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The Ron Receptor Tyrosine Kinase in Tumorigenesis and Metastasis

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Summary

The Ron receptor tyrosine kinase has been increasingly recognized for its tumorigenic potential in the last decade. Ron receptor activation leads to the activation of common receptor tyrosine kinase downstream signaling pathways, and most prominently in tumor models, activation of MAPK, PI3K and β -catenin. Numerous experimental models of mammalian tumorigenesis have demonstrated that increased Ron receptor activity correlates with increased tumorigenesis in a variety of organs of epithelial origin. The evidence for Ron as an oncogene in human tumor biology is growing. The Ron receptor is overexpressed and over-activated in a large number of human tumors, and overexpression of Ron correlates with a worse clinical outcome for patients in at least two human cancer states, namely breast and bladder cancer. Several experimental approaches have been taken to successfully block Ron activity and function, and given this convincing data, approaches to block Ron receptor activity in targeted human cancers should prove to be fruitful in the setting of future clinical research trials.

Keywords

receptor tyrosine kinases; Ron receptor; tumorigenesis; hepatocyte growth factor; therapeutics

Introduction

Activated growth factor receptor tyrosine kinases elicit a variety of important biological responses that regulate tumorigenesis. Several recent studies have documented Ron receptor overexpression and activation in a number of human cancers suggesting that Ron may be a critical factor in cellular transformation[1–7]. The Ron receptor, also referred to as macrophage stimulating 1 receptor (MST1R) or stem cell-derived kinase (Stk) in the mouse, belongs to the Met family of receptor tyrosine kinases. The protein contains a total of 1400

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amino acids and includes a signal peptide, an extracellular domain which contains a sema domain that is unique to this receptor family, a single pass transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular domain of Ron is 25% identical to the extracellular domain of Met while the tyrosine kinase signaling domain of Ron is 63% identical.

Ron expression is preferentially observed in epithelial cells and select macrophage populations. In tissues, Ron expression has been detected in the central nervous system, liver, kidney, testes, bone, lung, breast, and epithelia of the digestive tract. In macrophages, Ron signaling regulates select cytokine and chemokine production in response to inflammatory insults[8–12]. The Ron protein is synthesized as a single chain precursor of approximately 185 kDa which is proteolytically cleaved and presented on the cell surface as a disulfide linked heterodimeric glycoprotein containing an alpha (35 kDa) and a beta (150 kDa) chain[13]. Based on the signaling pathways elicited by Met and Ron, the activities elicited by this receptor family have been termed “invasive growth[14].” While information regarding the clinical significance of this receptor *in vivo* is limited, recent studies document the increasingly recognized importance of this protein in tumor biology[1–7, 15, 16].

The Ron Receptor Ligand, Hepatocyte Growth Factor-Like Protein

Hepatocyte growth factor-like protein (HGFL), also referred to as macrophage stimulating 1 (MST1) or macrophage-stimulating protein (MSP), is the ligand for the Ron receptor[17]. The name HGFL was given to the first isolated cDNA construct encoding for this protein, based on sequence similarity to hepatocyte growth factor[18]. HGFL is primarily expressed in hepatocytes and is secreted into the bloodstream as a biologically inactive single chain precursor of 80 kDa at a concentration of approximately 400ng/ml. Pro-HGFL is proteolytically cleaved by membrane-associated proteases such as matriptase or by members of the coagulation cascade. Recent studies have shown matriptase expression is sufficient for local HGFL cleavage leading to activation of Ron[19–22]. Interestingly, both Ron and matriptase are frequently upregulated in human cancers although coordinate upregulation of both proteins has not been documented. Proteolytic cleavage of HGFL results in the formation of a disulfide linked heterodimer composed of an alpha (50 kDa) and a beta (35 kDa) chain. The alpha chain of HGFL contains four kringle domains which are important for the function of HGFL while the beta chain of HGFL contains a serine protease-like domain and is primarily responsible for binding to Ron. HGFL was originally identified as a chemoattractant for macrophages but appears to regulate a diverse array of effects including cell proliferation, motility and invasion. However, gene-targeting studies in mice have shown that HGFL is not essential for embryogenesis, fertility, or wound healing. Unchallenged HGFL-deficient mice do exhibit lipid-containing cytoplasmic vacuoles in hepatocytes, however, this finding is of unclear significance[23].

Variant Forms of the Ron Receptor

In addition to full length Ron overexpression, a number of variants of Ron have been identified that are a result of either mRNA splicing or alternative initiation. Variants of Ron have been identified in both normal and malignant tissues. In mice, short-form (SF-)Ron is

naturally expressed in select cells and tissues including expression in the spleen and in splenic T cells, bone marrow, testes, lung, ovary, peritoneal macrophages, and in several hematopoietic cells including stem cells[24, 25]. SF-Ron is generated from an alternative start site located in intron 10 of the Ron gene and generates a Ron protein containing only a small extracellular portion along with the transmembrane and intracellular domains. A recent report has shown that the expression of full length Ron and SF-Ron may be the result of variations in the methylation patterns within two distinct CpG islands in the Ron proximal promoter, wherein widespread hypermethylation associates with lack of full length Ron expression whereas hypermethylation of the distal island associates with transcriptional activity of SF-Ron[26]. *In vivo*, SF-Ron confers susceptibility to Friend virus-induced erythroleukemia and SF-Ron kinase activity is required for erythropoietin-independent expansion of erythroid progenitors in response to Friend virus[25, 27]. SF-Ron is also expressed in human ovarian and breast cancers as well as in several human cancer cell lines derived from the pancreas, breast, ovary, lung and leukemias[24]. SF-Ron overexpression in epithelial cells results in an aggressive phenotype *in vitro* characterized by faster growth, motility, anchorage and contact independence and an alteration in morphology. Recent studies have also suggested that SF-Ron has nonredundant biological functions relative to full-length Ron in the progression of inflammatory immune responses *in vivo*. These observations suggest that the function of SF-Ron is biologically distinct from that of the full-length receptor and may participate in tumorigenesis[25].

In addition to SF-Ron, a number of other splice variants of Ron have been found in several cancers including lung, colon, and breast cancer. Four splice variants, referred to as Ron 165, Ron 160, Ron 155, Ron 55 based on their molecular weight, appear to have constitutive receptor activity and may induce oncogenic properties in cell lines[28]. However, the contribution of these variants to human tumorigenesis is unclear at present given that these studies had the potentially confounding concomitant overexpression of full length Ron. This is further supported by a recent study that suggests that the activity of SF-Ron may be affected by full length Ron expression[26]. Further studies are needed to clarify the clinical significance of Ron variants as causative factors in human tumorigenesis and as targets for therapeutic intervention.

Endogenous point mutations in Ron that induce receptor activity have not been identified or systematically explored. However, several constitutively active Ron receptor variants have been generated incorporating analogous naturally-occurring point mutations found in the Met, Kit and Ret receptor tyrosine kinases[29, 30].

Ron Receptor Signaling Mechanisms

Binding of HGFL to wild type Ron receptor leads to receptor dimerization, activation of the receptor's intrinsic tyrosine kinase activity and trans-autophosphorylation of several intracellular C-terminal tyrosine residues. These phosphorylated tyrosine residues serve as high-affinity docking sites for effector proteins containing Src homology 2 and phosphotyrosine-binding domains, including phosphatidylinositol 3-kinase (PI3K), phospholipase C γ , and growth factor receptor bound 2 (Grb2)[31, 32]. Grb2 and Sos recruitment to the Ron receptor can convert Ras-GDP into its active form, leading to

activation of PI3K/Akt and/or mitogen activated protein kinase (MAPK)[33–35]. FAK, Src, Jun kinase (JNK) and STAT3 are also involved in HGFL/Ron receptor signaling, but their direct upstream interactions with the Ron receptor have not yet been identified[36, 37]. Additional downstream components that mediate various HGFL-induced activities include activation of β -catenin and NF- κ B signaling pathways. In addition, several signaling pathways are activated when Ron is overexpressed in cell lines, such as β -catenin, Ras/MAPK, and PI3K. Thus, Ron activation leads to the recruitment of numerous signaling pathways that are capable of inducing a variety of pleiotropic responses.

β -catenin is part of the Wnt signaling pathway and β -catenin expression has been associated with several human cancers. A novel mechanism for activating β -catenin has recently been proposed whereby tyrosine phosphorylation of β -catenin by receptor tyrosine kinases leads to stabilization and nuclear translocation of β -catenin. This signaling interaction has been shown for the Ron, Met and VEGF receptors[38–40]. In one study, oncogenic activating mutants of Ron lead to activation of the β -catenin signaling pathway. In transfected cell lines, β -catenin activation (ie. tyrosine phosphorylation, nuclear translocation and increased expression of the downstream β -catenin target genes, *c-myc* and *cyclin D1*) occurs with M1231T and D1232V mutant Ron receptors in a ligand-independent manner, but not with wild type Ron receptor. Interestingly, both wild type and constitutively-active Ron receptors co-immunoprecipitated with β -catenin[41]. *In vivo*, mammary tumors derived from mice overexpressing Ron are associated with an increase in β -catenin and in *c-myc* and *cyclin D1* gene expression levels[16]. Lastly, human pancreatic cancer cells treated with HGFL also have increased nuclear β -catenin, and human colorectal carcinoma cells with suppressed Ron expression have decreased β -catenin expression[1, 42]. These studies suggest that receptor tyrosine kinase-induced tyrosine phosphorylation of β -catenin is a novel mechanism for stabilization and nuclear accumulation of β -catenin that occurs in human cancer[38–40]. Interestingly, increased tyrosine phosphorylation of β -catenin has been linked to disruption of cell adhesion and suggests that Ron activation of this pathway may function to regulate epithelial-mesenchymal transformation or cell migration leading to tumor metastasis[43].

Similarly, the MAPK and PI3K/Akt signaling pathways are also upregulated in a variety of cancers. In several human cancer cell lines containing high Ron expression, such as those derived from colon, prostate, lung, pancreas and stomach, activation of both of these pathways can occur. Activated MAPK or Akt can be abrogated using a neutralizing antibody against Ron.[5] In a mammalian model of tumorigenesis, mammary tumors from mice lacking functional Ron due to deletion of the tyrosine kinase domain of Ron also have decreased MAPK activation and decreased amounts of phosphorylated Akt[44]. Conversely, the addition of HGFL to several cell types results in increased MAPK activation[5, 37]. Taken together, these data demonstrate the importance of Ron expression in the regulation of signaling pathways that are often highly associated with human cancer initiation and progression.

The Ron receptor also participates in cross-talk with other receptor tyrosine kinases and integrins, which may be important in regulating the therapeutic response of human tumors. Ron has been shown to associate with the receptor tyrosine kinases Met and epidermal

growth factor receptor (EGFR) by immunoprecipitation and cross-linking experiments[3, 45, 46]. These associations result in receptor transphosphorylation, and in the case of Ron interactions with EGFR, co-immunoprecipitation and phosphorylation of PI3K[3, 45, 46].

Functional Importance of Ron in Murine Models of Tumorigenesis

To define the significance of Ron overexpression and activation *in vivo*, transgenic mice that overexpress a wild type or constitutively active Ron receptor in the mammary epithelium have been generated[16]. In this model, Ron overexpression was sufficient to induce mammary transformation in all animals and was associated with a high degree of metastasis, with metastatic foci detected in liver and lungs of >86% of all transgenic animals. These studies are the first to demonstrate that Ron overexpression can be a causative factor for metastatic breast cancer. In addition, this data correlates well with the observation that Ron overexpression in human breast cancer is associated with an aggressive cancer phenotype with decreased disease free survival time in patients and an increase in breast cancer metastasis[4]. Transgenic overexpression of Ron has also been performed in the murine pulmonary epithelium. In this model, 90–95% of the transgenic mice develop lung adenomas between 6 to 14 months of age[47].

In addition to Ron overexpression, two additional reports have documented the *in vivo* significance of Ron signaling in tumor initiation and progression by performing loss-of-function analyses[44, 48]. In one model, gene targeted mice lacking functional Ron were crossed with mice that are predisposed to breast cancer and metastasis by the mammary specific overexpression of polyoma virus middle T antigen (pMT)[44]. The pMT expressing mice develop aggressive mammary tumors that metastasize to the lung. The loss of Ron in this model results in a significant decrease in the rate of mammary tumor initiation, fewer numbers of tumors, and a decrease in mammary tumor size. In addition, tumors from the Ron-deficient mice exhibited less cellular proliferation and microvessel density compared to controls and had an increase in the amount of apoptotic cells. In the second model, the impact of Ron signaling was analyzed in a Ras-mediated model of chemically-induced skin carcinogenesis[48]. In this model, there were a greater number of benign skin papillomas produced in mice lacking Ron signaling. However, the absence of Ron resulted in papillomas that were smaller in size with significant decreases in cell proliferation compared to papillomas generated in control mice. Importantly, the loss of Ron in these papillomas leads to a significant reduction in the progression of the benign papillomas toward malignancy. Overall, these *in vivo* studies exemplify the significance of Ron in tumor formation, growth and metastasis.

Increased Expression of Ron in Multiple Human Tumor Types

Several human tumor types have increased Ron expression, including tumors of the breast, colon, lung, liver, kidney, ovary, stomach, pancreas, bladder and prostate[1–7, 49, 50]. Ron expression is also elevated in several cell types derived from these tumors. High expression of Ron is positively correlated with a poor prognosis in several types of human cancers, including those of the breast, bladder, prostate, colon, and pancreas; in addition, overexpression of both Ron and Met in breast and bladder cancers is associated with overall

decreased patient survival[2, 4, 5, 51]. Based on gene expression analyses performed using the Oncomine™ Cancer Profiling Database (www.oncomine.org), in breast cancer, increasing Ron expression is associated with metastatic disease, and in prostate cancer, Ron expression is correlated with advanced, androgen-independent cancers. Altogether, these data suggest that Ron is important in tumor progression and metastasis and as a clinical marker of advanced disease.

Targeting Ron as a Therapeutic Approach in Human Cancer

Given these encouraging results showing a significant role for Ron in mammalian tumorigenesis, a number of approaches have been advanced to target Ron signaling in an attempt to block Ron's tumorigenic impact. Several antibodies have been developed to neutralize the activities of Ron by preventing HGFL binding, and in addition, some of these antibodies may also have ligand-independent effects[5, 52]. These neutralizing antibodies would be anticipated to be effective in treating tumors containing high expression of Ron. In cell culture studies, antibodies directed against Ron are able to inhibit receptor-induced MAPK and Akt activation, as well as cellular migration. Moreover, in xenograph models of pancreatic cancer using Ron overexpressing pancreatic cells, antibody neutralization directed against Ron is able to reduce subcutaneous tumor growth[1, 5]. Moreover, antibody neutralization of Ron increases gemcitabine-induced apoptosis of Ron-expressing pancreatic cancer cells[51]. Thus, in several experimental settings, Ron activity has been effectively abrogated with the use of commercially available neutralizing antibodies.

In addition to neutralizing antibodies to target Ron, small molecule inhibitors have also been developed. A small peptide inhibitor encompassing five amino acids from the intracellular carboxy terminal of the Met receptor inhibits HGFL-induced Ron receptor phosphorylation, as well as Met-receptor induced cell scattering, invasion and branched morphogenesis (all properties shared between Met and Ron receptors)[53]. Also, a soluble peptide fragment encompassing a portion of the extracellular domain of Ron acts in a dominant-negative manner, inhibiting growth of HGFL-responsive cancer cells. This molecule is able to inhibit HGFL-induced phosphorylation of Ron and also has inhibitory effects on the growth of colon cancer cells[54]. Another possible treatment modality for negatively regulating receptor tyrosine kinase activity in human cancers include a class of anti-tumor drugs called geldanamycins, which have recently been shown to inhibit tumor cell growth by preventing proper folding and degradation of oncogenic proteins, including the Ron receptor[55]. These small molecules that negatively modulate Ron activity provide an alternative approach to targeting this receptor *in vivo*.

Tumor cell lines containing a knockdown of Ron through the use of small interfering RNA (siRNA) demonstrated that an efficient knockdown of this receptor could be achieved resulting in decreased cell proliferation, motility and an increase in cell death[42]. This finding suggests that targeting Ron through specific siRNA may also be effective for combating Ron overexpression and tumor growth inhibition.

As noted earlier, Ron receptor has been found to associate with other receptor tyrosine kinases. In addition to EGFR, Ron is also co-expressed with the Met receptor in bladder,

breast and liver cancers, and this co-expression has been linked with poor prognosis[2, 4, 50]. Combined, these studies suggest that targeting multiple receptor tyrosine kinases in these cancers might prove more effective than targeting either one individually.

Currently there are several ongoing human cancer clinical trials using small molecule compounds that target Ron in addition to other receptor tyrosine kinases[56, 57]. However, there are no human clinical trials underway that selectively target the Ron receptor itself. Given the capacity of Ron to associate with several other receptor tyrosine kinases involved in tumorigenesis, treatment directed against one member of a receptor association may be expected to interfere with the response of the corresponding partner receptor. Initial data from these trials suggests that the compounds are clinically well-tolerated and that they have impressive antitumor activity in patients with a broad range of metastatic tumor types who have failed prior treatment[56, 57]. The data outlined above suggests that Ron is a critical factor in human tumorigenesis, and that inhibition of Ron, either alone or in combination therapy, may prove to be beneficial in the treatment of high-risk cancer patients.

Future Perspective

Ten years ago there was little documented evidence in the literature to support a role for the Ron receptor in tumorigenesis aside from its sequence similarity to Met and Sea, which were both known oncogenes (the latter is the avian equivalent of the Ron receptor). In the brief period of time since its initial identification in 1993, Ron has been shown to be overexpressed, and/or over-activated in numerous human epithelial tumors, and multiple studies in experimental transgenic mouse models have substantiated a strong causative role for either overexpression of the wild type or constitutively active forms of this receptor tyrosine kinase in leading to *de novo* mammalian tumorigenesis.

In the upcoming decade, the impact of Ron receptor gain-of-function on human oncogenesis will be further substantiated in the literature, and more potential Ron-interfering compounds will be identified and investigated. Indeed, a strong case can be made to actively pursue therapeutic clinical study protocols aimed at blocking Ron receptor activity in human breast cancer with the data already available. Accumulating evidence will undoubtedly show this idea to be applicable to other human cancers.

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Executive Summary

Introduction

- Ron receptor structure and function is related to the *Met* receptor tyrosine kinase *proto-oncogene*.
- Ron receptor is expressed ubiquitously in epithelial cells and macrophages and regulates inflammatory insults and tumorigenesis.

The Ron Receptor Ligand, Hepatocyte Growth Factor-Like Protein

- The Ron receptor ligand is hepatocyte growth factor-like protein (HGFL), which circulates as an inactive precursor that requires proteases for activation.
- HGFL is not required for embryonic development but regulates a diverse array of proliferative and tumorigenic properties.

Variant Forms of the Ron Receptor

- Short-form Ron confers susceptibility to Friend Virus-induced erythroleukemia in mice.
- Additional Ron variants with constitutive receptor activity have been identified in human tumors and may contribute to tumorigenesis.

Ron Receptor Signaling Mechanisms

- Ron receptor activation draws upon commonly utilized receptor tyrosine kinase signaling pathways.
- Ron receptor participates in cross talk with other receptor tyrosine kinases.

Functional Importance of Ron in Murine Models of Tumorigenesis

- Ron loss-of-function leads to decreased tumor growth and malignant conversion in models of murine breast and skin tumorigenesis.
- Ron receptor gain-of-function leads to breast and lung tumorigenesis.

Increased Expression of Ron in Multiple Human Tumor Types

- Ron is overexpressed in multiple human tumor types and correlates with poor prognosis in breast and bladder cancer.
- Ron overexpression is associated with Met overexpression in breast, liver and bladder cancer.

Targeting Ron as a Therapeutic Approach in Human Cancer

- Several antibodies have been developed which are effective in blocking Ron activity in cell culture and *in vivo* models of tumorigenesis.
- Additional small molecule inhibitors of Ron activity have been identified that are effective in blocking Ron-mediated effects.

Future Prospective

- Ongoing clinical trials in human solid organ tumors utilizing compounds that nonspecifically inhibit Ron, as well as other receptor tyrosine kinases, are being performed with encouraging side effect profiles.
- In the future, targeted Ron receptor therapy may prove to be beneficial in human breast cancer, and other cancer types likely will follow suit.