/−</sup>). Mice expressing
the mutated Ras transgene and deficient in Ron signal function (Tg.AC +/- / Ron TK^-/-) and mice expressing the mutated Ras transgene with wild-type Ron signal function (Tg.AC +/- / Ron TK^+/+) were treated with 12-O-tetradecanoylphorbol-13-acetate (also known as TPA or PMA). This chemical treatment of the Ha-Ras-transgenic mice has been shown to induce the formation of papillomas, some of which undergo malignant conversion. Loss of Ron signal function resulted in an increased number of papillomas, but these papillomas showed significantly reduced growth. Most notably, loss of Ron signaling significantly reduced the number of papillomas that underwent malignant conversion, as well as reducing the number of other malignant tumor types found in these mice. The expression of Ron protein was found to be upregulated during TPA treatment. As had been found previously in the mammary carcinogenesis model, loss of Ron signaling impacted tumorigenesis through the MAPK and Akt signal pathways.

Gain of function mouse models for Ron overexpression in tumors

Two mouse models that overexpress Ron in different organ systems have been developed, and the effect of the overexpression of Ron on tumor development in those organs has been analyzed. One model overexpressed the human Ron gene in the lung by driving expression of Ron with the lung-specific surfactant C promoter (SPC) (78). Multiple adenomas developed at an early age in these mice. However, these adenomas did not progress to a malignant state. The adenomas were analyzed for point mutations in p53 and K-Ras, since mutations in these genes are frequently associated with lung tumors in mouse models, but no mutations were found in these genes in the time period under study. However, some indication of limited genomic instability was seen in individual tumors. In addition, the expression level of Ras, an important oncogene, was elevated in these adenomas. This data suggests that while Ron overexpression in the lung has oncogenic potential, progression to a malignant lesion may require additional genetic alterations in the lung.
A mouse model overexpressing murine Ron, driven by the mouse mammary tumor virus (MMTV) promoter, was developed in order to analyze the role of Ron overexpression in mammary tumorigenesis (15). These mice developed hyperplastic mammary glands by 12 weeks of age. Ron overexpression was sufficient for the development of mammary tumors in 100% of the female animals. The tumors overexpressing Ron were also found to be highly metastatic to liver and lung, and nearly 90% of the animals developed metastases. Ron overexpression was associated with receptor phosphorylation and kinase activity. The tumors were also found to overexpress cyclin D1 and c-myc, which have been associated with poor prognosis in human breast tumors. In addition, overexpressed Ron was associated with tyrosine phosphorylated β-catenin. The association of Ron and activated β-catenin, and the consequent upregulation of the β-catenin target genes cyclin D1 and c-myc, produces one plausible mechanism for the tumorigenic activity of Ron in breast cancer.

**Mechanisms of Ron induced tumorigenesis: signaling through the Ron receptor**

The pathways by which the Ron receptor conducts signals from the extracellular environment to the intracellular environment have been studied. However, the relationships of these different pathways to the specific biologic responses that are relevant to tumor formation are still poorly defined. Certain pathways appear to be commonly activated in many tumor types, whereas the responses of other signals may be cell-type specific. Ron activation by ligand binding and signaling via downstream adapter molecules has been shown to promote pleiotrophic effects dependent on cell type (79). The most prominent oncogenic pathways implicated in Ron signaling to date are activation of PI3-K/Akt, MAPK, Ras, Src and β-catenin. A preponderance of evidence in a number of tumor types indicates that a major mode of action of Ron in cancer is to promote cell survival via resistance to apoptosis. Both the MAPK and the PI3-K signal pathways have been implicated in this antiapoptotic action, with both pathways contributing to the effect generated by ligand stimulation of Ron (80). The activation of PI3-K leads to activation of Akt, which has been shown to enhance cell survival, but is not required for metastasis (81). Ligand binding of HGFL to the Ron receptor leads to phosphorylation of tyrosine residues on the C
terminus of the β chain of Ron. As with other receptor tyrosine kinases, activation of the kinase domain of Ron is thought to depend on receptor dimerization and trans-autophosphorylation of tyrosine residues. The phosphorylation of two tyrosine residues within the carboxyl-terminus (Y1353 and Y1360), are required for the biological activities of Ron (Figure 2). These tyrosine residues serve as docking sites for signaling molecules having Src homology-2 (SH2) and the phosphotyrosine binding domains (PTB). Tyr 1353 is important for Gab 1 binding whereas Tyr 1360 is critical for Grb2 binding. Recent studies have highlighted the importance of docking site Tyr 1353 being important in signal transduction and inducing cell migration downstream of Ron (82). HGFL stimulation induced recruitment and phosphorylation of Gab1 and Tyr 1353 was critical for this process. On the other hand Grb2 negatively regulated Ron signaling (82). Grb2 via its SH2 domain binds directly to the activated Ron receptor and allows recruitment of Son of sevenless (SOS) to the SH3 domain of Grb2. SOS activates Ras which recruits Raf to the membrane. Raf in turn activates MEK leading to Erk activation and the transcription of pro-proliferative genes (83). SHC via its PTB domain also binds directly to the phosphorylated tyrosine in the C terminal region of Ron and SHC-Grb2-SOS together can also activate the Ras pathway in response to HGFL. Grb2 may also act as an adapter to indirectly recruit multiple proteins to Ron (as is the case for Met) including the docking protein Grb2 associated binding protein-1 (Gab1) and Cbl ubiquitin ligases. Gab1 can also bind to membrane phosphotidyl-inositol 3,4,5- triphosphate (PIP3) via pleckstrin homology domain. When Gab1 and Ron are expressed in COS cells, Gab1 directly associates with tyrosine phosphorylated Ron through the Met binding domain (MBD) of Gab1. Gab1 can also directly associate with variety of signal transducers including PI3K, phospholipase-C (PLC-γ) and SHP2 phosphatase (84). Gab1 mediated signaling is important for inducing the branching morphogenesis (85). PI3K can also interact with Ron receptor either directly or through adaptor molecules (80). Activation of the Ras pathway is important for the program leading to invasive growth and PI3-K dependent activation of Akt activation is important for cell migration and survival (86-87) (Figure 2).
Ron is a strong inducer of both PI3-K and MAPK signaling pathways in vivo and in vitro. Prolonged activation of MAPK and Akt promotes cell differentiation, migration, survival, the functions intrinsic to Ron signaling (88). Tumor cell lines with a knockdown of Ron exhibit a diminution of basal phosphorylated MAPK and Akt (Wagh and Waltz, unpublished results). Moreover, in mammary tumors from mice expressing polyoma virus middle T antigen (pMT) under mouse mammary tumor virus promoter, loss of Ron receptor signaling leads to a significant decrease in pMAPK and pAkt in tumor lysates compared to mice with wild type Ron (14). These studies demonstrate the reliance of MAPK and PI3K/Akt signaling on Ron receptor expression.

Many human cancers have high cellular levels of β-catenin, and β-catenin plays a dual role in cell adhesion as well as acting as a transcription factor. Overexpression of activating Ron mutants M1254T and D1232V in NIH3T3 cells caused increase in cellular accumulation of β-catenin which thereby upregulated β-catenin responsive oncogenes c-myc and cyclin D1. Mutant Ron kinase caused tyrosine phosphorylation of β-catenin thereby increasing its stability and preventing degradation by the axin/GSK-3β complex (89).

The interaction of Ron with the extracellular matrix is important for the characteristic biological activity of Ron in promoting cell migration, and may also be important for its activity in promoting cell survival. Both of these biological activities, migration and enhanced survival, may contribute to the role that Ron plays in metastasis. Ron has been shown to directly interact with integrins (90). Cellular adhesion to extracellular matrix induced phosphorylation of Ron, and this activity was dependent on the kinase activity of Ron and of Src. In keratinocytes, HGFL stimulation of Ron was shown to lead to phosphorylation of the Ron receptor and also phosphorylation of α6β4 integrin (91). This interaction leads to the generation of 14-3-3 binding sites on Ron and the integrin, and the linkage of these molecules through the dimeric 14-3-3. This interaction is important for cell spreading and migration.

**Receptor cross-talk and Ron activity in tumorigenesis**
Another means of activating Ron signaling may be through the interaction of Ron with other receptors. This interaction between receptors of different types has been termed receptor cross-talk. Interaction between dissimilar receptors may play a role in stimulating receptor activity independent of ligand activity. However, receptor cross-talk may also retain responsiveness to ligand-induced activation. Both direct and indirect evidence exists that Ron interacts with other receptor types. This receptor cross-talk may be especially important for tumor progression since other interacting receptors have also been shown to be upregulated in tumors.

Ron is of course most closely related to the Met receptor, which is a known proto-oncogene. Accordingly, the regulation, expression, and interaction of Ron and Met have been studied in several normal tissue and tumor types. The regulation of expression of Met and Ron was examined in normal liver, hepatocellular carcinoma (HCC) tissues, and cell lines derived from HCC. Both Ron and Met were expressed in normal liver tissue. Both receptors were also overexpressed in a subset of HCC tumor tissues. The expression of Met and Ron was induced by the treatment of HCC cell lines with HGF, interleukin-1 and -6, and tumor necrosis factor alpha. Met and Ron expression appeared to be modulated in liver tumors by a similar cytokine network.

The interaction between Met and Ron was investigated by expressing full-length and kinase-inactive combinations of the two receptors in COS cells (92). When wild-type Met and Ron receptors were transiently expressed in COS cells, trans-autophosphorylation of tyrosine residues occurred in ligand-independent manner. However, treatment with either HGF or HGFL ligand increased the trans-autophosphorylation of the two receptors. By expressing a wild-type Ron receptor with a Met receptor in which the docking site tyrosines were deleted, or vice versa, it was demonstrated that transphosphorylation of Ron and Met occurred directly, rather than through a secondary signal transduction molecule. Through cross-linking of the proteins, Met-Ron complexes were detected on the cell surface, prior to ligand induced dimerization. Kinase dead Ron inhibited (mutant) Met induced
transforming ability of NIH3T3 cells, suggesting that Ron increases transforming ability of mutant Met (92).

The cross-talk between Ron and Met is also relevant to ovarian cancer (93). When a panel of human ovarian carcinoma tissues was evaluated, Ron and Met were significantly coexpressed in 42%. The mechanism by which cross-talk of Met and Ron could impact ovarian cancer was examined in vitro. The motility and invasiveness of ovarian cancer cells was stimulated by the addition of ligand for either receptor, but was synergistically enhanced by the co-administration of both ligands. The cross-talk between Ron and Met in ovarian cancers that overexpress both receptors may promote tumor progression.

A similar situation exists for cross-talk between Ron and Met in breast cancer (94). When Ron and Met expression was determined by immunohistochemistry on a panel of human invasive ductal breast carcinoma tissue samples, it was found that Ron and Met expression were independent predictors of distant metastasis. This clinical property correlates well with the observation that Ron influences cell scattering, motility, and invasiveness. Overall the synergism between Ron and Met can confer an aggressive phenotype to breast cancer. A multivariate retrospective analysis of clinical outcome was performed to determine the risk of the overexpression of Ron and Met in breast cancer. This analysis controlled for tumor size; tumor grade; and estrogen receptor, bcl-2, HER2/neu, and p53 status. In patients with overexpression of both Ron and Met, the likelihood of 10-year disease-free survival was only 11.8%, compared to 79.3% in patients with tumors that were negative for both receptors.

Decreased survival was also significantly associated with coexpression of Ron and Met in 19.1% of a cohort of 183 patients with transitional-cell bladder cancer (95). Overexpression of Ron in bladder cancer cell lines increased cell proliferation, motility, and survival. There is mounting evidence that cross-talk between Ron and Met may be a significant factor in subsets of various types of epithelial tumors.

Another tyrosine kinase receptor that is frequently overexpressed in many different tumor types, and has been a target for cancer therapeutic drug development for this reason, is the epidermal growth
factor receptor (EGFR). To determine the role of EGFR in Ron induced cellular transformation, a
dominant negative form of human EGFR was overexpressed in cells stably expressing mouse Ron (96).
This dominant negative EGFR dramatically reduced the scattering of these cells that is the normal
response to treatment with HGFL ligand. Cell scattering was also reduced when EGFR was chemically
inhibited. Co-transfection of dominant-negative Ron with wild-type EGFR, or co-transfection of
dominant-negative EGFR with wild-type Ron both produced significantly fewer transformed foci
compared to transfection of wild-type Ron or wild-type EGFR receptor alone. Trans-phosphorylation of
both receptors was induced when cells overexpressing murine Ron and expressing endogenous EGFR
were stimulated with either HGFL or the EGFR ligand epidermal growth factor (EGF). Co-
immunoprecipitation and activation of phosphatidyl inositol 3-kinase (PI3-K), a downstream signal
molecule that has been shown to play a role in cell motility, was observed after stimulation with either
ligand.

The coexpression of Ron and EGFR also has clinical significance in primary transitional cell
carcinoma of the bladder (97). In a cohort of bladder cancer patients, Ron and EGFR expression was
found in 33.3% of the tumor samples analyzed. Receptor coexpression was significantly associated with
tumor invasion, risk of local recurrence, and decreased survival. The interaction between Ron and EGFR
was also examined in a bladder cancer cell line that expresses high levels of both Ron and EGFR. The
interaction between Ron and EGFR was found to be ligand-independent. The knockdown of expression of
either Ron or EGFR via the transfection of siRNA reduced ligand-independent phosphorylation of both
receptors, although interestingly, the reduction of phosphorylation of EGFR by knockdown of Ron was
greater than the reverse. The inhibition of EGFR activity by either siRNA or by treatment with small
molecule inhibitors of EGFR also impacted biological effects mediated by Ron, with a reduction in
proliferation, migration, survival, and foci formation. In total, these results indicate that cross-talk
between Ron and EGFR may be an important mode of activation and stimulation of biological activities
mediated by Ron in both a ligand-dependent and ligand independent manner.
Ron cross-talk has also been shown to occur with two other classes of receptors that are less well characterized for relevance to cancer. Ron has been shown to interact with the interleukin-3 (IL-3) receptor common β chain (98). Cross-talk between these receptors after HGFL-ligand stimulation was shown to modulate downstream signal pathways through activation of the JAK2 signal transduction molecule, and to tip the balance of cellular activity toward shape change that is relevant to cell motility rather than to cell proliferation. Another class of receptors that may cross-talk with Ron are the plexins. Plexins are transmembrane receptors for semaphorins, a class of secreted molecules that were first characterized for axonal growth cone guidance. However, plexins are also overexpressed in variety of human cancers including pancreas, colon, and liver. Ron shares structural and functional similarities with plexins. Sema 4D, a ligand for B1 plexin, caused an increase in the invasiveness of NIH3T3 cells expressing Ron. Saturating concentrations of HGFL and 100 nM of Sema4D synergistically increased NIH3T3 cell invasion as compared to controls (99).

**Angiogenesis**

It has been well established that progressive tumor growth requires de novo blood vessel production, and that tumors produce angiogenic chemokines to fulfill the recruitment and growth of these blood vessels. The development of anti-angiogenic tyrosine kinase inhibitors, such as those that target vascular endothelial growth factor receptors (VEGFR), are an area of intensive research, and have moved rapidly into patient treatment (100). The role of the Ron receptor tyrosine kinase in mediating angiogenic signals is an intriguing area that has had little attention to date.

The first report that Ron may play a role in tumor angiogenesis was produced in an examination of Ron signal function in mammary carcinogenesis (14). Tumors induced by polyoma middle T expression, with or without Ron signaling, were examined for blood vessels by immunohistological staining. It was demonstrated that the ablation of Ron signaling was associated with a significant reduction in microvessel density.
Ron-mediated angiogenesis plays an important role in prostate cancer. The Ron receptor tyrosine kinase is overexpressed in human prostate tumors and cell lines, and that angiogenic chemokines from prostate cancer cells induce endothelial chemotaxis. Moreover, blocking Ron by receptor neutralizing antibody and by siRNA methods inhibits production of angiogenic chemokines in prostate cancer cells (101-102).

**Genomic instability and cell cycle disruption**

In recent work, the effect of Ron overexpression on genomic instability in the mouse model of mammary tumorigenesis has been examined (15). Primary cells derived from tumors were shown to display aberrant cell cycle kinetics and mitotic defects. These tumor-derived cells showed a high level of inherent DNA damage, as evidenced by the phosphorylation of substrates of ATM, and an accumulation of the cell cycle checkpoint protein Cdc25A. The accumulation of Cdc25A prompted the examination of Chk2, a cell cycle modulator of Cdc25A stability. Chk2 was also of interest, since point mutations in this gene have been shown to be a risk factor for human breast cancer. An interaction between Ron and Chk2 that converges on the Cdc25A protein was determined (103). This work explores a previously unexamined role for Ron in genomic stability in cancer.

**Ron expression in human tumors and tumor-derived cell lines**

The growing awareness of the potential role for Ron in human cancer has led to a recent examination of Ron expression in a range of human tumor types and tumor-derived cell lines (104). Panels of human tumor tissue were analyzed for the extent and intensity of Ron staining, and covered tumors of the breast, lung, prostate, gastric tissue, pancreas, and colon. The number of tumor tissues in these arrays ranged from 38 to 55. The percent of tissues that were positive for Ron expression ranged from 65% in colon cancer to 100% in breast cancer, with high staining intensities found in epithelial cells. A large number of cancer-derived cell lines were also analyzed for Ron expression, and positive cell lines were found that were derived from breast, lung, prostate, pancreas, and colon, ovary, stomach, and liver.
The involvement of overexpressed Ron in tumors of epithelial origin reflects its wide distribution in epithelial cells.

**Ron as a target of cancer therapy**

In the last ten years, progress has been made in developing new drug therapies for cancer by targeting specific overexpressed growth factor receptors that characteristically appear in solid tumors. Most of these growth factor receptors, like the Ron receptor, are activated by and transmit signal cascades by tyrosine phosphorylation. The drug therapies include both monoclonal antibodies and small molecule inhibitors. Some of the recently approved or experimental drug targets include the epidermal growth factor receptor (105), human epidermal receptor 2 (HER2/neu) (106-107), or drugs that target more than one receptor (108-109); and both platelet-derived growth factor (PDGFR) and vascular endothelial growth factor (VEGFR) receptors (110-111).

One of the first receptor tyrosine kinase that was targeted is the epidermal growth factor receptor. Both small molecule inhibitors and anti-EGFR antibodies have been approved for clinical use and have been used in combination, as well as therapies that combine targeted molecules with chemotherapy or radiation (112). The development of EGFR inhibitors is very important to future development of drug therapies against the Ron receptor, since Ron and EGFR have been shown to be closely connected and to interact. Combinatorial therapies have also been shown to be highly effective when targeting receptor tyrosine kinases. Combination therapy may involve small molecules that inhibit several receptor tyrosine kinases (111). The addition of receptor-targeted drugs to chemotherapeutic agents may also be an effective strategy. For instance, a combination therapy of tamoxifen, angiostatin and TIMP-2 (tissue inhibitor of metalloproteinase-2) administered to MMTV-neu mice significantly reduced primary tumor growth (90% inhibition, P=0.01) and metastasis free survival of up to 6 months in the experimental group as compared to 33% in control group, suggesting an overall survival advantage with this combinatorial therapy (113).
Biologic drugs that target the Ron receptor are in early stages of development. A humanized monoclonal antibody that blocks the interaction of Ron with HGFL has been developed: IMC 41A10 (ImClone systems, New York, NJ) (104). IMC41A10 not only inhibits the binding of HGFL to Ron, but also diminished Ron phosphorylation and its downstream signaling. IMC41A10 also significantly decreased tumor growth of murine xenografts from subcutaneously injected lung, colon and pancreatic cancer cell lines in nude mice.

The mechanism by which Ron promotes tumor growth, and a potential combination therapy, was examined in vitro using a different non-humanized monoclonal blocking antibody that is available (R&D systems). Treatment of BxPC-3 pancreatic cancer cells with this monoclonal antibody against Ron, followed by 0.1 \( \mu \text{mol/L} \) of gemcitabine, resulted in 32% increase in apoptosis as compared to gemcitabine alone (114). This interesting result suggests that the function of Ron in tumors may be to increase cell survival, and that blockage of Ron signaling might be used to increase apoptosis induced by classical chemotherapeutic drugs.

Additional antibodies have been used to block Ron signaling. Monoclonal antibodies named as ID-1 and ID-2 inhibited binding of HGFL to Ron and also diminished HGFL induced HT-29-D4 human intestinal cell migration, suggesting that these antibodies are efficient in blocking Ron mediated oncogenic signaling (115).

The extracellular region of Ron \( \beta \) chain contains the SEMA and the (plexin, semaphorins, integrins), PSI domains along with the four IPTs (Immunoglobulins like fold shared by plexins and transcription factors) (116). Both Ron-sema and Ron-PSI were able to inhibit binding of HGFL to Ron. In addition, they also blocked HGFL induced Ron tyrosine phosphorylation and inhibited growth of HCT116 colon cancer cells (117).

The chemotherapeutic agents that impair Hsp (Heat shock protein) function are geldanamycins. Hsp are important chaperone proteins that facilitate correct protein folding and assembly. Several
receptor tyrosine kinases including Ron are sensitive to these drugs (118). These drugs may be useful for combination therapy in concert with Ron-receptor-targeted drugs. Another approach to reducing Ron activity has used gene silencing. Use of a small interfering RNA (siRNA) against Ron expressed in human colorectal carcinoma significantly reduced cancer cell proliferation, motility, and increased apoptotic susceptibility of the cells (119).

Other types of combination therapy may also be beneficial. The MMTV-Ron driven mammary tumors in our mouse model are ER positive; hence combination of Ron inhibitory drugs along with tamoxifen may prove beneficial for treatment of patients having breast cancer (120). Since Ron driven tumors are highly metastatic, a combination of a Ron inhibitor along with angiostatin (drug that prevents tumor angiogenesis) may be efficient in reducing tumor growth and subsequent metastasis, because tumor cells can invade the primary site through newly formed blood vessels. Inhibitors of PI3-K and the NF-κB pathway in combination with Ron inhibitors may be a useful combinatorial therapy because activated PI3K/Akt can phosphorylate and inactivate GSK-3 beta and in turn activate beta-catenin signaling. Hence targeting both Ron and PI3K/Akt can be effective in cancers that over expresses both the genes.

Beta-Catenin regulation and function

Human beta-catenin (CTNNB1) gene is located on chromosome 3p21, a region that has loss of tumor suppressor genes and is implicated in tumor development (121). Beta-Catenin plays dual roles one in cell-cell adhesion and transcription. Beta-catenin pathway regulates cell proliferation, migration and differentiation, all of which are important in regulating embryonic development and tumorigenesis (122). In the absence of Wnt ligand, beta-catenin gets phosphorylated and degraded by Axin, APC, GSK-3beta and CK-1 thus targeting it for ubiquitin mediated degradation. Phosphorylation at N terminal serine threonine residues facilitate beta-catenin degradation. Tyrosine phosphorylation can also prevent beta-catenin degradation, leading to its cytoplasmic accumulation and nuclear transport. Nuclear beta-catenin interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF) to induce expression of its downstream
target genes (123). Some of the key downstream targets of beta-catenin that are important in cell migration, invasion and transformation are c-myc and cyclin D1. Thus increased nuclear beta-catenin correlates with increased beta-catenin activity. Downregulation of beta-catenin levels in nucleus is facilitated by its export by APC (124).

**Beta-catenin tyrosine phosphorylation**

One of the ways in which beta-catenin is regulated is via its tyrosine phosphorylation. There are 12 armadillo repeats in beta-catenin and about 19 tyrosine residues that span the structure. Phosphorylation at each tyrosine residue has a unique function. Beta-catenin phosphorylation at tyr 654, a residue in the 12th armadillo repeat causes its dissociation from E-Cadherin (125). This decreases E-Cadherin/beta-catenin interaction and increases its cytoplasmic accumulation. Phosphorylation at tyr 654 also enhances binding of beta-catenin to TATA binding protein (TBP) which aids in further increase in TCF-LEF activity. Fer and Fyn tyrosine kinases phosphorylate beta-catenin at tyr 142 and disrupt beta-catenin interaction with alpha-catenin (126). Other growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) also induce beta-catenin tyrosine phosphorylation (127). More interestingly, HGF-Met induced tyrosine phosphorylation of beta-catenin at residue 654 and 670. Abrogation of tyrosine to phenylalanine prevented beta-catenin TCF complex formation and DNA synthesis in response to HGF (128).

**Beta-Catenin and Breast Cancer**

Increased nuclear beta-catenin is seen in about 63% of breast cancer patients (129-130). Moreover beta-catenin expression and activation (nuclear expression) was reported in invasive breast cancers and was associated with poor outcome (131). Also reduced expression of membranous beta-catenin in breast cancer was associated with metastasis and worse outcome (132). Brca-1/p53 inactivated mouse breast tumors that develop basal like breast cancers which show beta-catenin accumulation in a small subset of tumors (133). Nuclear beta-catenin is also associated with ER, PR and HER-2 negativity, vimentin
expression and stem cell enrichment (130). Overexpression of N terminal deleted beta-catenin under MMTV promoter induced precocious alveolar development and mammary adenocarcinoma formation (124).

Conclusions

In conclusion, accumulating evidence shows that Ron plays an important role in human cancers. The data summarized above elucidates critical signaling pathways that are downstream of Ron and are important mediators of Ron induced tumorigenesis. In the future, more precise anti-cancer drugs that block Ron activity may be important additions to cancer therapy.


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Figure 1. Breast Cancer: Tumor-stroma interaction. Genetic and epigenetic changes in stem and progenitor cell population gives rise to different tumor subtypes including atypical ductal hyperplasia, ductal carcinoma in-situ and invasive ductal and luminal carcinoma. These changes cause abnormal interactions within the tumor microenvironment thereby inducing transformation of epithelial cells and giving rise to metastatic breast cancer. Macrophages are recruited to the site of tumor by chemotactic factors that then promote tumor progression and metastasis. (Figure is adapted from Nature Reviews Cancer 7, 659-672 (September 2007)).
Figure 2. The Ron and Met receptor tyrosine kinases exhibit certain similarities and differences between receptors. Structurally, Ron and Met are similar in that both receptors are single-pass, disulfide linked α/β...
heterodimers. However, the amino acid identity between Ron and Met is not high (34% overall) but the intracellular region involved in signal transduction is conserved (63%). The ligands for Ron and Met, HGFL and HGF respectively, also share a similar structure and have an overall amino acid identity of 45%. In contrast to their structural similarity, HGFL and HGF are secreted ligands, which originate from different cell types, with HGFL produced as an endocrine molecule secreted primarily from hepatocytes and HGF produced from mesenchymal cells operating in a paracrine fashion. Binding of HGFL or HGF to their corresponding receptor induces receptor dimerization and trans-autophosphorylation of tyrosine residues (1238/1239 Ron and 1234/1235 Met) in the tyrosine kinase domain, leading to the tyrosine phosphorylation of key C-terminal residues (1353/1360 Ron and 1349/1356 Met). Activation of either receptor results in recruitment of several downstream adaptor molecules and initiation of robust signaling responses. Signaling pathways that are impacted by these receptors include the PI3-K, Akt, β-catenin, Ras, MAPK and JAK/STAT pathways which induce pleiotropic biologic events such as proliferation, migration, invasion, cell scattering and branching morphogenesis.
CHAPTER II

Beta-catenin is required for Ron receptor induced mammary tumorigenesis

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Abstract

Our previous studies demonstrated that selective overexpression of the Ron receptor tyrosine kinase in the murine mammary epithelium leads to mammary tumor formation. Biochemical analysis of mammary tumor lysates showed that Ron overexpression was associated with increases in β-catenin expression and tyrosine phosphorylation. β-catenin has also been shown to be regulated through tyrosine phosphorylation by the receptor tyrosine kinases Met, Fer, and Fyn. However, the molecular and physiological roles of β-catenin and β-catenin tyrosine phosphorylation downstream of Ron are not known. To investigate this association, we show that Ron and β-catenin are coordinately elevated in human breast cancers. We also show, in both human and murine breast cancer cell lines, that Ron co-immunoprecipitates with β-catenin and that Ron activation induces the tyrosine phosphorylation of β-catenin. Ron activation, through binding of the Ron ligand, hepatocyte growth factor-like protein (HGFL), induces both β-catenin nuclear localization and transcriptional activity, with tyrosine residues Tyr 654 and Tyr 670 of β-catenin being critical for these processes. We also found that HGFL-dependent Ron activation mediates upregulation of the β-catenin target genes cyclin D1 and c-myc, and that expression of these target genes in breast cancer cells is decreased following inhibition of Ron and/or β-catenin. Finally, we show that genetic ablation of β-catenin in Ron-expressing breast cancer cells decreases cellular proliferation in vitro, as well as mammary tumor growth and metastasis following orthotopic transplantation into the mammary fat pad. Together, our data suggest that β-catenin is a crucial downstream regulator of Ron-mediated mammary tumorigenesis.
Introduction

Behind skin cancer, breast cancer is the most frequently diagnosed cancer in women in the United States. In 2010, it is estimated that over 200,000 new cases of invasive breast cancer will be diagnosed and over 40,000 women and men will die from this disease (American Cancer Society Statistics, 2010). While death rates attributed to breast cancer have slightly diminished over the past two decades, further efforts to understand the molecular pathways governing breast cancer initiation and progression are needed. In numerous cancers, alterations in the expression and activation of cell surface receptor tyrosine kinases and in the activation of signaling molecules downstream of these receptors have been shown to play important roles in cancer development. A number of receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR), Her 2, platelet derived growth factor, and the c-Met receptor, have been shown to be aberrantly expressed in human breast cancers (Coombes et al 1990, Graveel et al 2009, Shien et al 2005, Slamon et al 1989). Recently, several studies have also documented an increase in Ron receptor tyrosine kinase expression in human breast cancer (Lee et al 2005, Maggiora et al 1998, O'Toole et al 2006, Welm et al 2007).

The Ron receptor tyrosine kinase is overexpressed in about 50% of human breast cancers. In clinical studies with node-negative breast cancer specimens, Ron expression was shown to be an independent predictor of distant breast cancer metastases and breast cancer patients with high Ron expression had significantly worse 10-year disease-free survival (Lee et al 2005). In line with increased metastasis, activation of the Ron receptor signaling system, including Ron expression and expression of the Ron ligand, hepatocyte growth factor-like protein (HGFL), was shown to be a strong independent indicator of both metastasis and death in human breast cancer patients (Welm et al 2007). In murine breast cancer models, exogenous HGFL overexpression was able to promote breast tumor growth and broaden the spectrum of metastases (Welm et al 2007). Conversely, deletion of the Ron receptor in an oncogene-driven mouse model of breast tumorigenesis resulted in delayed tumor formation and decreased metastasis (Peace et al 2005).
To examine the significance of Ron receptor overexpression in breast cancers \textit{in vivo}, our laboratory previously generated a transgenic mouse model wherein the Ron receptor tyrosine kinase was overexpressed selectively in the mammary epithelium (Zinser et al 2006). Ron overexpression was sufficient to induce mammary tumors in 100\% of female mice and was associated with a high degree of breast cancer metastasis. We also showed that Ron overexpressing tumors exhibited elevated levels of tyrosine phosphorylated \(\beta\)-catenin. These studies suggested that Ron may be a causative factor in breast tumorigenesis and provided a model to dissect the molecular pathways downstream of Ron which are critical for tumor growth and metastasis.

\(\beta\)-catenin is a pivotal transcription factor that plays diverse roles in adherens junctions and in cell signaling events including cellular proliferation and motility [reviewed in (Brembeck et al 2006)]. Increases in \(\beta\)-catenin cytosolic and nuclear accumulation have been associated with a variety of malignancies including colon and breast cancer and elevated levels of \(\beta\)-catenin have been shown to be inversely correlated with patient survival (Apte et al 2006, Gavert and Ben-Ze'ev 2007, Kizildag et al 2008, Lin et al 2000, Nakopoulou et al 2006, Ranganathan et al 2005, Takahashi-Yanaga and Kahn 2010). Nuclear and cytosolic localization of \(\beta\)-catenin is also associated with stem cell enrichment, vimentin expression, and with an epithelial to mesenchymal transition (Micalizzi et al 2010, Takahashi-Yanaga and Kahn 2010). Interestingly, several reports have implicated \(\beta\)-catenin accumulation and activation downstream of receptor tyrosine kinase activation in multiple tumor types, although the \textit{in vivo} significance of these findings is still unclear (Apte et al 2006, Castellone et al 2009, Danilkovitch-Miagkova et al 2001, Gujral et al 2008, Lee et al 2010, Zinser et al 2006).

In this report we sought to examine the biological significance of \(\beta\)-catenin as a downstream mediator of Ron receptor activation in breast cancer. Our data shows for the first time that Ron and \(\beta\)-catenin are coordinately increased in human breast cancers and are associated with reduced survival and increased metastasis to lymph node. We show that Ron Kinase is able to tyrosine phosphorylate WT \(\beta\)-catenin but
not Y654/670F β-catenin. We also show that Ron activation induces the nuclear localization and transcriptional activation of β-catenin and β-catenin dependent target genes. We also demonstrate that tyrosine residues 654 and 670 of β-catenin are important in mediating Ron-induced β-catenin transcriptional activation and cell growth. Using in vivo loss of function model system we show that deletion of β-catenin significantly diminishes the growth of Ron expressing breast cancer cells. We also show that defect in breast cancer cell proliferation due to Ron loss can be rescued by treatment with GSK-3β inhibitor suggesting multiple modes for regulation of β-catenin downstream of Ron. These studies are first to identify β-catenin as a downstream effector molecule of Ron in inducing breast cancer proliferation in vitro and in vivo.
Results

Coordinate expression of Ron and β-catenin in human breast cancer tissue and their expression in breast cancer cell lines. Given that our prior studies showed increased β-catenin accumulation in mouse mammary tumors following Ron overexpression in the mammary epithelium, we sought to examine the potential correlation of Ron and β-catenin overexpression in human breast cancers and cell lines. To accomplish this, we performed immunohistochemical staining on serial sections of human breast cancer tissue arrays for Ron and β-catenin expression. A representative breast tumor having both high Ron and β-catenin expression is depicted in Figure 1A. In our analysis, we found that 34 of the 76 breast cancer samples exhibited high levels of Ron expression (Table 1). In addition, we observed a statistically significant correlation between high Ron expression and high β-catenin expression with 23 of the 34 samples that expressed high levels of Ron also exhibiting increased β-catenin staining (Table 1). Tissues with high β-catenin expression exhibited a heterogeneous mixture of membranous, cytosolic and nuclear β-catenin accumulation (Figure 1A and data not shown). In addition we found that patients with high Ron and high β-catenin expression had reduced survival and died due to cancer before 30 month follow-up period (4/5=80%) as compared to patients with low Ron and β-catenin (5/14=35.7%), *p<0.01. We also found that 2/5 (40%) patients with high Ron and β-catenin that died due to cancer had significantly higher lymph node metastasis as compared to patients (0/14=0%) with low Ron and β-catenin and that died due to cancer. We further analyzed Ron and β-catenin expression in a panel of human breast cancer cell lines compared to the murine R7 breast cancer cell line which was established from a mammary tumor from the MMTV-Ron mice (Zinser et al 2006). Figure 1B illustrates that that T47D human breast cancer cells expressed high levels of Ron, compared to MCF-7 and MDA-MB-231 breast cancer cell lines, with this cell line also expressing high levels of β-catenin.

Our prior studies on Ron overexpressing murine mammary tumors demonstrated an association of Ron and β-catenin by co-immunoprecipitation and with β-catenin in the immunoprecipitated tissue exhibiting tyrosine phosphorylation (Zinser et al 2006). To test for the ability of Ron to tyrosine phosphorylate β-
catenin, we immunoprecipitated Ron from mammary tumors of MMTV-Ron mice and used HEK-293 transfected Flag tagged WT and DM β-catenin as substrate in the kinase reaction. As shown in Figure 1C, Ron protein that specifically pulled down with Ron antibody compared to IgG antibody, was efficiently able to tyrosine phosphorylate WT β-catenin but not the DM β-catenin. Total input for Ron, β-catenin and flag tagged β-catenin is also shown in Figure 1C.

Tyrosine residues Tyr 654 and Tyr 670 are important in Ron-mediated β-catenin phosphorylation and nuclear localization. Previous studies have shown that β-catenin tyrosine phosphorylation at residues Tyr 654 and Tyr 670 is required for HGF-induced Met-mediated β-catenin nuclear translocation and ensuing transcriptional activation (Zeng et al 2006). Given the similarities between the Ron and Met receptor tyrosine kinases, we sought to examine the role of HGFL-induced Ron activation on β-catenin nuclear localization and the importance of β-catenin Tyr 654 and Tyr 670 in this process. As depicted in Figure 2A, we show that HGFL treatment of T47D cells induces nuclear localization of β-catenin compared to vehicle treated cells. Similar results were obtained in the murine R7 cells (data not shown).

To test the importance of β-catenin tyrosine residues Tyr 654 and Tyr 670 in HGFL-induced β-catenin nuclear localization, we utilized plasmids containing either a Flag tagged wild type β-catenin expression construct (WT) or a Flag-tagged expression construct containing a double mutant (DM) of β-catenin wherein Tyr residues 654 and 670 were replaced with phenylalanine (Zeng et al 2006). The WT and DM constructs were transfected into T47D cells and stable pools of T47D clones expressing similar levels of the Flag-tagged β-catenin were generated and labeled T47D-WT and T47D-DM (Figure 2B). To investigate the relevance of Tyr 654 and Tyr 670 phosphorylation of β-catenin on nuclear localization in response to Ron activation, the T47D-WT and T47D-DM cells were treated with HGFL or vehicle. Two hours after treatment, the cells were isolated into nuclear and cytoplasmic fractions. Each fraction, in addition to total cell extracts, was examined by Western analysis. As depicted in Figure 2C, HGFL treatment significantly increased the amount of WT β-catenin observed in the nuclear faction compared to vehicle treated cells. In contrast, only a limited amount of the β-catenin DM was able to localize to the
nucleus in the absence of HGFL stimulation compared to WT β-catenin and this level was not substantially increased by HGFL. Of note, the majority of exogenous WT and DM β-catenin protein was associated with the cellular membrane. **Figure 2D** is a representative quantification of the results from **Figure 2C** demonstrating that HGFL treatment increases the nuclear accumulation of WT β-catenin with minimal alterations in the nuclear amounts of DM β-catenin. Slight but consistent increases in β-catenin (either WT, DM or endogenous) levels were observed following HGFL treatment. Similar to the Met receptor, single mutations at Tyr 653 or Tyr 670 were not able to inhibit HGFL-induced nuclear localization of β-catenin suggesting that both tyrosine residues are required for this effect [data not shown and (Zeng et al 2006)].

**Ron activation promotes β-catenin mediated reporter activity and target gene expression.** To investigate the role of HGFL induced Ron activation on β-catenin dependent reporter activity, we utilized T47D and R7 breast cancer cell lines along with cell lines containing variations in Ron and β-catenin expression. Specifically, we generated T47D and R7 cells with a stable knockdown of Ron utilizing lentiviral constructs containing shRNA against Ron or utilized a nontargeting (NT) control shRNA sequence. The knockdown of Ron in each cell type was efficient with Ron protein levels reduced by approximately 60 to 90% (**Figure 3A**). T47D cells stably expressing WT Flag-tagged and DM Flag-tagged β-catenin were also utilized with **Figure 2B** depicting similar levels of exogenous WT and DM protein (Flag) and overall β-catenin levels in these cells. Each cell line was transiently transfected with a β-catenin/TCF dependent reporter plasmid (TOPFLASH) and 48 hours later the cells were stimulated with HGFL or vehicle. Two hours after stimulation, the cell were collected and examined for luciferase activity. The reporter activity for each vehicle treated cell line was normalized to 1. T47D NTsh and T47D shRon cells were similarly transfected with TOP-FLASH reporter followed by treatment with SB 216763 (GSK-3β inhibitor) for 16 hours. As shown in **Figure 3B**, HGFL treatment of both T47D and R7 cells leads to a significant induction of β-catenin-dependent reporter activity. Ron receptor expression is required for this induction as a knockdown of Ron in both cell types abrogates reporter activity. HGFL
was also able to induce β-catenin-dependent reporter activity in T47D-WT to levels over that of endogenous β-catenin. In contrast, however, overexpression of the 654/670 double tyrosine mutant β-catenin in T47D-DM cells abolished HGFL-induced reporter activity. Treatment with SB 216763 not only increased reporter activity in T47D NTsh cells compared to vehicle treatments, interestingly it also increased β-catenin reporter activity in T47D shRon cells, suggesting β-catenin can be activated via other pathways downstream of Ron. To examine the impact of HGFL on the production of well-established β-catenin target genes such as cyclin D1. T47D shNT and T47D shRon cells were treated in presence or absence of ligand HGFL for 2 hours and total extracts were examined by western analysis. We found that T47D cells with Ron knockdown showed an inability to induce cyclin D1 expression compared to control T47D cells with Ron expression when treated with HGFL (Figure 3D). Knockdown of β-catenin in the T47D cells also abolished the ability of HGFL to induce cyclin D1 levels (data not shown) demonstrating the specificity of HGFL-induced Ron activation on β-catenin for the induction of cyclin D1 under these conditions.

**β-catenin deletion in MMTV-Ron cells decreases their proliferation in vitro.** To examine the requirement of β-catenin downstream of Ron, mammary tumor cells lines were generated from MMTV-Ron mice that were bred to contain the β-catenin gene flanked by LoxP sites (Huelsken et al 2001). Three independent cell lines (labeled β-cateninF/F #1, #5 and #11) were generated as depicted in Figure 4A and express similar levels of Ron and β-catenin. To effectively eliminate β-catenin, the cell lines were transiently infected with an adenovirus dually expressing the Cre recombinase and GFP. Following infection, GFP-positive (β-catenin-/-) and negative cells (β-cateninF/F) were isolated by fluorescent activated cell sorting. As depicted in Figure 4B and 4C, β-catenin protein and RNA expression was efficiently deleted in GFP-positive cells. To examine the consequences of β-catenin loss in vitro, BrdU incorporation assays were performed. As quantified in Figure 4D, the β-catenin-/- cells exhibited a 50% reduction in BrdU-positive cells compared to β-cateninF/F. This decrease in proliferation was further supported by monitoring the growth of β-cateninF/F and β-catenin-/- cells over time (Figure 4E). These
data indicated that deletion of β-catenin dramatically decreases proliferation of MMTV-Ron derived breast cancer cells in vitro.

**Exogenous expression of WT β-catenin but not DM β-catenin rescues the proliferation defect observed in the β-catenin-/- cells.** To examine the ability of WT or DM of β-catenin to rescue the proliferation defect in the β-catenin-/- cells, the β-catenin-/- cells were transiently transfected with WT β-catenin, DM β-catenin or with the empty vector (EV) as a negative control. Figure 5A demonstrates similar levels of β-catenin expression in the WT and DM transfected cells. As shown in Figure 5B, expression of exogenous WT β-catenin was able to partly rescue the defect in cell growth observed in the β-catenin-/- cells. In contrast, expression of DM β-catenin did not change the growth of the β-catenin-/- cells. The growth rate of the β-cateninF/F cells, which express endogenous β-catenin, is shown for comparison. The partial rescue of cell growth observed with WT β-catenin expression in the β-catenin-/- cells is likely less than the β-cateninF/F cells due to the transient nature of our assays and the efficiency of transfection.

**β-catenin deletion abolishes the growth of MMTV-Ron derived breast cancer cells in vivo.** To examine the growth potential of β-cateninF/F and β-catenin-/- cells in vivo, equal number of cells were implanted orthopotically into the mammary fat pads of athymic nude mice. Tumor growth was measured biweekly by using digital calipers. As depicted in Figure 6A, the β-cateninF/F cells exhibited dramatic increases in tumor volume over the course of 6 weeks. In contrast, however, no growth was observed from the implanted β-catenin-/- cells (Figure 6A). At 6 weeks following orthotopic implantation, the mammary glands were harvested. Figure 6B demonstrates a significant increase in mammary gland weight from glands implanted with the β-cateninF/F compared to glands with β-catenin-/- cells. As an inset, a representative picture of each gland is provided. While these studies do not preclude the fact that β-catenin loss might simply hamper proliferation of any cancer cell line, independent of Ron signaling, they do show for the first time that β-catenin is essential for breast tumor growth in vivo in cells
transformed by Ron overexpression. Following histological analysis, representative mammary tumor histology is depicted for the β-cateninF/F cells (Figure 6C). Interestingly, the β-catenin-/- cells were still observed in the mammary glands of mice after 6 weeks suggesting that cells are viable but exhibit an inability to grow in vivo (Figure 6C). In addition to primary tumor formation, extensive metastasis was observed in mice implanted with the β-cateninF/F cells. Metastases were observed in various organs including colon, pancreas, kidney, lung, and liver (Supplemental Figure S2 and Table 2). No metastasis was observed in mice transplanted with the β-catenin-/- cells.
Discussion

Ron receptor overexpression in both human and murine breast cancers is associated with a highly aggressive tumor phenotype and with a high incidence of metastasis. Despite the importance of this receptor in breast cancer, the downstream effectors of Ron signaling in breast cancer have not been well-characterized. Previous studies in our laboratory have shown that mammary-specific Ron overexpression in mice leads to breast tumor formation and metastasis in vivo (Zinser et al 2006). We also demonstrated that Ron overexpression in these breast tumors was associated with elevated levels of tyrosine phosphorylated β-catenin. In this study, we examined the significance of Ron and β-catenin in human breast cancers. Our study is novel based on three unique findings. Firstly we show that Ron and β-catenin are coordinately elevated in human breast cancers and that overexpression of both leads to reduced overall survival and increased metastasis to lymph node as compared to patients with low Ron and β-catenin. Secondly we show that Ron can tyrosine phosphorylate β-catenin. Also HGFL mediated Ron activation leads to β-catenin nuclear localization and increased β-catenin reporter activity, and that Y654 and Y670 of β-catenin are important for these processes. Finally, using in vivo loss of function system we were able to show that targeted deletion of β-catenin in Ron expressing murine breast cancer cells significantly diminished mammary tumor growth and metastasis. Also β-catenin expression, in the context of Ron loss rescued the proliferation in breast cancer cells.

In human breast cancer, we showed for the first time that Ron receptor overexpression significantly correlates with elevated β-catenin levels. In addition to Ron, there is strong evidence implicating β-catenin signaling in breast tumorigenesis. In humans, breast tumors frequently exhibit elevated levels of β-catenin with higher expression levels correlating with decreased patient survival (Lin et al 2000). In mice, overexpression of an activated form of β-catenin leads to the development mammary hyperplasia and adenocarcinomas (Tsukamoto et al 1988). These studies suggest that Ron receptor overexpression and activation may contribute to β-catenin signaling in breast cancer and suggests β-catenin expression and activity may serve as a biomarker for assessing the prognosis of Ron overexpressing breast cancers.
Although our data set was limited in number, we found that overexpression of both Ron and β-catenin lead to reduced survival and increased lymph node metastasis before 30 months follow-up period compared to patients that had low Ron and β-catenin. Interestingly, overexpression of other receptor tyrosine kinases including Met, EGFR and RET have been associated with increased free β-catenin pools. Moreover, tyrosine phosphorylation of β-catenin has been shown to promote β-catenin escape from the APC/Axin/GSK3β-mediated destruction and disruption of E-cadherin association and the disassembly of adherens junctions (Castellone et al 2009, Roura et al 1999). Using flag tagged WT and mutant β-catenin, we demonstrate for the first time that Ron can tyrosine phosphorylate WT β-catenin and that mutations of tyrosine 654 and 670 to phenylalanine leads to diminished phosphorylation of β-catenin. Our data demonstrate that Ron receptor activation by HGFL stimulation leads to the tyrosine phosphorylation, nuclear localization and activation of β-catenin and β-catenin target genes in breast cancer cell lines. Furthermore, we show that mutation of β-catenin at residues 654 and 670 from tyrosine to phenylalanine leads to a loss of HGFL-induced, Ron-dependent β-catenin nuclear localization, transcriptional activity and target gene expression. We further show that targeted deletion of β-catenin in Ron expressing breast cancer cells results in decreased cell proliferation in vitro which can be rescued by expressing wild type β-catenin but not the 654/670 mutant of β-catenin. Interestingly, while the β-catenin knockout cells grew approximately 50% less efficiently than β-catenin expressing cell in vitro, β-catenin expression was required for growth and metastasis of Ron expressing breast cancer cells in vivo. We also found that β-catenin F/F cells that were highly tumorigenic and had broadened spectrum of metastasis, had no E-Cadherin expression and that deletion of β-catenin in these cells restored expression of E-Cadherin (date not shown), suggesting that loss of E-Cadherin may contribute of effects of β-catenin deletion in vivo. These studies are consistent with reports wherein knockdown of β-catenin expression was able to reduce RET-mediated tumor growth and invasiveness of RET overexpressing NIH 3T3 cells in subcutaneous xenograph models in nude mice (Gujral et al 2008). In addition, mutation of β-catenin at both tyrosine residues 654 and 670 was able to block hepatocyte growth factor (HGF) induced β-catenin nuclear translocation, activation and proliferation downstream of Met receptor activation in hepatic cancer cell
lines (Zeng et al 2006). Combined, these studies show that β-catenin is a critical factor downstream of a variety of receptor tyrosine kinases for promoting tumor cell proliferation. Further, our studies combined with previous reports on the Met receptor, demonstrating that WT β-catenin but not the 654/670 mutant of β-catenin can restore cellular proliferation, suggest a novel physiological role of receptor tyrosine kinases in inducing β-catenin-dependent proliferation. Moreover, our data support the contention that β-catenin is an important downstream effector or Ron-mediated cell proliferation in vitro and tumor growth in vivo.

In addition to β-catenin accumulation, we have also shown that HGFL-induced Ron activation results in increased levels of both cyclin D1 and c-myc. The expression of cyclin D1 and c-myc in response to HGFL was dependent on both Ron and β-catenin expression as knockdown of either Ron or β-catenin in the breast cancer cells was able to inhibit cyclin D1 and c-myc induction. In addition, expression of the β-catenin 654/670 double mutant of β-catenin was also able to blunt the induction of these genes in response to HGFL stimulation. Both c-myc and cyclin D1 have been shown to be overexpressed in different human cancers including breast cancer. While amplification of the cyclin D1 gene has been observed in about 15% of breast cancers, overexpression of cyclin D1 at the mRNA and protein levels is observed in about 50% of human breast cancers and is mostly associated with ER-positive tumors (Taneja et al 2010). Alterations in c-myc are a common event in breast cancer, although c-myc levels alone have been difficult to correlate as a predictive or prognostic factor based on the complexity of c-myc expression and activity (Hynes and Stoelzle 2009). Interestingly both c-myc and cyclin D1 are converging effectors of both estrogen and growth factor signaling cascades in breast cancer with the potential to mediate resistance to endocrine directed therapy. It is interesting to speculate that Ron receptor overexpression might be an important marker of breast cancer progression and endocrine responsiveness prompting the need for a greater understanding of Ron and Ron downstream mediators in breast cancer. In summary, our studies have identified a novel downstream pathway of Ron receptor activation that is important for breast cancer cell proliferation, tumor growth and spread. We show that HGFL stimulation of Ron induces tyrosine phosphorylation, nuclear localization and transcriptional
activation of β-catenin. This association of Ron and β-catenin is observed in both human and murine breast cancers and supports the potential of anti-Ron therapy in breast cancer due to the impact of Ron receptor signaling on tumor-established pathways.
Materials/Subjects and Methods

Cell Lines and Reagents. T47D, MDA-MB-231, and MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). The murine R7 breast cancer cell line was derived from a mammary tumor obtained from transgenic mice overexpressing Ron in the mammary epithelium (MMTV-Ron mice) (Zinser et al 2006). β-cateninF/F mammary tumor cell lines were generated from mammary tumors derived from MMTV-Ron that were crossed to into a homozygous β-catenin floxed (F/F) background (Huelsen et al 2001). Antibodies for Western analyses included anti-Ron β (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin (BD Biosciences, San Jose, CA and Cell Signaling Technology, Danvers, MA), anti- mouse cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-human cyclin D1 and c-myc (Thermo Scientific, Fremont, CA), anti-Flag antibody (Sigma Aldrich, St. Louis, MO), and anti-phosphotyrosine 4G10 (Millipore, Temecula, CA). Recombinant HGFL was purchased from R&D Systems (Minneapolis, MN). SB 216763 (GSK-3β inhibitor) was a kind gift from Dr. Jorge Moscat (University of Cincinnati).

Immunohistochemistry on Human Breast Cancer Specimens

Immunohistochemistry was performed on tissue microarrays (Cat. #CCB2 and CBA2, SuperBioChips Laboratories, New York, NY). Tissue staining and scoring were performed as previously described (Thobe et al 2010). Samples with no primary antibody or an IgG control antibody served as negative controls. High Ron expression was set at 190 or above and high β-catenin expression was set at 225 or above.

Plasmids. Wild-type (WT) β-catenin and Tyr 654/670 Phe (DM) β-catenin cloned into the p3XFLAG-CMV vector have been previously described (Zeng et al 2006). The human Ron shRNA (cat # RHS3979-9571732) and mouse Ron shRNA (cat # RMM3981-9590952) viral vector constructs were purchased from Open Biosystems (Huntsville, AL).
**Immunoprecipitation and Immunoblotting.** Cells were serum starved overnight and treated with HGFL (100 ng/ml) for the indicated times. Nuclear and cytoplasmic extracts were made as described previously (Dignam et al 1983). Immunoprecipitations and Western analyses were performed as previously described (Zinser et al 2006).

**Kinase Assay:** Flag tagged WT and DM β-catenin were transfected into HEK-293 cells. Flag WT and DM β-catenin were immunoprecipitated using 2 ug anti-Flag antibody (Sigma Aldrich, St. Louis, MO) from 500 ug total protein followed by incubation with protein G agarose beads (Upstate, Billerica, MA) overnight at 4°C. Ron protein was immunoprecipitated using 2 ug Mouse MSP-R Polyclonal antibody (R and D Systems, Minneapolis, MN) from 1mg total tissue lysate of mammary tumors from MMTV-Ron mice followed by incubation with protein G agarose beads overnight at 4°C. Immunocomplexes were washed 5 times in wash buffer followed by 2 washes in kinase buffer. For kinase reaction the immunocomplexes were combined together with γ-ATP-32P (12.5 μCi) for 45 minutes at 37°C. For positive control 20 ug dephosphorylated myelin basic protein (substrate, Upstate, Billerica, MA) was used. Reactions were terminated by addition of sample buffer and subjected to SDS-PAGE. The gel was fixed, dried and exposed to phosphoimager. Equal amounts of Flag WT and DM beads that were used as input for kinase reaction were also resolved on SDS-PAGE gel to confirm equal loading.

**Transient Reporter Assays.** The cells were transfected with the TCF reporter plasmid TOP-FLASH (Upstate Biotechnology, Lake Placid, NY) expressing firefly luciferase and pRLTX expressing Renilla luciferase (Clontech, Mountain View, CA) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 hours. The cells were then treated with HGFL (100 ng/ml) for 2 hours followed by a determination of luciferase activity according to the manufacturer’s instructions (Promega, Madison, WI).

**Viral Infection.** T47D and R7 cells were infected with human Ron shRNA and mouse Ron shRNA lentivirus, respectively. Cells were selected in media containing 0.5μg/ml and 5.0μg/ml of puromycin,
respectively, and polyclonal stable cell lines were generated. For genetic deletion of \( \beta \)-catenin, the \( \beta \)-cateninF/F cells were infected with Adenovirus Cre-GFP. The high GFP-expressing cells were isolated the following day utilizing a BD FACS Aria sorter.

**Cell Growth and Proliferation Assay.** To examine cell number over time, \( \beta \)-cateninF/F and \( \beta \)-catenin-/- cells were plated in triplicate on 24-well plates at a density of 5000 cells/well. Crystal violet staining was performed at 0, 24, 48, 72, and 96 hours after plating as previously described (Thobe et al 2010). For measurements of proliferation, \( 3 \times 10^4 \) \( \beta \)-cateninF/F or \( \beta \)-catenin-/- cells were plated on plastic coverslips. The cells were incubated with BrdU (3 \mu g/ml) for 4 hours, and BrdU staining was performed according to the manufacturer’s protocol (GE Healthcare, Piscataway, NJ). For rescue experiments, \( \beta \)-cateninF/F and \( \beta \)-catenin-/- cells were plated in 12-well plates at a density of \( 2.5 \times 10^4 \) cells/well. Cells were transfected utilizing the Nucleofector II kit (Amaxa Biosystems, Gaithersburg, MD) with WT- or DM-\( \beta \)-catenin. After 48 hours, MTT assays were performed.

**Quantitative Real-Time PCR.** To measure \( \beta \)-catenin transcript levels, quantitative real-time PCR was performed as previously described (Meyer et al 2009). For these experiments, the following primers were utilized: \( \beta \)-catenin 5’-TCCCTGAGACGCTAGATGAGG-3’ and 5’-CGTTTAGCAGTT TTGTCAGCTC-3’. Gene expression values were normalized to \( \beta \)-glucuronidase 5’-TTGAGAACTGTTATAAGACGCATCAG-3’ and 5’-TCTGGTACTCCTCCTGAACATGC-3’ as an internal control. Relative gene expression results are reported. Real-time analyses were repeated twice with similar results using samples from 3 individual isolations.

**Tumor Growth in Nude Mice.** Athymic female nude mice were purchased from the National Cancer Institute. \( 2 \times 10^6 \) \( \beta \)-cateninF/F (n=6) and \( \beta \)-catenin-/- (n=7) cells in 50% Matrigel (BD Biosciences, San Jose, CA) were injected into the mammary fat pad of the nude mice. Tumor measurements were collected and expressed as described previously (Miller et al 2006, Peace et al 2001). All the animal experiments
were done following a protocol approved by University of Cincinnati Institutional Animal Care and Use Committee.

**Statistical Analysis.** Statistical significance for all experimental analyses was determined by Student’s t-test or one-way ANOVA utilizing GraphPad Prism 4.0 software (GraphPad Software, Inc. La Jolla, CA). For the tissue microarray analyses, Pearson correlation analysis, $\chi^2$ tests and the Wilcoxon rank sum tests were all utilized (SAS® version 9.1.3, Cary, NC).
References


Figure 1. Ron and β-catenin expression and phosphorylation in human breast cancer.  

A, Serial sections from human breast cancer tissue arrays were stained for Ron and β-catenin. Representative images are shown. B, Human breast cancer cell lines were screened for Ron and β-catenin expression by Western analysis. Actin was used as a loading control. C, In vitro kinase assay associated with Ron using WT and DM β-catenin as substrate. WT and DM β-catenin were transfected in HEK293 cells. Kinase assay was performed by using immunoprecipitated Ron from mammary tumors of MMTV-Ron mice or a control IgG antibody and the substrates were immunoprecipitated WT or DM β-catenin. Dephosphorylated myelin basic protein (MBP, substrate) was used as positive control (data not shown). A representative kinase assay from three independent experiments with similar results is shown. Total input for Ron, β-catenin and Flag is shown as control.
Figure 2. Ron induces β-catenin nuclear localization and tyrosine phosphorylation. A, Ron activation induces nuclear localization of endogenous β-catenin. T47D cells were treated with HGFL (+) or vehicle (-). After 2 hours of treatment, nuclear and whole cell extracts were generated and examined by Western analysis for β-catenin levels. Expression of PARP and Tubulin were utilized as loading.
controls for the purity of the nuclear and whole cell extracts, respectively. B, T47D overexpressing WT and DM β-catenin were lysed and immunoblotted for Flag and β-catenin expression. Actin was used as loading control. C, Ron induces nuclear localization of exogenous WT β-catenin but not the DM form of β-catenin. T47D cells stably expressing exogenous WT and DM Flag tagged β-catenin were treated with vehicle or HGFL for 2 hours and fractioned cell extracts were examined by Western analysis as in A with the inclusion of an antibody recognizing Flag tagged exogenous β-catenin. D, Quantification of the level of exogenous WT or DM β-catenin normalized to controls (PARP or Tubulin) in the nuclear, cytoplasmic or total cell lysate of vehicle or HGFL treated cells. Note, the T47D cells transfected with DM β-catenin showed attenuated levels of β-catenin nuclear localization in response to HGFL compared to controls. All results are representative of at least 4 independent experiments.
Figure 3. *Ron activation induces β-catenin-dependent transcriptional activity.*  

**A**, Stable Ron knockdown in T47D and R7 breast cancer cells. T47D and R7 cells were transduced with Ron shRNA (shRon) or Non-target (NT) shRNA lentiviral plasmids. T47D and R7 cells transduced with shRon and shNT constructs were lysed and analyzed for Ron expression by immunoblotting. Actin was used as loading control.  

**B**, Ron activation by HGFL induced β-catenin-dependent TOP-FLASH reporter activity. T47D shNT, T47D shRon, T47D-WT β-catenin, T47D-DM β-catenin, R7 shNT and R7 shRon cells were transiently transfected with a β-catenin reporter plasmid (TOP-FLASH) and a control reporter plasmid pRL-TK for normalization of transfection efficiency. Following transfection, the cells were treated with vehicle or HGFL (100ng/ml) for 2 hours and a dual-luciferase assay was performed. Following transfection with TOP-FLASH reporter the T47D shNT and T47D shRon cells were also treated with DMSO or SB 216763 (5μM) for 16 hours and dual luciferase reporter assay was performed. Columns represent the average fold change of three independent experiments; bars represent Standard Error (SE).
*, P < 0.05 compared to the corresponding vehicle treated control group. These results are representative of three independent experiments with similar results.

C, HGFL-induced cyclin D1 expression in T47D cells is inhibited by knocking down Ron. Control T47D shNT cells and T47D shRon cells were treated with HGFL for 2 hours. Cell lysates were generated and examined for Ron and cyclin D1 expression. Actin was used as loading control. D, SB 216763 treatment rescues proliferation in Ron knockdown cells. T47D shNT, T47D shRon, R7 shNT and R7 shRon cells were plated in triplicate in 24 well plate and treated with DMSO or SB 216763 (5 μM) for 72 hours. Cell number was measured using MTT colorimetric assay. Columns represent the average fold change of three independent experiments; bars represent Standard Error (SE). *, P < 0.05 compared to the corresponding vehicle treated control group.
Figure 4. β-catenin deletion in breast cancer cell lines leads to a decrease in cell proliferation in vitro. A, Ron and β-catenin expression was analyzed by Western in three independent mammary tumor cell lines (β-cateninF/F) derived from MMTV-Ron expressing mice containing a Floxed β-catenin allele. Actin expression was used as a loading control. B, The β-cateninF/F cells were infected with an adenovirus expressing Cre recombinase and GFP. GFP-positive (β-catenin-/−) and negative (β-cateninF/F) cells were isolated by FACS sorting and analyzed for β-catenin expression Western analysis. C, Quantitative real-time PCR was utilized to examine β-catenin mRNA expression in β-cateninF/F and β-catenin-/− cells. Relative mRNA levels are shown normalized to an internal control. D, Deletion of β-catenin leads to decreased proliferation. β-cateninF/F and β-catenin-/− cells were incubated with BrdU for 4 hours and immunocyto-staining for BrdU incorporation was performed. The percent of cells staining positive for BrdU was quantitated from three independent high power fields and the data tabulated is a result from three independent experiments. *, P < 0.05. E, β-cateninF/F and β-catenin-/− cells were
plated in triplicate in 24 well plates. Cell number was measured at 0, 24, 48, 72 and 96 hours by crystal violet staining assays. The graph is representative of the three independent experiments. *, P < 0.05 compared to the corresponding β-catenin-/- cells.
Figure 5. Transfection of WT β-catenin but not DM β-catenin partly rescues cell growth of β-catenin-/- cells.  

A, β-catenin-/- cells were transiently transfected with Flag-tagged empty vector (EV), WT, or DM β-catenin. After 48 hours, cell lysates were generated and examined for Flag expression by Western analysis. Actin expression is provided as a loading control.  

B, β-catenin-/- cells were transfected with Flag-tagged empty vector (EV), WT, or DM β-catenin. After 48 hours, the cell number was quantiated utilizing an MTT assay. The experiment was performed three times in triplet and the data shown represents the mean of all experiments ± SE. *, P <0.05.
Figure 6. Deletion of β-catenin prohibits mammary tumor formation and metastasis following implantation in the mammary fat pad of nude mice. A, β-cateninF/F and β-catenin-/− cells were orthotopically transplanted into the mammary fat pads of nude mice. Mammary tumor growth was measured biweekly for up to 6 weeks and tumor volume was plotted over time. B, Mammary tumor weight of mice transplanted with β-catenin-/− and β-cateninF/F cells at 6 weeks. The inset depicts a representative picture of the mammary glands. *, P < 0.05 compared to the corresponding weight of the β-cateninF/F implanted glands. C, Hemotoxlyn and Eoisin staining of mammary tissue of glands implanted β-catenin F/F and β-catenin-/− cells. Bars=200 μm.
Supplemental Figure S1. Ron activation increased expression of β-catenin target genes. T47D, T47D-WT β-catenin, and T47D-DM β-catenin cells were treated with vehicle or HGFL (100 ng/ml) for 2 hours. Cell lysates were subsequently subjected to immunoblotting and probed for Flag, β-catenin, cyclin D1, cmyc, and Actin as loading control.
Two independent human breast cancer tissue arrays were stained for both Ron and β-catenin. The percent of Ron high and low expressing tissue samples is listed [Total Samples]. The number in parentheses shows the percentage of samples in the indicated group. There was a significant (p=0.0012) difference in the percentage of high β-catenin between the low and high Ron groups.

Table 1. Ron and β-catenin expression in human breast cancer specimens.

<table>
<thead>
<tr>
<th></th>
<th>Total Samples</th>
<th>β-cat High</th>
<th>β-cat Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ron High</td>
<td>34/76 (45%)</td>
<td>23/34 (68%)</td>
<td>11/34 (32%)</td>
</tr>
<tr>
<td>Ron Low</td>
<td>42/76 (55%)</td>
<td>15/42 (36%)</td>
<td>27/42 (64%)</td>
</tr>
</tbody>
</table>
Table 2. Metastatic dissemination of β-cateninF/F cells.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>100% (6/6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.7% (1/6)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>16.7% (1/6)</td>
</tr>
<tr>
<td>Lung</td>
<td>83.3% (5/6)</td>
</tr>
<tr>
<td>Liver</td>
<td>50% (3/6)</td>
</tr>
</tbody>
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The organ site of metastatic dissemination and percentage of mice exhibiting site specific metastatic tumor cell dissemination were determined in mice at 6 weeks post-orthotopic implantation of β-cateninF/F cells into the mammary fat pad. The percentage of mice injected which exhibited metastasis to the specific organ site is indicated. The number in parentheses represents the number of mice with site specific metastasis/number of mice examined.
CHAPTER III

Conditional Deletion of β-catenin in Mammary Epithelial Cells of Ron Receptor Overexpressing Mice Alters Mammary Tumorigenesis

This Chapter is conditionally accepted in Endocrinology, 2011.
Abstract

The Ron receptor tyrosine kinase is overexpressed in approximately 50% of human breast cancers. Transgenic mice that overexpress Ron in the mammary epithelium (MMTV-Ron mice) develop mammary tumors that exhibit upregulation of β-catenin and β-catenin target genes. β-catenin has been shown to be a mediator of mammary tumorigenesis in various breast cancer models including downstream of Ron. However, the in vivo impact of β-catenin downstream of Ron receptor activation on the development, growth and metastasis of mammary tumors has not been well-defined. To determine the significance of β-catenin in the context of Ron overexpression, we conditionally deleted β-catenin in mammary epithelial cells of MMTV-Ron mice. Conditional deletion of β-catenin in the mammary epithelium, through the use of whey acidic protein (WAP)-Cre transgenic mice, significantly delayed the onset of mammary hyperplastic nodules, the presence of palpable mammary tumors, and ultimately decreased liver metastasis. β-catenin loss in this model was also associated with decreased expression of cyclin D1. In total, studies support an important role for β-catenin downstream of Ron receptor signaling during the development of mammary tumorigenesis.
Introduction

Breast cancer is the second leading cause of cancer death among women in the United States (American Cancer Society, 2009-2010). Standard treatments for breast cancer include radiation therapy, chemotherapy, hormone therapy, and/or targeted therapy. While the use of specific monoclonal antibodies and tyrosine kinase inhibitors in combination with chemotherapy have shown promise in clinical studies, breast tumor reoccurrence and the lack of continued disease free survival persists (1-2). Therefore, the identification of new biomarkers and drug targets remains a high priority.

The Ron receptor tyrosine kinase is upregulated in about half of human breast cancers (3-4). In addition to breast cancer, Ron overexpression is also seen in several human epithelial cancers including lung, stomach, colon, pancreas, and prostate cancers (2, 5-7). Overexpression of Ron has been shown to induce proliferation and migration of cells in vitro and tumor formation in vivo (2, 8-9). Recently expression of Ron, hepatocyte growth factor-like protein (HGFL, the Ron ligand), and matriptase, a serine protease that cleaves pro-HGFL, were associated with poor overall survival among patients without metastatic disease at the time of primary tumor resection (10). Ron activation by HGFL has been shown to trigger activation of a number of signaling cascades including phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase (MAPK), c-Jun NH\textsubscript{2}-terminal kinase, and β-catenin, which affect cell proliferation, differentiation, and migration (6-7, 11-13).

We have previously shown that overexpression of Ron in the mouse mammary epithelium induces tumor formation in 100% of female mice and is associated with elevated levels of β-catenin and the upregulation of β-catenin target genes, including cyclin D1 and c-myc (9). Increased β-catenin, localized to the nuclear and cytoplasmic compartments, is observed in about 40% of primary breast tumors and is associated with poor prognosis and poor patient survival (14). Dysregulated β-catenin signaling leads to perturbation of mammary stem and progenitor cell dynamics as well as mammary tumor formation in mice (15-18). The Ron receptor is also an important regulator of pubertal mouse
mammary gland development as mice with a targeted deletion of the Ron tyrosine kinase domain exhibit alterations in mammary ductal extension and branching morphogenesis (19).

Based on these reports, we hypothesized that Ron-induced β-catenin activation is important for mammary tumor initiation, progression and metastatic disease in vivo. Herein, we show that decreasing the expression of β-catenin within the mammary epithelium of transgenic mice overexpressing Ron induces a delay in palpable mammary tumor development compared to control mice. These differences were associated with a delay in the onset of mammary hyperplasia and the downregulation of the β-catenin target gene cyclin D1. Our findings demonstrate and further support an important link between the Ron receptor and its downstream signaling mediator, β-catenin, in regulating mammary gland tumorigenesis in vivo.
Results

Deletion of β-catenin delays the onset of mammary hyperplasia in MMTV-Ron mice. To determine the impact of altering β-catenin expression within the mammary epithelium on Ron-induced mammary tumorigenesis, we utilized the MMTV-Ron transgenic mice, which we previously showed develop aggressive and highly metastatic breast cancer as a result of Ron receptor overexpression in the mammary epithelium (9). We subsequently crossed MMTV-Ron mice with mice containing a floxed β-catenin allele (β-cat<sup>F/F</sup>) (20). MMTV-Ron β-cat<sup>F/F</sup> mice were then crossed with the well-characterized WAP-Cre mice to delete β-catenin during pregnancy and lactation (21). To generate study animals, MMTV-Ron β-cat<sup>F/F</sup> mice were bred to WAP-Cre β-cat<sup>F/F</sup> mice and corresponding offspring utilized.

To define the impact that deletion of β-catenin has on the incidence and initiation of hyperplasia and palpable mammary tumors in MMTV-Ron mice, we isolated mammary glands at 2.5, 4, 6, 8, and 10 months of age from MMTV-Ron β-cat<sup>F/F</sup> (control) and MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre mice. Glands were subsequently examined by whole mount and histological analysis for the presence of hyperplastic nodules. Hyperplasia was determined by the presence of a congregated cell mass around the ducts of the mammary gland. Hyperplastic areas were identified in control MMTV-Ron β-cat<sup>F/F</sup> mammary glands as early as 2.5 months of age with the majority of animals exhibiting mammary hyperplasia by 4 months of age (Figure 1A). In contrast, no hyperplasia was evident in MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre glands at 2.5 months and only sparsely found by 4 months of age (Figure 1B). A significant difference in the percentage of mice with hyperplastic nodules was found at 4 months comparing the MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre versus the MMTV-Ron β-cat<sup>F/F</sup> control mice. The percentage of MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre animals with hyperplastic glands remained less throughout the time course, but did not reach statistical significance. By 10 months of age, the extent of hyperplasia in the mammary glands of MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre mice still had not approached that of the controls in which all mice exhibited mammary hyperplasia at this time frame. In total, mammary glands from MMTV-Ron β-cat<sup>F/F</sup> WAP-Cre
(Figure 1B) mice had a delay in the onset of mammary gland hyperplastic nodules compared with control mice at every time point examined with statistical differences observed at 4 months between groups.

**β-catenin expression is reduced in MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice during involution.** To examine the extent of β-catenin in MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice, mammary glands from MMTV-Ron β-cat\(^{FF}\) and MMTV-Ron β-cat\(^{FF}\) WAP-Cre were isolated from age-matched female mice during their first involution cycle at involution day 6. As depicted in Figure 2A, mammary glands from MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice exhibited significantly reduced β-catenin expression as analyzed by Western analyses compared to control mice. Immunohistochemical staining of the mammary glands from these mice demonstrated reduced β-catenin expression in MMTV-Ron β-cat\(^{FF}\) WAP-Cre glands as compared to controls (Figure 2B) although a mosaic pattern of β-catenin expression was observed. No overt abnormalities were identified in the involuting mammary glands of MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice compared to controls based on whole mount and histological analyses (Figure 2C).

**WAP-Cre-induced deletion of β-catenin delays palpable mammary tumor development in MMTV-Ron mice.** Mammary tumor kinetics as a result of β-catenin loss by WAP induced expression of Cre is depicted in Figure 3A. While mammary tumors form in 100% of the mice from both groups, palpable tumor development was significantly delayed in the MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice versus the control mice. The MMTV-Ron β-cat\(^{FF}\) WAP-Cre mice had a median time to palpable mammary tumor formation of 312 days versus the MMTV-Ron β-cat\(^{FF}\) mice having a median of 283 days.

**WAP-Cre-induced β-catenin deletion results in an altered mammary tumor phenotype.** WAP-Cre-induced β-catenin deletion produced mammary tumors which displayed a different histology compared to mammary tumors from MMTV-Ron β-cat\(^{FF}\) mice. MMTV-Ron β-cat\(^{FF}\) WAP-Cre mammary tumors had
a high propensity to contain keratin pearls which consisted of concentric layers of abnormal squamous cells with high deposition of cytokeratins (Figure 3B). This phenotype was present in 12 out of 18 tumors analyzed (67%). Mammary tumors from MMTV-Ron β-catFF mice displayed phenotypes consistent with adenocarcinomas with varying degrees of large cells and regions of desmoplasia as previously described (9).

Characterization of β-catenin expression in mammary glands from MMTV-Ron β-catFF and MMTV-Ron β-catFF WAP-Cre mice at 4 months of age. A large percentage of control mammary glands showed the presence of hyperplastic nodules by 4 months of age. In contrast, only 15% of the mammary glands from MMTV-Ron β-catFF WAP-Cre mice displayed hyperplasia at this time point. To correlate this difference to changes in β-catenin expression, mammary glands from 4 month old MMTV-Ron β-catFF and MMTV-Ron β-catFF WAP-Cre mice were isolated. A significant decrease in β-catenin mRNA and protein expression was observed in MMTV-Ron β-catFF WAP-Cre glands as compared to MMTV-Ron β-catFF glands at this time frame (Figure 4A and 4B). Immunohistochemical staining for β-catenin was also performed on MMTV-Ron β-catFF and MMTV-Ron β-catFF WAP-Cre glands (Figure 4C), which also showed decreased β-catenin expression in the MMTV-Ron β-catFF WAP-Cre glands as compared to the controls. Interestingly, at this early time point, there were no differences observed in the percentage of cells staining positive for BrdU incorporation or TUNEL staining between genotypes (Figure 4D and 4E). These results show efficient β-catenin deletion in the mammary epithelium at 4 months of age, a time point when hyperplastic nodules are less prevalent in the MMTV-Ron β-catFF WAP-Cre glands even though proliferation and apoptotic rates are not significantly different between genotypes.
Reduced β-catenin expression in mammary glands from MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice at 8 months. Hyperplastic nodules were present in a majority of the mammary glands assessed from MMTV-Ron β-cat<sup>FF</sup> and MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice by 8 months of age. To determine the extent of β-catenin deletion at this time point, mammary glands from MMTV-Ron β-cat<sup>FF</sup> and MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice were isolated and examined for β-catenin expression by qRT-PCR (Figure 5A), Western analysis (Figure 5B) and by immunohistochemistry (Figure 5C). A significant reduction in β-catenin mRNA and protein expression was observed in MMTV-Ron β-cat<sup>FF</sup> WAP-Cre glands as compared to controls. In addition, MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mammary glands displayed significantly reduced proliferation, as indicated by less BrdU incorporation, and reduced apoptosis, quantitated by TUNEL staining in comparison to control glands at this time point (Figure 5D and 5E).

β-catenin expression in end stage mammary tumors from MMTV-Ron β-cat<sup>FF</sup> and MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice. By 10 months of age, mammary tumors with desmoplastic regions and a morphologically distinct keratin pearl phenotype were evident in the MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice (Figure 3B). Tumors were isolated from mice of each genotype when they reached end stage (defined as when the tumors represented approximately 10% body weight) to determine the extent of β-catenin loss. As shown in Figure 6A and 6B, end stage mammary tumors from MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mouse exhibited a significant loss of β-catenin mRNA and protein expression compared to tumors from control mice. Similarly, mammary tumors from MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice displayed a corresponding decrease in Cyclin D1 expression, a β-catenin target gene, compared to controls (Figure 6A and 6B). Immunohistochemical staining for β-catenin within end stage tumors also demonstrated a significant reduction in β-catenin expression in the MMTV-Ron β-cat<sup>FF</sup> WAP-Cre tumors compared to controls, although β-catenin expression was observed to be mosaic in regions of the MMTV-Ron β-cat<sup>FF</sup> WAP-Cre tumors (Figure 6C). At this late stage of tumor development, there were no significant differences observed in mammary tumor cell proliferation or cell death between groups (Figure 6D and 6E).
Decreased metastatic burden to the liver in MMTV-Ron mammary tumors with β-catenin deleted.

Serial sections of the lung and liver from control MMTV-Ron β-cat$^{F/F}$ and MMTV-Ron β-cat$^{F/F}$ WAP-Cre mice were examined histologically for the presence of mammary tumor metastasis. A high frequency of metastasis to the lungs and liver was observed in both groups (Table 1). Interestingly, the incidence of liver metastasis in MMTV-Ron β-cat$^{F/F}$ WAP-Cre mice was significantly reduced compared to that observed in the MMTV-Ron β-cat$^{F/F}$ controls. Figure 7 shows representative images of lung and liver metastases from control MMTV-Ron β-cat$^{F/F}$ and MMTV-Ron β-cat$^{F/F}$ WAP-Cre mice.
Discussion

In this study we examined the effect of β-catenin deletion in the context of Ron overexpression in the mammary gland. Here, we show that the development of mammary hyperplasia is delayed in MMTV-Ron mice when β-catenin is deleted through the use of WAP-Cre transgenic mice. The onset of palpable mammary tumor formation in MMTV-Ron β-catF/F WAP-Cre was also significantly delayed compared to the control mice with a median tumor latency of 312 days in the MMTV-Ron β-catF/F WAP-Cre mice compared to 283 days in the control mice. This increase in the time to develop palpable tumors is consistent with our data showing a decrease in the proliferation rate of the MMTV-Ron β-catF/F WAP-Cre hyperplastic nodules, corresponding with the loss or reduction in β-catenin and cyclin D1 expression in the mammary tumors of the MMTV-Ron β-catF/F WAP-Cre mice compared to controls. Additionally, liver metastases in the MMTV-Ron β-catF/F WAP-Cre were also significantly reduced compared to control mice. However, metastasis to the lung was not different between the two groups. These data may imply that lung metastases occur as an earlier event in the MMTV-Ron mice compared to liver metastases or that β-catenin expression may be important in the establishment or growth of liver metastases. In total, these studies suggest that continued suppression of β-catenin within the mammary epithelium results in a delay in mammary tumor onset and decreased overall metastatic tumor burden.

Histologically, MMTV-Ron β-catF/F WAP-Cre mammary tumors were also highly associated with foci of central keratinization found within concentric layers of abnormal squamous cells. Breast cancer from patients and mice with alterations in BRCA1 frequently exhibit basal-like phenotypes which contain metaplastic elements consisting of neoplastic spindle cells or squamous cells with the presence of keratin pearls (23-25). These similarities suggest that mammary tumors from the MMTV-Ron β-catF/F WAP-Cre mice may have attributes of a murine model of basal-like breast cancer, although further investigation into these tumors is warranted to determine if this models may prove useful for examining new treatment modalities that target BRAC1-driven and/or basal-like carcinomas.
While the tumors in both the MMTV-Ron β-cat$^{FF}$ and MMTV-Ron β-cat$^{FF}$ WAP-Cre mice were very aggressive, the disparate tumor phenotypes in the MMTV-Ron β-cat$^{FF}$ and MMTV-Ron β-cat$^{FF}$ WAP-Cre models may be due to a combination of factors. While efficient β-catenin loss was observed in MMTV-Ron β-cat$^{FF}$ WAP-Cre mammary tumors compared to those from MMTV-Ron β-cat$^{FF}$ mice, it is important to note that the pattern of β-catenin deletion was mosaic. This point is important as β-catenin has been shown to play pivotal roles in the mammary gland regulating stem cell self renewal and multipotency, as well as promoting proliferation and cell survival during various stages of mammary gland development (17, 26-27). Further studies are needed to determine if the phenotypes observed reflect β-catenin loss in different mammary epithelial cell types, the precise timing of this loss and/or the extent of deletion of this protein during MMTV-Ron induced tumorigenesis.

Overall, our data support prior studies which demonstrate that Ron overexpression leads to the development of highly aggressive, metastatic mammary tumors (9, 28). More specifically, prior work showed that Ron overexpression in the mammary epithelium of mice lead to mammary tumors which exhibited increases in β-catenin expression, activation and tyrosine phosphorylation. Recently published studies have further identified an important role of the Ron receptor tyrosine kinase, and the Ron ligand hepatocyte growth factor-like protein (HGFL), in the activation of β-catenin in breast cancer cells through tyrosine phosphorylation of tyrosine residues 654 and 670 in β-catenin (28). An absolute requirement for β-catenin expression was also shown for the growth of breast cancer cell lines in vivo following orthotopic transplantation into the mammary glands of nude mice. Despite efficient deletion of β-catenin in the studies herein, complete loss of β-catenin was not observed. Combined with our prior studies in breast cancer cell lines, these data suggest that β-catenin may be an essential protein in the mammary gland wherein reduced levels of this protein are permissive for tumor growth.

In an examination of human breast cancer specimens, a statistically significant correlation between high Ron expression and high β-catenin expression was observed (28). Breast cancer patients with high Ron and high β-catenin expression exhibited significantly reduced survival within a 30-month
follow-up period and more lymph node metastasis compared to patients with low Ron and β-catenin expression. In this report, we show that tumor formation in MMTV-Ron mice is regulated by β-catenin in vivo wherein reduced β-catenin expression leads to increases in tumor latency, decreases in tumor growth and lessened metastasis. The extent and timing of β-catenin expression may play an important role in tumor growth, may regulate aspects of tumor histology, and may possibly alter ensuing responses to breast cancer treatment. Further understanding of the complex spatiotemporal expression of β-catenin will be required to provide insights into the development and eventual treatment of patients which exhibit activation of this pathway in breast cancer.
Materials and Methods

Generation of mice. Mice with mammary specific Ron overexpression, referred to as MMTV-Ron mice and mice with a floxed β-catenin allele were previously described (9, 20). β-catenin deletion in the mammary glands of MMTV-Ron β-cat\(^{F/F}\) mice was achieved by crossing MMTV-Ron β-cat\(^{F/F}\) mice to mice that expressed the WAP-Cre transgene (21). To promote tumor development in the MMTV-Ron mice, all study animals were continuously mated starting at approximately 8 weeks of age until tumor development. Female MMTV-Ron transgenic mice are unable to nurse their offspring to weaning. Therefore, all pups were removed within 1-2 days from the transgenic dams. Genotyping of the transgenic mice was performed by PCR analysis with the following primer sets: β-catenin primers forward 5’-ACT GCC TTT GTT CTC TTC CCT TCT G-3’, reverse 5’-CAG CCA AGG AGA GCA GGT GAG G-3’; WAP-Cre forward 5’- CAT CAC TCG TTG CAT CGA CC-3’, reverse 5’-TAG AGC TGT GCC AGC CTC TTC-3’. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Tumor Latency. Animals were palpated weekly for over 400 days to assess mammary tumor development. Tumor development in MMTV-Ron β-cat\(^{F/F}\) and MMTV-Ron β-cat\(^{F/F}\) WAP-Cre mice were compared as previously described (9, 11).

Tissue Histology. Tissues were processed as described previously (9). Briefly, mammary glands, lungs, and liver samples were fixed, paraffin embedded, and cut into 4μm sections. Sections were stained with hematoxylin and eosin for routine histological examination. For whole-mount analysis, mammary glands were fixed in Carnoy's fixative and stained overnight in carmine alum. Samples were dehydrated, cleared in xylene, mounted, and examined on a stereoscope equipped with an Axiovert digital camera.
Quantitative Real-Time (qRT)-PCR and Western Analysis. Total RNA was isolated from tissues using Trizol reagent (Invitrogen, Carlsbad, CA). qRT-PCR was performed, as previously described (19, 22), using the following primer sets: β-catenin forward 5’-TCCCTGAGACGCTAGATGAGG-3’ and reverse 5’-CGTTTAGCAGTTTTGTCAGCTC-3’; and Cyclin D1 forward 5’-GCGTACCCTGACACCAATCTC-3’ and reverse 5’-CTCCTCTTCGCACTTCTGCTC-3’. Gene expression values were normalized to β-glucuronidase: forward 5’-TTGAGAACTGGTATAAGACGCATCAG-3’ and reverse 5’-TCTGGTACTCCTCCTACTGAACATGC-3’ as an internal control. Relative gene expression is reported. qRT-PCR analyses were performed on samples from six independent isolations per genotype. Protein was isolated from tissues as described previously (9). Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). After transfer, the membranes were probed with a rabbit polyclonal anti-β-catenin antibody (1:1000, Cell Signaling, Danvers, MA). The membranes were stripped and reprobed with an anti-cyclin D1 antibody (1:1000, Neomarkers, Fremont, CA). Actin or tubulin expression was used as the loading control.

Proliferation and TUNEL Analysis. For BrdU analysis, mice were injected with 5-bromo-2’-deoxyuridine (BrdU) 2 hours prior to sacrifice and immunohistochemistry was performed on paraffin-embedded mammary tumor tissue sections as previously described (9, 11). The proliferation index was measured by quantification of BrdU-positive nuclei at 400X magnification and counting at least three independent fields per slide from at least three different tumors from each group. An In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer’s instructions to stain mammary tumor sections for the quantification of cell death as previously described (9, 11). Cell death was quantified by counting the number of terminal deoxynucleotidyl transfer mediated
dUTP nick end labeling (TUNEL)-positive nuclei in mammary tumor tissue at 400× magnification from three different tumors of each genotype.

**Statistical Analysis.** Statistical analysis for hyperplasia was determined using $\chi^2$ test. Statistical significance for BrdU incorporation, TUNEL analysis, qRT-PCR and tumor metastasis was determined by a Student's t-test using GraphPad Prism software (San Diego, CA). Data on tumor development was subjected to Kaplan-Meier analysis using GraphPad Prism software (GraphPad Software, San Diego, CA).
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Figure 1. Time course to assess the onset of mammary hyperplasia in MMTV-Ron β-cat\textsuperscript{FF} and MMTV-Ron β-cat\textsuperscript{FF WAP-Cre} mammary glands. Mammary hyperplasia was evaluated from inguinal #4 mammary glands taken at 2.5, 4, 6, 8, and 10 months of age in MMTV-Ron β-cat\textsuperscript{FF} (A) and MMTV-Ron β-cat\textsuperscript{FF WAP-Cre} (B) mice by whole mount and histological analysis (H&E, hematoxylin and eosin staining). The number of mice which displayed mammary hyperplasia by either whole mount or histological analysis is indicated along with the total number of mice evaluated per group. The numbers in parentheses indicated the percent of mice which exhibited mammary hyperplasia at the time point indicated. Although a reduction in the percentage of mice with hyperplastic nodules was consistently observed throughout the time course in the MMTV-Ron β-cat\textsuperscript{FF WAP-Cre} mice, only at 4 months was there a significant difference in the percentage of mice with hyperplastic nodules compared to MMTV-
Ron β-cat^{FF} control mice. *P<0.05 compared to the corresponding time point from MMTV-Ron β-cat^{FF} mice.
Figure 2. Efficient deletion of β-catenin in MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice at involution day 6. Mammary glands from age matched MMTV-Ron β-cat<sup>FF</sup> and MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice were isolated at involution day 6 and examined for the extent of β-catenin deletion by Western analysis (A) and immunohistochemistry (B). Insets show a 2-fold magnification of a representative mammary duct stained for β-catenin. Whole mount and H&E analysis of corresponding mammary glands from MMTV-Ron β-cat<sup>FF</sup> and MMTV-Ron β-cat<sup>FF</sup> WAP-Cre mice displayed no appreciable differences in mammary gland morphology (C). Scale bar=50 μm.
Figure 3. β-catenin loss in MMTV-Ron β-cat^{FF} WAP-Cre mice delays the onset of palpable mammary tumor formation in MMTV-Ron mice. A, β-catenin deletion in WAP-Cre transgenic mice significantly delayed mammary tumor formation. The percent of tumor free mice is plotted over time and results in a statistically significant delay in palpable tumor formation between groups (P≤0.05). Tumor incidence in MMTV-Ron β-cat^{FF} control and MMTV-Ron β-cat^{FF} WAP-Cre mice was similar with 100% of the animals developing mammary tumors. The median time to palpable mammary tumor formation in MMTV-Ron β-cat^{FF} WAP-Cre mice (n=26) was 312 days compared to 283 days in the control MMTV-Ron β-cat^{FF} mice (n=29). B, Representative images of mammary tumors from MMTV-Ron β-cat^{FF} and MMTV-Ron β-cat^{FF} WAP-Cre mice are shown and were taken from mice when tumors represented approximately 10% body weight in each genotype. Note the presence of keratin pearl deposits in tumors from MMTV-Ron β-cat^{FF} WAP-Cre mice versus well-differentiated mammary tumor histology in tumors from MMTV-Ron β-cat^{FF} mice. Scale bar=200μm.
Figure 4. Decreases in β-catenin expression are observed in mammary glands isolated from MMTV-Ron β-cat^{F/F} WAP-Cre mice at 4 months of age. **A**, Quantitative real-time (qRT)-PCR of β-catenin expression was performed on mammary glands isolated at 4 months of age from MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice. mRNA expression values were normalized to β-glucuronidase as an internal control and the relative expression of β-catenin is depicted. *P<0.05. **B**, Western analysis for β-catenin expression was performed on mammary glands isolated at 4 months of age from MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice. Actin was used as a loading control. **C**, Immunohistochemical staining for β-catenin was performed on mammary glands from MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice isolated at 4 months of age. Representative images are shown. Insets show a 2-fold magnification of a representative mammary duct stained for β-catenin. **D** and **E**, Mammary glands from 4 month old MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice
were examined for the extent of epithelial cell proliferation by BrdU immunohistochemistry (D) or for epithelial cell apoptosis by TUNEL staining (E). The number of cells positive for BrdU and TUNEL staining was quantitated per 0.25mm$^2$ field. Reduce proliferation and TUNEL staining is observed in the mammary epithelium from the MMTV-Ron β-cat$^{FF}$ WAP-Cre compared to controls, although this trend is not statistically significant at this time frame.
**Figure 5.** β-catenin expression is significantly reduced in mammary glands from 8 month old MMTV-Ron β-cat^{FF} WAP-Cre mice compared to controls. **A**, qRT-PCR of mRNA isolated from mammary glands of MMTV-Ron β-cat^{FF} and MMTV-Ron β-cat^{FF} WAP-Cre mice isolated at 8 months of age. **B**, Total protein extracts from mammary glands of MMTV-Ron β-cat^{FF} and MMTV-Ron β-cat^{FF} WAP-Cre were analyzed for β-catenin expression. **C**, Tissue sections of mammary glands from 8 month old mice were utilized for immunohistochemical analysis of β-catenin. Insets show a 2-fold magnification of a representative mammary duct stained for β-catenin. The extent of epithelial cell proliferation by BrdU immunohistochemistry (**D**) or for epithelial cell apoptosis by TUNEL staining (**E**) was examined in 8 month old mammary glands from MMTV-Ron β-cat^{FF} and MMTV-Ron β-cat^{FF} WAP-Cre mice. *P*<0.05. Scale bar=50μm
Figure 6. End stage tumors from MMTV-Ron β-catF/F WAP-Cre mice maintain β-catenin loss. qRT-PCR analysis of β-catenin (A) and Cyclin D1 (B) expression was performed on mRNA isolated from end stage mammary tumors of control and MMTV-Ron β-cat F/F WAP-Cre mice. *P<0.05. B, Western analysis was performed on mammary tumor lysates from control and MMTV-Ron β-catF/F WAP-Cre mice. The tumor lysates were analyzed for expression of β-catenin and Cyclin D1. Tubulin was used as the loading control. C, Representative immunohistological images of end stage mammary tumors from MMTV-Ron β-catF/F and MMTV-Ron β-catF/F WAP-Cre mice showing β-catenin expression are depicted. Insets show a 2-fold magnification of the noted area stained for β-catenin. D, Quantification of BrdU
incorporation to assess proliferation rates of mammary tumors in MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice is shown. \(E\), Quantification of TUNEL positive cells to assess the apoptotic rate in end stage mammary tumors isolated from MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice is shown. Scale bar=50µm
Figure 7. Metastatic dissemination in MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice. Representative images of metastatic lesions within the lungs and livers from MMTV-Ron β-cat^{F/F} and MMTV-Ron β-cat^{F/F} WAP-Cre mice are shown. Arrows indicate metastatic foci. Scale bar=200 μm.
Table 1. Number of mice with metastasis to the lung and liver represented by genotype.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>MMTV-Ron β-cat^{F/F} WAP-Cre</th>
<th>MMTV-Ron β-cat^{F/F} WAP-Cre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>28/31 (90%)</td>
<td>22/23 (95.6%)</td>
</tr>
<tr>
<td>Liver</td>
<td>21/24 (87.5%)</td>
<td>14/22 (63.6%)*</td>
</tr>
</tbody>
</table>

The number of mice with metastasis to the particular tissue/total number of mice evaluated for the select tissue is indicated. The percentage of mice with metastasis to the select tissue is provided in parenthesis.

*P<0.05 compared to liver metastasis observed in the MMTV-Ron β-cat^{F/F} mice.
CHAPTER IV

HGFL is important for Ron dependent mammary tumorigenesis
Ron overexpression in the mammary epithelium of mice lead to mammary tumors with 100% incidence and were associated with a high degree of metastasis to the lung and liver. However, the requirement of HGFL in tumor formation and metastatic dissemination has not been directly examined. To determine the significance of HGFL in Ron dependent mammary tumorigenesis, we examined mammary tumor growth and metastasis in MMTV-Ron expressing mice with or without a targeted deletion of HGFL. Our studies show that HGFL loss significantly delayed mammary tumor initiation in this model. These changes were associated with a decrease in Ron receptor kinase activity based on kinase assays, a reduction in mammary tumor proliferation and decrease in beta-catenin expression. Additionally we show that HGFL is expressed by tumor epithelial cells and macrophages (normal as well as tumor associated). Macrophages were predominantly localized within the tumor in HGFL proficient tumors; in contrast macrophages were present only in the peripheral regions in HGFL deficient tumors. Taken together, our data demonstrates that HGFL is required for Ron mediated tumor initiation, growth and metastasis and suggests that the HGFL/Ron pathway is a potential therapeutic target for breast cancer.
Introduction

Breast cancer is a second leading cause of cancer deaths among women in United States and about 39,520 breast cancer deaths are expected to occur in 2011 [1]. Breast tumors from triple negative breast cancer patients that are Estrogen receptor (ER), progesterone receptor (PR) and HER2 negative, cannot be treated with hormonal therapies or HER2 targeted therapies and are associated with poor prognosis [2]. Some of the current chemotherapy options that have shown success in clinics are treatment with cetuximab along with taxol [3], Poly (ADP-Ribose) Polymerase Inhibitors [4] and Imatinib for inhibition of PDGFR and c-Kit [5]. However these treatment options are not completely effective, largely because of tumor heterogeneity. Hence early screening, diagnosis, validation of biomarkers and treatment decision remains a challenge. Progress in understanding disease mechanism and identification of biomarkers to customize anti-cancer therapy is a need.

Ron receptor tyrosine kinase, a member of Met family of Receptor tyrosine kinases is upregulated in fifty percent of human breast cancers [6]. Ron receptor and its ligand hepatocyte growth factor like protein (HGFL) is located on chromosome 3p21, a region often amplified in breast cancer [7]. Ron receptor is expressed on both epithelial cells and macrophages [8-9]. Given its expression in different cell types, the receptor plays important role during embryogenesis [10], wound healing [11], tissue remodeling [12] and inflammation [13]. Ron elicits its tumorigenic potential by regulating cell proliferation, motility, adhesion and anoikis [14]. More importantly this activity of Ron in some cancer cells is ligand dependent [7, 15-18]. Ron overexpression in mammary gland of mice under MMTV promoter (MMTV-Ron) induces mammary tumors in 100 percent of female mice. The tumors are highly aggressive and metastasize to lung and liver [8]. These tumors also have activation of β-catenin and its target genes [16].

HGFL is a secreted protein, primarily produced by hepatocytes, it gets cleaved by proteases and then binds to the Ron receptor in paracrine manner to activate it. MT-SP1 or matriptase activates pro-HGFL into HGFL. Matriptase is expressed on macrophages and breast cells and is upregulated in cancer [19]. A
regulatory region 135bp upstream of HGFL translational start site along with sequences in first intron/exon is important in HNF-4 binding and required for liver specific expression of HGFL gene [20-21]. HGFL has both pro-apoptotic and anti-apoptotic functions [22-23]. HGFL acts as a chemoattractant for resident macrophages [12]. Ron activation on macrophages promotes tumor growth through increased Arg1 expression, thus highlighting HGFL’s role in aiding in epithelial-stromal crosstalk [9]. Macrophages aid in matrix remodeling, angiogenesis and stimulate tumor growth through synthesis of growth factors [24]. Overexpression of HGFL in a mammary tumors driven by polyoma-Middle T-antigen (PyMT) caused increased early tumor growth and broadened the spectrum of metastasis compared to vector control transduced tumors. In addition overexpression of all three genes in the pathway was independent predictor of metastasis and death in breast cancer patients [25]. However the importance of HGFL in the context of Ron induced mammary tumorigenesis remains unknown.

Here we show that MMTV-Ron mice lacking HGFL have delayed mammary tumor initiation. The delay in tumor initiation is due to delayed mammary hyperplasia and decreased Ron kinase activity in HGFL deficient tumors. Moreover the HGFL proficient tumors have increased β-catenin expression, proliferation and decreased death compared to HGFL deficient tumors. We also show for the first time that HGFL is produced by macrophages in normal mammary gland as well as in tumor associated and that HGFL produced by macrophages is important for growth of MMTV-Ron mammary tumor cells. This study highlights the importance of HGFL in the context of Ron driven mammary tumorigenesis and warrants further investigation into the mechanism of macrophage derived HGFL in promoting Ron induced mammary tumorigenesis.
Results

HGFL is important for mammary tumor initiation in MMTV-Ron mice

To determine whether HGFL loss changes kinetics of mammary tumorigenesis in MMTV-Ron mice, we crossed the MMTV-Ron, HGFL+/+ mice to mice that were deleted for HGFL. The HGFL null mice develop normally and have not phenotypic abnormalities except presence of lipid filled vacuole in the hepatocytes and delayed macrophage activation [26]. MMTV-Ron, HGFL−/− mice developed mammary tumors with 100% incidence. Tumor latency curve is plotted over time. We found that HGFL loss significantly delayed mammary tumor initiation in these mice however the rate of mammary tumor progression was similar in both the genotypes (Figure 1). The median time for palpable tumor formation increased from 283 days in control MMTV-Ron mice (n=29) to 311 days in MMTV-Ron, HGFL−/− mice (n=19) (Figure 1, inset). Protein extracts from mammary tumors from MMTV-Ron, HGFL+/+ mice showed presence of HGFL in mammary tumor lysates in contrast to HGFL deficient tumor lysates (Figure 1B)

HGFL is important for development of mammary hyperplasia

We determined whether MMTV-Ron, HGFL−/− mice had delayed mammary ductal hyperplasia a step preceding mammary tumor formation compared to control MMTV-Ron, HGFL+/+ mice. We isolated inguinal mammary glands (#4) at 2.5, 4, 6 and 8 months and analyzed them for presence of ductal hyperplasia. Aberrant mammary lesions in carmine red stained whole glands and H&E were found in both genotypes (Figure 2A). However interestingly, MMTV-Ron, HGFL−/− mice had deferred ductal hyperplasia at each time point compared to control MMTV-Ron, HGFL+/+ mice (Figure 2B). The percentage of control mice at 2.5, 4, 6 and 8 months that developed hyperplasia was 10%, 71%, 89% and 100% respectively. In contrast the percentage of MMTV-Ron, HGFL−/− mice that developed hyperplasia was 0%, 36%, 50% and 57% respectively.

HGFL is important for Ron Phosphorylation and Kinase Activity
To determine if presence of tumor derived HGFL is important for Ron activity, we performed kinase assays with immunoprecipitated Ron or IgG control from mammary tumor lysates of MMTV-Ron, HGFL \(^{+/+}\) and MMTV-Ron, HGFL \(^{-/-}\) mice and myelin basic protein (MBP) as a substrate. We found that immunoprecipitated Ron from MMTV-Ron, HGFL \(^{+/+}\) tumors could robustly phosphorylate MBP however immunoprecipitated Ron from MMTV-Ron, HGFL \(^{-/-}\) tumors showed decreased phosphorylation of MBP (Figure 3A). Using a pRon specific antibody, we found that there was increased pRon (Y1238/Y1239) receptor in MMTV-Ron, HGFL \(^{+/+}\) mammary tumors as compared to MMTV-Ron, HGFL \(^{-/-}\) mammary tumors. Input for total Ron is shown as loading control. Ron Kinase activity was normalized to Ron expression in the tumors and quantification for that is shown in Figure 3B.

**Decreased \(\beta\)-catenin expression in mammary tumors of MMTV-Ron, HGFL \(^{-/-}\) mice**

We have previously published that \(\beta\)-catenin is an important downstream mediator of Ron induced mammary tumorigenesis [8, 16]. So we determined whether \(\beta\)-catenin levels were altered in mammary tumors of HGFL deficient mice. mRNA from MMTV-Ron, HGFL \(^{+/+}\) and MMTV-Ron, HGFL \(^{-/-}\) tumors were analyzed for \(\beta\)-catenin expression. We found decreased \(\beta\)-catenin mRNA expression in MMTV-Ron, HGFL \(^{-/-}\) tumors as compared to HGFL proficient tumors (Figure 4A). Protein extracts from MMTV-Ron, HGFL \(^{+/+}\) and MMTV-Ron, HGFL \(^{-/-}\) tumors were analyzed for \(\beta\)-catenin expression and tumors from MMTV-Ron, HGFL \(^{-/-}\) mice had decreased \(\beta\)-catenin levels compared to MMTV-Ron, HGFL \(^{+/+}\) tumors (Figure 4B).

**HGFL deficient tumors have decreased proliferation and increased apoptosis**

To determine if the delay mammary tumor initiation and subsequent hyperplasia is due to lack of proliferation and increased death, we performed BrdU and TUNEL analysis. End stage mammary tumors from MMTV-Ron, HGFL \(^{+/+}\) and MMTV-Ron, HGFL \(^{-/-}\) mice were analyzed for proliferation using BrdU incorporation. We found that mammary tumors from MMTV-Ron, HGFL \(^{+/+}\) mice had significantly more BrdU incorporation than MMTV-Ron, HGFL \(^{-/-}\) mice (Figure 4A). A representative image of BrdU
stained tumor is shown in Figure 4B. We also examined cell death in the end stage tumors using TUNEL staining. The mammary tumors from MMTV-Ron, HGFL^{+/+} mice had significantly less TUNEL positive cells than tumors from MMTV-Ron, HGFL^{-/-} mice (Figure 4C). A representative image of TUNEL positive mammary tumor section is shown in Figure 4D.

**HGFL expression in tumor epithelial cells and tumor associated macrophages (TAM)**

To examine the compartment in mammary gland that is responsible for HGFL production, we performed RT-PCR of HGFL in normal mammary gland, normal mammary epithelial cells and macrophages. We used CD11b antibody to pull-down macrophages and performed immunofluorescence for F4-80 to confirm specificity. Majority of cells in CD11b pull down fraction were F4-80 positive (data not shown). We found macrophages had highest expression levels of HGFL in normal mammary gland. In contrast, fat tissue and normal mammary epithelial cells contributed to little or no HGFL (Figure 6A). Immunohistochemistry on normal mammary gland also showed HGFL expression in stromal compartment but not in epithelium (Figure 6B). We determined if tumor epithelial cells and TAM contributed to HGFL produced in mammary tumors of MMTV-Ron, HGFL^{+/+} mice. By RT-PCR analysis, we found that both tumor epithelial cells as well as TAM contributed equally to the amount of HGFL in produced by mammary tumors (Figure 6C). Immunohistochemistry of HGFL on mammary tumor sections is shown in Figure 6D. To confirm our RT-PCR data, we also performed dual-immunofluorescence of F4-80 and HGFL on CD11b pull down fraction from normal and tumor bearing glands. We found that 92% of F4-80 positive cells were HGFL positive (Figure 6E).

**Differential macrophage location in HGFL proficient and deficient mammary tumors**

Macrophages play important role in tumor initiation and progression directly by secreting mutagenic compounds that induce DNA damage and indirectly by secretion of angiogenic and other growth factors [27]. To determine if there was any differences in macrophage recruitment and location, we performed F4/80 staining on mammary tumor sections from MMTV-Ron, HGFL^{+/+} and MMTV-Ron HGFL^{-/-}
mammary tumors. We found that in MMTV-Ron, HGFL<sup>+/+</sup> tumors F4/80 was located within the tumor and stromal regions. However in MMTV-Ron HGFL<sup>-/-</sup> tumors we found that F4/80 was exclusively localized in the stromal regions (Figure 7).
Discussion

While some of the previous studies highlight the importance of HGFL in mammary tumor model, the requirement of HGFL downstream of Ron receptor was not elucidated. This is the first study that shows importance of HGFL downstream of Ron overexpression. We demonstrate that Ron overexpressing mice that have lack of HGFL get delayed mammary tumors. This delay in mammary tumorigenesis was associated with reduced Ron Kinase activity and decreased proliferation. Moreover the mice that had loss of HGFL also had decreased β-catenin expression. We also demonstrate for the first time that HGFL is produced by normal as well as tumor associated macrophages that may play important role in promoting Ron receptor induced mammary tumor formation.

We have shown that HGFL is important for Ron mediated mammary tumor initiation and that HGFL loss in MMTV-Ron mice increased tumor latency and delayed mammary hyperplasia. Previous studies from our laboratory have demonstrated the in vivo tumorigenic potential of Ron receptor in PyMT breast tumor model [28] as well as TRAMP prostate tumor model [29]. In both these models loss of Ron decreased tumor burden at least in part due to decreased tumor proliferation. The activity of Ron in tumors is context and cell type specific. Ron activity in prostate tumors cells is ligand independent [30], however its activity breast cancer cells that have Ron overexpression is ligand dependent [16]. Overexpression of HGFL in mammary tumors of PyMT mice increases the growth of the PyMT tumors as compared to vector control transduced tumors [25], suggesting proliferative role of HGFL in context of tumors that have Ron overexpression [28]. However our study addresses the critical question of whether ligand is important for Ron mediated breast tumorigenesis and requirement of HGFL in promoting Ron activity in those tumors.

Transgenic mice that overexpress HGF in mammary gland under WAP promoter developed mammary hyperplasia that progressed to form invasive mammary tumors and displayed increased proliferation as compared to WT mice [31]. Coherent with role of hepatocyte growth factors (HGF) our data also demonstrates importance of HGFL in promoting ductal hyperplasia in MMTV-Ron mice. HGF
overexpressing mammary tumors had increased phosphorylation of c-Met receptor. Similarly using kinase assays and p-Ron antibody, we have observed increased phosphorylation and activity of Ron receptor in MMTV-Ron, HGFL \( ^{+/+} \) tumors compared to MMTV-Ron, HGFL \( ^{-/-} \) tumors.

\( \beta \)-Catenin a component of Wnt signaling pathway is important for transcription as well as cell-adhesion. In breast cancer cells, Ron activation by HGFL induces \( \beta \)-catenin nuclear accumulation [16]. Also loss of \( \beta \)-catenin in Ron overexpressing breast cancer cells reduced proliferation in vitro and in vivo tumor growth of orthotopically transplanted cells [16]. \( \beta \)-catenin nuclear accumulation was also observed in WAP-HGF mammary tumors [31]. Similar to prior observations, we found \( \beta \)-catenin expression was decreased in MMTV-Ron, HGFL \( ^{-/-} \) mammary tumors. Concomitantly we also observed increased BrdU incorporation and decreased TUNEL staining in MMTV-Ron, HGFL \( ^{+/+} \) tumors. Thus suggesting that Ron maybe driving proliferation in MMTV-Ron, HGFL \( ^{+/+} \) tumors through activation of \( \beta \)-catenin.

HGF is a fibroblast derived growth factor that acts in a paracrine as well as autocrine manner to activate the c-Met receptor [32]. HGFL however is normally secreted by hepatocytes and acts in paracrine manner to activate the Ron receptor. Recent literature shows elevated expression of HGF [33] as well as HGFL [25] in human breast cancers that is associated with poor prognosis. Our data shows for the first time that HGFL is produced by macrophages and tumor epithelial cells. This is important as stromal derived HGFL can crosstalk with Ron on epithelial cells to promote mammary tumorigenesis.
Materials and Methods

**Generation of Mice.** Mice with mammary specific Ron overexpression were crossed to HGFL knockout mice [8, 26]. To study mammary tumor initiation and progression, these mice were continuously mated starting at 8 weeks of age until their tumors reached 20% of their body weight by which the animals were euthanized. At harvest, thoracic and inguinal mammary glands, lung and liver were collected. Genotyping of the transgenic mice was performed by PCR analysis with the following primer sets: Primers used for genotyping mice were as follows: MMTV-Ron forward 5’TGG GTG GTG AGG TCT GCC AAC ATG A3’, reverse 5’CCG TCT TCG GGA GTT AAA GAT CAG GG3’. HGFL forward and Reverse. All animal procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

**Tumor Latency.** Animals were palpated weekly for over 400 days to assess mammary tumor development. Tumor development in MMTV-Ron, HGFL+/+ and MMTV-Ron, HGFL−/− mice were compared as previously described [8].

**Tissue Histology.** Tissues were processed as described previously [8]. Briefly, mammary glands, lungs, and liver samples were fixed, paraffin embedded, and cut into in 4μm sections. Sections were stained with hematoxylin and eosin for routine histological examination. For whole-mount analysis, mammary glands were fixed in Carnoy's fixative and stained overnight in carmine alum. Samples were dehydrated, cleared in xylene, mounted, and examined on a stereoscope equipped with an Axiovert digital camera.

**Kinase assay.** Kinase assay was performed according to published protocols [8]. In brief, Ron was immunoprecipitated from 500 μg mammary tumor lysates of MMTV-Ron, HGFL+/+ and MMTV-Ron, HGFL−/− mice with myelin basic protein as substrate. Samples were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the gels were fixed, dried and imaged on a phosphoimager.
**Real Time PCR and Western analysis:** MMTV-Ron, HGFL<sup>+/+</sup> and HGFL<sup>-/-</sup> mammary tumors, tumor epithelial cell line and macrophages (normal and tumor associated) were used for RNA isolation. RNA was isolated from frozen tissues or cells using TRIzol Reagent according to the manufacturer's instructions. cDNA was generated from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit. qRT-PCR analysis was then performed using the Applied Biosystems 7300 Real Time PCR System and Sequence Detection Software Version 1.3.1. Each cDNA sample was run in duplicate and amplified by Power SYBR Green PCR Master Mix. qRT-PCR was performed using following primer set: β-catenin forward 5’-TCCCTGAGACGCTAGATGAGG-3’ and reverse 5’-CGTTTAGCAGTTTTGTCAGCTC-3’. HGFL forward and reverse. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). After transfer, the membranes were probed with goat polyclonal anti-HGFL antibody (1:1000, Santa Cruz, Santacruz, CA). The membranes were stripped and reprobed with an anti-beta catenin antibody (1:1000, BD Biosciences). Actin or tubulin expression was used as the loading control.

**Proliferation and TUNEL Analysis.** For BrdU analysis, mice were injected with 5-bromo-2’-deoxyuridine (BrdU) 2 hours prior to sacrifice and immunohistochemistry was performed on paraffin-embedded mammary tumor tissue sections as previously described [8, 34]. The proliferation index was measured by quantification of BrdU-positive nuclei at 400X magnification and counting at least three independent fields per slide from at least three different tumors from each group. An In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer’s instructions to stain mammary tumor sections for the quantification of cell death as previously described [34]. Cell death was quantified by counting the number of terminal deoxynucleotidyl transfer mediated dUTP nick end labeling (TUNEL)-positive nuclei in mammary tumor tissue at 400X magnification from three different tumors of each genotype.

**Statistical Analysis.** Statistical analysis for hyperplasia was determined using z-test. Statistical significance for BrdU incorporation, TUNEL analysis, qRT-PCR and tumor metastasis was determined
by a Student's t-test using GraphPad Prism software (San Diego, CA). Data on tumor development was subjected to Kaplan-Meier analysis using GraphPad Prism software (GraphPad Software, San Diego, CA).
References


Figure 1. Effect of HGFL deletion on mammary tumorigenesis in MMTV-Ron mice. A, Kaplan Meier Curve shows tumor incidence in MMTV-Ron, HGFL $^{+/+}$ (n=29) and MMTV-Ron, HGFL $^{-/-}$ (n=19) mice. Inset shows median time for tumor formation. B, Mammary tumors from MMTV-Ron, HGFL $^{+/+}$ and MMTV-Ron, HGFL $^{-/-}$ mice were probed for HGFL. Tubulin was used as loading control. p<0.05, Log-Rank (Mantel-Cox) test.
Figure 2. Mammary hyperplasia in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{-/-} mice. A, Representative whole mount and H&E from MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{-/-} mice at 8 month. B, Quantification of mammary hyperplasia observed in inguinal mammary glands (#4) and H&E at 2.5, 4, 6 and 8 months in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{-/-} mice. The number (n) of MMTV-Ron, HGFL^{+/+} mammary glands analyzed for 2.5, 4, 6, and 8 months were 10, 7, 9 and 8 respectively. For MMTV-Ron, HGFL^{-/-} mice the number of mammary glands analyzed were 6, 11, 4 and 7 respectively. p<0.05 at 8 months, Z-test.
Figure 3. Ron Kinase activity in MMTV-Ron, HGFL<sup>+/+</sup> and MMTV-Ron, HGFL<sup>-/-</sup> tumors. A, A representative Kinase activity of Ron receptor immunoprecipitated from MMTV-Ron, HGFL<sup>+/+</sup> and MMTV-Ron, HGFL<sup>-/-</sup> tumors was assayed using [γ-<sup>32</sup>P] and myelin basic protein (MBP) as a substrate is shown. Phosphorylated MBP was detected after SDS-PAGE by autoradiography. The immunoprecipitated Ron was also probed with pRon (Y1238/Y1239) and total Ron is shown as input. B, Graph shows quantification of average Ron Kinase activity from three independent kinase assays with n=3 samples per group in each assay. The kinase activity is normalized to total immunoprecipitated Ron. p<0.05, Students t-test.
Figure 4. β-catenin expression in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{−/−} mammary tumors. A, Real-Time PCR for β-catenin expression in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{−/−} mammary tumors. Graph shows average transcript expression from 4 independent samples repeated twice. The β-catenin expression was normalized to mGUS internal control. B, SDS-PAGE analysis of β-catenin expression in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{−/−} mammary tumors. Actin was used as loading control. p<0.05. Students t-test.
Figure 5. Increased proliferation and decreased death in MMTV-Ron, HGFL^{+/+} mammary tumors.

A, Immunohistochemistry was performed on end stage mammary tumor sections using anti-BrdU antibody. Graph shows quantification of average BrdU positive cells in MMTV-Ron, HGFL^{+/+} (n=4) and MMTV-Ron, HGFL^{−/−} (n=4) mammary tumors. B, Representative immunohistochemistry of BrdU positive cells (indicated in brown) per 100 μm. C, Graph shows quantification of average Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells in MMTV-Ron, HGFL^{+/+} (n=4) and MMTV-Ron, HGFL^{−/−} (n=4) end stage mammary tumors. D, Representative immunohistochemistry of TUNEL in MMTV-Ron, HGFL^{+/+} and MMTV-Ron, HGFL^{−/−} mammary tumors. p<0.05. Students t-test.
Figure 6. HGFL is expressed by tumor epithelial cells and macrophages. A, Real-Time PCR of HGFL in normal mammary glands. Graph of HGFL mRNA expression from normal mammary whole
Figure 7. Differential macrophage localization in MMTV-Ron, HGFL+/+ and MMTV-Ron, HGFL-/- mammary tumors. Representative immunohistochemistry of F4/80 on mammary tumor sections. N=4 mammary tumors in each genotype were analyzed for macrophage localization.
CHAPTER V

HGFL neutralization decreases mammary tumor growth of MMTV-Ron cells that is associated with decreased levels of MAPK and cyclin D1
Abstract

Ron Receptor and its ligand are overexpressed in breast cancer. HGFL induced Ron activation in breast cancer induces cancer cell proliferation, migration and invasion. Therefore inhibition of HGFL binding is important for reducing several pleotropic effects downstream of Ron. Hence to test the utility of HGFL neutralization on inhibiting mammary tumor growth in vivo, MMTV-Ron expressing mammary tumor cells were orthotopically implanted into the breast of syngenic, immunocompetent, wild type mice. Cohorts of mice were then treated with either a control antibody or a HGFL neutralizing antibody. Our data show that blockade of HGFL leads to decreased mammary tumor growth that was associated with decreased levels in the Ron downstream signaling targets cyclin D1 and MAPK activation. Taken together, our data demonstrates that HGFL is required for Ron mediated tumor initiation, growth and metastasis and suggests that the HGFL/Ron pathway is a potential therapeutic target for breast cancer.
Introduction

Ron Receptor and its ligand HGFL are overexpressed in 50 and 20 percent of breast cancers respectively. Ligand dependent Ron activation induces Ron tyrosine phosphorylation and mammary cell proliferation and migration (1). Downregulation of Ron receptor in cancer cells leads to decreased proliferation (2). Ron receptor decreases tumor growth in transgenic models of polyoma middle T-antigen (PyMT) and Ras (3-4). Transgenic mice overexpressing the Ron receptor in mammary epithelium lead to mammary tumor formation that was highly aggressive, metastasizing to tissues such as lung and liver (5). Ron also synergizes with other receptors such as c-Met and EGFR to enhance signaling (6-7). Thus Ron along with it’s highly homologous receptor Met are implicated in tumor progression and metastasis and are a potential antineoplastic targets.

Inhibition of Ron activity and in vivo tumorigenesis of HT-29 colon cancer cells has been previously achieved using a small molecule kinase inhibitor (8). O’Toole and colleagues developed an antibody that binds to human Ron receptor which inhibits ligand binding. They showed that HGFL neutralization decreased subcutaneous growth of HT-29 colon cancer cells. Further treatment of pancreatic cancer stem cells with anti-Ron antibody Zt/e9 reduced their viability and induced rapid internalization of Ron receptor (9).

Unlike in prostate cancer, Ron activity in breast cancer seems to be ligand dependent (10-11). Hence utilizing an HGFL neutralizing antibody would be a better approach to target the Ron receptor and its downstream signaling events in breast cancer. Utilizing a murine R7 cells that were derived from our MMTV-Ron transgenic mammary tumors which overexpresses the Ron receptor, we show that neutralization of HGFL (targeting murine Ron) was efficient in decreasing orthotopic growth of the R7 tumors. We also found that the antibody was also efficient in blocking HGFL/Ron dependent MAPK and cyclin D1 expression.
Results

Murine HGFL neutralizing antibody decreased MAPK and Cyclin D1 expression in R7 Ron overexpressing cells

To determine the in vitro efficacy of HGFL neutralizing antibody, we utilized R7 breast cancer cells. We treated R7 cells with either vehicle or HGFL in combination with the neutralizing antibody. We found increased pMAPK expression compared to vehicle control. However, treatment with neutralizing antibody and HGFL decreased the levels of pMAPK as compared to control IgG antibody and HGFL. MAPK and Akt are important signaling molecules downstream of Ron. Similar to changes seen in pMAPK expression, we saw reduced cyclin D1 expression after HGFL neutralization in R7 cells. Cyclin D1 is upregulated in response to beta-catenin activation (Figure 1).

Murine HGFL neutralizing antibody decreased R7 mediated mammary tumor initiation

We determined orthotopic tumor growth of R7 cells after treatment with either IgG control or HGFL neutralizing antibody. We injected R7 cells in mammary fat pad of mice at day 0 and administered the antibodies starting at day 3. We treated mice twice a week. At about 40 days we harvested the mice and found that mice treated with HGFL neutralizing antibody had significantly reduced mammary tumor growth at 4.5 week post tumor cell injection as compared to IgG treated mice (Figure 2).

Murine HGFL neutralizing antibody decreased R7 mediated mammary tumor progression

We injected R7 cells in mammary fat pad of FVB/N mice at day 0 and started administering the HGFL neutralizing antibody or IgG at day 14 when tumor size was about 100 mm$^3$. The mice we sacrificed at day 40 when the tumors reached 2000mm$^3$. Starting at week 2, HGFL neutralization significantly decreased orthotopic growth of R7 cells compared to IgG treated mice (Figure 3).
HGFL/Ron pathway is deregulated in breast cancer. Previous studies targeting Ron receptor were performed using established cell lines. For instance, Ron inhibition using ImClone 41A10 antibody reduced subcutaneous as well as orthotopic tumor growth of L3.6pl pancreatic cancer cells (12). ImClone 41A10 was also reduced subcutaneous growth of HT-29 colon, NCI-H292 lung and BxPC-3 pancreatic cancer cells (13). However there were a number of pitfalls with the previous studies. Firstly, most of the tumor xenografts were done in nude mice. We now know that immune cells play crucial role in modulating tumor microenvironment to sustain growth of tumor cells. Hence our study is first to examine the Ron inhibition in immunocompetent syngeneic model that has intact microenvironment. Secondly, all the previous studies were performed using established cell lines. Our study is the first to examine the effects of HGFL neutralization on tumor cells that were established in context of Ron overexpression. Thirdly, most previous studies were performed using subcutaneous model. Our study examined Ron inhibition in cells that were implanted orthotopically, a more relevant model that mimics human breast cancer. Last but not the least, previous studies used human cancer cells to examine HGFL neutralization. The drawback with such study is that murine HGFL cannot bind to human Ron receptor, hence it is important to determine HGFL neutralization using murine breast cancer cells that can bind to ligand in vivo.

Our study is the first to examine HGFL neutralization using murine breast cancer cells that were implanted orthotopically. A significant tumor growth reduction was observed after HGFL neutralization (Figure 2 and 3). This growth reduction observed in vivo might be due to decreased expression of Ron target genes like MAPK (ERK1/2) and cyclin D1 (Figure 1). MAPK and cyclin D1 promote tumor cells proliferation. Hence the mechanism by which Ron mediates tumor growth of R7 cells maybe through activation of MAPK and cyclin D1. Thus HGFL/Ron pathway is critical mediator of breast tumorigenesis and a potential therapeutic target for tumors that have HGFL/Ron overexpression.
Materials and Methods

Generation of mammary tumors. 1X10⁵R7 mammary tumors cells (from MMTv-Ron mice) were resuspended in 50 μl of serum free media and injected in the mammary fat pad of FVB/N mice. Tumor cells were injected either at day 0. The HGFL neutralizing antibody (98E12) or IgG antibody was administered intraperitoneally twice a week. Tumor volume was measured twice a week using following equation: \( \pi \times L \times W^2 \).

Western analysis. For these experiments R7 cells were plated in complete media with 5% FBS at approximately 1x10⁴ cells/well in a 12-well tissue culture dish and allowed to adhere overnight. The next day, cells were serum starved for 3 hours in DMEM:F12. Antibodies were then added to the dish at the concentration of 5ug/ml and incubated for 15 minutes, HGFL was then added at a final concentration of 100 ng/ml and cells were incubated for an additional 15 minutes. Total cell lysates were prepared and 20 ug run out on a 10%tris-glycine gel and transferred to nitrocellulose for further analysis.
References


Figure 1. HGFL neutralization decreases MAPK activation and cyclin D1 expression. R7 cells were serum starved and treated with either vehicle or HGFL by itself or with and without HGFL neutralizing antibody. HGFL neutralization decreases levels of pMAPK and cyclin D1 in R7 cells.
Figure 2. HGFL is important for tumor initiation of R7 cells. R7 cells were implanted orthotopically in WT-FVB mice. Intraperitoneal treatment twice a week with HGFL neutralizing antibody and control IgG antibody was started at day 3 when the tumors were not established. Tumor volume measurements were performed twice a week with digital calipers until the mice were sacrificed at day 40 or when the tumors reached 2000 mm$^3$. 
Figure 3. HGFL is important for growth of R7 cells in vivo. R7 cells were implanted orthotopically in WT-FVB mice. Intraperitoneal treatment twice a week with HGFL neutralizing antibody and control IgG antibody was started when tumor size reached 100 mm$^3$. Tumor volume measurements were performed twice a week with digital calipers until the mice were sacrificed at day 40 or when the tumors reached 2000 mm$^3$. Treatment with HGFL neutralizing antibody diminished tumor growth of R7 cells as compared to IgG2A control antibody.
Chapter VI. Conclusions and Discussion
Conclusions and Discussion

**Ron Receptor Overexpression and its impact on mammary tumorigenesis**

Breast cancer is associated with abnormal RTK expression due to protein overexpression, gene amplification and transcript deregulation. (1-2). The Ron receptor tyrosine kinase is overexpressed in approximately 50% of human breast cancers and increased Ron expression is associated with aggressive phenotype in node negative breast cancers (3-4). Ron expression is low in normal breast epithelium and in benign lesions (3). Patients with Ron expression in breast tumors had significantly worse 10 year disease free survival as compared to patients with Ron deficient breast tumors (4). Ligand binding to Ron induces receptor homodimerization creating docking sites for adaptor proteins that in turn activate downstream signaling cascades such as RAS, MAPK, PI3K and Akt (5-6). Ligand independent Ron activation can occur via receptor heterodimerization with EGFR that induces cell scattering and transformation (7). In addition to Ron receptor function in promoting tumorigenesis, Ron has diverse biological effects in different cell types. Ron and HGFL regulate inducible nitric oxide synthase (1) in macrophages, liver injury, and in keratinocytes regulate wound healing, migration and proliferation (8-10). Ron overexpression has been shown to increase foci formation, proliferation and migration in vitro and tumor formation in vivo. Mice lacking the tyrosine kinase domain of Ron (Ron TK−/− mice) when crossed with transgenic mice expressing polyoma middle T antigen (PyMT), specifically in mammary epithelium resulted in increase tumor latency, decreases mammary tumor growth and metastasis (11). Loss of Ron in MMTV-PyMT, mice decreased pMAPK and pAKT levels in tumors compared to control mammary tumors. Further transgenic mice that overexpress Ron in mammary epithelium under the MMTV promoter develop mammary tumors with short latency and metastatic phenotype (12). Mammary tumors from MMTV-Ron transgenic mice have increased Ron expression and kinase activity as compared to non-transgenic controls. Moreover tumors from transgenic mice have increased expression of beta-catenin and its target genes such as cyclin D1 and c-myc (12). Thus Ron overexpression induces pleiotropic effects and induces activation of pathways that are important for tumor initiation and
progression. Ron receptor and its ligand HGFL are located on chromosome 3p21.3. Interestingly beta-catenin is also located on chromosome 3p21. This region of the chromosome is altered during tumor development and exhibits both, loss of heterozygosity as well as amplification in various tumors and cancer cell lines (13-15). About 15-42% of breast cancers have amplification of this chromosomal region which may explain at least some of the overexpression observed for these proteins. However the most likely reason for Ron overexpression is transcript upregulation. HGFL null mice develop normally and have no obvious phenotypic defects, except for presence of lipid-filled cytoplasmic vacuoles in their hepatocytes (16).

Hence to investigate the molecular mechanism of Ron receptor mediated mammary tumorigenesis our laboratory created mice that selectively overexpressed this receptor in the mammary epithelium of mice under the MMTV promoter (12). We determined the impact of beta-catenin loss and its effects downstream of Ron receptor (Chapter 2 and 3).

**Beta-catenin activation and requirement downstream of Ron**

Previous studies have shown that Ron and beta-catenin are independently important in mammary tumorigenesis, however no direct link was established between these two molecules in breast cancer. Our study shows for the first time that Ron and beta-catenin are coordinately upregulated in human breast cancers (17) (Chapter 2, Figure 1 and Table 1). We also show that patients with Ron and beta-catenin overexpression had reduced survival and lymph node metastasis as compared to patients with low Ron and beta-catenin. Mammary tumors from transgenic Ron overexpressing mice have increased tyrosine phosphorylated beta-catenin and total beta-catenin as compared to controls (12). Tyrosine kinase such as RET, Met and EGFR are known to tyrosine phosphorylate beta-catenin. Tyrosine phosphorylation of beta-catenin is important for regulation of beta-catenin activity. Human beta-catenin has about 17 tyrosine residues. Phosphorylation of some tyrosine residues results in beta-catenin escape from Axin/APC/GSK-3 beta complex and ultimately leading to its increased nuclear accumulation and transcriptional activity. We
demonstrate that beta-catenin associates with Ron and gets directly phosphorylated by Ron at tyrosines 654 and 670 (12, 17) (Chapter 2, Figure 2). Further, we show that tyrosines 654 and 670 are important in nuclear translocation and beta-catenin activity in breast cancer cells (Chapter 2, Figure 2 and 3). However we do not which residues in beta-catenin are important for its association with Ron. Our kinase assay using WT and tyr 654/670 mutant beta-catenin would suggest that Ron phosphorylates beta-catenin at tyr 654 and 670. Human pancreatic cancer cells that have activated Ron show reduced E-Cadherin expression and increased beta-catenin nuclear accumulation (18). Another hypothesis is that in breast cancer cells, Ron forms a complex with E-Cadherin at the cell membrane and induces tyrosine phosphorylation of beta-catenin that is associated with E-Cadherin. This hypothesis can be tested using T47D breast cancer cells, as these cells contain high levels of Ron, beta-catenin and E-Cadherin. Interestingly, in murine breast cancer cells that have Ron overexpression (cells derived from MMTV-Ron tumors), we observe little to none E-Cadherin expression (Wagh and Waltz, unpublished data). Given the role of Ron in inducing invasion and metastasis, implicates that these cells have characteristics of EMT and are very metastatic (Chapter 2, Supplemental Figure S2). This suggests that Ron promotes tumor growth through activation of beta-catenin. Transcription factors like snail, slug, twist, siah interacting protein (SIP), and WISP3 are suppressors of E-Cadherin transcription (19). Hence it will be worthwhile to study if Ron can directly activate any of these transcription factors to modulate beta-catenin. In addition Ron activation induces expression of cyclin D1 and c-myc. Cyclin D1 is a critical downstream target of beta-catenin and it induces proliferation in response to mitogenic stimuli. The expression of cyclin D1 and c-myc in breast cancer cells was dependent on Ron and beta-catenin (Figure 3). Knockdown of Ron or beta-catenin was capable of inhibiting induction of cyclin D1 and c-myc (Chapter 2, Figure 3 and data not shown). Both cyclin D1 and c-myc are upregulated in 40% of human breast cancers (20-21). In thyroid cancer, RET mediated Akt activation, is able to phosphorylate and inactivate GSK-3beta leading to beta-catenin nuclear translocation (22). Since Ron activation also leads to activation of Akt in breast cancer (Wagh and Waltz unpublished), it would be interesting to investigate if Ron-Akt and beta-catenin fall in a linear pathway in breast cancer (23). Similar to activation of Akt, Ron also induces activation of MAPK
(ERK1/2) in breast cancer. Activated ERK can phosphorylate and inactivate GSK-3beta which in turn activates beta-catenin. We do not know if Ron dependent beta-catenin activation is dependent on ERK. Thus it is possible that beta-catenin may co-operate with other signaling cascades activated by Ron to mediate mitogenic activity.

Loss of tumor suppressor gene such as protein phosphatase and tensin homolog (PTEN) enriches normal and malignant human mammary stem/progenitor cells by activation of beta-catenin pathway (24). It would be interesting to investigate if Ron overexpression promotes PTEN loss through activation of beta-catenin pathway. We have shown in breast cancer cells that have Ron overexpression, beta-catenin activation is Wnt independent (Chapter 2, Figure 3). Further our lab has previously demonstrated that total beta-catenin levels were upregulated in transgenic mammary tumors as compared to non-transgenic controls. Also MMTV-Ron, HGFL−/− mammary tumors have decreased beta-catenin mRNA levels as compared to HGFL proficient tumors, thus it may be possible that HGFL mediated Ron activation may also regulates beta-catenin levels through transcriptional activation of a downstream mediator.

Mechanism of beta-catenin activation in breast cancer involves downregulation of some Wnt inhibitors. Since breast cancer cells that have hyperactivated Ron and beta-catenin signaling, overexpression of the Ron receptor may directly inhibit Wnt inhibitors like Dickkopf (DKK) or secreted Frizzled related protein (sFRP).

Breast cancer cell lines T47D, MCF-7 and MDA-MB-231 have high levels of activated and total beta-catenin. Beta-catenin is an important regulator of cell proliferation in these human breast cancer cells, as knockdown of beta-catenin dramatically reduced the cell number as compared to control cells (25). Similar to its role in human breast cancers, beta-catenin is also important for growth of MMTV-Ron cells, as knockdown of beta-catenin in these cells reduced growth in vitro and in vivo (Figure 4 and 6). Although beta-catenin null cells had 50% less proliferation in vitro, they failed to show any growth in vivo. Interestingly, WT beta-catenin could rescue the proliferation in beta-catenin null cells; however the
mutant beta-catenin could not rescue this defect (Chapter 2, Figure 5). In summary Ron/beta-catenin pathway is a critical contributor to breast cancer cell proliferation and tumor progression.

Targeting beta-catenin for an anti-cancer therapy is currently being explored. Therapies currently used are conditionally replicative adenovirus, treatment with ICG-001 inhibitor (targets CREB and beta-catenin complex), Prostaglandin E2 inhibitor (targets PG E2 mediated beta-catenin signaling), nonsteroidal anti-inflammatory drug (NSAID) and small molecule inhibitors such as PKF115-584 (disruption of beta-catenin/Tcf complex) (26-27). PKF115-584 efficiently reduced levels of cyclin D1 and c-myc in HCT116 colon cancer cells. Interestingly, treatment of R7 murine Ron overexpressing breast cancer cells with PKF115-584 dramatically reduced the growth of R7 cells in dose dependent manner (Zinser and Waltz, unpublished data). Thus targeting Ron mediated beta-catenin activation could have therapeutic potential in breast cancer.

**HGFL/Ron activation and function**

HGFL is a serum glycoprotein involved in peritoneal macrophage activation and migration. HGFL also promotes proliferation and migration of epithelial cells that overexpress Ron. HGFL can also induce apoptosis and stimulate bone resorption (28). The function of HGFL may vary with cell type. HGFL secreted by hepatocytes in liver is released as an inactive protein and cleaved by cell surface proteases or proteases from blood coagulation cascade into a disulphide linked heterodimer. HGFL mediates inflammatory response through macrophage activation (9). Tissue-specific maturation of resident peritoneal macrophages causes release of proteases and induction of protein realignment which causes migration of mononuclear cells towards inflammatory stimuli. In this context, HGFL null mice have delayed resident peritoneal macrophage activation (16). Although HGF and HGFL have similar structural homology, there in vivo functions are very different. HGF does not bind to murine Ron receptor and HGFL cannot bind to Met receptor. Matriptase (MT-SP1) which is expressed on epithelial cells and macrophages can cleave pro-HGFL into HGFL. MT-SP1 and HGFL are overexpressed in 40% and 20%
of breast tumors, respectively (29-30). HGFL functions are dependent on its binding and activating Ron receptor.

Our data shows that HGFL loss delays mammary tumor initiation in MMTV-Ron mice (Chapter 4, Figure 1). HGFL deficient mammary tumors have decreased proliferation and increased apoptosis. Beta-catenin expression is decreased in HGFL deficient tumors as compared to HGFL proficient tumors.

**HGFL and its role in tissue morphogenesis and macrophage chemotaxis**

TAMs are recruited to the site of tumor by growth factors often produced by tumor epithelial cells themselves. This is in lieu with our working hypothesis that HGFL secreted by Ron overexpressing epithelial cells attracts macrophages to promote mammary tumorigenesis (Chapter 4, Figure 6). We also show that HGFL is produced by TAMs (Chapter 4, Figure 6). Tumor associated macrophages (31) have M2 activated phenotype that is involved in tumor development through release of IL-10 and PG-E2 which are suppressors of inflammatory reaction to the tumor (32). TAM can produce pro-angiogenic chemokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), transforming growth factor (TGF beta) and IL-6 (33-34). TAMs induce chronic inflammation through secretion of reactive oxygen species and nitric oxide which in turn induce DNA damage and other chromosomal abnormalities. In breast cancer, the abundance of TAMs is associated with poor prognosis (35). Macrophages in mammary gland both produce and respond to local estrogens thus increasing risk for breast cancer. Macrophages also play an important role during mammary gland development and morphogenesis. Mice deleted for mononuclear phagocytic cells such as macrophages have impaired mammary branching morphogenesis (36). Based on our data about macrophage infiltration at the site of tumor in HGFL proficient mice, we speculated that HGFL promotes macrophage chemotaxis just as colony stimulating factor-1 (CSF-1). CSF-1 secreted by tumor cells promotes progression of solid tumors through recruitment of TAMs. CSF-1 is overpressed in breast cancer and is associated with poor prognosis and dense leukocyte infiltration (37-38). More importantly
CSF-1 locally blocks maturation of dendritic cells so they are unable to present antigens and promote development of immunosuppressive TAMs, a hallmark of deregulated tumor microenvironment in cancer (39). So it can be hypothesized that HGFL promotes mammary tumor initiation by inducing immunosuppression and macrophage recruitment.

TAMs also play critical role in migration and invasion of tumor cells by cleaving basement membrane (33). Tumor epithelial cells also respond to TAMs produced growth factor such as EGF to promote their growth, chemotaxis and invasion (40). Treatment with HGFL neutralizing antibody along with anti-inflammatory drugs should prevent macrophage chemotaxis and their activation that will inhibit tumor growth and invasion. Thus targeting HGFL produced by both tumor epithelial cells as well as macrophages will be an attractive therapeutic target for breast tumors that have HGFL and Ron overexpression. Some of HGFL/Ron targeting strategies that are discussed in Chapter 5, were successful in inhibiting growth of tumors that were driven by cells that had Ron overexpression. HGFL neutralization also inhibited activation of MAPK, AKT and cyclin D1 some of the important downstream signaling molecules of Ron. It is important to dissect the mechanism of reduced tumor growth in mice that were administered HGFL neutralizing antibody. It will be interesting to know whether HGFL neutralization on tumor epithelial cells or macrophages was important in reducing mammary tumor growth. Another important aspect will be to determine whether HGFL neutralization on tumor epithelial cells or macrophages affected macrophage recruitment and activation at the tumor site. Role of macrophage specific HGFL can be dissected by selectively deleting HGFL in macrophages (HGFL^{ff} X LysM Cre) and then determine the orthotopic growth of R7 cells in those mice. Alternatively, bone marrow transplants of HGFL WT and knockout mice can help to answer macrophage specific role of HGFL. Epithelial specific role of HGFL can be dissected by overexpressing or knocking down HGFL in R7 cells and determining their growth in HGFL proficient and deficient mice.

Our working model is that, HGFL binds to Ron receptor on tumor epithelial cells to activate the Ron receptor. Ron activation leads to phosphorylation of intercellular tyrosine residues that induce
phosphorylation of beta-catenin. Beta-catenin in turn can translocate into nucleus to activate its downstream targets such as cyclin D1 and c-myc to induce proliferation. HGFL/Ron pathway is important for mammary tumorigenesis, as genetic ablation of HGFL or pharmacologic inhibition of HGFL reduced mammary tumor growth by decreasing cellular proliferation and Ron kinase activity.
References


Figure 1. Model for HGFL/Ron activation in mammary tumor growth and metastasis. HGFL produced in tumor microenvironment through autocrine as well as paracrine manner promotes Ron activation on tumor epithelial cells which then activates beta-catenin and its downstream signaling molecules.