

# The Macrophage Stimulating Protein/Ron Pathway as a Potential Therapeutic Target to Impede Multiple Mechanisms Involved in Breast Cancer Progression

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**Abstract:** Macrophage Stimulating Protein (MSP) is the only known ligand for the receptor tyrosine kinase Ron. The MSP/Ron pathway is involved in several important biological processes, including macrophage activity, wound healing, and epithelial cell behavior. A role for MSP/Ron in breast cancer has recently been elucidated, wherein this pathway regulates tumor growth, angiogenesis, and metastasis. Here, we review the recent literature surrounding MSP/Ron function in tumor cells, inflammatory cells, and osteoclasts – cell types that often coexist in breast tumor microenvironments. We discuss the potential implications of MSP/Ron activity occurring concurrently in these cell types on tumor progression and metastasis. Lastly, we outline the potential for targeting MSP/Ron as a novel therapy for breast cancer, and for other cancer types.

**Keywords:** Breast cancer, macrophage stimulating protein, metastasis, MSP, MST1R, osteolysis, Ron, therapeutic target.

## INTRODUCTION

Breast cancer has a relatively low case-fatality rate, but approximately 20% of women diagnosed with breast cancer eventually develop metastatic disease. Because breast cancer is highly prevalent and metastatic breast cancer is rarely curable, a significant number of women, about 40,600 in the U.S. per year, will die of the disease [1]. Although the incidence of breast cancer is much higher in women over 50, breast cancer is the major cause of death from all reasons in women age 35-50 and represents a major health care and societal problem.

The goal of initial treatment is to reduce the risk of both local and systemic recurrence. Initial treatment for localized breast cancer is designed to reduce the risk of in-breast and regional disease; local recurrence in breast, skin, subcutaneous tissues and axilla rarely causes death but often results in significant morbidity and can give rise to systemic metastases [2]. Local therapy begins with surgery: either lumpectomy to remove the tumor from the intact breast, or mastectomy. Radiation therapy reduces the risk of local recurrence and is almost always recommended after lumpectomy. Radiation is also generally recommended after mastectomy if the tumor is large or involves regional lymph nodes. Initial treatment of breast cancer often also includes systemic therapy designed to eradicate occult metastatic disease that is not clinically evident, but which may eventually cause relapse and death. Treatment given in this setting is termed “adjuvant therapy” and, because of the near-universal fatality of metastatic breast cancer, is recommended for the majority of women newly diagnosed with breast cancer in order to improve disease-free survival [3].

Systemic therapy for breast cancer may be given either as adjuvant therapy or as treatment of metastatic disease, and includes hormonal therapy, chemotherapy, and/or biological agents. Hormonal therapy is used if the malignant cells express estrogen and/or progesterone receptors. Chemotherapy of several different classes can be effective in all types of breast cancer, independent of hormone receptor expression. Targeted therapies used in breast cancer include trastuzumab, a monoclonal antibody approved for use in combination with chemotherapy for HER2 positive breast cancer, and lapatinib, a small molecule used for HER2 positive metastatic breast cancer. Bevacizumab, a VEGF inhibitor, is also approved for use in metastatic breast cancer [3]. Numerous other agents are being evaluated as potentially effective adjuvant therapies, notably bisphosphonates, which are best known for blockade of osteoclast function. Various bisphosphonates are in late-phase clinical trials and may reduce the risk not only of skeletal metastases, but visceral metastases as well [4].

Patients with metastatic breast cancer have a median survival of approximately two years [5]. Women with hormone-sensitive metastases limited to bone and soft tissue have on average a longer survival, while those with extensive parenchymal organ involvement usually have a shorter survival, particularly if the tumor does not express hormone receptors or HER2. These latter tumors are aggressive, and there are no targeted therapies available for this subtype of breast cancer. Clearly, there is a need for new therapies with greater efficacy against this disease, and a particular need for therapies that might reduce or prevent the growth of metastatic lesions. Targeted therapies are generally less toxic than other anti-cancer agents and, when effective, are invaluable in achieving the goal of treatment of metastatic breast cancer: to achieve disease control while avoiding toxicity due to therapy. Here, we discuss the exciting potential for a new therapeutic target in breast cancer: the receptor tyrosine kinase Ron.

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## THE MET/RON FAMILY OF RECEPTOR TYROSINE KINASES

The receptor tyrosine kinase Ron (also known as human MST1R, for macrophage stimulating 1 receptor, and as murine Stk1, for stem cell kinase 1) is the cell surface receptor for macrophage stimulating protein (MSP; also known as MST1, for macrophage stimulating 1, and as HGFL, for hepatocyte growth factor-like). In humans, Ron is one of only two members of a distinct receptor tyrosine kinase (RTK) family that also includes Met. The highest amino acid identity between Ron and Met is located within the kinase domain (63% identity); the other regions are not highly conserved (34% overall). The respective ligands for Ron and Met are also similar; MSP is 45% identical to hepatocyte growth factor (HGF), the Met ligand. Both ligands are glycoproteins that are secreted as inactive single-chain peptides and are proteolytically processed into active, disulfide-linked  $\alpha/\beta$  heterodimers [6]. HGF binds and activates Met and MSP binds and activates Ron [7-9]; although there is crosstalk between Ron and Met intracellular signaling [10, 11] the ligands and receptors are not interchangeable [12].

Like their ligands, Met and Ron are cleaved disulfide-linked heterodimers. The mature receptors consist of extracellular  $\alpha$  and  $\beta$  chains, involved in ligand binding, and the intracellular portion of the  $\beta$  chain, which is responsible for signaling. Binding of ligand causes receptor homodimerization and phosphorylation of two tyrosine residues within the catalytic site, which regulates kinase activity [8, 13]. Activation of kinase activity results in phosphorylation of the carboxy-terminal docking site of the receptor. The docking site is essential for downstream signaling through direct and indirect binding of SH2 domain-containing adaptor proteins such as Grb2, PI3K, and Src [14]. Ron and Met are both expressed in a variety of tissues during development and, in adults, are expressed mainly on epithelial cells and in the nervous system. However, Ron is also highly expressed on adult macrophages and osteoclasts.

Although the signaling pathways that are activated by Ron and Met are similar, they culminate in related, yet distinct, cellular functions. Both are known to induce “scattering,” a phenomenon in which cells detach from one another and migrate away from the central colony [10, 15, 16]; both promote proliferation through the MAPK pathway and survival through both MAPK and PI3K/AKT pathways [17-19]; and both have the ability to promote an epithelial-mesenchymal transition, albeit in distinct situations [20, 21]. However, a major effect of MSP/Ron signaling is on the motility and activation of macrophages – a function clearly divergent from that of Met.

Terminally differentiated macrophages express Ron and were first noted to respond to MSP by rapidly altering their shape and increasing chemotactic and phagocytic ability [22, 23]. More recently, it has been realized that MSP/Ron also plays a critical role in attenuation of the inflammatory response. Mice lacking Ron activity display defects in the inflammatory process, most notably the inability to downregulate TNF $\alpha$  and nitric oxide production in response to infection or injury [24-27]. Thus, MSP/Ron signaling plays a dual role in regulating inflammation: initial stimulation of chemotaxis and phagocytosis – important features of “classical” macrophage activation – and, more critically, resolution

of the inflammatory response by promotion of the “alternatively activated” macrophage state (discussed in more detail below).

MSP belongs to a group of kringle domain-containing proteins that diverged from an ancient family of serine proteases involved in blood coagulation and fibrinolysis [28]. Amino acid substitutions in the catalytic domain during evolution rendered MSP inactive as a protease, although it retained the feature of being cleaved and activated by other serine proteases. Such cleavage is, in fact, required for the conversion of pro-MSP to the mature, active form of MSP that can bind and activate Ron.

Activation of pro-MSP was originally discovered in wound exudates, where it resulted in stimulation of macrophage activity [23]. A serine protease responsible for activating pro-MSP was localized to macrophage membranes [29] and later identified as matriptase [30]. However, other proteases, such as hepatocyte growth factor activator, also appear to cleave and activate pro-MSP *in vivo* [31]. Pro-MSP is predominantly secreted from the liver, and exists in the blood plasma in its biologically inactive form at a concentration of about 5nM, and is thus poised to initiate Ron signaling upon cleavage [23].

Functional consequences of MSP/Ron signaling are not limited to macrophages or the inflammatory process. Ron, like Met, is upregulated in many types of epithelial cancer, and they are occasionally co-upregulated [32, 33]. Although the role of Met in cancer has been investigated for more than 20 years, culminating in development of multiple targeted therapies now in clinical trials (for a recent review, see [34]), Ron has more recently been recognized as a major player in progression of human epithelial cancers. This report focuses on the significance of the MSP/Ron pathway in breast cancer, and the ensuing opportunities for therapeutic intervention.

## RON EXPRESSION AND FUNCTION IN BREAST CANCER

Ron is expressed at very low levels in normal human breast epithelium, but becomes overexpressed in a large proportion of breast tumors (Table 1). Interestingly, Ron mutation is not associated with breast cancer, suggesting that overexpression of the wild type protein is sufficient to contribute to tumor development or progression. The reason for overexpression has not yet been established. Both MSP and Ron are located on chromosome 3p21.31, and the 3p21 region is often altered in cancer. Specifically, 3p21 undergoes both loss of heterozygosity [35] and amplification in various tumors and cancer cell lines [36, 37]. This region was amplified in 15-42% of lung, renal, and breast cancers examined [36], which suggests that amplification could contribute to MSP and/or Ron overexpression in breast cancer. Consistent with this, MSP is also overexpressed by up to ~20% of early stage human breast tumors [38].

Mouse models have been instrumental for elucidating the contribution of Ron signaling to breast cancer. When overexpressed under the mouse mammary tumor virus (MMTV) promoter, Ron caused mammary hyperplasia by 12 weeks of age, followed by development of adenocarcinoma

in 100% of female mice [39]. Tumors initiated by Ron exhibited spontaneous metastasis to liver and/or lung in ~90% of animals, which is remarkable given the relatively limited metastatic potential of other transgenic mouse models of breast cancer [40]. Confirmation that the Ron pathway is a significant contributor to breast tumor metastasis was obtained by examining 457 breast cancers from two independent patient cohorts [38]. In these studies, co-overexpression of MSP, its activating enzyme matriptase, and Ron (collectively referred to as MSP/matriptase/Ron) was used as a surrogate indicator of Ron signaling activity, and was a significant independent prognostic factor for metastasis and reduced survival. Importantly, overexpression of MSP or Ron mRNA alone did not significantly correlate with patient outcome, suggesting that Ron function in metastasis of breast cancer could be largely ligand-dependent, even when the receptor is overexpressed. Indeed, activation of Ron by overexpression of MSP in a mouse model of mammary cancer (transgenic mice expressing the polyomavirus middle T antigen under the mouse mammary tumor virus promoter; MMTV-PyMT [41]) was sufficient to cause spontaneous metastasis to lung, lymphatics, and bone. Patients whose tumors expressed MSP/matriptase/Ron also exhibited significantly more metastasis to lung, liver, brain, and bone (bone was the most frequent site of metastasis). Furthermore, MSP-induced bone metastases in the mice were osteolytic, as they are in human breast cancer patients, and appear to be the first example of spontaneous metastasis of a primary (non cell line-derived) tumor from the mammary gland to the bone in mice [38]. Thus, MSP/Ron activity exerts a gain-of-function effect in breast cancer, promoting tumor metastasis to clinically relevant sites.

#### OPPORTUNITIES FOR RON INHIBITOR THERAPY IN BREAST CANCER

Consistent with the knowledge that breast cancer is a complex and remarkably heterogeneous disease, single agent targeted therapy has generally not been effective long term, even in combination with standard chemotherapy. This is particularly true for metastatic breast cancer, which is still considered incurable [42]. Our ever-increasing understanding of mechanisms involved in tumor progression suggests that the ability to simultaneously abrogate several independent processes that are critical for cancer progression would hold great promise for new therapeutic approaches.

It is now realized that many of the processes that contribute to tumor progression and metastasis are not actually intrinsic to the tumor cells. Rather, the tumor microenvironment plays a key role in critical processes such as angiogenesis [43], growth factor production [44], tumor inflammation and immunoeediting [45], invasion and intravasation [46], as well as modifying the metastatic site to create a hospitable environment [47]. In fact, it has become clear that tumor progression and metastasis in mouse models can be severely restricted or even eliminated by limiting tumor inflammation [48, 49].

Data obtained *in vivo* using sophisticated mouse models and primary human breast cancer specimens strongly suggest that the Ron pathway is an exciting new target for therapy against solid tumors. MSP/Ron not only plays a causal role

in tumor development and progression, but also plays a critical role in the type of inflammation that is known to occur in tumor microenvironments [50, 51]; this is discussed in detail below. Based on the known function of Ron in tumor cells, macrophages and osteoclasts, we suggest that Ron inhibition would simultaneously block essential processes both *intrinsic* and *extrinsic* to the tumor cells: tumor growth and angiogenesis, promotion of metastasis by 'alternatively activated' macrophages, promotion of the wound healing process, and osteolysis due to breast cancer bone metastasis. The specific functions of Ron in each of these processes in the 'normal' state are discussed in detail below, followed by a discussion of the implications for Ron activity in the setting of cancer.

#### TUMOR-INTRINSIC ACTIVITIES: MSP/RON IN TUMOR GROWTH AND ANGIOGENESIS

Investigation of Ron activity in epithelial cancer cell lines has revealed roles in cell proliferation, survival, migration, and epithelial-mesenchymal transition [15, 52-54]. Selective Ron inhibitors have been generated and were reported to affect these processes, indicating that blockade of Ron function is achievable at least in certain settings [18, 55].

As described above, gain of function studies in mouse models have shown that activation of Ron through either overexpression of MSP [38] or overexpression of Ron [39] was sufficient to increase tumor growth as well as both the frequency and tissue tropism of metastasis in mice, and overexpression of MSP/matriptase/Ron significantly correlated with increased metastasis and death in breast cancer patients [38].

Conversely, loss of Ron function has been demonstrated to affect tumor growth, angiogenesis, and metastasis in a mouse model of breast cancer. Mice lacking the tyrosine kinase domain of Ron (Ron TK-/- [56]) were crossed to MMTV-PyMT mice, which resulted in decreased mammary tumor growth and reduced metastasis to lung – the only site of metastasis in the MMTV-PyMT model. This effect occurred in parallel with decreased vasculature and increased apoptosis in the tumors [57]. Selective Ron inhibitors have also shown some efficacy in xenograft models for other types of cancer (see below). Together, these data suggest that abrogation of Ron activity can impair tumor growth and reduce the likelihood of metastasis, and that pre-clinical studies using Ron inhibitors have shown promising results.

#### TUMOR-EXTRINSIC ACTIVITIES: MSP/RON IN MACROPHAGE ACTIVITY

Ron is expressed on terminally differentiated resident macrophages, but not on mononuclear phagocytes or circulating monocytes; Ron is upregulated during macrophage differentiation [58]. Ron is expressed on many different types of resident macrophages including alveolar macrophages, microglia, peritoneal macrophages, and dermal macrophages from either normal or wounded skin [23, 59, 60].

As suggested by its name, MSP does function to stimulate macrophages. Activated MSP increases the ability of

macrophages to undergo chemotaxis; stimulation of Ron by MSP leads to rapid changes in cell shape and motility [30, 61]. MSP/Ron activity also promotes rapid phagocytosis of C3bi coated erythrocytes *via* complement receptor 3 [62].

Consistent with its function in macrophage stimulation, *in vivo* experiments demonstrate that the Ron pathway is important in protection against Gram-positive bacteria. When Ron *-/-* mice were challenged with *Listeria monocytogenes* they showed increased bacterial burden and increased susceptibility to the infection - a phenotype similar to that of interferon-gamma (IFN $\gamma$ ) knockout mice and tumor necrosis factor-alpha (TNF- $\alpha$ ) knockout mice. Lack of Ron function may manifest itself in the inability of macrophages to efficiently eliminate the bacteria, as rapid clearance by macrophages *via* the complement receptor is known to be essential in preventing *Listeria* infections [62].

Studies of MSP/Ron signaling in macrophages indicate that, although the Ron pathway is involved in macrophage activation and protection from particular microorganisms, it is *critical* for resolution of inflammation in many models. Mice lacking Ron activity are viable and fertile but have noteworthy defects in macrophage function; Ron is necessary to limit inflammatory responses [56, 63]. Peritoneal macrophages isolated from Ron deficient mice produce increased levels of nitric oxide in response to lipopolysaccharide (LPS) stimulation, and when Ron TK-*-/-* mice are challenged with sub-lethal doses of LPS, they are more susceptible to LPS-induced endotoxic shock [56].

The mechanisms by which MSP/Ron signaling functions to resolve inflammation are elegantly studied. One important function of Ron is to downregulate interleukin 12 (IL-12) production in macrophages. The inability of Ron TK-*-/-* mice to downregulate IL-12 leads to increased IFN- $\gamma$  production by natural killer cells, and a prolonged inflammatory reaction [50]. *In vitro*, MSP is sufficient to polarize macrophages from the "classically activated" to the "alternatively activated" state [64] (also known as the M1 and M2 states, respectively, and further described below). MSP/RON signaling also suppresses inflammation through several other routes: activation of suppressors of cytokine signaling, down-regulation of IFN- $\gamma$ , reduction of major histocompatibility complex class II cell surface expression, and reduction of IFN- $\gamma$ -induced STAT1 phosphorylation [50]; downregulation of inducible nitric oxide synthase (iNOS) [64]; increased production of the anti-inflammatory cytokine IL-10 [65]; and downregulation of cyclooxygenase-2 (COX-2) expression through inactivation of Nuclear Factor kappa B (NF $\kappa$ B) [66].

Taken together, the published data indicate that the MSP/Ron pathway plays a dual role in inflammation: a role in initial macrophage activation, as well as an important role in downregulating the inflammatory response. Ron activity results in increased migration of macrophages to sites of infection, and stimulates phagocytosis early in the infection process. Later, Ron is required to resolve inflammation by downregulating iNOS and IL-12 and by upregulating IL-10. Still, much is to be learned: the different roles for Ron signaling in infections elicited by gram-positive versus gram-negative bacteria indicate that MSP/Ron function is context dependent, and the interaction of MSP-stimulated

macrophages with cells of the acquired immune system is yet to be discovered.

## AN INTERSECTION OF INTRINSIC AND EXTRINSIC FACTORS: MSP/RON IN WOUND HEALING

Skin wound repair is essential for tissue homeostasis and involves three phases: inflammation, proliferation, and remodeling (for review see [67]). Inflammatory cells play a crucial role in the wound healing process. Macrophages remove dead tissue, stimulate the growth of new blood vessels, regulate fibroblast recruitment, re-growth of the epithelium, and remodeling of connective tissue (for review see [68]). Classically activated macrophages (M1 macrophages) are present early during the wound healing process and function to remove pathogens and stimulate the immune response, whereas alternatively activated (M2) macrophages predominate later in the repair process. M2 macrophages fail to present antigen to T cells, produce minimal amounts of pro-inflammatory cytokines and nitric oxide, and are less efficient than M1 macrophages at killing microbes. Instead, M2 macrophages secrete extracellular matrix (ECM) proteins and polyamines, which influence production of cytokines, inhibit clonal expansion of lymphocytes, and stimulate proliferation of epithelial cells [69]. M2 macrophages are characterized by downregulation of iNOS and upregulation of arginase 1, which metabolizes arginine to urea and ornithine. Consequently, there is increased arginase activity in experimental rat wounds, along with increased ornithine levels [70]. Importantly, MSP/Ron signaling is instrumental in the switch from expression of iNOS to expression of arginase [64].

Successful wound repair entails resolution of the inflammatory response and, as discussed above, MSP is both necessary and sufficient to induce M2 macrophage polarization, which assists in attenuation of inflammation. MSP/RON signaling is involved at various steps of the wound healing process. In experimental excisional wounds in rats, immunostaining revealed both MSP and Ron within the wound, where maximum staining occurred between 7 and 21 days post-wounding [71]. There are also increased levels of activated MSP in fluids collected from burn wounds in humans, and RON-expressing macrophages are scattered throughout the dermis.

In addition to resolving inflammation in wounds, Ron plays a role in repairing wounded skin. This process, referred to as re-epithelialization, involves migration and proliferation of epidermal keratinocytes. Cells at the wound margin loosen their extracellular matrix (ECM)-cell and cell-cell interactions in order to migrate across the wound (for review, see [72]). Ron is upregulated by proliferating and differentiated populations of keratinocytes [23], and MSP promotes keratinocyte migration in mouse wounds and in wound healing assays *in vitro*. In primary keratinocytes, the 14-3-3 protein associates with Ron in response to MSP signaling, which induces spreading and improved migration on laminin 5 ECM [73]. It is notable, however, that MSP deficient mice do not show any defects in specific skin wound healing models [74], suggesting that functional redundancies exist for this important biological process.

The role of MSP/Ron in other models of injury has also been investigated. In two different models of lung injury, Ron proved to be essential for protection from unregulated inflammation. When injected with intrapulmonary LPS, mice lacking Ron function display increased lung injury and damage due to overproduction of nitric oxide and TNF $\alpha$  through the NF $\kappa$ B pathway. Again, MSP/Ron function was deemed necessary to suppress NF $\kappa$ B activation *in vivo* [75]. In a nickel-induced acute lung injury model, in which mice are exposed to aerosolized nickel particles, mice lacking RON function exhibited significantly decreased survival times compared to control mice. The mice showed increased levels of IL-6 and the chemokines Chemokine (C-C motif) ligand 2 (CCL2) and Chemokine (C-X-C motif) ligand 12 (CXCL12), as well as increased serum nitrite levels. These effects were commensurate with earlier onset of pulmonary inflammation, edema and lethality [76]. Gene expression analysis indicated that genes responsible for inflammation, edema and lymphocyte function were significantly altered in mice lacking Ron activity [77].

Paradoxically, in a model of LPS-induced acute liver failure in galactosamine-sensitized mice, RON deficient mice are actually protected from liver injury. This finding was based on histological analysis as well as serum alanine amino transferase levels, and was associated with decreased number of liver cells undergoing apoptosis [78].

The last three examples of Ron involvement in injury indicate that the cytokine milieu and the type of injury likely influence the outcome of MSP/Ron signaling. Data from mice lacking Ron activity indicate that blocking MSP/Ron signaling therapeutically may not adversely affect healing of common skin wounds, but could be a concern for life-threatening infections. An important consideration, however, is whether acute loss of Ron function (as would occur with therapeutic blockade of Ron signaling) would have different effects than the chronic lack of function that develops in genetically engineered mice.

### INFLAMMATION AND CANCER: DUAL FUNCTION OF MSP/RON?

A growing body of evidence supports the idea that inflammation contributes to cancer development and progression (for review, see [45]). The risk of developing cancers of the esophagus, colon, pancreas, lung, and gallbladder is heightened by the presence of chronic inflammatory diseases. Chronic inflammation, like chronically unhealed wounds, is characterized by a prolonged cycle of tissue damage, cellular proliferation and tissue repair [79]. The inflammatory environment is enriched with macrophages that generate high levels of reactive oxygen and nitrogen species to fight infection. However, when unregulated, these agents can react with DNA and cause mutations in proliferating epithelial and stromal cells (for review, see [80]).

Increased tumor associated macrophage (TAM) density is associated with tumor progression and metastasis (for review, see [81]). TAMs have many characteristics of an M2 activation phenotype, and are thought to contribute to tumor development by releasing IL-10 and PGE<sub>2</sub>, which suppress the inflammatory reaction to the tumor [82]. TAMs also

release pro-angiogenic factors such as vascular-endothelial growth factor (VEGF), endothelin 2 and plasminogen activator, and pro-proliferative factors such as epidermal growth factor (EGF), fibroblast growth factor, HGF, platelet-derived growth factor, transforming growth factor  $\beta$  (TGF $\beta$ ), and IL-6 [83, 84]. TAMs are also thought to facilitate tumor cell invasion and metastasis by releasing MMP2 and MMP9, which modify the ECM and basement membrane, and by facilitating a paracrine loop of EGF and colony stimulating factor-1 signaling to promote metastasis [85]. Thus, TAMs endow tumors with an environment that enhances the survival, migration and proliferation of epithelial cells and are a large contributor to the observation that tumors are much like chronically unhealed wounds [86].

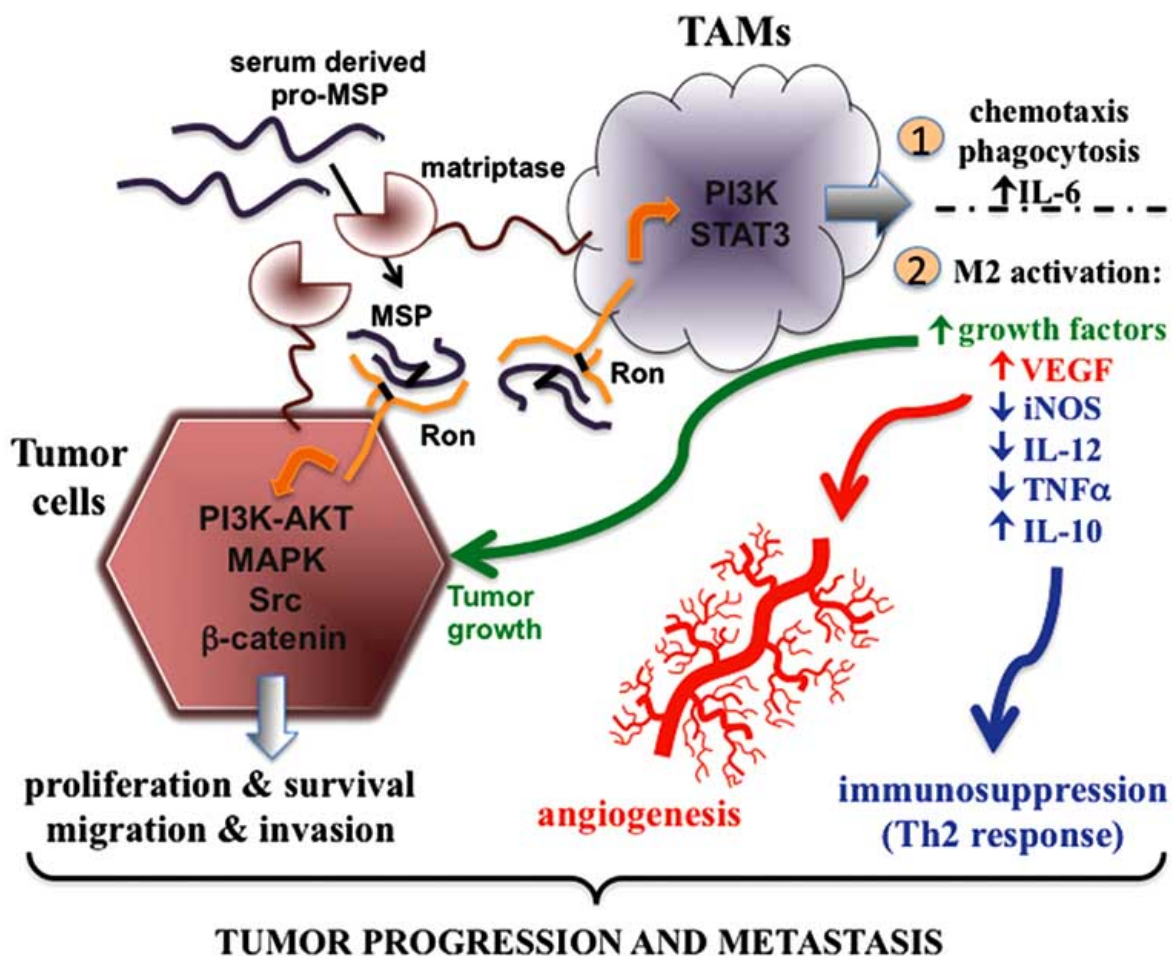
Although there is insufficient evidence at this time to suggest that MSP/Ron-induced inflammation *directly* participates in cancer progression, studies indicate that MSP is able to evoke dose-dependent superoxide anion production in human alveolar macrophages *via* src, MAPK, and p38 signaling pathways [59]. In human alveolar macrophages from either smokers or non-smokers, MSP efficiently activates NF- $\kappa$ B. However, MSP evokes superoxide production, cytokine release and NF $\kappa$ B activation to significantly higher levels in cells from smokers versus those from non-smokers, indicating that MSP may enhance inflammation due to cigarette smoke [65]. Although this may contribute to tumorigenesis, there is another likely, and potentially more impactful, role for MSP/Ron in tumor progression and metastasis: polarization of TAMs to an M2 phenotype.

### CONSEQUENCES OF MSP/RON ACTIVATION IN TUMORS

As described above, pro-MSP is present in high concentrations in serum, and conversion of pro-MSP into MSP occurs locally at sites of inflammation [23]. A serine protease that was shown to cleave and activate MSP, matriptase, is normally present on macrophages, but is also upregulated in a large percentage of breast cancers [30]. In addition, RON is overexpressed to high levels (and Ron is phosphorylated) in ~50% of breast cancers [87]. It is reasonable to presume that activation of MSP locally, at sites of inflammation in tumors, would not only lead to activation of Ron on TAMs, but also on the tumor epithelium, where Ron has been shown to induce proliferation, survival, cell migration, EMT, invasion, and metastasis (see above).

Although the result of Ron signaling in TAMs is still unclear, MSP/Ron activates signaling pathways in macrophages that are known to be involved in tumor progression. Ron activation causes phosphorylation of the signal transducer and activator of transcription 3 (STAT3) protein [88], which is required for the immunosuppressive and tumor promoting effects of TAMs. In fact, STAT3 knockout mice [89] show similar inflammatory phenotypes as RON deficient mice, and several infectious agents are known to cause inflammation-induced cancer *via* STAT3 activation [90]. Furthermore, the MSP/Ron-induced cytokine IL-6 activates STAT3 in both inflammatory cells and epithelial cells [90].

In addition to the immunosuppressive effects of MSP/Ron *via* STAT3 activation, the MSP/Ron pathway also



**Fig. (1).** Model for the contribution of MSP/Ron function in tumor progression and metastasis through both cell autonomous (tumor cell proliferation, survival, migration and invasion) and non-cell autonomous (macrophage activation and polarization) functions.

down-regulates STAT1 activity [27], which is involved in anti-tumor immune responses through upregulation of IL-12 [90]. STAT1 and STAT3 clearly act in opposing roles with regard to immune responses against tumors; genetic deletion of STAT3 in immune cells leads to upregulated STAT1 activity and increased anti-tumor properties [91]. Thus, it is likely that both STAT3 activation and STAT1 inhibition by MSP/Ron may manifest in immune tolerance to the tumor, in addition to the potential function of MSP/Ron in promoting secretion of pro-growth and pro-angiogenic factors by M2-polarized TAMs. The potential consequences for MSP/Ron activation in tumors are summarized in Fig. (1).

### MSP/RON IN OSTEOLYTIC BONE METASTASIS

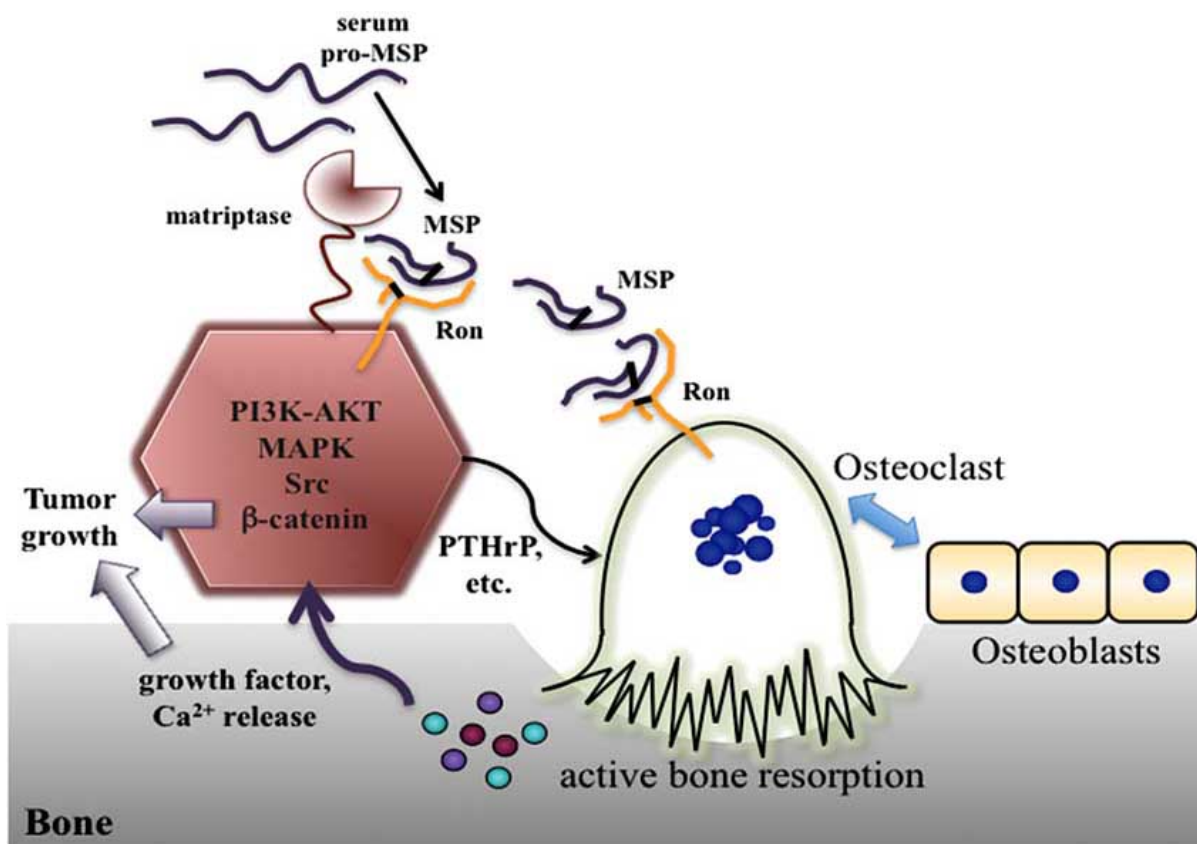
In addition to its expression and activity in macrophages, Ron is also expressed on osteoclasts, the specialized macrophages of bone. Ron becomes expressed on the surface of multinucleated osteoclast-like cells when human bone marrow cells are differentiated *in vitro*, and MSP activates osteoclasts, causing bone resorption [92]. *In vivo*, Ron is highly expressed on osteoclasts but does not appear to play a critical role in bone development, since mice lacking Ron function have no overt bone defects [56].

The role of MSP in osteoclast activation is highly relevant to breast cancer, since bone metastasis occurs in 70-

80% of patients and is therefore the most common site for relapse [93, 94]. Osteoclasts are activated *in vitro* by breast cancer cells that express MSP, and to a much greater extent than that induced by control tumors [38]. Furthermore, mice with mammary tumors expressing MSP spontaneously developed osteolytic bone metastasis, and breast cancer patients with high MSP/matriptase/Ron experienced significantly more metastasis to bone than those without high MSP/matriptase/Ron [38]. A model for the role of MSP/Ron activity in osteolytic bone metastasis is shown in Fig. (2). A specific understanding of whether the MSP/Ron pathway contributes to the “vicious cycle” of breast tumor growth in bone that was previously proposed by Guise and Mundy (for recent reviews on breast cancer bone metastasis, see [93, 95]), or whether MSP/Ron activation defines a new mechanism for osteolysis remains to be determined. Understanding the role of the MSP/Ron pathway in breast cancer bone metastasis would have important clinical implications.

### MSP/RON AS A THERAPEUTIC TARGET

The ability to simultaneously block several key pathways that contribute to tumor progression might lead to more efficacious therapy. We suggest that the MSP/Ron pathway holds promising potential in this regard, since it is upregulated in a large proportion of cancers and contributes



**Fig. (2).** Model for the role of MSP/Ron activity in osteolytic bone metastasis as a complication of breast cancer. MSP can directly activate osteoclast activity through Ron stimulation. The ensuing bone resorption can release calcium and growth factors that stimulate tumor growth and perpetuate a “vicious cycle” [93].

to proliferation, survival, migration, and invasion of tumor cells. In addition, though, MSP/Ron activity also promotes M2 macrophage polarization, potentially leading to secretion of immunosuppressive cytokines as well as growth and angiogenic factors that support the tumor. Blockade of MSP/Ron function might therefore interfere with critical tumor-promoting pathways in the tumor itself *and* in the tumor microenvironment.

### Strategies for Ron Inhibition

One can imagine several potential strategies to interfere with MSP/Ron function, including prevention of pro-MSP activation, blockade of MSP-Ron interaction and/or receptor dimerization, and inhibition of Ron kinase activity. Inhibition of MSP activating enzymes such as matriptase is unlikely to be effective due to redundancy between several serine proteases capable of activating MSP *in vitro* and *in vivo* [30, 31, 96]. Strategies to prevent ligand-receptor interactions and/or receptor downregulation could be achieved through generation of monoclonal antibodies (mABs). mABs can also have the added benefit of inducing antibody-mediated cellular cytotoxicity, analogous to that achieved by the HER2 antibody trastuzumab (Herceptin) in breast cancer. One Ron inhibitory antibody has been described, and was shown to be effective in slowing growth of colon, lung, and pancreatic cancer xenografts [18].

Inhibition of kinase activity may be less specific, due to high conservation of kinase domains within receptor tyrosine kinases, but would have the added benefit of oral availability and potentially lower cost. One advantage of targeting Ron with a small molecule kinase inhibitor is that Met kinase inhibitors are already available, some of which are being tested in clinical trials [34]. Since the kinase domains of Ron and Met are 68% identical, it is very likely that a Met inhibitor will also block Ron, at least to some extent. A dual Ron/Met inhibitor recently showed promising results in xenograft studies using Met-dependent cell lines or colon cancer cells expressing an endogenous, hyperactive form of Ron (see below) [55].

### Challenges for Drug Development Against MSP/Ron

As with all potential new therapies, there are great challenges. The MSP/Ron pathway, in particular, may be even more confounding due to dual effects on the tumor and on the host immune system. One hurdle in drug development for oncology is pre-clinical testing in animal models, and immunodeficient mice are routinely used for initial studies. However, if MSP/Ron functions to promote tumor progression and/or metastasis through alteration of immune function, the results would be very difficult or impossible to discern in such a model. Use of syngenic, immunocompetent mouse models such as the one we developed [38] can overcome the problem of immune involvement, but pre-

cludes testing species-specific drugs such as anti-human Ron antibodies. Likewise, if activation of host macrophages is a key component of MSP/Ron function in tumors, drugs that recognize and inhibit both the human and murine Ron proteins would be required for validation in xenograft models.

The existence of multiple isoforms of Ron poses another challenge for drug development. There are a number of alternative Ron isoforms described. These include hyperactive splice variants [97, 98], and an N-terminally truncated form of Ron, termed short form Ron (sfRon). sfRon is generated through utilization of a second, internal promoter within intron 10 of *RON*, creating a constitutively active form of the receptor that does not require ligand binding for activity [99]. sfRon is expressed in cell lines originating from multiple cancer types, and has been detected in primary breast cancers [99]. In mice, sfRon is required for transformation of erythroblasts by the Friend virus, and mice with a naturally occurring polymorphism in the sfRon promoter are resistant to this form of erythroleukemia [100]. Since the human sfRon promoter is relatively uncharacterized, it is unknown whether polymorphisms exist and are relevant to tumorigenesis. However, methylation of the main *RON* promoter may contribute to expression of sfRon in cancer cell lines [101]. Upregulation of active forms of Ron in cancer could serve as an important contributor toward resistance to therapies designed to interfere with ligand binding, for example. Thus, in our view, Ron kinase inhibitors may hold the greatest promise for targeted therapy against this pathway in breast cancer.

## RELEVANCE OF THE MSP/RON PATHWAY IN OTHER CANCERS

The Ron pathway may also be an excellent therapeutic target in cancers other than breast. Ron is overexpressed in a wide variety of human cancer tissues (Table 1) and, although its function in the epithelial compartment is not understood for all of these malignancies, the function of MSP/Ron in tumor inflammation is likely to be conserved. MSP and matriptase are also upregulated in many cancers [30, 38, 102, 103], further supporting the idea that both autocrine and paracrine pathways could contribute to tumorigenesis and/or progression of malignancy.

Ron is overexpressed in small cell lung carcinoma (SCLC) cell lines, a pulmonary carcinoid cell line, and in non-small cell lung carcinoma (NSCLC) [102, 103]. While MSP expression is low to undetectable in both SCLC and pulmonary carcinomas, MSP is expressed in NSCLC primary tumors and cell lines [103, 104]. Addition of MSP to NSCLC cell lines expressing Ron resulted in increased cell motility [103]. In addition, overexpression of Ron in distal lung epithelial cells results in the development of lung adenomas *in vivo* [105].

Expression of full length Ron, as well as various isoforms of Ron, has been demonstrated in human colon cancer cell lines as well as primary adenocarcinomas [97, 106, 107]. Ron is highly expressed in 60% of colorectal adenocarcinomas and its expression correlates with the degree of differentiation of these tissues [106, 107]. The constitutively active splice variants *RON* $\Delta$ 155, *RON* $\Delta$ 160, and *RON* $\Delta$ 165

are most notably expressed in colon cancers. Expression of *RON* $\Delta$ 155 or *RON* $\Delta$ 160 in NIH3T3 cells lead to tumor formation *in vivo* [97, 106-108], and expression of full length Ron in colon epithelial cells results in an increase in cell motility and invasiveness, while protecting the cells from apoptosis [108]. Silencing Ron expression by RNAi in colon cancer cell lines led to decreased cell proliferation and motility, with an increase in apoptosis [109]. Silencing of Ron also reduced tumorigenesis *in vivo*, suggesting that Ron expression is required to maintain the tumorigenic phenotypes of colon cancer cells [109].

**Table 1. Expression of Ron in Primary Human Cancer Tissues**

| Cancer Type  | % of Tumors Expressing Ron   | Reference(s) |
|--------------|------------------------------|--------------|
| Breast       | 50                           | [87]         |
|              | 32                           | [32]         |
|              | 100                          | [18]         |
|              | 8-20*                        | [38]         |
| Lung         | 93                           | [18]         |
|              | 50 (NSCLC <sup>#</sup> only) | [103]        |
| Colon        | 60                           | [106, 107]   |
|              | 65                           | [18]         |
| Pancreatic   | 93                           | [114]        |
|              | 79-93                        | [115]        |
|              | 69                           | [18]         |
| Bladder      | 33                           | [116]        |
| Ovarian      | 56-60                        | [118]        |
| Prostate     | 92                           | [18]         |
| Liver        | 29 (HCC <sup>†</sup> only)   | [123]        |
| Gastric      | 73                           | [18]         |
| Glioblastoma | 82                           | [121]        |

\* MSP/matriptase/Ron co-overexpression

<sup>#</sup> Non small cell lung carcinoma

<sup>†</sup> Hepatocellular carcinoma

While mutations in Ron have not been identified in cancers other than in a single lung tumor [110], two alterations have been identified which may have a role in Crohn's disease [111]. A genome wide linkage study performed with a cohort of Crohn's disease patients identified strong linkage disequilibrium with two non-synonymous single nucleotide polymorphisms (SNPs) within the *RON* gene [111]. The first SNP, rs2230590, results in an Arg523Gln substitution while the second, rs1062633, results in a Gly1335Arg substitution. Further evidence for a role for the MSP/Ron pathway in inflammatory bowel diseases (IBD) comes from another genome wide linkage study performed on a cohort of IBD patients. This study identified significant linkage disequilibrium with a SNP located within the *MSP* gene [112]. This nonsynonymous SNP, rs3197999, results in an Arg698Cys coding variant, which is predicted to interfere with the ability of MSP to bind to Ron [112]. Importantly, this coding variant showed association with both Crohn's disease and ulcerative colitis, suggesting that the MSP/RON pathway

may have an important role in multiple forms of inflammatory bowel disease - though the mechanisms for this role have yet to be elucidated [112]. Associations between *RON* and *MSP* SNPs in inflammatory bowel diseases, which predispose patients to colon cancer (for a recent review, see [113]), provide further support for a connection between MSP/Ron function in inflammation and tumor progression (Fig. 1).

Ron is overexpressed in 79-93% of human pancreatic tissue samples, and in 83% of metastatic lesions [114, 115]. Activation of Ron by MSP in pancreatic cell lines leads to activation of Erk and Akt pathways, as well as induction of EMT characteristics such as increased cell migration, invasion, and loss of E-cadherin [114, 115]. Inhibition of Ron by a neutralizing antibody resulted in inhibition of the cell migratory and invasive phenotypes [115].

Ron is overexpressed in 33% of primary bladder tumors, where Ron levels correlated with poor grade as well as tumor size and stage [116]. Overexpression of Ron in a uroepithelial cell line led to proliferation, motility, and increased survival [116]. Ron also cooperated with Met and EGFR in these cells; co-expression of Ron and Met was significantly associated with decreased survival and metastasis-free survival in 19% of patients [116]. Co-expression of Ron and EGFR was found in 33% of patients and significantly associated with invasion, risk of recurrence, and decreased patient survival [117].

Ron expression was detected in 56% of ovarian cancers and 60% of borderline ovarian tumors [118]. The level of Ron expression also significantly correlated with decreased survival in ovarian cancer patients [119]. A correlation between overexpression of Ron and concomitant expression of Met was demonstrated, and stimulation of ovarian cancer cell lines *in vitro* by MSP and/or HGF lead to increased motility and invasion [118, 120].

Full length Ron, and several splice variants, were expressed in primary human glioblastomas and glioblastoma cell lines. Of the glioblastoma patient samples analyzed, 82% expressed some form of Ron, while 100% of the glioblastoma cell lines analyzed demonstrated Ron expression [121]. MSP was also expressed in glioblastoma cell lines, where it functions to increase cell migration [121]. A novel splice variant, RON $\Delta$ 90 was also identified, which inhibited MSP-induced phosphorylation of Ron as well as cell migration.

Ron was expressed in 92% of prostate tumor tissues and is overexpressed in prostate cancer cell lines [18]. Ron expression correlates with the stage of disease in the primary tumor and is expressed in prostate metastases. Levels of angiogenic chemokines correlate with Ron expression, and knockdown of Ron resulted in a decrease in angiogenic factors, NF- $\kappa$ B, and endothelial cell migration *in vivo*. Knockdown of Ron also resulted in decreased tumor growth and microvessel density, indicating that Ron may play an important role in the angiogenic process in prostate cancer [122].

Ron has been shown to be overexpressed in two out of seven hepatocellular carcinoma (HCC) tissue samples. The cytokines IL-1 $\alpha$ , IL-6, and TNF $\alpha$ , as well as the growth factor HGF were shown to increase Ron expression in a

HCC cell line. These factors are commonly upregulated in liver disease and may therefore play a role in liver carcinogenesis through the upregulation of Ron [123]. Notably, the liver is the primary site of MSP production and therefore may contribute to increased Ron activity in the liver.

## CONCLUSIONS

In summary, the MSP/Ron pathway appears to be active in a large number of solid tumors from various organs, and MSP/Ron activity correlates with aggressive disease and poor outcome. The known roles for this pathway strongly suggest that MSP/Ron could play a significant, dual role in tumor progression by acting directly on tumor cells and indirectly through inflammatory cells. Thus, inhibition of Ron may provide a promising new avenue for cancer treatment by simultaneously affecting at least two critical aspects of tumor progression. In breast cancer, blockade of Ron function may succeed in decreasing tumor growth, metastasis, and destruction of bones through MSP-driven osteolysis.

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