Met-Related Receptor Tyrosine Kinase Ron in Tumor Growth and Metastasis

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The Ron receptor is a member of the Met family of cell surface receptor tyrosine kinases and is primarily expressed on epithelial cells and macrophages. The biological response of Ron is mediated by binding of its ligand, hepatocyte growth factor-like

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protein/macrophage stimulating-protein (HGFL). HGFL is primarily synthesized and secreted from hepatocytes as an inactive precursor and is activated at the cell surface. Binding of HGFL to Ron activates Ron and leads to the induction of a variety of intracellular signaling cascades that leads to cellular growth, motility and invasion. Recent studies have documented Ron overexpression in a variety of human cancers including breast, colon, liver, pancreas, and bladder. Moreover, clinical studies have also shown that Ron overexpression is associated with both worse patient outcomes as well as metastasis. Forced overexpression of Ron in transgenic mice leads to tumorigenesis in both the lung and the mammary gland and is associated with metastatic dissemination. While Ron overexpression appears to be a hallmark of many human cancers, the mechanisms by which Ron induces tumorigenesis and metastasis are still unclear. Several strategies are currently being undertaken to inhibit Ron as a potential therapeutic target; current strategies include the use of Ron blocking proteins, small interfering RNA (siRNA), monoclonal antibodies, and small molecule inhibitors. In total, these data suggest that Ron is a critical factor in tumorigenesis and that inhibition of this protein, alone or in combination with current therapies, may prove beneficial in the treatment of cancer patients.

I. 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 (Ronsin *et al.*, 1993). 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) (Iwama *et al.*, 1994). Met and Ron are the only two members of this RTK family, in contrast to other receptor tyrosine kinase families with multiple members (Manning *et al.*, 2002). Ron was classified based upon 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 (Huff *et al.*, 1993; Huff *et al.*, 1996). Sea is activated by chicken macrophage stimulating-protein (MSP) (Wahl *et al.*, 1999). To date, homologs of Ron and its ligand have been identified by sequence analysis in many mammalian species including *Rattus norvegicus* (rat), *Canis lupus* (dog), *Bos taurus* (cow), *Equus caballus* (horse), and *Macaca mulatta* (rhesus monkey) (BLAST sequence analysis, 2007). Homologs of Ron have also been found in nonmammalian species, including *Fugu rubripes* (puffer fish) and *Strongylocentrotus purpuratus* (sea urchin) (Cottage *et al.*, 1999; Lapraz *et al.*, 2006).

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 precursor glycosylated protein and is further processed by furin-like proteases before being delivered as a mature receptor to the cell surface (Gaudino *et al.*, 1994). 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 contains the extracellular, transmembrane, and intracellular regions of the receptor (Gaudino *et al.*, 1994). 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 sequence analysis, 2007) (Fig. 1). Human and murine Ron cDNAs share about 74% identity overall, with about 88% identity in the intracellular domains (Iwama *et al.*, 1994). 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 (Wei *et al.*, 2005). 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 binding sites for NF-kβ, Ets-1, and the estrogen receptor (Waltz *et al.*, 1998).

II. Ron LIGAND STRUCTURE AND FUNCTION

The ligand for Ron is hepatocyte growth factor-like (HGFL) protein and is also known as 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 (Han *et al.*, 1991). The protein sequence of the isolated gene 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 HGF, the ligand for the Met receptor. By sequence comparison, however, HGF and HGFL are only about 45% identical (BLAST sequence analysis, 2007) (Fig. 1). 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 (Degen *et al.*, 1991). The mouse homolog of HGFL was predicted to display the same domain structure as human HGFL and to be about 80% identical. The expression pattern of HGFL was determined by Northern analysis of tissues in the pregnant rat. The liver represented the primary site of expression for HGFL, with low levels detected in lung, adrenal gland, and placenta. Another group similarly cloned a cDNA for MSP from a library prepared from HepG2 cells, a human hepatocarcinoma

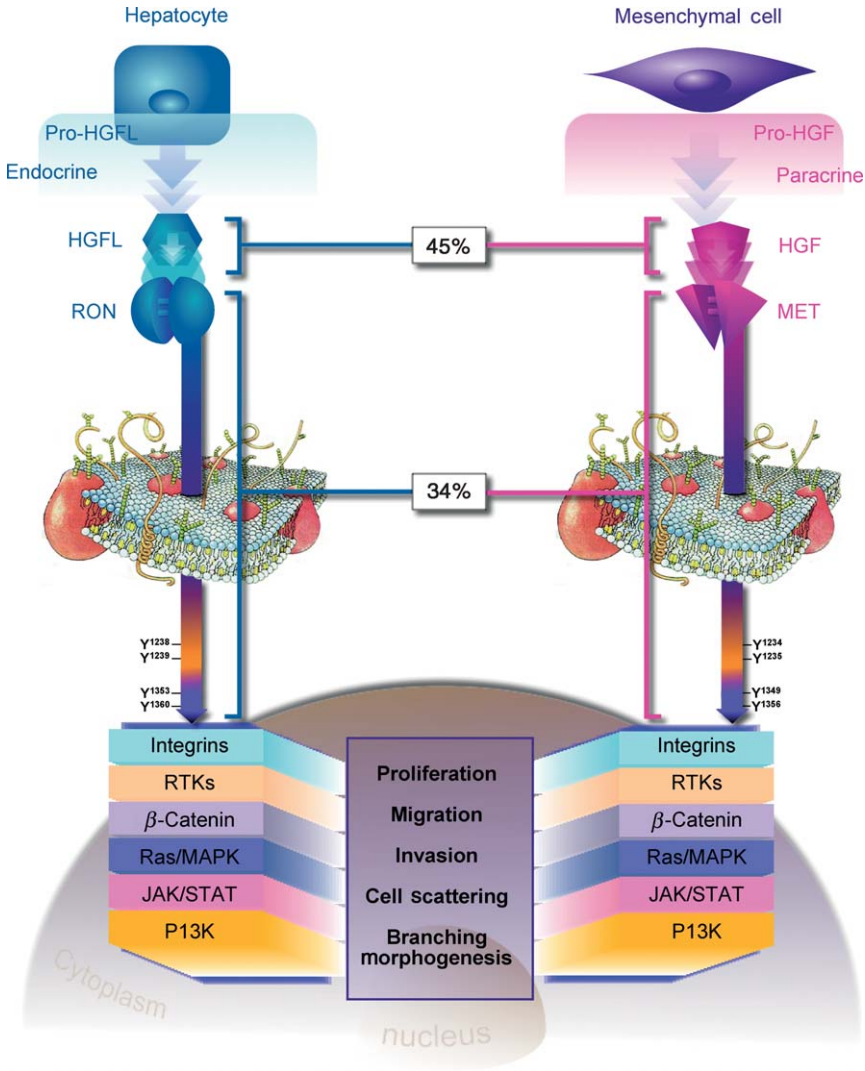

Fig. 1 The Ron and Met receptor tyrosine kinases exhibit important similarities and differences between receptors. Structurally, Ron and Met are similar in that both receptors are single-pass, disulfide-linked α/β heterodimers. However, the amino acid identity between Ron and Met is not high (34% overall) 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 mesechymcal cells operating in a paracrine fashion. Binding of HGFL or HGF to their corresponding receptor induces receptor dimerization and trans-autophosphorylation of tyrosine residues

cell line (Yoshimura *et al*., 1993). 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, like HGFL, to be most similar to HGF. HGFL and MSP were soon determined to be identical (Shimamoto *et al*., 1993). Two independent groups later determined HGFL to be the ligand for the Ron receptor. Further, in spite of sequence similarities, no cross activation is seen between HGFL and Met, or HGF and Ron (Gaudino *et al*., 1994; Wang *et al*., 1994b).

Despite the structural similarity of HGF and HGFL, their production and mechanism of action differ. 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 400 ng/ml, and works in an endocrine fashion at distant sites to activate Ron (Fig. 1). 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 (Sakamoto *et al*., 1997). Dysregulation of the spatial localization of Ron and HGFL, as well as dysregulation in the quantity of the receptor and ligand, may be important in tumor tissue growth.

Since the identification of HGFL, further work has elucidated details about the promoter sequence associated with its gene. Promoter analyses have suggested that the transcription factor hepatocyte nuclear factor-4 is important for the liver-specific expression of HGFL (Waltz *et al*., 1996). Specific elements in the first intron of HGFL have also been found to regulate liver- and kidney-specific expression (Wetzel *et al*., 2003). In addition, experiments performed in one cell type derived from large-cell lung carcinoma have demonstrated the ability of mutant p53 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 (Zalcenstein *et al*., 2006). Further experiments will elucidate whether this effect is conserved in other cell lines.

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

(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.

it bind the receptor. Proteolytic cleavage results in the formation of a disulfide-linked heterodimer of HGFL composed of an alpha (50 kDa) 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 the functional activities of Ron whereas the beta chain is important for binding of HGFL to its receptor (Danilkovitch *et al.*, 1999a; Waltz *et al.*, 1997).

Proteases of the coagulation cascade, such as kallikrein, factor XIIa, and factor XIa, are capable of cleaving pro-HGFL into HGFL (Wang *et al.*, 1994c). Membrane bound proteases produced by macrophages were shown to have specific and nonspecific pro-HGFL proteolytic activity, such that both activation and degradation of pro-HGFL occurred at the cell surface (Wang *et al.*, 1996c). The inhibitor of the HGFL degrading enzyme was identified as alpha 1-antichymotrypsin (Skeel and Leonard, 2001). Interestingly, increased expression of alpha 1-antichymotrypsin in human breast tumors, which might allow for the increased activation of HGFL, was associated with significantly poorer prognosis of patients with grade 2 and 3 breast adenocarcinomas (Hurlimann and van Melle, 1991). Estradiol treatment of breast cancer cells has also been shown to increase the production of alpha 1-antichymotrypsin (Massot *et al.*, 1985).

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 (Bhatt *et al.*, 2007). Matriptase is highly expressed in many breast, ovarian, prostate, and colon cancer cell lines (Bhatt *et al.*, 2003). An examination of 330 node-negative breast cancer specimens showed an association between expression of matriptase and poor patient outcome (Kang *et al.*, 2003). An analysis of microarray gene expression data from 162 primary tumors was also analyzed for expression of Ron, HGFL, and matriptase. Overexpression of all the three was associated with significantly shorter relapse-free survival when compared with other patients. The overexpression of all three genes also significantly improved the accuracy of prediction of a 70-gene signature predicting poor outcome (Welm *et al.*, 2007).

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 (Welm *et al.*, 2007). In this model system, orthotopically transplanted cells expressed the polyoma middle T antigen under the control of the mouse mammary tumor virus (MMTV) 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.

III. Ron CHROMOSOMAL LOCATION AND CANCER

Interestingly, the genes for each of the two receptor–ligand pairs, that is Met and its ligand HGF and Ron and its ligand HGFL, are 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 3p21.31 (Human Protein Atlas Version: 3.0, 2007). Both the murine Ron gene and the HGFL murine counterpart are also located on chromosome 9qF2 (UCSC Genome Browser, 2007). The human chromosome 3p21 region has been frequently observed to undergo loss of heterozygosity in cancer specimens and cell lines, suggesting that 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 (Senchenko *et al*., 2003). Aberrations in the 3p21 chromosome region have also been examined in lung cancer cell lines, and renal cell and breast carcinoma biopsy material. Amplification of 3p is a common event in these cancers, occurring in 15–42.5% of the samples examined (Senchenko *et al*., 2004). 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 in these human tumors has not yet been produced.

IV. 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 changes, altered response to chemoattractants, and stimulated phagocytosis in macrophages (Skeel *et al*., 1991). Through absorption studies, it was determined that HGFL was binding to a receptor and activating mature resident macrophages (Skeel and Leonard, 1994). Further studies demonstrated Ron to be expressed on

human alveolar, peritoneal macrophages, and monocyte-derived macrophages, but not on circulating human monocytes (Brunelleschi *et al.*, 2001). Ron, through HGFL stimulation, was shown to play an inhibitory role in regulating nitric oxide production by macrophages (Wang *et al.*, 1994a). Further, mice with a defect in Ron signaling have altered inflammatory responses *in vivo* (Waltz *et al.*, 2001).

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 (Federico *et al.*, 2007; Perwez Hussain and Harris, 2007). Many papers have described the ability of nitric oxide, a known mediator of inflammation, to alter neoplastic effects (Hussain *et al.*, 2004). Ron has been shown to be a negative regulator of nitric oxide in epithelial cells as well as macrophages (Hess *et al.*, 2003b). Moreover, in macrophages, the various effects of Ron, including superoxide anion production, apoptotic resistance, and phagocytosis, are induced through interactions with diverse signal molecules, including Src, Erk, p38, and PI3-K/Akt, which have been implicated in tumorigenesis (Brunelleschi *et al.*, 2001; Chen *et al.*, 1998; Lutz and Correll, 2003).

V. 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 (Gaudino *et al.*, 1995; Quantin *et al.*, 1995). 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 to 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 (Wang *et al.*, 1996a). Several other keratinocyte cell lines showed equivalent binding levels. In contrast, a receptor binding study was used to estimate the number of epidermal growth factor receptors (EGFRs) in NIH3T3 fibroblasts to be about 70,000 per cell (Roque *et al.*, 1992).

Ron expression has been seen in the glandular epithelium of the gastrointestinal tract, including the 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 (Chodosh *et al*., 2000; Hess *et al*., 2003b). Ron protein is also expressed in tumor cells from the breast, colon, pancreas, liver, gastric system, kidney and lung, and haematopoietic cells (Gaudino *et al*., 1994). 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 is expressed during the process of mouse embryo implantation and placentation. *In vivo*, Ron is expressed in the invading ectoplacental cone and trophoblast giant cell regions surrounding the implanting embryo. Using several murine trophoblast cell lines, HGFL stimulation has been shown to increase invasion through a basement membrane component material (Matrigel) and to enhance cell survival (Hess *et al*., 2003a). In liver progenitor cells, the Ron receptor induces additional cell responses in response to ligand stimulation, including cell scattering (motility), DNA synthesis, and extracellular matrix invasion (Medico *et al*., 1996). These normal cellular responses are also mechanisms by which tumor cells propagate, invade, and metastasize.

VI. EPITHELIAL TO MESENCHYMAL TRANSITION

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. Cells undergoing EMT demonstrate a transition from cuboidal to 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, characteristics resembling 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 alterations in cell motility (Wang *et al*., 2004). 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.

VII. 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. Cells expressing a point mutation in the kinase domain (M1231T) and those expressing wild-type Ron showed equivalent tumor latency and 100% tumor formation in the nude mice. To determine whether 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 (Peace *et al.*, 2001).

The oncogenic potential of similar point mutations in the human Ron gene has also been investigated (Williams *et al.*, 1999). The point mutations D1232V and M1254T in the tyrosine kinase domain of the Kit and Ret receptor respectively are found in human malignancies 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 these mutant receptors in fibroblasts induced constitutive Ron receptor phosphorylation. Phosphorylation and constitutive activation of Ron also led to activation of its downstream target, the mitogen-activated protein kinase (MAPK) (Santoro *et al.*, 1998).

A constitutively active form of Ron has also been produced as a Tpr-Ron chimera that mimics the oncogenic form Tpr-Met (Santoro *et al.*, 1996). 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.

VIII. LOSS OF FUNCTION MOUSE MODELS FOR Ron

To dissect the function of Ron *in vivo*, several different mouse models with defects in Ron were produced. 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 strategy knocks-out completely a large genomic region of Ron containing Ron 5′-flanking sequences, the extracellular domain, the transmembrane domain, and a portion of the intracellular domain of the Ron gene. Strikingly, mice with this large deletion of Ron are lethal at an early stage of embryo development (e7.5) (Muraoka *et al.*, 1999). Mice that were hemizygous for this deletion 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 mutant line made it impossible to further 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 eight 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 (Waltz *et al.*, 2001). Homozygous mice with this germline deletion, referred to at the $TK^{-/-}$ mice, 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 also been used to examine the role of Ron signaling in oncogene-mediated tumorigenesis.

A similar enhanced response to inflammation was observed in another mutant Ron mouse model (Correll *et al.*, 1997). In this case, the gene targeting strategy inserted a β-galactosidase gene into exon 1 of the mouse Ron gene, so that transcription of the reporter would arise from the endogenous reporter and would be translated from its own start site. With this

strategy, homozygous mutant mice were produced that were viable and phenotypicaly normal. In this model, 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 in all three mutant mouse models demonstrates that the Ron gene plays a significant role in the negative regulation of inflammatory responses.

The expression of the ligand for Ron, HGFL, has also been deleted in a mouse model ($HGFL^{-/-}$) (Bezerra *et al.*, 1998). 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 $HGFL^{-/-}$ 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.

IX. 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 $TK^{-/-}$) were crossed with mice predisposed to mammary cancer through expression of polyoma virus middle T antigen (pMT) under the control of the MMTV promoter (MMTV-pMT) (Peace *et al.*, 2005). 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 $TK^{-/-}$) markedly impacted mammary tumor latency, tumor growth, and metastasis compared to mice with intact Ron signal function (MMTV-pMT/Ron $TK^{+/+}$). 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. In this model, the experiments demonstrated that Ron impacted tumorigenesis through the MAPK and Akt signaling pathways.

Loss of Ron signaling was also examined in the context of skin carcinogenesis using a model of chemically-induced Ras-mediated skin cancer (Chan *et al.*, 2005). Mice expressing a mutated Ras transgene (v-Ha-Ras; Tg.AC) were crossed to mice deficient in the Ron tyrosine kinase domain ($TK^{-/-}$). 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 signaling 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.

X. 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) (Chen *et al.*, 2002). 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. These data suggest 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 MMTV promoter, was developed in order to analyze the role of Ron overexpression in mammary tumorigenesis (Zinser *et al.*, 2006). 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.

XI. 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 pleotrophic effects dependent on cell type (Iwama *et al.*, 1996). 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 (Danilkovitch *et al.*, 2000). The activation of PI3-K leads to activation of Akt, which has been shown to enhance cell survival, but is not required for metastasis (Hutchinson *et al.*, 2001).

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) is required for the biological activities of Ron (Fig. 1). These tyrosine residues serve as docking sites for signaling molecules having Src homology-2 (SH2) and the phosphotyrosine binding domains (PTB). 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 (Li *et al*., 1995). 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 (van den Akker *et al*., 2004). Gab1-mediated signaling is important for inducing the branching morphogenesis (Maroun *et al*., 2000). PI3K can also interact with Ron receptor either directly or through adaptor molecules (Danilkovitch *et al*., 2000). 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 (Danilkovitch *et al*., 1999b; Wang *et al*., 1996b).

Ron is a strong inducer of both PI3-K and MAPK signaling pathways *in vivo* and *in vitro*. 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 pMT under MMTV promoter, loss of Ron receptor signaling leads to a significant decrease in pMAPK and pAkt in tumor lysates compared with that in mice with wild-type Ron (Peace *et al*., 2005). 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 (Danilkovitch-Miagkova *et al*., 2001).

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 (Danilkovitch-Miagkova *et al*., 2000). 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 $\alpha 6\beta 4$ integrin (Santoro *et al.*, 2003). 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.

XII. 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 protooncogene. Accordingly, the regulation, expression, and interaction of Ron and Met have been studied in several normal tissues 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 (Follenzi *et al.*, 2000). 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 (Follenzi *et al*., 2000).

The cross-talk between Ron and Met is also relevant to ovarian cancer (Maggiora *et al*., 2003). 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 coadministration 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 (Lee *et al*., 2005). 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 (Cheng *et al*., 2005). 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 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 (Peace *et al*., 2003). This dominant-negative EGFR markedly 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. Cotransfection of dominant-negative Ron with wild-type EGFR, or cotransfection 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. Transphosphorylation 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). Coimmunoprecipitation 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 (Hsu *et al.*, 2006). 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 either Ron or EGFR expression via the transfection of small interfering RNA (siRNA) reduced ligand-independent phosphorylation of both receptors, although interestingly, the reduction in 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 (Mera *et al.*, 1999). 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 NIH-3T3 cells expressing Ron. Saturating concentrations of HGFL and 100 nM of Sema 4D synergistically increased NIH3T3 cell invasion as compared to controls (Conrotto *et al.*, 2004).

XIII. 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 antiangiogenic 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 (Kesisis *et al.*, 2007). 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 (Peace *et al.*, 2005). Tumors induced by polyoma middle T expression, with or without Ron signaling, were examined in blood vessels by immunohistological staining. It was demonstrated that the ablation of Ron signaling was associated with a significant reduction in microvessel density. Further studies are required to define the significance of Ron in tumor angiogenesis.

XIV. 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 (Zinser *et al.*, 2006). 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. This work explores a previously unexamined role for Ron in genomic stability in cancer.

XV. RON EXPRESSION IN HUMAN TUMORS AND TUMOR-DERIVED CELL LINES

The growing awareness of the potential role for Ron in human cancer has lead to a recent examination of Ron expression in a range of human tumor types and tumor-derived cell lines (O'Toole *et al.*, 2006). 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. The following sections will briefly describe the current information that is available about Ron expression in different tumor types.

A. Breast Cancer

The most compelling information about the overexpression of Ron in tumor tissue is demonstrated in breast cancer. The first report examining the expression of Ron in human breast tumor tissue showed that Ron is overexpressed in about 50% of breast tumors. Its expression is very low in normal mammary gland and in benign lesions but is significantly higher in primary breast carcinomas (Maggiora *et al.*, 1998). Ron receptor is highly expressed in epithelial breast cancer cells including T47D and ZR 75-1 cells (Gaudino *et al.*, 1994). HGFL is able to induce Ron activation in T47D cells, and stimulation of Ron receptor in ZR 75.1 cells causes increased cell proliferation, invasion, and about a 12-fold increase in migration (Gaudino *et al.*, 1994; Maggiora *et al.*, 1998). Interestingly, a feline form of Ron was found to be overexpressed in about 33% of archival feline mammary carcinoma samples tested (De Maria *et al.*, 2002). Mouse models have also demonstrated an important role for Ron in mammary tumorigenesis (Peace *et al.*, 2005; Zinser *et al.*, 2006).

B. Prostate Cancer

The recent work showing that Ron is expressed in breast cancer suggests that Ron may be important in prostate cancer as well. A recent survey of Ron expression in human cancer showed that Ron is expressed in 92% of the prostate tumor tissues examined, and that Ron was highly expressed in several prostate cancer cell lines including PC-3, DU145, and LnCAP (O'Toole *et al.*, 2006). The Waltz laboratory has examined a panel of human prostate tissue in order to determine the relationship of Ron expression to tumor stage. Preliminary data indicates that Ron expression is limited in the normal prostate epithelium, and expression increases progressively with stage of disease in benign prostate hyperplasia, compared to prostate

adenocarcinomas and prostate metastases. Preliminary results also indicate that Ron may play an important role in prostate cancer *in vivo* as well.

C. Pancreatic Cancer

Since Ron appears to be associated with ductal epithelium, such as breast tissue, the expression and function of Ron in pancreatic cancer was examined. Ron receptor is highly expressed in several human pancreatic cell lines, including BxPC-3, CFPAC-1, FG, and L3.6 pl and murine pancreatic cancer cell lines 4964PDA, 4964LM, 5143PDA, and 5143LM (Camp *et al*., 2007; Thomas *et al*., 2007). Ron activation in the human pancreatic cell line L3.6 pl leads to activation of the Erk and Akt pathways that are downstream of Ron and showed characteristics of EMT, including HGFL-induced L3.6 pl cell shape changes, migration, and invasion. Migration and invasion in these cell lines in response to ligand stimulation was blocked by a neutralizing monoclonal antibody against Ron. The L3.6 pl cells also showed loss of E-cadherin and increased nuclear translocation of β-catenin in response to HGFL stimulation. Monoclonal antibody blockage of Ron signaling successfully decreased subcutaneous and orthotropic tumors growth formed by injecting human L3.6 pl cells into nude mice (Camp *et al*., 2007). The response of migration and invasion after ligand stimulation was also shown in several other pancreatic cell lines (Thomas *et al*., 2007). Ron expression was also examined in human pancreatic tumor tissue samples by immunohistochemistry. Ron expression was very low in normal ductal epithelia, and significantly increased in invasive and metastatic cancers. In one report, 93% of the human pancreatic cancer tissues showed overexpression of Ron relative to normal ductal epithelium (Camp *et al*., 2007). In another report, 79% of the primary pancreatic cancers and 83% of the metastatic lesions overexpressed Ron. In addition, 100% of eight invasive carcinoma tissue specimen tested for phophorylated Ron receptor had positive staining (Thomas *et al*., 2007). A mouse model of pancreatic cancer (PdxCre/LSL-KRAS^{G12D}) was also examined for overexpression of Ron by immunohistochemistry at 6 months of age. In this model, the increase in Ron expression with the progression of disease was similar to that seen in human tissue samples, with normal pancreatic ducts showing very low-level Ron expression that increased with tumor grade (Thomas *et al*., 2007).

D. Renal Tumors

Both Ron and HGFL have been shown to be expressed in normal human renal tissue (Rampino *et al*., 2002). Although the liver is the primary site of HGFL ligand production, it has also been shown that HGFL is produced by

cultured tubular cells of the kidney *in vitro*, and that cultured human mesangial cells express Ron and are activated by HGFL from tubular cell supernatant. This ligand stimulation was found to induce proliferation, migration, and invasion of the mesangial cells, properties which are important in tumorigenesis. The expression of Ron was then studied in a number of different renal tumor types (Rampino *et al.*, 2003). Ron was strongly expressed in a phosphorylated form in oncocytomas, a benign tumor, and was not found in renal carcinomas. The mechanism by which Ron promoted this tumor growth appeared to be predominately by opposing apoptosis rather than inducing proliferation.

E. Bladder Cancer

Immunohistochemical analysis of a panel of bladder cancer specimens showed that Ron was overexpressed in 32.8% of the primary tumors, and 23.3% of these positive tumors showed high levels of expression. Overexpression of Ron *in vitro* in a bladder cancer cell line increased cell proliferation, motility, and resistance to apoptosis (Cheng *et al.*, 2005). Ron crosstalk with the Met receptor and with the EGFR receptor was shown in bladder cancer cell lines (Cheng *et al.*, 2005; Hsu *et al.*, 2006).

F. Ovarian Cancer

Ron expression was detected in 55% of the human ovarian cancer tissues specimens that were examined. HGFL stimulation was examined in this tissue type *in vitro*, and caused increased motility and invasion of SK-OV3 ovarian carcinoma cells that have high-level Ron expression (Maggiora *et al.*, 2003).

G. Lung Cancer

Ron is expressed in normal lung, and is localized to the apical surface of ciliated epithelium. Stimulation of Ron with its ligand HGFL increased ciliary beat frequency, and therefore Ron may play a role in mucociliary lung clearance. Ron has also been identified in small cell carcinoma of the lung (SCLC), in a pulmonary carcinoid cell line, and in a SCLC cell line (Willett *et al.*, 1997). Ron was also examined in nonsmall cell lung cancer (NSCLC) (Willett *et al.*, 1998). Ron was expressed in both primary human tumor tissue and in NSCLC cell lines. *In vitro* tests of Ron activity in these cell lines showed that ligand stimulation induced Ron phosphorylation,

showing that Ron was active, and that ligand stimulation increased cell motility, an important component of metastasis.

H. Gastrointestinal Tumors

The distribution and expression level of Ron in normal human gastrointestinal organs was examined by immunohistochemistry, and a comparison was made of expression between adult and fetal tissue (Okino *et al.*, 2001). High-level expression was seen in the esophagus, small intestine, and colon, but in gall bladder was negative. Immunoreactivity for Ron was strong in fetal stomach and pancreas, but was faint in these organs in the normal adult tissues. This result suggests that Ron may be associated with differentiation in these organs, and that it may function as an oncofetal protein. Several splicing variants of Ron were initially isolated from gastrointestinal origin cell lines and tissues. The first was isolated from a gastric cancer cell line, and was termed ΔRon (Collesi *et al.*, 1996). This splicing variant has a molecular weight of 165 kDa. It dimerizes in the intracellular compartment and is constitutively active. The same form was later identified in normal and malignant human colonic tissues (Okino *et al.*, 1999). The expression of Ron was in general related to the degree of differentiation of the tissue. Other splice variants, RonΔ160 and RonΔ155, were also originally isolated from colorectal cancer cells, and then identified in human primary adenocarcinomas (Wang *et al.*, 2000; Zhou *et al.*, 2003) These variants caused cellular transformation as tested by focus-formation assay when expressed in NIH3T3 cells. RonΔ165 and RonΔ155 stable transfectants also gave multiple colonies when grown in soft agar, showing their transforming potential. These activated forms of Ron also showed transforming potential *in vivo*. RonΔ160 and RonΔ155 expressed in NIH3T3 cells formed tumors when xenografted on the flank of nude mice.

I. Liver Cancers

Ron mRNA and protein is expressed in normal human liver, and its expression has been localized to hepatocytes and Kupffer cells, the resident macrophage population of the liver. Indeed, ablation of Ron receptor activity negates the detrimental effect of bacterial lipopolysaccaride (LPS) in a murine model of acute liver failure, a process induced by the action of LPS on Kupffer cells (Leonis *et al.*, 2002).

Ron has also been shown to be overexpressed in 2 of 7 HCC tissue samples (Chen *et al.*, 1997), and approximately one-half of 45 hepatoblastoma tumor specimens analyzed (Leonis and Waltz, unpublished observations).

Various cytokines, such as IL-1α, IL-6, and TNF-α, and growth factors like HGF, increase Ron expression in the HCC cell line HepG2. These cytokines are known to be upregulated in liver disease, including the LPS-induced murine model of acute liver failure described above, and thus alterations in the production of these cytokines may play an important role in inducing liver tumors, in part by modulating Ron receptor expression (Chen *et al.*, 1997; Leonis *et al.*, 2002).

J. Short Form Ron

Another truncated form of the Ron gene was first identified in mice from the locus that confers susceptibility to Friend virus-induced erythroleukemia (Persons *et al.*, 1999). This short form of the receptor is deleted at the C terminus, but retains the transmembrane and intracellular domains of the protein. In mice, the short form Ron was found to interact with the envelope glycoprotein of Friend virus (Nishigaki *et al.*, 2001). An equivalent truncated short form has also been identified for the human Ron gene (Bardella *et al.*, 2004). This form has been identified in both normal and cancer cells, including ovarian, pancreatic, gastrointestinal, and leukemic cells. Expression of this short form Ron induces characteristics of EMT, including shape change, motility, and anchorage-independent growth. This short form may be responsible for cell motility (Ghigna *et al.*, 2005). A specific involvement of a virus equivalent to Friend virus in humans has not been identified.

XVI. Ron AS A TARGET OF CANCER THERAPY

In the last 10 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 EGFR (Cohen *et al.*, 2004), human epidermal receptor 2 (HER2/neu) (Barlesi *et al.*, 2005; Rabindran *et al.*, 2004), or drugs that target more than one receptor (Plosker and Keam, 2006; Wong *et al.*, 2006); and both platelet-derived growth factor (PDGFR) and vascular endothelial growth factor (VEGFR) receptors (Caponigro *et al.*, 2005; Izzedine *et al.*, 2007).

One of the first receptor tyrosine kinase that was targeted is the EGFR. Both small molecule inhibitors and anti-EGFR antibodies have been

approved for clinical use and have been used together, and in combination with chemotherapy or radiation (Huang *et al*., 2004). 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 (Izzedine *et al*., 2007). 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 mice with breast tumors, the 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 (Sacco *et al*., 2003).

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 (O'Toole *et al*., 2006). This antibody not only inhibits the binding of HGFL to Ron, but also diminished Ron phosphorylation and its downstream signaling. In addition, this antibody 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 commercially-available mouse monoclonal blocking antibody (R&D systems). Treatment of BxPC-3 pancreatic cancer cells with this monoclonal antibody against Ron, followed by 0.1 μmol/l of gemcitabine, resulted in 32% increase in apoptosis as compared to gemcitabine alone (Thomas *et al*., 2007). 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 (Montero-Julian *et al*., 1998).

The extracellular region of the Ron β chain contains a Sema domain, a plexin, semaphorins, and integrins (PSI) domain, and also four IPTs (immunoglobulins like fold shared by plexins and transcription factors) domains (Danilkovitch-Miagkova, 2003). 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 (Angeloni *et al*., 2004).

Chemotherapeutic agents that impair Hsp (heat shock protein) functions are geldanamycins. Hsps are important chaperone proteins that facilitate correct protein folding and assembly. Several receptor tyrosine kinases including Ron are sensitive to these drugs (Germano *et al.*, 2006). 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 siRNA against Ron expressed in human colorectal carcinoma significantly reduced cancer cell proliferation, motility, and increased apoptotic susceptibility of the cells (Xu *et al.*, 2004).

Other types of combination therapy may also be beneficial. 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 can be a useful combinatorial therapy, since these pathways are upregulated in different cancers in response to Ron activation.

XVII. CONCLUSIONS

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

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